Biotechnological Applications of *Jatropha curcas* Seeds for Bioenergy Carriers and Bioactive Compounds

By

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Thesis

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IN

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By

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Islamabad
2020

DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this thesis/work elsewhere for any other degree.

Abdul Haq

DEDICATED

TO

My Lala G, Addy and family

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CONTENTS

List of abbreviations

ACE: acid catalyzed esterification

AD: anaerobic digestion

ANOVA: analysis of variance

AOAC: Association of Official Analytical Chemists

AR: aqueous residues

ASTM: American Society for Testing and Materials

ATCC: American type culture collection

AV: acid value

BGP: biogas potential

C/N: carbon to nitrogen ratio

CCA: canonical correspondence analysis

CFU: colony forming units

CHNS: carbon, hydrogen, nitrogen and sulphur

COD: chemical oxygen demand

CTAB: cetyl trimethylammonium bromide

DMSO: dimethyl sulfoxide

DPPH: 2, 2-diphenyl 1-picrylhydrazyl

EV: ester value

FAME: fatty acid methyl esters

FFA: free fatty acids

FIC: fractional inhibitory concentration

FICI: fractional inhibitory concentration index

FTIR: fourier transform infrared radiation

GAE: gallic acid equivalent

GC-MS: gas chromatography coupled with mass spectrometry

HRT: Hydraulic retention time

JO: Jatropha oil

JPC: Jatropha pressed cake

JSC: Jatropha seed cake

JWS: Jatropha whole seed

K: Kelvin

Kg: kilogram

L: liter

LB: Luria broth

LCFAs: Long chain fatty acids

m: meter

M: molar

MDR: multidrug resistant

ME: methanolic extract

MFC: minimum fungicidal concentration

MHA: Mueller Hinton agar

MIC: minimum inhibitory concentration

mL: milliliter

MP+JSK: mango peel plus *Jatropha curcas* seed kernel

MR: Methanolic residues

MRSA: methicillin-resistant *Staphylococcus aureus*

NARC: National Agriculture Research Council

NL: normalized liter

NmL: normalized milliliter

NR: *n*-hexane residues

OD: optical density

OLR: organic loading rate

OTU: operational taxonomic unit

Pa: pascal

PBS: phosphate buffer saline

PE: paired–end

PNP: para nitrophenol

PNP-L: para-nitro phenyl Laurate

QE: Quercetin equivalent

QIIME: Quantitative insights into molecular ecology

rpm: revolution per minute

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

SK+SC(H_3PO_4): *Jatropha curcas* deoiled seed kernel mixed with dilute acid treated seed coat

SN: saponification number

TAG: triacylglycerides

TGs: triglycerides

TS: total solids

VFA: volatile fatty acids

VS: volatile solids

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Abstract

Currently, the energy and other value added commodities around the globe come from fossil fuels based refineries. However, the consumption of fossil fuels for energy and other materials has resulted in the creation of various undesirable and complicated problems, for example, the emission of greenhouse gases (global warming), steady increase in the prices of fossil fuel products and reduction in their reserves. Therefore, it has become critical to seek for alternative sustainable, environmental friendly, cost-effective and more importantly, renewable energy resources for biofuels production and valuable bio-based commodities. Biomass is one of the ideal alternatives that can be used in biorefinery context to fulfill the world demands and replace the fossil fuels refineries. Biomass can be further categorized into edibles and non-edible feedstocks. Edible feedstocks face challenges such as competition between the lands required for fuels and food supply ultimately increasing the food prices. While, non-edible feedstocks such as *Jatropha curcas* are the ideal, renewable and sustainable options for biorefinery concept, having no fuels versus feed competition. In the current study, *J. curcas* seed was used as feedstock for biorefinery to produce sustainable energy carriers (biogas and biodiesel) as well as commodities through jointly applied conversion technologies. Biodiesel production from *J. curcas* seed oil was the most feasible option, and biogas production from pressed cake after methanolic extraction was the best option for biogas production. The methanolic extract of pressed cake showed good antimicrobial, and antioxidant activities and also the extraction proved to enhance biogas production and reduced negative effect on microbial profile in the reactor. Biodiesel production from the oil extracted from *J. curcas* seed was evaluated using chemical (two-step process) and biological approach (lipases based). The pressed cake obtained after the oil extraction from seed was also used for biogas production. However, pressed cake is composed of antimicrobial phytochemicals that inhibit the microbial communities during anaerobic digestion (AD). But in this study, it was interesting that the extraction of methanolic extracts from pressed cake enhanced the biogas production, also the microbial evenness/richness and relative abundance in the reactor. Phytochemical analyses of *J. curcas* de-oiled pressed cake extracts was carried out using GC-MS and FTIR. *J. curcas* oil and different extracts from de-oiled pressed cake were evaluated individually as well as in combination with commercial antibiotics against various bacterial clinical pathogenic and multidrug resistant strains. In addition, the de-oiled pressed cake extracts and seed oil were evaluated for their antioxidant, cytotoxic, enzyme inhibition and antifungal activities against phytopathogenic fungal strains. The microbial communities and biogas yield during AD in

continuous reactor was evaluated when reactors were fed with whole seed, seed oil, pressed cake and methanolic residues (*J. curcas* pressed cake after the methanolic extraction). The antimicrobial effects of *J. curcas* seed was evaluated on the biogas yield and microbial communities involved in AD process in continuous setup using high throughput Illumina MiSeq sequencing. The effect of carbon to nitrogen ratios on biomethane yield during anaerobic co-digestion of *J. curcas* de-oiled seed kernel and mango peels was evaluated in continuous reactors. *J. curcas* seed oil was also used for chemical (two-step process) and bacterial (lipase) based biodiesel production. For bacterial based biodiesel production, lipase producing indigenous bacterial strains were isolated. Plackett-Burman and central composite designs were used to optimize various factors during bacterial based transesterification of *J. curcas* seed oil.

J. curcas de-oiled pressed cake extracts and seed oil were rich in various phytochemicals. In antibacterial activities, methanolic extracts remained more active compared to seed oil, *n*-hexane and aqueous extracts individually as well as in combinatorial activities against clinical and multidrug resistant (MDR) bacterial strains. Methanolic extract in combination with rifampicin showed the highest synergism against various MDR and clinical bacterial isolates. The methanolic, *n*-hexane, aqueous extracts and seed oil in various combinations with antibiotics showed 45, 33, 9 and 26% synergism, respectively. Similarly, methanolic extract was highly potent against selected fungal strains compared to the other extracts and seed oil. The methanolic extract was also found significantly potent in antioxidant activities compared to the other treatments.

In batch process, higher biogas yield was obtained from methanolic residues compared to pressed cake, aqueous and *n*-hexane residues. The methanolic residues were easily biodegradable and therefore accumulation of VFAs was observed when OLR was increased. Therefore, at higher organic loading rates, methanolic residues were evaluated for biogas production in two stage continuous anaerobic digesters to ensure efficient and stable biogas production. Methanolic extract significantly inhibited the hydrolysis phase of AD and decreased biogas yield by 35.5%. It was confirmed by studying the effect of methanolic extract on microbial communities in continuous reactor in which the relative abundance of fermentative bacteria was higher in reactors fed with methanolic residues (Jatropha pressed cake after methanolic extraction) compared to the one fed with Jatropha pressed cake. Jatropha oil and whole seed did not exhibited inhibitory effects on methanogens during AD. The effect of operational parameters (organic loading rate and hydraulic retention time) on microbial

communities in continuous reactors showed that higher relative abundance of methanogenic and lower abundance of fermentative bacterial communities was observed in all reactors at hydraulic retention time 20 compared to 15 and 10 days. The biomethane yield of co-digested mango peel and seed kernel (1:4 weight ratio based on volatile solids) was 61, 50, 36, and 25% higher compared to the biomethane yields of mango peel, seed kernel, mango peel:seed kernel (2:1) and mango peel:seed kernel (1:1), respectively. The co-digestion of mango peel and seed kernel at 1:4 ratio resulted in the highest actual biomethane yield, followed by 1:1 and 2:1 ratios with yields of 52, 39 and 32% of the theoretical yields, respectively, illustrating the importance of adjusting C/N ratio by co-digestion of the right amounts of co-substrate.

Biodiesel production from *J. curcas* seed oil was feasible using chemical and biological means. Different variables such as oil to methanol ratio, catalyst concentration, temperature, reaction time and agitation were optimized for biodiesel production and the highest volumetric biodiesel yield obtained was 97-98% for chemical and bacterial based biodiesel production at optimized conditions. In case of lipase mediated biodiesel production, the highest volumetric yield of biodiesel (~97%) was obtained by using *Brevibacterium* SB11 MH715025 and *Pseudomonas* SB15 MH715026, strains at optimum conditions. The fuel properties of biodiesel produced by the chemical method and selected strains from *J. curcas* seed oil were in line with quality standards specified by ASTM D6751 and EU-14103.

The study concludes that *J. curcas* seed is a multipurpose substrate and could be used for production of a number of products including antimicrobials, antioxidants, cytotoxic and bioenergy carriers (biogas and biodiesel). Therefore, using *J. curcas* in a biorefinery context rather than simply for biofuel production is an ideal solution to increase the economic value of *J. curcas* plant for biofuel and pharmaceutical industrial sectors. Moreover, by extracting the antimicrobials, the seeds toxicity is reduced, increasing the efficiency and economic value for biofuel production.

List of Papers

This thesis is based on the work contained in the following papers, referred to by their Roman numerals in the text. The start page of published paper is attached at the end of appendices

Published Papers

I. **Haq, A**., Siddiqi, M., Batool, S.Z., Islam, A., Khan, A., Khan, D., Khan, S., Khan, H., Shah, A.A., Hasan, F. and Ahmed, S and Badshah. M. 2019. Comprehensive investigation on the synergistic antibacterial activities of *Jatropha curcas* pressed cake and seed oil in combination with antibiotics. *AMB Express, 9*(1), p.67.

Submitted Papers

I. **Haq, A.,** Rehman M.L.U and Badshah, M. 2019. Two-Step process optimization for biodiesel production from non-edible *Jatropha curcas* seed oil with high free fatty acids (**Submitted to Energy Sources, Part A: Recovery, Utilization, and Environmental Effects**).

II. **Haq,** Adeel, S and Badshah, M. 2019. Lipase mediated biodiesel production from *Jatropha curcas* seed oil using indigenous bacteria of oil contaminated soil**. (Submitted to Bioenergy Research).**

To be submitted

III. **Haq, A.,** de los Reyes III, F. L and Badshah, M. 2019. Enhancement of biogas yield during anaerobic codigestion of different parts of *Jatropha curcas***.**

IV. **Haq, A.,** Malik, A., Weaver, J., Wang, L., de los Reyes III, F. L and Badshah, M. 2019. Effect of operational parameters and methanolic extracts from *Jatropha curcas* pressed cake on biogas production and microbial communities during anaerobic digestion.

V. **Haq, A.,** Mushtaq, S and Badshah, M. 2019. Phytochemical analyses, bioactivities and antifungal activity of *Jatropha curcas* seed cake extracts and oil.

CHAPTER 1

1. Introduction

Currently, the demands of energy, transportation, fuels and commodities around the globe mostly depend on the consumption of existing fossil fuels which resulted in various economic and environmental issues. Some of the major issues include depletion in the fossil fuel reservoirs, increase in fuel prices and emission of greenhouse gases that ultimately is leading towards global warming effects and climate change. These alarming threats have diverted the focus of scientists to explore alternative sustainable, renewable energy resources for biofuels and valuable bio-based products in biorefinery context (Saini et al., 2019). In biorefinery, the biofuels, renewable energy and value added products are produced from plant biomass in an integrated sustainable approach by joining various production methods and technologies. The biorefinery concept is similar to the petrochemical based refinery in which the plant biomass is treated in a way to produce multiple products such as biofuels, value added biomolecules and biomaterial (Moncada et al., 2015). The renewable biofuels and commodities which are derived from easily available living matter (biomass) through biological processes and their production can help reduce the dependency on fossil fuels based refineries that result in the greenhouse gas emissions (Maity, 2015). Biomass is an abundant source of renewable energy that is formed by storing the sunlight energy in plants and animals. The biomass simply means organic matter which may be in the form of crops, woody plants, algae, animals or their wastes. The green plants absorb sunlight energy and stored it in chemical form. It may be mainly in the form of carbon, oxygen or hydrogen. The main components of biomass are cellulose (polymer of glucose), hemicellulose (hetero-polymer of different pentose and hexose sugars), lignin, proteins, ash, simple sugars, starches, water and other compounds. The energy acquired form the biomass is called biomass energy (Wyman et al., 2019). The plant biomass has been categorized into different groups such as first generation feedstocks and second generation feedstocks. The first generation feedstocks are mostly starch based edible material such as sugarcane, corn, soybean and rapeseed. Using edible feedstock for renewable bioenergy and biomaterial production is now commercially established techniques. The increase in biofuel production and value added products from edible feedstocks in the recent years has led to the debate on “feed versus fuel competition”, a dilemma regarding the risk of diverting farmland or crops for biofuels production and commodities to the detriment of the food supply. Biofuel production and bio-products from edible feedstocks may cause food scarcity in developing countries.

Therefore, the second generation feedstocks, mostly non-edibles and lignocellulosic in nature, are considered ideal for biorefinery concept, as it does not directly affect the food chain and supply. Non-edibles are further divided into two main categories; (i) those which grow on arable land only such as Castor and Linseed and rubber tree and their use for biorefinery may indirectly lead to food insecurity and (ii) those which can grow both on arable and non-arable land (wasteland) such as *Jatropha curcas* having no food versus fuel competition. *J. curcas* plant is considered ideal for biofuel and value valued added biomaterials production because it can grow under a wide range of environmental conditions, including saline and barren lands (Islam et al., 2018). The genus Jatropha belongs to *Joannesieae* of *Crotonoideae* in Euphorbiaceae family. It is commonly called Physic nut, Purging nut, Purge nut, black vomit nut and Jamal gota. It has round about 176 known species (Grover et al., 2019). *J. curcas* is a tropical perennial plant that can grow in low to high rainfall areas. It is a multipurpose non-edible pest and drought resistant shrub that typically reach to a height of 5 m, and have the capability to reach up to 10 m under favorable conditions (Mohammad et al., 2019). It is a fast growing plant which can grow on marginal lands with low nutrient availability. After planting, the shrub starts producing fruits from second year onward, and if properly managed, can produce 4-5 kg of seeds after the fifth year and over its life span of 40-50 years (Singh et al., 2008). *J. curcas* seed composed of 35-40% seed coat, 35-40% oil content and 55-60% seed kernel. Moreover, the seed is highly rich in crude protein content (19 to 31%), neutral detergent fibers (3.5-6.1%), lipids (43-59%) and inorganic contents (3.4-5%) (Makkar et al., 1997, Mastan et al., 2019). The unsaturated fatty acids account for more than 75% of the total fatty acids composition of *J. curcas* seed (Achten et al., 2008). The seeds of *J. curcas* plant could be exploited in sustainable way for the production of various biofuels (biodiesel and biogas) and other bio-products such as pharmaceuticals and fertilizers, as a result increasing its economic viability (Nahar and Sunny, 2014, Atabani et al., 2013). Utilization of *J. curcas* seed in biorefinery context will play an important role in economic, social and environmental sustainability development because it will produce energy carriers (biogas, biodiesel) and other value added products. The *J. curcas* seed oil can be used as an efficient source for biodiesel production and antimicrobial activities. The Jatropha pressed cake after the oil extraction has higher potential for biogas production. However, the antimicrobial phytochemicals present in pressed cake might inhibit the microbial communities in anaerobic digester. Therefore, it would be wise to extract the phytochemicals from *J. curcas* de-oiled pressed cake and afterwards, utilize it for biogas production. A number of solvents can be used to extract phytochemicals from *J. curcas* seeds such as methanolic, aqueous and n-hexane solvents. The methanolic

solvents had been reported with higher efficiency of phytochemical extraction from *J. curcas* de-oiled pressed cake compared to *n*-hexane and aqueous solvents (Haq et al., 2019). In the current study, the *J. curcas* seed has been treated in biorefinery context as given in Figure 1.1.

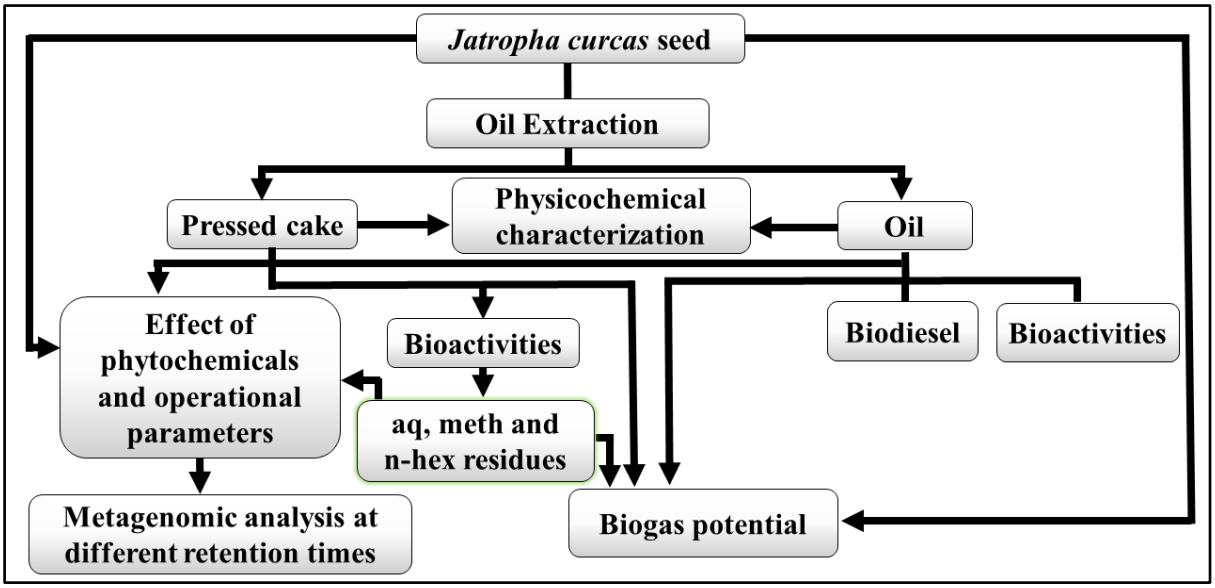

Figure 1.1. Study design for utilization of *Jatropha curcas* seeds in biorefinery context.

Moreover, the de-oiled pressed cake, wood, fruit and seed husks of *J. curcas* shrub can also be used as potential sources for biofuels and additional renewable energy products, making it a zero-waste bioenergy source. The *J. curcas* de-oiled pressed cake can also be used as biofertilizer. In addition, the anaerobic digestion of organic waste of *J. curcas* shrub was shown to produce biogas comprising mainly methane that can be used as potential renewable energy fuel (Kumar and Sharma, 2008). From biofuel aspects, many investors, policy makers and green project developer have chosen *J. curcas* as an important plant to partially solve the energy crises and mitigate global warming problems like greenhouse gas emission (Openshaw, 2000). A number of studies have been conducted on *J. curcas* plant for production of various energy carriers (Singh et al., 2008, Gunaseelan, 2009, Vyas and Singh, 2007). *J. curcas* has been highly investigated for biodiesel production using transesterification reaction. In transesterification, the triglycerides (oils) are allowed to react with methanol in the presence of catalyst (alkaline base) producing esters (alkyl esters) and glycerol as the end products. *J. curcas* can produce 1.8-2.4 ton oil and approximately 0.5-12 tons of seeds per hectare depending on the various agronomic conditions. After the oil extraction from *J. curcas* seed, the remaining de-oiled seed cake can be used as by-products in the form of biofertilizer. This de-oiled seed cake makes about 50-75% of the total weight of seed (Staubmann et al., 1997). A 1.0 kg seed cake is equivalent to 0.15 kg chemical fertilizer which contain nitrogen,

phosphorus and potassium in 40:20:10 ratios by weight (Openshaw, 2000). *J. curcas* pressed cake has 94% volatile solids and 93% total solids. The *Jatropha curcas* de-oiled cake is a potential environmental pollutant, and anaerobic digestion has been identified as a possible solution to this problem (Raheman and Mondal, 2012). While it has been documented that the biogas yield of *J. curcas* seed cake is 60% higher than that of cattle manure (Hessami et al., 1996). The de-oiled seed cake is rich in carbohydrates, proteins and fats due which it can be used as a potential source for biogas production using methanogenic consortia during anaerobic digestion (Staubmann et al., 1997, Singh et al., 2008). But anaerobic digestion of the de-oiled pressed cake has two main limitations, low C/N ratio and presence of phytochemicals that leads to reactor instability and inefficient biogas production. Monodigestion of *J. curcas* seed cake still results in a low biogas yield, because of the relatively low carbon to nitrogen (C/N) ratio of about 9:1 (Raheman and Mondal, 2012). This suggests that codigestion with other substrates having a high C/N ratio (e.g., 20:1 to 30:1 for cow dung) (Hessami et al., 1996) is needed. A possible co-digestion substrate is fruit waste, such as mango peels, with a reported C/N ratio of 45.4 (Suryawanshi et al., 2013). *J. curcas* de-oiled seed cake in codigestion with pig manure produced 0.446 m^3 biogas per kg of dried mass with 70% methane content using pig manure as inoculum (Staubmann et al., 1997). Similarly, the mechanically and solvent extracted de-oiled seed cake produced 0.6 m^3 and 0.5 m^3 biogas/kg dried mass, respectively (Radhakrishna, 2007).

J. curcas seeds are also composed of toxic compounds, including trypsin inhibitor, saponin, lectin, phytates and phorbol esters (Martinez-Herrera et al., 2006). It has extensively been reported for its various bioactivities including antioxidant, cytotoxic, anti-inflammatory, antitumor, antimicrobial and antiviral activities (Abdelgadir and Van Staden, 2013, Saetae and Suntornsuk, 2010, Oskoueian et al., 2011a, Oskoueian et al., 2011b). Traditionally, *J. curcas* plant has been used for the treatment various ailments such as rheumatoid arthritis, jaundice, goiter, fever, mouth and skin infections (Aiyelaagbe et al., 2007, Bhaskarwar et al., 2008). Moreover, its seed oil, stem bark and roots, leaves have also been reported for their antibacterial and antifungal activities (Nwosu and Okafor, 1995, Saetae and Suntornsuk, 2010, Igbinosa et al., 2009).

The process of anaerobic digestion is carried out by the complex interactions of microbial communities in which organic feedstocks are converted into CH_4 and CO_2 through intermediates. The polymeric compounds such as carbohydrates, proteins and lipids are converted into CH_4 and CO_2 through various important steps including hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fitamo et al., 2017, Xu et al., 2018a). Mostly,

the hydrolysis and methanogenesis have been reported as the rate limiting steps. For easily degradable substrates the methanogenesis is the rate limiting step. If the substrate is complex and not easily degradable, in that case the hydrolysis is the rate limiting step (Schnurer and Jarvis, 2010). The process efficiency and stability is totally dependent on the interactions and syntrophic activities of the diverse and highly abundant microbial communities during anaerobic digestion (Li et al., 2009, Werner et al., 2011). There are a number of operational and environmental parameters which affect the efficiency and stability of reactor during anaerobic digestion. These operational parameters are highly correlated to microbial community composition in anaerobic digester. For example organic loading rate (OLR), hydraulic retention time (HRT), pH, temperature and pretreatment have tremendous effect on the microbial communities during anaerobic digestion (Xu et al., 2018b). Culture dependent techniques have been extensively used for the identification of specific microbial communities involved in anaerobic digestion process. However, these techniques are mostly unable to explore majority of microbial communities and time consuming (Su et al., 2012, Amann et al., 1995). The culture independent techniques have resulted into enormous exploration of microbial communities during anaerobic digestion. These techniques have broadened the horizon on the dynamics of various metabolically important microbial communities during anaerobic digestion, ultimately leading to reactor stability and efficiency (Amann et al., 1995, Werner et al., 2011, Sundberg et al., 2013, Xu et al., 2018b). The culture-independent techniques have explored new dimensions in microbial diversity and familiarized us with many previously uncharacterized dominant microorganisms in anaerobic digestion (Werner et al., 2011, Sundberg et al., 2013).

The most important step in understanding the microbial communities function and configuration is the taxonomic and phylogenetic classification of DNA sequences. Few decades ago, a revolutionary increase was observed in the studies regarding molecular analysis using small subunit of 16S rRNA. The PCR and high throughput sequencing further strengthened this area of research among the scientific community (Campanaro et al., 2018). The traditional Sanger sequencing is highly cumbersome, needing pure microbial culture for 16S rRNA sequencing. Conversely, the advanced next generation sequencing (NGS) platform made it feasible to sequence directly the environmental samples. It is relatively cost effective with high throughput. The high throughput sequencing technologies such as Illumina and pyrosequencing platforms for sequencing 16S rRNA gene amplicons have increased scope of studying microbial consortia in anaerobic digesters (Sundberg et al., 2013, Ziganshin et al., 2013). The high throughput sequencing technologies have resulted in exploring previously

unrecognized level of microbial diversity (Nelson et al., 2011, Sundberg et al., 2013). The microbial diversity by high throughput technologies leads to explaining how a process can respond to changes in anaerobic digestion. An advantage of increased in-depth community profiling is that it helps in studying the unique microbial communities with low abundance and their potential role in sustaining process stability and efficiency in anaerobic reactor. Pyrosequencing (454 Roche platform) has been extensively used for metagenomic analysis of microbial diversity and their function during anaerobic digestion analysis. However, with the passage of time the impact of this technology gradually decreased due to homopolymer errors that led to over estimation of rare microbial communities in a process (Carvalhais et al., 2012, Bokulich et al., 2013). Pyrosequencing has the capability to generate 250-500 bp short reads of 16S rRNA gene (Morozova and Marra, 2008) and its taxonomic alignment mostly limited to genus level. On the other hand, Illumina MiSeq platform is going to cover the market due to its relatively lower cost, higher throughput and higher efficiency in terms of time. It can generate 200-300 bp paired end reads with ten times higher abundance compared to pyrosequencing (Caporaso et al., 2012).

Renewable energy sector has gradually developed especially in developed countries. The developing countries have also a higher potential for renewable energy production especially from biomass. South-east Asia including; Pakistan has an abundant resources of biomass. Although, Pakistan is an agricultural country and about 60% of its population is dependent on the agriculture sector. Around 62% of the rural areas people use biomass as primary source of household cooking and heating in conventional ways (Naqvi et al., 2018). Pakistan has around 80 million acre barren land in marginal and coastal areas of Sindh and Balochistan that could be used for cultivation of *J. curcas*. It will help in capture of atmospheric CO_2 resulting in greenhouse gas mitigation. So utilizing these areas for cultivation of *J. curcas* plants will increase its economic viability and also play a role in developing the socioeconomic level of the local population in terms of employment and investment. Pakistan has settled its target of 10% blends of biodiesel with petro-diesel fuels till 2025 and it is estimated that the energy demands of Pakistan will increase three times till 2050 (Ahmad et al., 2015). Moreover, around 5380 small scale biogas reactors has been installed in rural areas of Pakistan that producing 12-16 million cubic meter biogas per day that can easily support the local household's requirements. Recently, the government has invested 356 million to import further 1400 biogas plants for rural areas (Naqvi et al., 2018). This study will help in increasing the sustainability of biofuel sector in Pakistan and the production of biofuels along various

bioactive products from *J. curcas* could be exploited to minimize the cost of biofuel technology and increase its economic value.

1.1. Research Hypothesis

Renewable bioenergy resources are considered the potential options to decrease dependency on the fossil fuel reserves. Among the renewables, biomass is an abundant source for biofuel production (Tun et al., 2019). But the biofuels are controversial due to large land required for production, food versus fuel competition and marginal economic viability in the lack of subsides (Gressel, 2008, Mulpuri et al., 2019). Most of the energy feedstocks like *J. curcas* have the potential to be utilized for multiple purposes including pharmaceutical, agricultural and energy production. But mostly it is used for the production of one kind of product either biofuel (gaseous or liquid) or other commodities that is considered non-sustainable. Based on our research hypothesis, the production of biofuels and commodities from *J. curcas* seed in biorefinery context is more sustainable than the production of a single product and will enhance the economic viability of biofuel sector. Moreover, the extraction of antimicrobial phytochemicals from *J. curcas* seed will enhance the stability and efficiency of anaerobic digestion process in terms of microbial communities and biogas yield. Therefore, *J. curcas* seed was utilized for the production of multiple energy carriers (biogas and biodiesel) along with other value added compounds in biorefinery context.

1.2. Research Questions

1. Does *J. curcas* seed has a higher potential for biofuel production?
2. How *Jatropha curcas* seed could be efficiently used for the production of multiple products in biorefinery context?
3. Does *Jatropha curcas* seed's toxicity affect the microbial communities in the reactor during anaerobic digestion process and what could be potential application of the substances that could be inhibitory for anaerobic digestion process?

1.3. Aim and objectives of the study

Aim

The aim of the study is the production of energy carriers (biogas, biodiesel), extraction of bioactive compounds from *J. curcas* seed and evaluation of the effects of extracts on microbial communities during its anaerobic digestion.

Objectives

The objectives of the current study are

- To study the antimicrobial, antioxidant and cytotoxic activities of seed oil, aqueous, n-hexane, and methanolic extracts of *J. curcas* de-oiled seed cake
- To evaluate biogas potential of *J. curcas* seed and the effects of methanolic extracts of de-oiled seed cake on different stages of anaerobic digestion
- To determine the effect of dilute phosphoric acid pretreatment on biogas yield of different parts of *J. curcas* fruit
- To determine the impact of manipulating the C/N ratio on biogas yield by codigestion of *Jatropha curcas* seed kernel and mango peels.
- To assess the effect of operational parameters (organic loading rate and hydraulic retention time) on anaerobic digestion of *J. curcas* seed
- To study microbial diversity in anaerobic digestion of *J. curcas* seed at different retention times in continuous reactor
- To optimize biodiesel production from *J. curcas* seed oil

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CHAPTER 2

2. Literature Review

Fossil fuels based refineries play a hallmark role in the production of energy and many other industrial products and they play a vital role in economic development and are considered important factors of human life (Keyuraphan et al., 2012, Saini et al., 2019). However, fossil fuels resources are depleting at a higher rate and their consumption is majorly contributing to global warming. The emission of harmful gases as a result of fossil fuel consumption indirectly leads to the melting of glaciers, rise in sea level, climate change and reduction of biodiversity. Increase in the demand of the fossil fuels for daily consumption in industries and transportation is the major factor in the hike of crude oil prices, ultimately affecting the global economy (Agarwal, 2007, Amoah et al., 2019). In last few decades, a number of alternatives have been introduced, where the production of biofuel and other value added compounds using biomass in biorefinery context is considered as an ideal and efficient source from the socio-economic and environmental perspectives. The utilization of biomass for biofuels and value added compounds can significantly shrink the reliance on fossil fuels based refineries (Weldemichael and Assefa, 2016, Pessoa et al., 2019). Biomass have fewer advantages over fossil fuels in biorefinery context. Biofuels being biodegradable in nature and other associated products are easily acquirable from biomass and they are eco-socio and environmental friendly due to which they are expected to cover the global fuel market in near future for biorefinery development (Demirbas, 2008, Kim and Dale, 2005).

2.1. First and second generation feedstocks

Biomass mainly categorized as (i) first generation feedstocks and (ii) second generation feedstocks. The former are edible feedstocks while later are non-edible feedstocks. The well-known first generation feedstocks which can be used for biofuels production and commodities are edible food crops such as maize, wheat, soybean, sunflower, corn and rapeseed comprising of starch, proteins and oils. The most common first generation biofuels are bioethanol, biodiesel and starch based biogas. Bioethanol is produced from fermentation of different feedstocks containing fermentable sugars and carbohydrates. The feedstocks used for bioethanol are sugarcane, sugar beet and starch crops including wheat and maize. Worldwide bioethanol production was around 45-50 Millions of tonnes of oil equivalent (Mtoe) (de Souza Abud and de Farias Silva, 2019). United States of America (USA) is the leading country in bioethanol production (69.32 billion litres per year) producing mainly (about 95%) from maize and only 3% from other cereals including sorghum, barley or wheat starch. Brazil is the second

largest producer of bioethanol with 27.25 billion litres per year, using the sugarcane. European Union (EU) is the third largest bioethanol producing country based on sugar beet (Ballesteros and Manzanares, 2019). Biodiesel is one of the most commonly available biofuel which is produced from edible fats and oil based crops including soybean, sunflower, rapeseed and palm. Worldwide biodiesel production has reached to around 31.6 billion litres in 2015. The largest biodiesel producing economies are EU, USA and Brazil that are producing 13.5, 4.8 and 4 billion litres biodiesel per year, respectively (Naylor and Higgins, 2017). The recent worldwide estimate of biogas production is unknown. However, the worldwide biogas production in 2014 was around 1.28 EJ representing around 59 billion m^3 biogas (35 m^3 biomethane) production. USA and EU are at the top in biogas production and the other countries also rapidly increasing the production of biogas. In the EU primary energy production from biogas was around 654 PJ in 2015 with biomethane volume of 18 million m^3 equivalent (Association, 2015, Eurostat, 2017). The most important advantages of edible feedstocks for biorefinery purpose are higher sugar and fats contents, low recalcitrance and easy conversion into biofuels and associated products. However, edible feedstocks have gained attention worldwide but the feasibility of these feedstocks have certain challenges such as competition for food supply and land acquisition for biofuels and commodities, which ultimately increased the cost of food. This competition creates ethical, political and environmental concerns regarding their use.

An alternative strategy has been made to produce biofuels and associated products from the second generation non-edible feedstocks which are considered ideal options for biorefinery development. Non-edible feedstock includes various lignocellulosic energy crops, herbaceous biomass woody biomass, forest residues, and agricultural waste residues, municipal solid waste and animal fats. Second generation feedstocks are diverse and abundant in nature and various technologies can be used to process them for biofuel production. Different kinds of technologies including thermochemical, flash pyrolysis, enzymatic are used to produce syngas or other gaseous as well as liquid biofuels. The biofuels and value added compounds acquired from second generation feedstocks have certain advantages over the first generation in terms of land use, efficiency and environmental performance (Searcy and Flynn, 2008, Fleming et al., 2006). Although, 2^{nd} generation feedstocks required for the production of biofuels and value added compounds are relatively cheaper, widespread and easily available, but still the technologies using them as feedstock are in unestablished or in pre commercialization phase. These technologies, however, can be grown rapidly and soon will cover the global market

(Kamm et al., 2005). There are various non-edible crops that are used as 2nd generation feedstocks for biofuel production and commodities including Karanja (*Pongamia pinnata*), Mahua (*Madhuca longifolia*), Neem (*Azadirachta indica*), Moringa (*Moringa oleifera*), Polanga (*Calophyllum inophyllum*) and Jatropha (*Jatropha curcas*). These feedstocks have been used for biodiesel and biogas production in various studies worldwide (Islam et al., 2018, Chinnici et al., 2018). However, most of these non-edible feedstocks are cultivated on arable lands that indirectly lead to the feed vs fuel competition to some extent. Conversely, *J. curcas* plant has certain advantages over these other non-edible feedstocks in biorefinery context as it can grow in drought, hot, arid, semi-arid, sandy, saline and slightly acidic conditions.

2.2. *Jatropha curcas* a non-edible second generation feedstock

There are about 170 species of the genus *Jatropha*. Among them, *Jatropha curcas* is a multipurpose oil rich plant belonging to *Euphorbiaceae* family and can grow in marginal and wastelands on the coastal sides. However, it cannot grow in loamy soils and is not suitable for flood susceptible areas. Therefore, in order to determine the criteria for land acquisition for *J. curcas* cultivation, agriculturally and commercially important lands should be avoided for their cultivation. *J. curcas* is intolerant to waterlogged areas (Negussie et al., 2013). It can reach a height of 5 m and afterwards yields 7.5-12 tons of seeds per hectare per year (Ramesh et al., 2006). *J. curcas* is pest resistant and has higher potential for biofuel production due to its ecological and environmental benefits. The utilization of *J. curcas* seed for biofuel production will help in mitigation of greenhouse gas emissions and will partially replace the petro-diesel. Its fruits are 2.5 cm long ovoid, black in colour and have two to three halves. The fruit comprised of 35-40% shell and 60-65% seeds (Singh et al., 2008). There are about 400-425 fruits per kg corresponding to 1580-1600 seeds per kg weight. The fruits are further de-hulled to expose the shell, and the seed husks are usually de-corticoid for oil extraction (Singh et al., 2008, Achten et al., 2008). There are about 80% unsaturated fatty acids and 20% saturated in *J. curcas* seeds. The seed contain about 25-40% oils based on total weight of seeds. The seed oils can be extracted using different methods including; solvents or by mechanical expeller. It has been observed that the oil recovery using solvents is higher compared to the mechanical extraction. The solvent extracted oil yield of *J. curcas* seed is around 95%, while the mechanical expeller gave 85% oil yield (Singh et al., 2008). The previous literature had reported that the *J. curcas* seed oil mainly comprised of oleic and linoleic acids, linolenic acids, stearic acid and palmitic acid (Moser, 2009). The seed oil could be used as potential source for

biodiesel production. To sum up *J. curcas* seed can be used an ideal feedstock for bioenergy production other value added compounds in biorefinery context as shown in Figure 2.1.

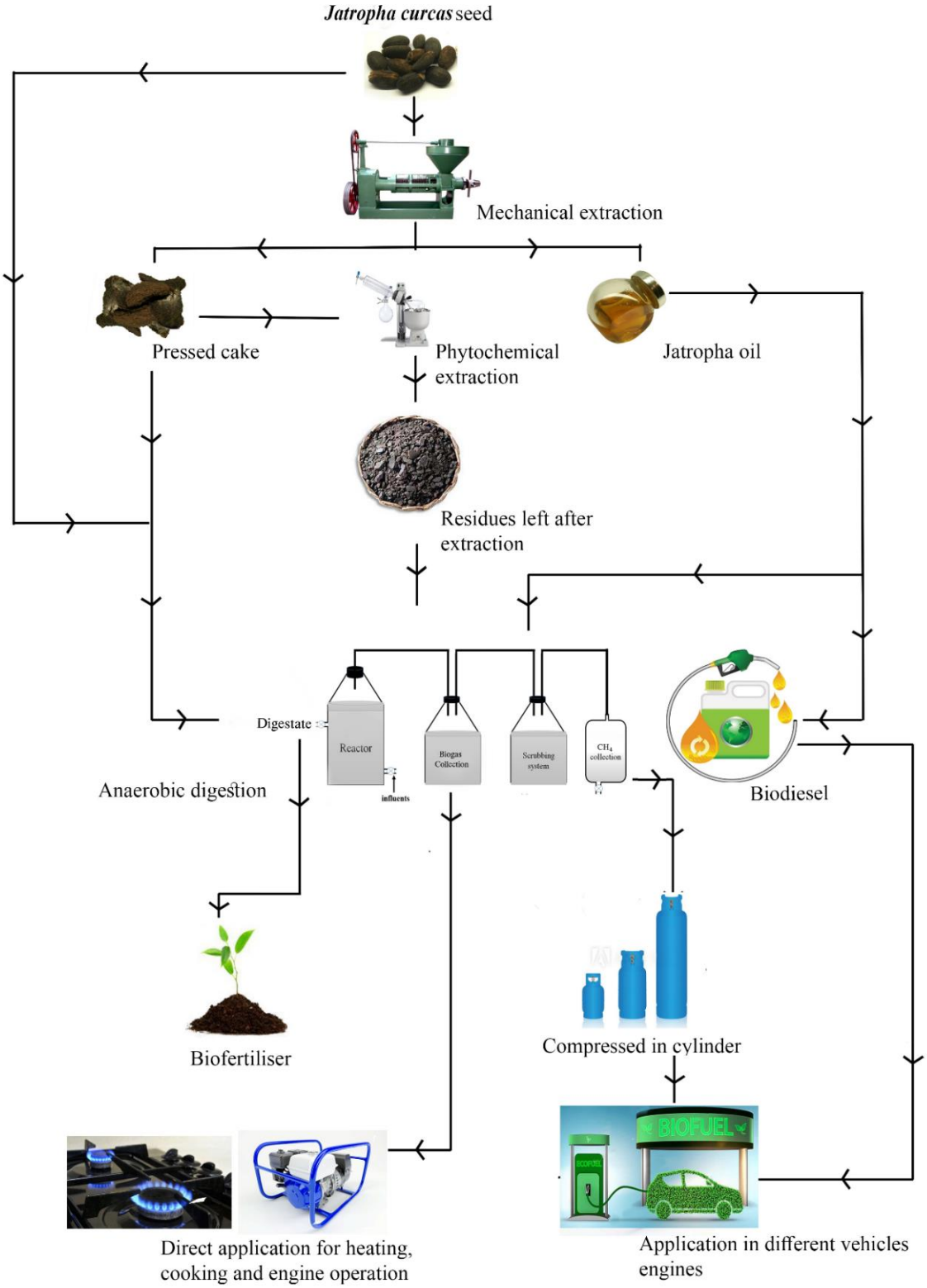

Figure 2.1. Sustainable utilization of *Jatropha curcas* seeds for bioenergy and value added compounds in biorefinery context

2.3. *Jatropha curcas* in biorefinery context

J. curcas is a dynamic plant and has the potential for production of a number of products such as energy carriers (biogas, biodiesel, bioethanol and biohydrogen), biomaterials, fertilizers and protein diet for animals. *J. curcas* seed is highly rich in oil comprising 25-40% oil that could be extracted by a number of techniques, the most commonly used are solvent and mechanical extraction methods (Nahar and Ozores-Hampton, 2011). A 100 kg seed results in 28 to 30 kg oil using mechanical expeller unit. The mechanical extraction of seed results in pressed cake and oil. The resulting oil is utilized for biodiesel production producing glycerol as byproduct. This glycerol is non-toxic, biodegradable and can be used in a number of processes. The seed oil can also be used as fuel directly but its higher viscosity is the major barrier in their use as fuels in ignition engine. Therefore, conversion of *J. curcas* oil in biodiesel is necessary in order to reduce oil viscosity (Singh and Singh, 2010). The biodiesel can be produced via acidic, alkaline or lipase mediated transesterification and this biodiesel can be used as blend in combination with petro diesel (Singh et al., 2008). Pakistan has settled its target of 10% biodiesel blends with petro diesel until 2025 (Chakrabarti et al., 2012). The seed also contain many other products such as glucose, fructose, galactose, saccharous, raffinose, stachylose, and proteins. The *J. curcas* seed contain curcin, phytic acids, curcasin and many other long chain fatty acids including; arachidic, myristic, oleic, palmitic, stearic and linoleic acids (Nahar and Ozores-Hampton, 2011). The pressed cake can be used as an animal feed but the removal of toxic compounds is needed prior to its use as animal feed. The phorbol esters are mostly considered the most toxic compounds in the pressed cake and if they are not removed the animals might be harmed by consuming this feed. Moreover, a number of phytochemicals are present in pressed cake. They can be used as medicinal products to treat various diseases after the extraction and purification using sophisticated techniques (Islam et al., 2011). The pressed cake can be used for biogas production during anaerobic digestion and the resulting slurry from biogas reactor can be used as potential fertilizer in fields (Staubmann et al., 1997). The biogas can further be purified into biomethane that can be used for electricity generation and as fuel in vehicle's engines for transport. This approach may increase its potential viability for biorefinery purpose. The utilization of *J. curcas* in biorefinery context will increase the sustainability of biofuel sector and will boost the country's economy (Giwa et al., 2018). All of the bioenergy carriers and value added products which can be obtained from *J. curcas* seed are discussed in detail as follows.

2.4. Biodiesel Production

Biodiesel are the long chain alkyl fatty acid esters that are produced by the reaction of triglycerides with alcohol (methanol or ethanol) in the presence of a catalyst (alkaline, acidic or enzymatic). Biodiesels are non-toxic, biodegradable, almost sulfurless, non-aromatic and environmental friendly fuels. The transesterification reaction basically occurs in following three reversible steps, (i) triglycerides are converted into diglycerides; (ii) the diglycerides are converted into monoglycerides and (iii) glycerine is produced.

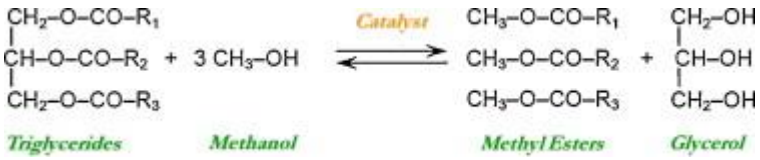

Figure 2.2. Transesterification reaction for biodiesel production (Borges and Díaz, 2012)

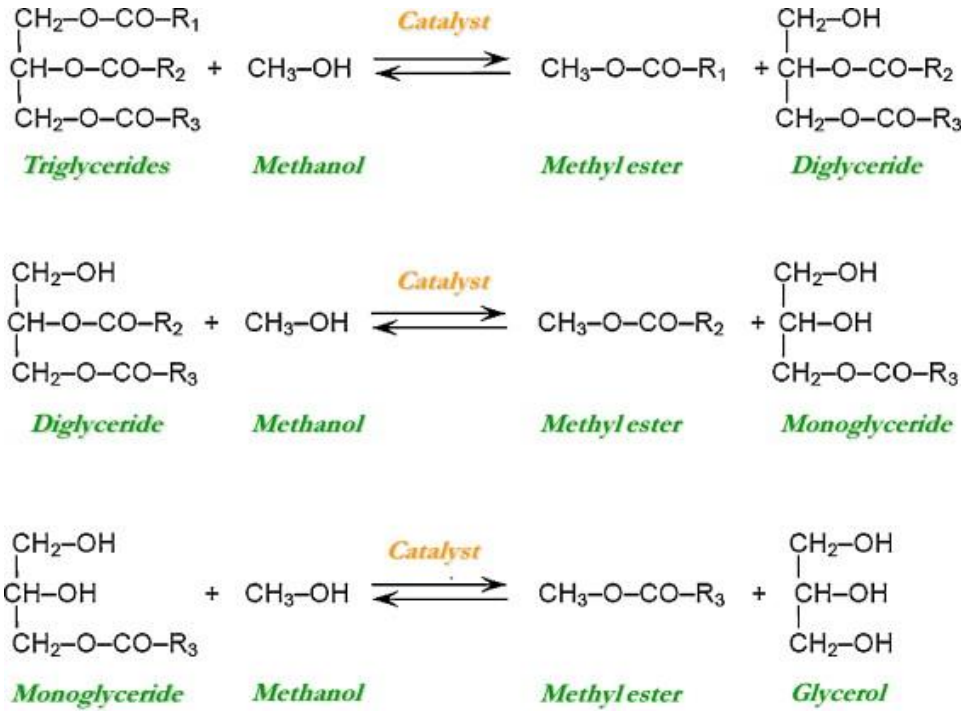

Figure 2.3. Three steps of transesterification reaction for biodiesel production (Borges and Díaz, 2012)

During all these steps, biodiesel esters (methyl esters) are produced. The stoichiometric ratio between triglycerides and alcohols are 1:3. However, it is preferable to use higher alcohol to oil ratio in order to continue reaction in forward direction and provide sufficient alcohols for reaction completion due to its highly volatile nature. There are many alcohols reported for biodiesel production but on commercial scale, only methanol and ethanol have gained more

attention due to their potential characteristics (cost-effective, easily soluble and rapid reaction with triglycerides and NaOH during transesterification). Catalyst is used to make the reaction rate faster and increase the biodiesel yield. An alkaline transesterification process is mostly favored for biodiesel production as the alkaline catalysts are cost effective, comparatively more efficient, require lower reaction temperature and pressure than acid catalysts. However, base alkaline catalyst may react with free fatty acids (FFA) to form soap which may consume the catalyst leading to reduced reaction efficiency (Yaakob et al., 2013). Sodium and potassium hydroxides have been extensively reported as alkaline catalysts due to their higher efficiencies. Sodium hydroxide as less expensive than potassium hydroxide. However, potassium hydroxide is highly efficient and require relatively less time for reaction completion during biodiesel production process (Yusuf et al., 2011). The alkaline transesterification has been extensively reported for biodiesel production from non-edible feedstocks. Ideally, alkaline transesterification process is carried out smoothly and without any hindrance when the FFA are lower than 1 or 2%. However, *J. curcas* seed oil are rich in free fatty acids (FFA) accounting for about 14% of the total oil (Tiwari et al., 2007). There are a number of pretreatments to reduce FFA and enhance biodiesel production. Especially, acid pretreatment most commonly carried out by using H_2SO_4 as catalyst which not only reduces the free fatty acids contents but also convert the triglycerides into biodiesel. But the problem with acidic transesterification is the use of higher molar ratio of alcohol to oil in reaction/process which ultimately increase the cost of the process. In order to reduce the cost of the process and FFA contents of *J. curcas* seed oil, two step transesterification process is used in which the acid (H_2SO_4) is used in initial phase to reduce the FFA and esterify the oil. The resultant product is purified and proceeded for alkaline transesterification process in which the pretreated product is converted into fatty acids alkyl esters in the presence of an alkali catalyst (KOH or NaOH). In two step transesterification process more than 95% biodiesel yield is obtained (Patil and Deng, 2009). The transesterification process is also carried out using microbial cells or their enzymes. Especially, the lipase producing microorganisms are involved in transesterification process. The lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are used to convert oil or fats into biodiesel using transesterification or esterification reactions. Lipases belong to alpha beta hydrolase fold super-family. They have a network of hydrogen bonding in active site comprising of serine, aspartic acid and histidine amino acids (Laachari et al., 2015, Farrokh et al., 2014, Thakur et al., 2014). Lipases are highly abundant on industrial scale and have been categorized at third position in biotechnological applications after amylases and proteases.

Lipases are involved in hydrolysis of triglycerides into fatty acids and glycerol at the interface of oil and water. This reaction is reversible in aqueous and non-aqueous media (Hama et al., 2018). Lipases also exhibit enantioselective features including; esterification, transesterification, interesterification, acidolysis and aminolysis (Hasan et al., 2009). The substrates used for lipases are usually water insoluble and are, therefore, first dissolved in organic solvent followed by buffer to increase its solubility. The lipases have an advantage of being soluble in aqueous phase and are able to catalyze the reaction both in aqueous and organic phases. However, organic solvents mostly influence the structural conformation of lipases and thus affecting their catalytic performance (Guo et al., 2015). Secondly, they require relatively higher time in process completion and are expensive compared to the alkaline and acidic catalysts (Kapoor and Gupta, 2012). Lipase based biodiesel production is efficient, energy saving and environmental friendly alternative option to that of the conventional chemical based transesterification processes (Fjerbaek et al., 2009). All organisms produced lipases but the microbial lipases including fungal and bacterial have been extensively used for biodiesel production. *Candida* are well-known lipase producing yeasts which had been reported for biodiesel production (Sankaran et al., 2016). Similarly, the bacterial strains *Streptomyces, Burkholderia, Pseudomonas*, *Brevibacterium*, *Acinetobacter* and *Bacillus* are the well-known bacterial species utilized for biodiesel production (Hama et al., 2018). They have tremendous potential of biodiesel production. Lipases have certain advantages over the conventional chemical based biodiesel production processes such as easy recovery of biodiesel and glycerol. In addition, they have broader range of reactivity. i.e. esterification and transesterification simultaneously, conversion of FFA and are resistant to lower pH. They also high production in non-aqueous media, require low temperature and pressure that ultimately reduce the energy consumption to complete the process. Therefore, lipases are considered more economical compared to conventional chemical processes for biodiesel production (Mittelbach, 2015, Hama and Kondo, 2013, Sankaran et al., 2016, Ashfaq, 2014). For evaluation of biodiesel quality certain standard rules have been adopted and are named as ASTM D 6751 (in USA) and EN 14214 (in Europe). All biodiesel fuels must comply with these standards before their commercialization. Biodiesel can be used in engine with little or no modifications. Viscosity is one of the most important feature which greatly influenced the performance of an engine. The vegetable oils are usually converted into esters to reduce its viscosity. The viscosity increases as the chain length and number of double bonds increases in any hydrocarbon. It play a critical role in engine combustion. The fuels with lower viscosities get easily sprayed

by engine injector, separate into mixtures and get combusted quickly due to small diameter of droplet and maximum air contact (Canakci and Sanli, 2008). The density is also playing the same role as the viscosity. Generally, fuels with lower densities and viscosities are easily sprayed, separated into mixtures and get combusted in combustion chambers due to small diameter of droplets. The density of a fuel has direct relation with droplet size. So generally biodiesel produced from vegetable oils, or any kind of fats have a little bit higher density than the conventional diesel. However, these densities are acceptable by the international standards (Canakci and Sanli, 2008). Cetane number is the opposite of octane number. Cetane number is basically used to show the ignition delay time which is the time taken by a fuel from injector to combustion chamber during engine operation. The fuels with higher cetane number have lower ignition delay resulting in higher heating of engine injector, ultimately resulting in the plugging of injector nozzle. Biodiesel have higher cetane number than conventional diesel fuels but they are within the acceptable range of international standards specified by ASTM D 6751 and EN 14214. Cetane number increases with increasing chain length, decreased branching and double bonds (unsaturation) of esters. Flash point can be defined as the temperature at which fuel get combusted when it comes in contact with fire. It is very important from safety point of view and directly correlated with volatility of fuel. Generally, fuels with lower volatility and higher viscosities are considered bad for engine performance due to misfire, ignition delay and bad start up. Biodiesel feedstocks have higher flash point and are normally reduced through transesterification reaction but still they are higher compared the conventional diesel fuels (Szybist et al., 2007). Cold flow properties are also very important in selection of fuels. Cold flow properties are measured by finding the cloud point and pour point of a fuel. Cloud point is the temperature at which crystals and wax formation occurs and pour point is the lowest possible temperature at which a liquid can flow and pumped (Lee et al., 1995). Usually fuels have higher cloud points than pour point. Biodiesels have higher cloud and pour points than conventional diesel fuels. It is the most critical obstacle in widespread usage of biodiesel as fuels. The cloud and pour point increase with increasing chain length and decreased with increasing unsaturation (Leung and Guo, 2006). Oxidation stability is another important feature of fuels which determine its ability to resist the oxidation. The oxidation usually creates peroxides which are subsequently converted into aldehydes, ketones and short chain acids producing unpleasant odor. The oxidation decreases the fuel properties due to gum formation which does not ignite during the engine operation and start deposited in engine combustion chambers. On oxidation the

biodiesel becomes more viscous and its cetane number increases. Therefore oxidized biodiesel burns earlier than the non-oxidized one and result in higher nitrous oxide emission (Ma and Hanna, 1999, Monyem et al., 2001).

2.5. Factors affecting biodiesel production

The efficiency of biodiesel process depends upon certain variables such as molar ratio of alcohol to oil, reaction time, catalyst concentration and agitation.

2.5.1. Molar ratio

Alcohol to triglycerides molar ratio play an important role in biodiesel production. The stoichiometric ratio of alcohol to triglycerides is 3:1 for biodiesel production, but practically an excess of alcohol is needed to carry out the reaction in forward direction and reduce the chances of evaporation of alcohols. In most of the previous reports, a molar ratio of alcohol to oil (6:1) has been determined to be the optimum ratio for biodiesel production. If the molar ratio alcohol to oil is increased than the optimum ratio (6:1), it does not increase the yield, rather cost of the process is increased. However, higher molar ratios of alcohol to oil up to 15:1 are required for the feedstocks having higher concentration of free fatty acids, especially in case of acid catalysts (Yaakob et al., 2013).

2.5.2. Reaction time

The conversion of triglycerides into esters also depends upon the reaction time. Initially, the reaction proceeds slowly due to the mixing and dispersion of alcohols in fats/oils. Later on, the reaction rate is increased reaching maximum at optimum reaction time. It has been reported that the biodiesel yield reaches maximum at reaction time $\leq$ 90 min and increasing time beyond this limit did not improve the esters yield (Alamu et al., 2007). Moreover, the increase in reaction time beyond the optimum range reduce the product yield, ultimately leading to esters loss and formation of FFA to form more soap (Yaakob et al., 2013, Eevera et al., 2009).

2.5.3. Reaction temperature

The transesterification reaction temperature also influence the biodiesel yield. The increase in temperature reduces the viscosity of oil and also the reaction time. However, if the reaction temperature is increased beyond the optimum range, it will lead to the formation of soap. The temperature must not be increased than the boiling point of alcohol because it will

lead to rapid alcohol evaporation. Normally, the optimum temperature for biodiesel production ranges from 50 to 60 °C depending upon the type of oil (Leung and Guo, 2006, Ma and Hanna, 1999).

2.5.4. Catalyst concentration

The catalyst concentration plays an important role in increasing the esters yield. An increased amount of catalyst is required for an efficient biodiesel production process. However, if the catalyst is increased beyond its optimum limit, further addition will lead to the formation of soap resulting in esters loss and higher cost of the process. A number of studies had reported 1.5% as an optimum catalyst concentration for an increased biodiesel yield (Eevera et al., 2009, Leung and Guo, 2006).

As the *J. curcas* seed oil has higher potential for biodiesel production that has been discussed earlier. The *J. curcas* seed also has higher potential for biogas production which is discussed below.

2.6. Biogas production

Biogas is a mixture of different gases produced by anaerobic and facultative anaerobic bacteria in the absence of oxygen. Crude biogas is composed of different gases such as CH_4 (40-75%), CO_2 (15-60%), H_2S (0.005-2%) and other trace gases including N_2 (0-2%), water vapors (5-10%), CO (< 0.6%), etc. (Ryckebosch et al., 2011). *J. curcas* seed cake has been used in a number of studies for biogas production. Jatropha seed were reported to yield about 60% more biogas than cow dung. The previous literature showed that *J. curcas* produced 355 L biogas per kg of seed cake with a 70% methane content (Raheman and Mondal, 2012). Similarly, the biogas produced by *J. curcas* seed cake codigested with paddy straw corresponding to C/N ratio (27:1) was 10.5% higher than monodigestion of seed cake (Raheman and Mondal, 2012). In another study, it was reported that the biogas yield of *J. curcas* seed cake was 30.7% higher at 10% TS than 15% TS (Singh et al., 2008). A number of technologies are used for biogas production from agricultural residues and waste materials. However, anaerobic digestion is one of the most commonly used process for biogas production.

2.7. Anaerobic digestion

Anaerobic digestion is a highly complexed process carried out by facultative and obligate anaerobic consortia. These microbial communities are synergistically dependent on one another to maintain the process stability for efficient biogas production. Basically, biogas

or biomethane formation occurs through a number of steps including hydrolysis, acidogenesis, acetogenesis and methanogenesis as shown in Figure 2.4. A number of studies have reported hydrolysis as the rate limiting phase as various complex intermediate compounds and volatile fatty acids are produced during this phase that are inhibitory to microbial communities at the concentration higher than their optimum range. On the other hand, some studies also suggesting methanogenesis as the rate limiting phase, especially when the feedstocks are easily degradable during anaerobic digestion (Lu et al., 2008). Broadly, the anaerobic digestion process is divided into two distinct phases including; the fermenting phase and methanogenic phase. The microbial communities involved in these two phases are distinct from each other in terms of physiology, growth kinetics, nutritional requirements and sensitivity to environment. In most of the cases, it remains highly critical to keep balance between the microbial communities of these two phases because any perturbation in their behavior or performance may lead to reactor instability and lower biogas yield. These two groups might be separated physically using membranes, pH or kinetic controls (Adekunle and Okolie, 2015).

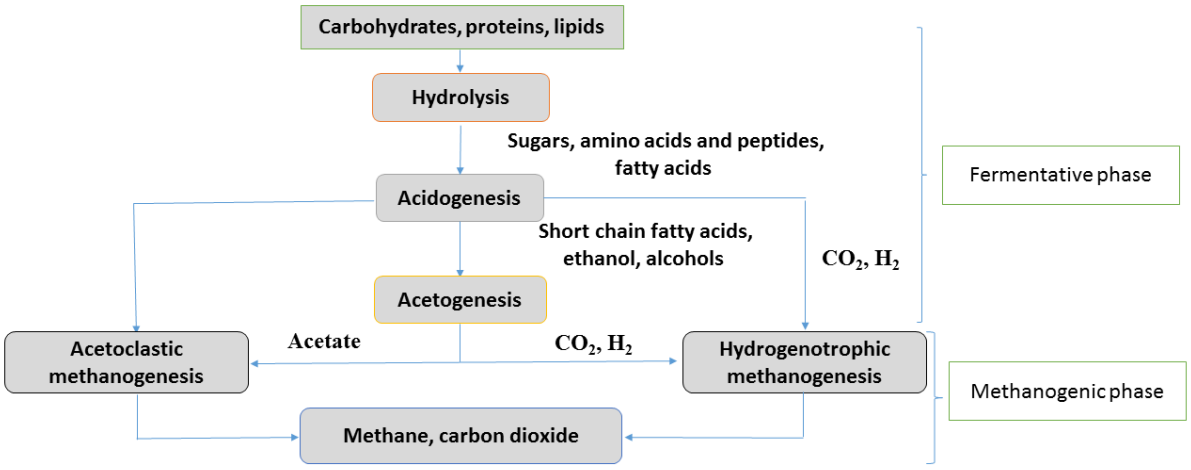

Figure 2.4. Phases of anaerobic digestion process

2.7.1. Hydrolysis

Hydrolysis is the first phase in anaerobic digestion process in which highly complexed suspended/insoluble polymeric organic compounds are converted into simpler soluble forms through different enzymes. The complexed polymeric compounds are cellulose, hemicellulose, proteins and fats and the soluble compounds are mostly monosaccharides, amino acids and fatty acids. These soluble compounds are further utilized by microorganisms as source of energy or cell carbon. This step is carried out by facultative and strict anaerobic bacteria

including *Bacteroides*, *Clostridia*, *Streptococci*, *Micrococci*, *Streptococci*, *Butyrivibrio* and *Fusobacteria* and some others, that secrete different enzymes including cellulases, amylases, lipases, proteases and xylanases (Schnurer and Jarvis, 2010, Cirne et al., 2007a). The hydrolysis phase is very important because the larger polymeric compounds are converted into simpler forms to increase their bioavailability to microbial communities during anaerobic digestion process. The biodegradation of all these polymeric compounds occurs through different enzymes which create cuts in these complexed structure and convert them into intermediate or simpler forms. Some of the microbial communities produced a single specialized type of enzymes which degrade only specific substrates such as sugars or proteins. While other microorganisms secrete different enzymes that could convert a number of compounds into simpler soluble forms to be used as source of energy or nutrition. The sugars degrading microorganisms are called saccharolytic and the proteins degraders are called proteolytic bacteria. There are a number of enzymes that are secreted by hydrolytic bacterial communities such as lipases, amylases, proteases, pectinases, cellulases and hemicellulases. All of these enzymes are involved in different reactions catalyzing lipids, carbohydrates, sugars, proteins, cellulose and hemicellulose. The hydrolysis rate is highly dependent on the nature of substrate. Generally, the biodegradation of cellulose and hemicellulose take longer time compared to proteins (Schnurer and Jarvis, 2010).

2.7.2. Acidogenesis

Acidogenesis and hydrolysis are ten times faster steps in anaerobic digestion process than other steps. Acidogenesis is the fastest step in anaerobic digestion. In acidogenesis, the monomers (sugars, amino acids and long chain fatty acids) are converted into intermediate products such as H_2, CO_2, alcohols and short chain organic acids including; propionic acid, butyric acid, acetic acid, valeric acid and other short chain fatty acids. The acidogenic microbial communities are strict or facultative anaerobes including *Salmonella*, *Lactobacillus*, *Escherichia coli*, *Bacillus* and S*treptococcu*s species. The production of these short chain organic acids favors the lower pH ranging from 4 to 5.5 at which acidogenic and hydrolytic bacteria are active (Demirel and Yenigün, 2002). The concentration of the hydrogen produced as an intermediate at this stage further influences the upcoming products formation. The higher the partial pressure of hydrogen, the lower the yield of reduced compounds (Gerardi, 2003).

2.7.3. Acetogenesis

The products of acidogenic phase are converted into other intermediate products including alcohols, acetate, hydrogen and CO_2. The optimum pH required by acetogenic bacteria is 6 and they used acetyl Coenzyme A pathway for the production of acetate, H_2 and CO_2. The enzymes involved in this pathway are highly sensitive to O_2, operational and environmental parameters. The well-known microbial communities performing acetogenic reactions are *Syntrophomonas wolfeii*, *Syntrophobacter wolinii* that produce CO_2, H_2 and acetate (Mah, 1982). The microbial communities involved in this phase usually carried out anaerobic oxidation reactions. The syntrophic acetogenic relations occurs in this phase, in which the product of one microbial group are converted into substrates utilized by the other microbial group (methanogenic consortia). This interaction is dependent on the partial pressure of hydrogen. During anaerobic oxidation, protons are used as the terminal electron acceptors, which ultimately lead to the production of H_2 molecules. However, these oxidation reaction are only feasible when the partial pressure of hydrogen is low, explaining why the interaction with methanogenic consortia is important that consume hydrogen to produce methane. Acetogens are obligate hydrogen producing microorganisms that cannot survive at higher partial pressure of hydrogen. Therefore, these hydrogen producing microorganisms are present in symbiotic relations with other hydrogen consumers, maintaining a balanced process. In conclusion, during this phase inter-species symbiotic collaboration occurs through hydrogen transfer (Gerardi, 2003).

2.7.4. Methanogenesis

During this phase, the intermediate products from other phases are converted into CH_4 and CO_2 by methanogenic bacteria or archaea. There are two types of methanogenic bacteria; acetoclastic methanogens and hydrogenotrophic methanogens. Acetoclastic methanogens (*Methanosaeta* and *Methanosarcina thermophila*) consume the acetate as substrate and hydrogenotrophic methanogens (*Methannospirillum hungatei*, *Methanoculles receptaculi*) produce CH_4 by reducing H_2 and CO_2 (Aslanzadeh, 2014). Hydrogenotrophic methanogens are fast growing (doubling time 6 h) than acetoclastic (doubling time about 2.6 days) (Christy et al., 2014). In methanogenic phase, about 70% of methane is produced by acetoclastic methanogens from acetate and 30% is produced through redox reaction of hydrogen and carbon dioxide by hydrogenotrophic bacteria. Methanogens have slow generation time from hours to days. Especially the acetoclastic methanogens are highly vulnerable to fluctuation caused by

higher concentration of volatile fatty acids produced in acidogenic and acetogenic phases. Therefore, this step is also considered as the limiting phase due to slow growing rate. If the intermediate products (VFAs) in the other phases are increased beyond the acceptable limit, the methanogens are inhibited resulting in reactor instability and lower biogas yield. Therefore, the speed of all phases during anaerobic digestion must be in coordination in order to have consistently efficient and stabilized process resulting into higher biogas yield. For efficient process, these fermentative and methanogenic bacterial consortia must be in equilibrium state. It has been reported that the methanogens are highly vulnerable to higher acidity in the reactor. A pH range from 6.5 to 8 is considered ideal for normal and efficient performance of methanogenic communities (Schmidt et al., 2009). The acidity of the reactor is increased if the organic acids production rate is higher than the consumption by methanogens, as a result the pH goes down and creating unfavorable conditions for methanogenic bacteria (Jain et al., 2015).

2.8. Factors affecting anaerobic digestion

Anaerobic digestion process is a very complex process and the performance of microbial communities, stability of the reactor and biogas yield are dependent on a number of operational and environmental parameters. These parameters have been extensively studied and all of them have certain optimum ranges. The decrease or increase than the optimum range of these parameters causes reactor instability and perturbations.

2.8.1. pH value and alkalinity

The pH of feedstock changes at different stages of anaerobic digestion. Initially in hydrolysis and acidogenesis phases, the pH is relatively lower due to acid formation in the form of organic acids and volatile fatty acids. The reactor pH during initial days is round about 6 and increases gradually with passage of time as the fatty acids and organic acids starts converting into methane and CO_2. There must be an equilibrium between the fermenting phases and methanogenic phases to increase the biogas yield and reactor stability. The normal pH of anaerobic digester ranges from 6.5 to 7.5, the microbial communities efficiently perform their functions in this range (Palatsi et al., 2009). If the pH is in optimized range, the digesters buffering capacity is higher and as a result the microbial communities are active resulting in higher biogas yield. The pH lower than the normal range indicates over-acidification due to short chain volatile fatty acids that inhibit the methanogenic bacteria during anaerobic digestion. If the pH is increased than the optimum range it will lead to higher alkalinity that

usually occurs due to higher ammonia production. The ammonia produced above the acceptable limit imbalances the performance and stability of the reactor and the methanogens. Especially, acetoclastic methanogens are highly sensitive to higher ammonia production. Therefore, an optimum pH range is the first indicator of reactor stability and should always be monitored to check the performance (Palatsi et al., 2009, Zonta et al., 2013). Alkalinity is also an important factor playing role in stability of the process and enhancement of biogas yield. Alkalinity is the measure of basic (alkaline) substances in the reactor. The higher the alkalinity, the better will be the reactor performance and stability. Usually the reactors work best at neutral pH but sometimes they also work very well above the neutral pH such 7.5 to 8.5 due to higher alkalinity values. The biodegradation of protein rich organic matter causes an increase in the level of ammonia. This ammonia further reacts with carbon dioxide to produce ammonium bicarbonates. There are two types of alkalinity inside a biogas reactor; total alkalinity (TA) and bicarbonate alkalinity (BA). The bicarbonate alkalinity usually for a stable process ranges from 3000 to 15000 mg HCO_3/L. Low alkalinity mostly occurs due to acid production resulting in lowering the pH of the reactor. The increase in acidity of the reactor usually occurs in startup phase or due to overload or temperature fluctuations. Sometimes, the inhibition of microbial communities by toxic feedstocks also reduce the alkalinity of the reactor. If the alkalinity of a biogas reactor fluctuates, it is a sign of inhibition warning. Alkalinity and pH of a reactor are sometimes adjusted by various agents (Schnurer and Jarvis, 2010).

2.8.2. Temperature

A balanced temperature is imperative for an efficient microbial performance and higher stability of the reactor. There are normally two ranges of operating temperatures, thermophilic and mesophilic. The thermophilic temperature ranges from 45 to 60 °C. In most of the studies, the 55°C has been reported the optimum thermophilic temperature. The optimum range of mesophilic temperature is around 35 °C. The solid state anaerobic digesters perform well at thermophilic compared to mesophilic range. Moreover, at thermophilic range the pathogen reduction is also higher and the reaction completes in shorter time compared to mesophilic range. In case of liquid state anaerobic digestion, the mesophilic range is preferred and the microbial communities perform well leading to higher biogas yield. However, thermophilic temperatures are not preferred in normal liquid state anaerobic digesters due to higher energy requirements and extreme heating of the reactor with lower biogas yield. Moreover, there is a third range of temperature for anaerobic digestion of lignocellulosic biomass, psychrophilic

range below 20 °C. In colder regions, the psychrophilic anaerobic digestion is highly needed with an efficient microbial performance and higher reactor stability (Jain et al., 2015).

2.8.3. Total solids content

Total solid contents play an important role in stabilizing the anaerobic digestion process. In liquid state anaerobic digestion, the reactor is more stable and efficient in terms of biogas yield at total solid (TS) contents less than 10%. The solid state anaerobic digesters normally operated at higher TS in order to achieve higher conversion of organic compounds into methane and CO_2. However, in solid state anaerobic digestion setup, the biogas yield decreased with increasing the TS beyond a certain limit. For example, a study has reported that the methane yield was 17% less at TS content 30% than at TS 20% during solid state anaerobic digestion process. Another study has also reported that when the solid state anaerobic digestion process was changed from 20% to 30%, the chemical oxygen demand (COD) was reduced from 80.69% to 69.05% (Fernández et al., 2008).

2.8.4. Organic loading rate

Organic loading rate (OLR) is an important parameter in anaerobic digestion process. Organic loading rate can be defined as, the amount of raw feedstock added or fed to a reactor at a specific time per unit volume. For organic loading rates, the TS and volatile solids (VS) of the organic matter must be known to have an accurate loading rate at a specified time. The TS is the solid part that remains after the water evaporation. The VS is the organic part of the solid matter or substrate. Most of the studies reported optimum OLR 0.5-1.6 kg volatile solids per m^{-3} per day for municipal solid wastes digestion. If the OLR is increased than the optimum range of a reactor, the volatile fatty acids will tremendously increase and accumulated resulting in over-acidification of the reactor. Conversely, the higher OLR has also an advantage of lower volume requirement for the degradation of a substrate during anaerobic digestion. Therefore, the rectors operating at higher OLRs are cost effective and are more economically viable compared to lower OLRs (Luste and Luostarinen, 2010, Rai, 2000). Sometimes, several months are needed in achieving the desired OLR because the methanogens are slowly growing. Usually methanogens take few days to double their numbers, so if in initial a sudden higher load is provided there will not be that much methanogenic bacteria to convert this matter into biogas as a result the organic acids and VFAs are accumulated resulting in over-acidification and unbalancing the process. The highly degradable substrates loading in high concentration also cause the accumulation of VFAs which subsequently inhibit methanogens resulting in the loss

of methane. In thermophilic reactors, an OLR 4-5 kg VS per m^{-3} per day is used. While in mesophilic reactors, this loading rate is lower i.e. 2-3 kg VS/m^{-3}/day (Richards et al., 1991). In some studies a load of 13.5 kg VS/m^{-3}/day has been recorded in thermophilic reactors. Similarly, in mesophilic reactor a load of 6 kg VS/m^{-3}/day with retention time 20 days has been reported. The organic loading rate should be kept constant as much as possible over the time and must not be changed more than 10-15% per week (Angelidaki et al., 2006, Schnurer and Jarvis, 2010).

2.8.5. Retention time

Retention time of substrate is defined as the time taken by it to replace all the organic matter present in a reactor. It may also be defined as the time spent by the organic matter in a specific volume of reactor. During anaerobic digestion, the organic matter (hydrocarbons) are converted into CH_4 and CO_2. The organic matter is continuously reduced and the new organic matter is added subsequently at equal intervals to maintain the same volume replacing the old one. Both of these organic matters are converted into biogas. Typically more solid substrate is added and less is removed because prior to removal, the solid organic matter is also converted into biomethane and carbon dioxide. The added and removed volume of organic matter is regulated by liquids. The removed solid organic matter is composed of partial water having dissolved salts and organic matter that has been degraded inside the reactor during anaerobic digestion. This residue with dissolved salts and organic matter in water sometimes called digestate. Usually, the anaerobic digesters have 10 to 25 days HRT but it can be increased depending upon the substrate. If the substrate is easily degradable, a shorter HRT is required. In such cases, the methanogenesis is mostly limiting phase because the organic matter in the hydrolytic and acidogenic phases quickly degraded and accumulated in the form of intermediate compounds called short chain volatile fatty acids (VFAs). These VFAs are inhibitory to methanogens at higher concentration beyond the acceptable limit. If the substrates is recalcitrant such as fiber or cellulose rich organic matter, a longer retention time is needed to break down this organic matter completely. In such cases, the hydrolysis is considered the limiting step because the complex organic matter are hard to degrade into simpler form by extracellular enzymes. In Germany, many reactors treating lignocellulosic biomass are operated at 50 to 100 HRTs in order to completely degrade the organic matter. More time spent by a substrate will result in more biogas yield due to higher contact time. The degree of digestion is defined as the conversion of organic matter into methane and carbon dioxide in a

specific time. Generally, the batch processes have higher digestion rate than continuous processes. The degree of digestion in a batch process might be theoretically 100% but practically it impossible because a lot of organic matter is remained as residues and does not convert into biogas. The degree of digestion also depends upon the substrate type. The easily degradable substrates have >90% digestion rate and the recalcitrant mostly have ≥60% during the specified time (Edström, 2004, Rohstoffe eV, 2005).

2.8.5. Microbial seeding

The two main groups, fermentative and methanogenic bacteria play an important role in stabilizing the anaerobic digestion process. Usually, the fermentative (acidogenic and hydrolytic) bacteria grow rapidly compared to methanogens. While the methanogens are slow growers and required higher time for doubling their numbers. So in order to maintain a stable process, both the fermentative and methanogenic bacteria must be in an equilibrium to increase the stability and efficiency of the reactor in terms of biogas yield. It would be desirable if the number of methanogens are maintained higher compared to fermentative bacteria during anaerobic digestion. However, the increase in number of methanogens beyond a certain limit decrease the biogas yield due to the lower concentration of feedstock (Wang et al., 2012).

2.8.6. Uniformity in feeding

In order to maintain the reactor performance more efficient, a uniform feeding of the same quantity and quality is highly desirable to increase the biogas yield. If the feeding is done at regular intervals, it will keep the microbial communities at a constant concentration. The change in feeding time, quality and concentration highly affect the anaerobic digestion process due to sudden shocks. Therefore, there must be a balance in the feeding to maintain a stable process with higher biogas yield (Wang et al., 2012, Giuliano et al., 2013).

2.8.7. Diameter to depth ratio

Diameter to depth ratio is also very important in maintaining the microbial communities active during anaerobic digester. The reactors with 0.66-1 diameter to depth ratio gave higher biogas yield, but these reports are not confirmed from the field studies conducted earlier. However, esthetically the reactor with 16 feet depth and 4-5 feet diameter would be more stable and producing higher biogas yield. Mostly the actively growing microbial communities lie in the lower half of the reactor and the external day/night temperature changes do not affect them due to higher depth (Kwietniewska and Tys, 2014, Jain et al., 2015).

2.8.8. Carbon to nitrogen ratio

Carbon and nitrogen are the two most important elements required by microbial communities present in anaerobic digester. Carbon is needed for energy production and nitrogen is used for building proteins. There must be an overall balance between these two elements during anaerobic digestion. The optimum range of carbon to nitrogen (C/N) ratio is 20-30. If the carbon is much higher than the nitrogen in a reactor, the microbial strains would be unable to build their biomass due to scarcity of nitrogen required for protein building. At higher carbon contents, the VFAs are accumulated at higher concentration resulting in microbial inhibition during anaerobic digestion. At higher nitrogen level, the total ammonia nitrogen (TAN) production is increased beyond the acceptable range resulting in inhibition of methanogenic communities. The NH^{4+}-N at concentration of 2-3 g/L is the threshold limit for ammonia inhibition. If the ammonia is increased beyond this range, the microbial inhibition occurs but in most of the cases even at much higher ammonia level than the threshold level does not cause any inhibition on microbial communities. This is due to adaptability of certain microbial communities to higher level of ammonia in reactor. The free ammonia when accumulated at higher level, it mostly target the acetate consuming methanogens and penetrate through their cell membrane into cytoplasm where it attract the hydrogen ions to form ammonium ion and as a result the microbial cell efflux the potassium ions already present inside the cells and bring inside more hydrogen ions for reacting with free ammonia due to which the pH of the cells tremendously dropped and cell inhibition or death occurs. The acetoclastic methanogens already have lower concentration of potassium ions compared to hydrogenotrophic methanogens inside the cells (Schnurer and Jarvis, 2010). The methanogens are highly sensitive to increase level of TAN. The C/N ratio varies with feedstocks. Some feedstocks have lower C/N ratios and some of them have higher C/N ratios. The C/N ratio of *J. curcas* seed cake is 9:1 (Raheman and Mondal, 2012). Similarly, the C/N ratio of manure is around 20:1. On the other hand, the mango peels have higher C/N ratios around 45.4:1 (Suryawanshi et al., 2013).

2.8.9. Mixing

Mixing is also an important parameter for maintenance of stable conditions in anaerobic digester. Mixing increases the chances of contact of substrate to the microorganisms inside the digesters resulting in an efficient biogas production. Usually, the reactors contents are mixed by propeller or pumps during anaerobic digestion. Some of the microbial communities thrive

in close proximity with each other in the form of aggregates that is necessary for transfer of hydrogen among them. However, too high intensity of mixing may also reduce the biogas yield due to shearing of microbial aggregates during anaerobic digestion process. In addition, the appropriate mixing also reduce the uneven loading during anaerobic digestion process. Mostly the organic compounds are settled down without mixing and concentrated at the bottom resulting in lower chances of microbial contact with these solids. So an appropriate mixing is highly appreciated to evenly distribute the solids and microbial communities inside the reactor.

2.9. Toxicity

There are a number of substances which inhibit the anaerobic microbial communities inside the biogas reactor. Inhibitory substances might enter the reactor due to poorly managed or contaminated materials or by using a substances that do not cause any inhibition in initial phases. All of the toxic substances behave in a certain unique way and the response of process to these compounds depends on certain parameters such as concentration of the toxic compound, retention time, temperature, pH and types of microorganisms.

There are different levels of toxicity which are influenced by different factors as mentioned earlier. They might be antagonistic, synergistic, complexed or adaptable inhibitions (Chen et al., 2008, Chynoweth, 1987), as described below:

1. Antagonism: When the level of inhibition of combined toxic compounds is lower than their individual level.
2. Synergism: Synergism can be defined as the increase in the inhibition level of toxic compounds than their individual level.
3. Complex formation: when the inhibitory compounds combined together with similar or dissimilar compounds and become invisible to microbial communities during anaerobic digestion
4. Adaptation or acclimatization: Sometimes, the microbial communities start to adapt the toxicity of the compounds and use them as substrate or protect themselves by acquiring certain changes in their composition. This phenomena is called acclimatization.

Some inhibitions are reversible and some are non-reversible. In non-reversible inhibitions, the microorganisms are unable to recover from the inhibition even when the inhibitory compounds are removed. In such conditions, these microorganisms must be replaced by the new actively growing consortia or the process must be re-started. On the other hand, in case of

acclimatization, the microorganisms gradually adopt to the toxic compounds and go into lag phase in which they restrict their growth and started learning how to cope with these inhibitory compounds. In order to avoid the breakdown of complete process, it is mandatory to reduce the load and extend the retention time. Otherwise the microbial communities will be washed off soon. It is also important to reduce the concentration of toxic compounds or to refresh the inoculum from some other efficient anaerobic digester to increase the biogas yield. There are a number of inhibitory substances that have been reported to inhibit the anaerobic digestion process.

2.9.1 Inhibitory substances

Ammonia (NH_3) is released when protein rich substances are biodegraded during anaerobic digestion and usually inhibit methanogenic bacteria. Ammonia and ammonium ions are mostly present in equilibrium during anaerobic digestion. The inhibitory form of nitrogen containing compound is ammonia and increase in its production depends upon various parameters such as pH and temperature. Ammonia is inhibitory at concentration above 30 mg/L but some studies have reported a stable anaerobic digestion process even at ammonia concentration more than 100 mg/L (Fricke et al., 2007). Normally, the concentration of ammonia is monitored by measuring ammonium nitrogen ($NH\ 4^{+}$-N) which is the composite form of ammonia and ammonium. The ammonium nitrogen is considered inhibitory at concentration ranging from 1.5 to 14 g/L (Fricke et al., 2007, Chen et al., 2008). Ammonium nitrogen at the level above 3 g/L have been reported with microbial inhibition in various studies (Schnurer and Jarvis, 2010).

2.9.2. Long chain fatty acids

Fats are energy rich compounds and could be a source of higher energy and methane production but an increase in fats level beyond a certain level could be problematic for microbial communities inside the reactor. Fats are mainly composed of fatty acids and glycerol. These fatty acids may be saturated, mono-unsaturated or poly-unsaturated. Saturated fatty acids are present in meat and dietary feeds, while poly-unsaturated are present in fish and corn oil and mono-unsaturated fatty acids are present in vegetable oils or nuts. Triglycerides are the most common type of fats that are hydrolyzed into long chain fatty acids during anaerobic digestion. There are different types of long chain fatty acids but all of them are composed of more than 18 carbon atoms (Schnurer and Jarvis, 2010). These long chain fatty acids including stearic acid, meristic acid, linoleic acid, linolenic acid, palmitic acids, etc. are released during

the lipid biodegradation in anaerobic digestion process and have been reported to possess inhibitory effects on both bacterial and methanogenic communities. The fatty acids surrounds the microbial cell membranes and stop the transport of materials through their cell membranes both inward and outward. Thermophilic microbial communities have been reported with higher sensitivity to LCFAs compared to mesophilic communities. Long chain fatty acids are able to have an acute or chronic inhibition of microbial cells. Even sometimes, they kill the microbial communities during anaerobic digestion process (Chen et al., 2008). The toxicity of long chain fatty acids, in most of the cases, is recoverable but the recovery time might be longer (Chen et al., 2008). The LCFAs have been reported with possible biodegradability of up to 5 g COD-LCFAs/ g VS. Long chain fatty acids are hydrolyzed by lipases but the increase in lipase concentration at higher level also intensifies the inhibitory effects on microbial communities during anaerobic digestion (Cirne et al., 2007b, Rigo et al., 2008). The microbial communities during the lag phase (days to weeks for LCFAs) stick to these acids and are sometimes washed off along with effluents. These LCFAs leads to the foam formation due to surface active nature inside the reactor (Albertsson, 2007).

2.9.3. Antibiotics

Antibiotics are the substances produced by microbial strains that inhibit the other microbial communities. It has been reported that methanogens are more sensitive to antibiotics compared to the other bacterial communities but this concept is mostly related to biogas production i.e. mostly in the presence of antibiotics like chloramphenicol and chlortetracycline the methane production has been reduced 50% at 20 and 40 mg/L. Thiamphenicol has been reported to reduce 60% methane production at 80 mg/L concentration (Schnurer and Jarvis, 2010). Sometimes the animals also get antibiotics in their feed such as monensin and rumensin that have strong inhibitory effects on methanogenic communities (Zitomer et al., 2007). However, methanogenic communities have also been reported for biodegradation of various antibiotics during anaerobic digestion process (Gartiser et al., 2007).

2.9.4. Heavy metals

Metals are non-biodegradable and can be accumulated in reactor resulting in microbial inhibition. Heavy metals, for example cadmium, uranium and mercury can be toxic while cobalt, nickel, molybdenum and zinc can be non-toxic. The heavy metals may inhibit the microbial cells enzymes activity. Low concentration of certain heavy metals are required for enzymes activity. To date, there is no specific limit of metals toxicity but the inhibitory

concentration of heavy metals mostly lies in 100 mg/L (Chen et al., 2008). Some metals are non-toxic and may appear in the reactor with several hundred grams per litres without causing toxicity. Heavy metals may chelate various compounds such as sulphides and become invisible to the microbial communities. Thus leaving the process without any inhibition. They may also chelate bentonite and citrate (Chen et al., 2008). Metals may interact with each other with either synergistic effects or antagonistic effects. For example, nickel has been reported for antagonistic (reduced inhibition) effects on cadmium and zinc and synergistic effects with copper, molybdenum or cobalt (Chen et al., 2008).

2.9.5. Detergents

Detergents are the compounds that are used to reduce surface tension. They mostly cause inhibition of anaerobic bacteria and also reduce the biogas or biomethane yield. The most commonly reported surfactant is linear alkylbenzenes sulphonates (LAS) that inhibit both bacteria and methanogens and is slowly degraded during anaerobic digestion process. The level of inhibition is concentration dependent and the upper limit of LAS for inhibition inside the biomass is 14 mg/L.

2.9.6. Sulphides

Sulphides also known for inhibition of microbial communities during anaerobic digestion process. The hydrogen sulphide (H_2S) and hydrosulphide (HS^-) are present in equilibrium during anaerobic digestion process but sometimes the equilibrium shifted towards H_2S with decreasing pH. An increase in the H_2S concentration beyond a certain limit causes inhibition of microbial communities inside the reactor resulting in lower biomethane yield. The inhibitory concentration range of H_2S is 50-400 mg/L (Chen et al., 2008). Sulphide ions (S^{2-}) also have higher affinity towards metals and bind with them forming precipitates and the microbial communities are unable to utilize the essential metals for their activities during anaerobic digestion process. Hydrogen sulphide is liberated from sulphur containing amino acid (cysteine and methionine) fermentation as well as sulphate reduction by sulphate reducing bacteria. The level of sulphides are reduced practically in biogas plant by adding $FeCl_2$ or $FeCl_3$ forming iron sulphide and is removed (Schnurer and Jarvis, 2010).

2.10. Microbial communities' analyses during anaerobic digestion

Anaerobic digestion is a dynamic process in which a highly complex network of microbial communities survive and function in syntrophic relations with one another. These

microbial communities are involved in the production of a number of products such as fatty acids, amino acids, sugars, volatile fatty acids, hydrogen ions, acetate, etc. and all of these products are produced by different microbial groups including, hydrolytic, acidogenic, acetogenic and methanogenic communities. The stability and efficiency of biogas production process under anaerobic conditions is entirely dependent upon the syntrophic relation of microbial communities belonging to different or same functional groups within same reactor (Werner et al., 2011). Culture dependent techniques have been utilized for the identification and investigation of key metabolic activities of microbial communities during anaerobic digestion process but a large number of microbial communities are unable to be studied using these techniques due to lack of facilities and difficulties in culturing those microorganisms especially, obligate anaerobes are hard to culture *in vitro* (Su et al., 2012). The microbial communities' structure could be studied via PCR amplification and analysis of conserved marker genes. The 16S rRNA gene has been most extensively studied and has highly developed reference databases (Su et al., 2012). Traditional molecular techniques include denaturing gradient gel electrophoresis, single strand conformation polymorphism, terminal restriction fragment length polymorphism and Sanger sequencing using 16S rRNA gene. These techniques have been utilized for years to investigate microbial structures and function inside the reactor. However, all of these techniques are time-consuming and give low throughput (Talbot et al., 2008, Delmont et al., 2012). So studying microbial communities using only culture dependent techniques will lead to incomplete and biased understanding of microbial physiology and ecology. The interaction of anaerobic microbial communities with abiotic or biotic factors would remained unresolved. Therefore, culture independent techniques such as next generation sequencing including (454-pyrosequecing and Illumina sequencing) are considered ideal options in order to get full insights into the reactor configurations, microbial functions, overall performance efficiency and stability during anaerobic digestion process (Zarraonaindia et al., 2013). These sequencing technologies utilized 16S rRNA gene based microbial analyses. Pyrosequencing (454-Roche) has been extensively used for microbial analysis in anaerobic digesters but it has the problem of homopolymers synthesis which misinterpret the microbial abundance. It could generate short reads with length ranging from ~250 to 500 bp that span variable region of 16S rRNA gene (Morozova and Marra, 2008). The Illumina sequencing technology gaining more attention as it could produce ten times more sequences with relatively lower cost and can sequence long paired end sequences (2 × 300 bp reads) (Caporaso et al., 2012). Metagenomic is the random sampling and sequencing from any

environment using high throughput techniques. Metagenomic has gone beyond 16S rRNA gene based analyses of microbial communities and gained insights into microbial physiology and functions. The next generation sequencing technologies have resulted in wide adoption of this approach as a large data is acquired that is proportional to complex microbial configuration inside the reactor (Shakya et al., 2013, Vanwonterghem et al., 2014). These sequencing technologies in combination with stable isotopes imaging had been used in various studies regarding anaerobic digestion process. Culture independent technologies have revealed both phylogenetic and metabolic diversity (Vanwonterghem et al., 2014, Lee et al., 2012, Pervin et al., 2013, Sundberg et al., 2013). The next generation sequencing technologies have tremendously decreased the cost and also increased the yield resulting sequencing of ten to hundreds of samples in a single run and simultaneously examined the temporal and spatial resolution of microbial communities during anaerobic digestion process. These analyses increased the power of statistical analysis of such a complex microbial community network. Various correlations of microbial communities with operational parameters including; OLR, HRT and feedstock composition have been studied that influence the microbial community structure and change the pathways involved in anaerobic digestion of wastes (Ziganshin et al., 2013, Xu et al., 2018). The culture dependent technologies just revealed 69 operational taxonomic units (OTUs) while the culture independent high throughput technologies have revealed thousands of OTUs in anaerobic digestion process (Lee et al., 2012, Riviere et al., 2009). Deep microbial analyses could revealed insights into rare OTUs that are involved in some specific function and could increase our understanding of microbial community profiling linked with reactor behaviors. Metagenomic is the random sampling and sequencing from any environment using high throughput techniques. Metagenomic has gone beyond 16S rRNA gene based analyses of microbial communities and gained insights into microbial physiology and functions. The next generation sequencing technologies have resulted in wide adoption of this approach as a large data is acquired that is proportional to complex microbial configuration inside the reactor (Shakya et al., 2013, Vanwonterghem et al., 2014). The *J. curcas* seeds have antimicrobial phytotoxins that inhibit the microbial communities involved in anaerobic digestion. The effect of the inhibition of these phytochemicals on microbial communities can be studied best using high throughput sequencing and molecular tools. The medicinal values of *J. curcas* seeds are discussed as follows.

2.11. *Jatropha curcas* as a medicinal plant

Jatropha curcas is a multipurpose shrub with higher potential for biofuels and medicinal compounds production. A number of studies had been conducted on different parts of *J. curcas* plant for antimicrobial activities. Especially, *J. curcas* leaves, latex, bark, stem and roots have been used for various antibacterial and antifungal activities. In addition, it have antioxidant, cytotoxic and antitumor activities (Islam et al., 2011). Traditionally, *J. curcas* has been used for treatment of various illnesses including rheumatoid arthritis, goiter, dysentery, diarrhea, fever, jaundice and constipation. *J. curcas* plant have a number of phytochemicals in its different parts including; alkaloids, phenolic, flavonoids, tannins, steroids, glycosides, curcin, long chain fatty acids, phorbol esters and phytic acids. All of these phytochemicals have been reported for antibacterial, antifungal, cytotoxic, antiviral and insecticidal activities. These phytochemicals could either be used as base for developing a new medicine or could be used as phytomedicine to treat different diseases (Iwu, 2014). Jatropha leaves have various phytochemicals that include flavonoids, steroids, alkaloids, glycosides, vitexin and isovitexin and various sterols. *J. curcas* leaves have been used in folk medicine for treatment of vaginal bleeding, rheumatism, to promote breast lactation, as purgative and also for wound healings (Islam et al., 2011). Stem, bark, branches and twigs of *J. curcas* plant are rich in various phytochemicals including; alkaloids, saponins, tannins, steroids, glycosides, phenolic and flavonoids, etc. and are involved in various antimicrobial and anticancer activities (Igbinosa et al., 2009). Tannins have been reported to form irreversible complexes with proline rich proteins resulting in its inhibition. Tannin containing compounds are mostly used to treat diarrhea and dysentery diseases (Dharmananda, 2003). Flavonoids have been extracted from *J. curcas* bark and are known for antimicrobial, antioxidant, cytostatic and anti-inflammation and analgesic activities (Hodek et al., 2002). *J. curcas* latex has been used as traditional medicine to treat various diseases. It has been reported for use of toothache and haemostatic wound dressings. Latex contains tannins, saponins, wax, resin and proteolytic enzyme curcain that is known for wound healing activities in mice. A novel compound curcacycline A has been reported in *J. curcas* latex that is used to inhibit the pathway of classical human complement system and proliferation of human T-cells. The curcacycline B is known to enhance the rotamase activity of cycophilin B. The latex is also known to treat eczema, ringworm and scabies etc. (Islam et al., 2011). Hence, *J. curcas* plant could be used as potential source of various phytochemicals that will increase its economic viability.

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CHAPTER 3

Chapter 3: Antibacterial activities of *Jatropha curcas* seed

Paper 1

Title: Comprehensive Investigation on the Synergistic Antibacterial Activities of *Jatropha curcas* Pressed Cake and Seed Oil in Combination with Antibiotics

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3.1. Abstract

Synergistic combinations of various antimicrobial agents are considered ideal strategies in combating clinical and multidrug resistant (MDR) infections. In this study, antibacterial potential of *Jatropha curcas* crude seed extracts, seed oil, commercially available antibiotics, and their combinations were investigated for their synergistic effect against clinical, MDR and ATCC bacterial strains by agar well diffusion assay. Methanolic extracts remained more active against *Staphylococcus aureus* (ATCC), with zone of inhibition (ZOI) of 21 mm, than clinical and methicillin-resistant *S. aureus* (MRSA) strains (ZOI range ~15.0-17.0 mm). Molecular docking demonstrated that beta-monolaurin from methanolic extract exhibited greater affinity conformation for UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine (MurF) ligase's active pocket with binding energy of -7.3 kcal/mol. Moxifloxacin exhibited greater activity against *Escherichia coli* (ATCC) (ZOI ~50.0 mm), followed by ofloxacin against *Pseudomonas chlororaphis* (47.3 mm), moxifloxacin against *P. monteilii* (47 mm), *P. aeruginosa* (46.3 mm) and MRSA2 (46 mm) and ofloxacin against *S. aureus* (ATCC) strains (45.7 mm). Methanolic extract in combination with rifampicin showed the highest synergism against MRSA strains, *A. baumannii*, *E. coli*, *E. faecalis*, *S. aureus*, and *P. aeruginosa*, *A. baumannii* (MDR strain), *P. chlororaphis*, *E. coli* ATCC25922 and *S. aureus* ATCC25923. In combinations, moxifloxacin exhibited the highest antagonism. The methanolic, *n*-hexane, aqueous extracts and seed oil in various combinations with antibiotics showed 44.71, 32.94, 9.41 and 25.88% synergism, respectively. The current study showed that potency of antibiotics was improved when screened in combination with *J. curcas* seed's components, supporting the drug combination strategy to combat antibacterial resistance.

Keywords: *Jatropha curcas*, antibiotics, multidrug resistant bacteria, combinatorial therapy, fractional inhibitory concentration index, synergism.

3.2. Introduction

Over the last few decades, the exhaustive over-prescription, and self-medication of clinically available antibiotics and consequently the long-term exposure of pathogenic microorganisms to these antibiotics has led to the development of antibiotic resistance (Harbottle et al. 2006). The mechanism behind the antibiotic resistance, as a result of long-term exposure, is the accumulation of multiple genes each coding for resistance to a single drug. This mechanism within a single bacterial cell has added in the emergence of multidrug resistant (MDR) bacteria. MDR bacteria use horizontal gene transfer to spread the antibiotic resistance genes among themselves (Odonkor and Addo 2011). A number of diseases caused by MDR bacterial strains are incurable and fatal due to their high resistance rate towards most of the clinically available antibiotics (Nikaido 2009). Currently, more than 70 percent of the pathogenic bacteria are reported to have acquired resistance against antibiotic therapies (Harvey et al. 2015). In this context, the development of novel, efficacious, cost-effective and non-cross resistant antibiotics has become the only alternative to treat bacterial infection and remains a great challenge for pharmaceutical industries in terms of exploring novel and efficacious drugs as well as drug development expenditure (Sharma et al. 2016).

Historically, medicinal plants or their extracts have been used as traditional medicine to treat various infectious. Numerous plants or their extracts have been reported to possess antimicrobial properties (Rachana et al. 2012). Plants or their products may act as bactericidal as well as bacteriostatic agent against microbial efflux pump, quorum sensing and biofilm formation (Savoia 2012). During the last few decades, investigations on the antimicrobial potential of natural products remained a great focus for drug discovery around the world (Rios and Recio 2005). However, only a few medicinal plants or their extracts were managed to reach clinical trials. As of now, not even a single antimicrobial agent derived from medicinal plants has been officially approved. Different technical challenges have been sought and possible recommendations have been proposed for development of drugs derived from natural compounds (Cos et al. 2006). Mixtures of commercially available pharmaceutical and herbal remedies against different ailments have been reported for use traditionally in self-medication (Buchness 1998; Donaldson 1998). Plant remedies used in combination with pharmaceutical drugs have certain herb-to-drug interactions and the possible outcomes of these interactions include synergistic amplification of the antimicrobial potential and reduction in the adverse side effects of synthetic drugs. These combined effects have certainly reduced the chances of lower efficacy of drugs used alone to treat a microbial infection for a long time (Borchers et al.

1997). Based on the traditional herb-to-drug combination strategy, the ineffective synthetic antibiotics at present can be used in combination with the inexpensive, handy and harmless medicinal plants. The herb-to-drug combination strategy may lead to the discovery of novel antibiotics and the re-use of those antibiotics towards which bacteria have developed resistance. (Saklani and Kutty 2008).

Jatropha curcas is a multipurpose shrub belonging to *Euphorbiaceae* family and its seeds contain oil that can be used for biodiesel production and assayed for antimicrobial potential as well. It can sustain itself in sub-tropical, semi-arid, saline and acidic soil regions. Traditionally, it has a long history of medicinal use and has been greatly utilized in treating bacterial as well as fungal infections. Various extracts of *J. curcas* were phytochemically analyzed and reported to have antimicrobial activities against different human pathogens (Ajayi 2018; Arekemase et al. 2011). However, only a few reports are available on antimicrobial activities of pressed cake (de-oiled seed) of *J. curcas* and that is mostly restricted to standard cultures such as American type culture and collection (ATCC) strains.

In the present study, *J. curcas* pressed cake and seed oil were investigated for their phytochemical constituents analysis and antibacterial potential against clinical bacterial pathogenic isolates, MDR and ATCC bacterial strains. Moreover, for the first time, the fractional inhibitory concentrations (FIC) of various extracts of pressed seed cake and seed oil of *J. curcas* in combination with the various commercially available antibiotics against selected bacterial strains have been studied in order to investigate their synergistic, antagonistic, indifference and additive effects.

3.3. Materials and Methods

3.3.1. *J. curcas* seed oil extraction

The local variety of *J. curcas'* seeds was obtained from local dealer and identified at the Department of Plant Sciences, Quaid-i-Azam University, Islamabad. Oil was extracted from whole seeds of *J. curcas* plant using mechanical oil expeller. After extracting oil, the de-oiled seed cake was preserved in sterile zipper bags at 4 °C and the oil was stored in the dark for further use.

3.3.2. Preparation of extracts, commercial antibiotics' solutions and their combinations

De-oiled seed extracts of *J. curcas* plant were prepared as previously described (Basri and Fan 2005). 100 g of fine powdered de-oiled seed cake of *J. curcas* was dissolved in 500 mL of water, methanol or *n*-hexane and incubated at 30°C for 48 h, in a shaking incubator at 100 rpm. The solution was filtered through Whatman filter paper and the filtrate was concentrated at 45°C under reduced pressure in rotary evaporator. The concentrated filtrate was allowed to dry at room temperature. The yield of methanolic, *n*-hexane and aqueous extracts were 17.0, 10.39 and 9.04%, respectively. Each extract (200 mg) was dissolved in 1 mL DMSO for further use. Stock solutions of the selected commercially available antibiotics (powder form) including ciprofloxacin (Global Pharmaceuticals), cefotaxime (Global Pharmaceuticals), rifampicin (Pfizer Laboratories Limited) and moxifloxacin (Bio Labs Pak (Pvt) Limited) were prepared at a concentration of 100 µg/mL in de-ionized water. Ofloxacin (GlaxoSmithKline Pakistan Limited), the broad spectrum antibiotic that is active against various Gram positive and Gram negative bacteria, was used as a positive control. The *J. curcas* seed oil, seed extracts and the antibiotics were filtered using sterile syringe filter (0.2 µm pore size). Commercially available antibiotics were used in combination with *J. curcas* seed oil and its de-oiled seed cake extracts. For combinatorial activities, each extract and antibiotic solution was taken in1:1 volume in sterile tubes. 100 µL of seed oil, each extract and antibiotic was, individually as well as in combinations, were spread on MHA and the plates were incubated at 37°C for 24 h to confirm sterility.

3.3.3. Preliminary phytochemical screening

The preliminary qualitative phytochemical screening of *J. curcas* seed oil and de-oiled seed cake was carried out for qualitative identification of balsams, flavonoids, saponins, glycosides, saponin glycosides, cardiac glycosides, steroids, volatile oils and tannins by methods previously reported (Amina et al. 2013; Arekemase et al. 2011; Sajjad et al. 2015).

3.3.4. Characterization of *J. curcas* seed oil and de-oiled seed extracts

The *J. curcas* seed oil and seed extracts were analyzed by FTIR (Bruker Tensor 27) absorption spectra registered for *J. curcas* seed oil and de-oiled seed extracts in the range of 4000-400 cm^{-1}.

The chemical composition of *J. curcas* seed oil and de-oiled seed cake extracts (aqueous, methanolic and *n*-hexane extracts) was also analyzed by gas chromatography coupled with mass spectrometry (GC-MS) technique (GC-MS – QP5050A, Shimadzu, Europe) according to the previously described methods (Mu'azu et al. 2013; Oskoueian et al. 2011) with some modifications as discussed below. For GC-MS analysis of *J. curcas* seed oil and de-oiled seed cake extracts, some of the conditions were varying and then a 2 µL of each sample (12.5 mg/mL) was injected in column using automated injector with a split ratio of 1/48 and 1/25 for *J. curcas* de-oiled seed cake extracts and seed oil, respectively. A DB-5 column was used with a length of 30 m, internal diameter of 0.25 mm and thickness of 0.25 µm while flow rate was maintained at 1 $mL.min^{-1}$ and 1.8 $mL.min^{-1}$ for de-oiled seed cake extracts (methanolic, *n*-hexane and aqueous) and seed oil, respectively. Thermal conductivity detector was used for detection of analytes. The identification of the peak was based on computer matching of mass spectra with National Institute of Standards and Technology library. The mass to charge scanning ranged from 40-600 amu.

3.3.5. Collection and maintenance of bacterial cultures

Three types of bacterial strains including commonly occurring Gram positive and Gram negative human pathogenic clinical isolates (*Acinetobacter baumannii, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Enterococcus faecalis and Staphylococcus aureus*), MDR Gram positive and Gram negative bacterial strains (*Pseudomonas monteilii, Pseudomonas chlororaphis, Klebsiella pneumoniae, Acinetobacter baumannii* (MDR), and methicillin-resistant *Staphylococcus aureus* (MRSA) strains (MRSA1, MRSA2, MRSA3, MSSA4 and MRSA5) were selected. These strains were selected because they are considered most challenging in terms of antibiotic susceptibility and cause various infections in a large population. All the strains were obtained from Pakistan Institute of Medical Sciences, Islamabad. In addition, the two ATCC strains, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as reference strains (positive controls). Each strain was grown and maintained on nutrient agar media at 4°C and sub-cultured on fresh media at regular intervals. The antibiotic resistance profiling of MDR strains was confirmed by disc diffusion method (Appendix 1 Table S1).

3.3.6. Preparation of bacterial culture for antibacterial assay

Bacterial cultures were prepared for antibacterial assay according to the method of Gahlaut and co-workers (Gahlaut and Chhillar, 2013). The bacterial strains, under aseptic conditions, were incubated and grown in nutrient broth at 37°C for 24 h and centrifuged at 4000 rpm for 5 min. Supernatants were discarded and pellets were re-suspended in 20 mL sterilized normal saline, followed by centrifugation at 4000 rpm for 5 min. The pellet obtained was suspended in sterile normal saline, labeled accordingly and its optical density (OD) was measured at 600 nm wavelength using ultraviolet visible spectrophotometer (8453 UV-Visible spectrophotometer). The bacterial suspension was diluted with normal saline until the OD was in range of 0.5-1.0 that corresponds to 5×10^6 CFU.mL^{-1} (Sarker et al. 2007).

3.3.7. Antibacterial assay

Antibacterial potential of *J. curcas* seed oil, de-oiled seed extracts and the selected commercially available antibiotics was evaluated using agar well diffusion method of Boakye and co-workers (Boakye–Yiadom 1979). The standardized inocula (5×10^6 CFU.mL^{-1}) were swabbed onto respective plates containing Mueller Hinton agar (MHA) growth media using sterile cotton swabs. A sterile copper borer of 8 mm diameter was used to create wells in the solidified growth medium in the plates. Each well was properly labeled and filled with 100 µL of *J. curcas* seed oil, de-oiled seed extracts and the selected commercially available antibiotics, independently. Ofloxacin was used as a positive control. DMSO (99.9%) and de-ionized water was used as negative control for seed extracts and antibiotics. The inoculated petri plates were left for an hour at room temperature to allow for diffusion of treatments before the bacterial growth commenced. The plates were then incubated at 37°C for 24 h, followed by the measurement of zones of inhibition (ZOI) around the wells. The results of the antibacterial activity of negative control (DMSO or de-ionized water) was subtracted from that of the extracts or antibiotics.

3.3.8. Molecular docking with AutoDock

An AutoDock 4 on Intel was used for molecular docking of chemical compounds detected in methanolic residues by GC-MS analysis. The docking was carried out into the active sites of *Acinetobacter baumannii* UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine (MurF) (MurF) receptor (PDB, ID, 4QDI). A 32 bit operating system Intel Core ™ i5 CPU M 540 @ 2.53 GHz was used and in order to cover the active sites, the grid was set

manually and centrally along the X, Y and Z axis as -29.60, 2.43 and -2.59 with 10 Å dimensions accordingly. All other parameters were kept at default.

3.3.9. Minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) of all treatments (*J. curcas* seed oil, de-oiled seed extracts and antibiotics) were determined using agar well diffusion method. The said treatments were diluted by two-fold dilutions. The seed oil, de-oiled seed extracts and antibiotics dilutions prepared were in the range of 0.097-100 mg/mL and 0.049-100 µg/mL, respectively. A standardized inoculum (5×10^6 CFU.mL^{-1}) of each target bacterial strain was swabbed on the MHA plate, a 100 µL of each treatment was added in the respective well and incubated overnight at 37°C for 24 h. The lowest dilution of each treatment that gave a clear inhibition zone was considered as MIC of the respective treatment. Sterility of solutions was maintained throughout the experiments.

3.3.10. Synergistic antibacterial assay

Synergistic antibacterial potential of each *J. curcas* de-oiled seed extract and its seed oil in combination with ofloxacin, ciprofloxacin, moxifloxacin, cefotaxime and rifampicin was determined against selected bacterial strains by checkerboard method as previously described (Farooqui et al. 2015) with some modifications. The MHA plates were swabbed with standardized (standardized) inoculum (5×10^6 CFU.mL^{-1}) followed by the addition of 100 µL of various serial dilutions of the combination of respective drugs (mixture of 50 µL of *J. curcas* seed oil or de-oiled seed extract and 50 µL of selected antibiotic) in respective wells. The plates were left at room temperature for an hour in order to allow the combined drugs to properly diffuse in media before incubation at 37°C for 24 h. The final seed extract or antibiotic concentration for clinical, MDR and reference strains was about 0.097 to 200 mg/mL or 0.049 to 100 µg/mL, respectively. Fractional inhibitory concentration index (FICI) for each combination was determined using Eq. I.

$$\sum \text{FICI} = \text{FIC}\,[\text{A}] + \text{FIC}\,[\text{B}] \qquad \text{(I)}$$

Where [A] = *J. curcas* seed oil or de-oiled seed extracts; [B] = antibiotics; FIC [A] = MIC of agent A in combination/MIC of agent A alone and FIC [B] = MIC of agent B in combination/MIC of agent B alone

The FICI for each combination was determined as described in the literature (Hossain et al. 2016) and is given as follows: FICI ≤0.5 = Synergy; FICI >0.5 or ≤1 = additive; and FICI >1 or ≤4 = indifference; and FICI >4 = antagonism.

3.3.11. Statistical analysis

All the assays were carried out in triplicate and data was presented as the mean of three independent experiments ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by *Bonferroni's post-test* for multiple comparisons were applied to compare the antimicrobial activities of individual extracts and antibiotics independently as well as in combination using GraphPad Prism Software version 6.0 (La Jolla, CA, USA).

3.4. Results

3.4.1. Chemical characterization of *J. curcas* seed oil and de-oiled seed extracts

In the present study, a number of conventional phytochemical analyses were carried out to elucidate the composition of *J. curcas* seed oil and de-oiled seed cake. Different qualitative chemical tests were performed to investigate the major phytochemical compound/groups present in *J. curcas* de-oiled seed cake and seed oil. During conventional phytochemical screening, different phytochemicals such as flavonoids, tannins saponin glycosides and steroids were found in de-oiled seed cake. Similarly, in seed oil, the phytochemicals such as flavonoids, tannins, glycosides and steroids were found. In addition, characterization of chemical composition of *J. curcas* pressed cake and seed oil was carried out using different spectroscopic techniques. In addition, the analyses of FTIR spectra (Appendix 1 Figures S1-S4) obtained for *J. curcas* de-oiled seed extracts (methanolic, *n*-hexane and aqueous) also revealed the presence of a broad range of compounds such as ester linkages, amide linkages, carbon-hydrogen bonds, aromatic functional groups and carbonyl linkages that correspond to the presence of carbohydrates, cellulose, hemicellulose and lignin components. The corresponding different functional groups present in all extracts of *J. curcas* extracts are shown in Tables S2-S5. FTIR spectroscopy is used to identify specific functional groups present in an organic, polymeric, inorganic compound or other material (Chen et al. 2015).

Moreover, the GC-MS spectrophotometric analyses of *J. curcas* seed oil and de-oiled seed cake extracts (Figure 3.1) showed the presence of a wide range of bioactive compounds. About 16 different saturated and non-saturated long chain fatty acids were determined in *J. curcas* seed oil (Appendix 1 Table S6). Similarly, in *n*-hexane extract, both saturated and unsaturated fatty acids were identified such as oleic acids, 9,12-octadecadienoic acid (Z,Z)-, palmitic acid and myristic acid (Appendix 1 Table S7). Conversely, GC-MS analysis of methanolic extract presented diverse compounds such as I-(+)-ascorbic acid 2,6-dihexadecanoate, 9-hexadecenal, beta-monolaurin, bis (tridecyl) phthalate, 1-docosanol and

diacetone alcohol (Appendix 1 Table S8). The GC-MS analysis of aqueous extract also indicated the presence of different compounds such as 1,4-dithiane, dodecanoic acid methyl ester, methyl tetradecanoate, vitamin D3, methyl ester, palmitic acid, isopropyl linoleate and di-n-octyl phthalate (Appendix 1 Table S9). In addition, the identified structures, areas and heights of the peak are given in Tables S6-S9.

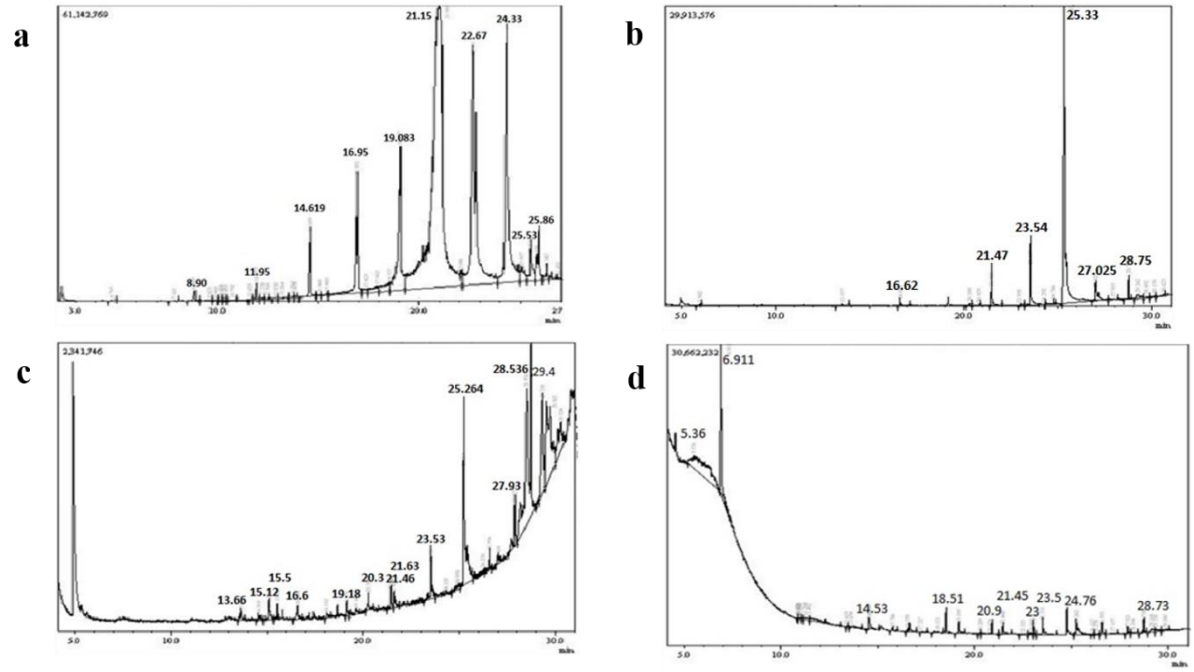

Figure 3.1 Gas chromatography coupled with mass spectrometry chromatogram obtained for *J. curcas* de-oiled pressed cake extracts and seed oil, (a) GC-MS chromatogram obtained for *J. curcas* seed oil, (b) GC-MS chromatogram obtained for *n*-hexane extract of *J. curcas* de-oiled seed cake, (c) GC-MS chromatogram obtained for methanolic extract of *J. curcas* de-oiled seed cake, (d) GC-MS chromatogram obtained for aqueous extract of *J. curcas* de-oiled seed cake.

3.4.2. Antibacterial assay

In the current study, antibacterial activities of *J. curcas* seed oil, de-oiled seed cake extracts, and commercially available antibiotics (ofloxacin, ciprofloxacin, moxifloxacin, cefotaxime and rifampicin) were used against the selected clinical pathogenic isolates, MDR and standard ATCC bacterial strains. In addition, synergistic activities of all these components in combination with aforementioned, antibiotics was also investigated against each selected bacterial strain.

When the extracts and seed oil were individually evaluated against clinical isolates, the methanolic extract exhibited significant ($P < 0.001$) antibacterial activity (ZOI 15 mm) against

S. aureus as compared to those of seed oil, *n*-hexane and aqueous extracts (Figure 3.2a). While for *n*-hexane and aqueous extracts or seed oil, the *S. aureus* was found to be the most resistant bacterial strain with no antibacterial activity against it. Among the clinical isolates, *A. baumannii* strain was found to be susceptible to all extracts but the activity of methanolic extract was significantly higher as compared to that of seed oil ($P < 0.05$) as shown in Figure 3.2a. The ZOI exhibited by seed oil, aqueous, *n*-hexane and methanolic extracts against *A. baumannii* in (Figure 3.2a) were 10, 12, 12 and 13 mm, respectively. Overall, the methanolic extract, against clinical isolates, remained more active than other de-oiled seed extracts and oil. In addition, for ATCC reference strains, the highest antibacterial activity was exhibited by methanolic extract with ZOI 21 mm against *S. aureus* (ATCC 25923) (Figure 3.2a). The antibacterial activity of methanolic extract was more significant ($P < 0.001$) than *n*-hexane, aqueous extract and seed oil against *S. aureus* (ATCC 25923).

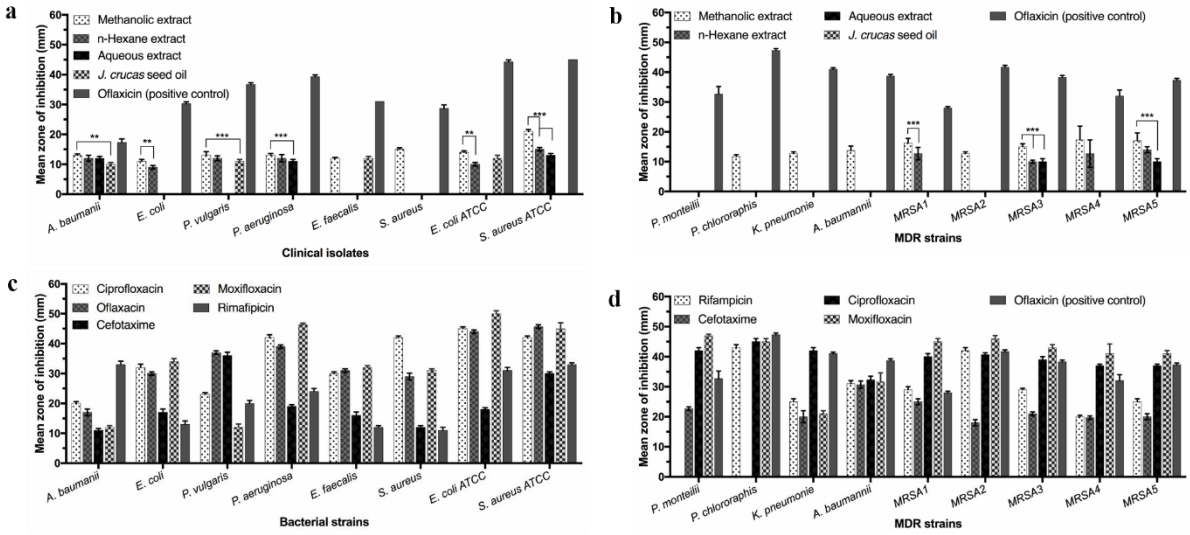

Figure 3.2 Antibacterial activities of *J. curcas* de-oiled pressed cake extracts (200 mg/mL DMSO), seed oil (200 mg/mL DMSO) and commercially available antibiotics (100 µg/mL in de-ionized water) against clinical, MDR and ATCC bacterial strains. **(a)** Antibacterial activities of *J. curcas* de-oiled pressed cake extracts and seed oil against clinical and ATCC bacterial strains, **(b)** Antibacterial activities of *J. curcas* seed oil and de-oiled seed cake extracts against selected MDR strains, **(c)** Antibacterial activities of commercially available antibiotics against selected clinical and ATCC bacterial isolates, **(d)** Antibacterial activities of commercially available antibiotics against selected MDR bacterial isolates. 100 µL of solution of each extract, seed oil, antibiotic and positive control (ofloxacin) was added to the respective wells punched in MHA plates, pre-swabbed with respective clinical isolates and incubated at 37°C for 24 h. All the tests were carried out in triplicate. The mean zones of inhibition (mm) created by the

respective treatments against each bacterial strain were recorded. The bigger the mean zone of inhibition (mm), the higher was considered the susceptibility of bacterial strains. Data presented are means of three independent experiments ± SD. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ (for comparisons of all treatments by one-way ANOVA, followed by Bonferroni's post test).

Similarly, in case of MDR strains, the highest antibacterial activity was exhibited by methanolic extract (Figure 3.2b) with ZOI ~17 mm against methicillin resistant *S. aureus* (MRSA4 and MRSA5) strains and was found significantly higher ($P < 0.001$) than that exhibited by seed oil and aqueous extract. The *n*-hexane extract was found second most active extract with ZOI of 13 mm against MRSA5 (Figure 3.2 b) and was significantly more active ($P < 0.001$) than seed oil. Overall, methanolic extract exhibited the most potent antibacterial activities (Figure 3.2 b) compared to seed oil, *n*-hexane and aqueous extracts against MDR strains. *J. curcas* seed oil did not exhibit any antibacterial activity against any MDR strain. The most resistant MDR strain was *P. monteilii* against which none of the extracts and seed oil exhibited any antibacterial activity.

In case of antibiotics for clinical isolates, the highest antibacterial activity was exhibited by moxifloxacin with a ZOI ~46 mm against *P. aeruginosa* (Figure 3.2 c) and was significantly higher ($P < 0.001$) than that of ciprofloxacin, cefotaxime, rifampicin and ofloxacin (control drug). Moxifloxacin exhibited comparatively more potent antibacterial activities against all clinical strains except *P. vulgaris* and *A. baumannii* (Figure 3.2 c). Rifampicin and cefotaxime had the least potent antibacterial activities against all selected clinical isolates (Figure 3.2 c) with the only exception of *A. baumannii* and *P. vulgaris* against which rifampicin and cefotaxime exhibited higher antibacterial activities with ZOI 33 and 36 mm, respectively. Rifampicin exhibited significantly higher ($P < 0.001$) antibacterial activity compared to other antibiotics against *A. baumannii*. Moxifloxacin and ciprofloxacin mostly exhibited potent antibacterial activities showing slight variation with ofloxacin (positive control) against clinical isolates. Conversely, in reference strains, moxifloxacin exhibited significantly higher ($P < 0.001$) antibacterial activity compared to all other antibiotics against *E. coli* (ATCC 25922) strain.

Against MDRs, among the test drugs, moxifloxacin was the most potent antibiotic exhibiting the highest antibacterial activity with ZOI 47 mm against *P. monteilii* (Figure 3.2 d) and was significantly higher ($P < 0.001$) compared to all other antibiotics except ciprofloxacin. Overall, ofloxacin (positive control) was the most potent one and exhibited slightly higher antibacterial activity than moxifloxacin and ciprofloxacin with ZOI ~47.33 mm against *P.*

chlororaphis. Cefotaxime was found to be the least potent among other antibiotics against MDRs (Figure 3.2 d).

3.4.3. Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of a drug that inhibits the growth of a specific microorganism.

Table 3.1 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of methanolic extract and each antibiotic and their combination against clinical pathogenic and reference bacterial isolates.

		MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		FIC	FICI	Output
Strain	Compound	Alone	Combined			
A. baumannii	Me/Ctx	100/0.19	0.39/0.195	0.0039/1	1.0	Indifference
	Me/R	100/12.5	12.5/0.39	0.125/0.031	0.15	Synergistic
	Me/Of	100/12.5	12.5/6.25	0.125/0.5	0.6	Indifference
	Me/Cip	100/6.25	12.5/6.25	0.125/1	1.12	Indifference
	Me/Mox	100/6.25	6.25/3.125	0.0625/0.5	0.5	Synergistic
E. coli	Me/Ctx	100/100	100/50	1/1	2.0	Indifference
	Me/R	100/100	1.56/0.78	0.0625/0.0156	0.0781.0	Synergistic
	Me/Of	100/50	0.39/3.125	0.0039/0.0625	0.06	Synergistic
	Me/Cip	100/100	6.25/0.195	0.0625/0.0039	0.0664	Synergistic
	Me/Mox	100/3.125	25/12.5	0.25/8.01	4.25	Antagonistic
E. faecalis	Me/Ctx	100/50	100/50	1/1	2.0	Indifference
	Me/R	100/100	6.25/3.125	0.0625/0.031	0.09	Synergistic
	Me/Of	100/12.5	25/0.39	0.25/0.0312	0.28	Synergistic
	Me/Cip	100/50	0.78/12.5	0.0078/0.25	0.25	Synergistic
	Me/Mox	100/1.56	25/12.5	0.25/8.01	8.26	Antagonistic
P. vulgaris	Me/Ctx	100/0.19	12.5/6.25	0.125/32.05	32.17	Antagonistic
	Me/R	100/6.25	12.5/6.25	0.125/1	1.125	Indifference
	Me/Of	100/12.5	12.5/3.125	0.125/0.25	0.375	Synergistic
	Me/Cip	100/6.25	6.25/6.25	0.0625/1	1.062	Indifference
	Me/Mox	100/6.25	100/50	1/8	8.26	Antagonistic
S. aureus	Me/Ctx	100/50	0.78/0.39	0.0078/0.0078	0.0156	Synergistic
	Me/R	100/6.25	0.39/0.195	0.0039/0.0312	0.0351	Synergistic
	Me/Of	100/50	0.39/1.56	0.0039/64.10	64.106	antagonistic
	Me/Cip	100/12.5	3.125/0.39	0.0312/0.0312	0.062	Synergistic
	Me/Mox	100/3.125	3.125/1.56	0.0312/0.49	0.53	Synergistic
P. aeruginosa	Me/Ctx	100/50	50/3.125	0.06/0.06	0.1	Synergistic
	Me/R	100/6.25	100/1.56	0.03/0.2	0.2	Synergistic
	Me/Of	100/12.5	25/50	0.003/4	4.0039	Antagonism
	Me/Cip	100/3.125	100/0.39	1/0.1	1.1	Indifference
	Me/Mox	100/0.39	25/3.125	0.06/8	8	Antagonistic
***S. aureus* (ATCC)**	Me/Ctx	50/50	0.78/0.39	0.008/256	0.023	Synergistic
	Me/R	50/6.25	0.39/0.195	0.007/0.03	0.03	Synergistic
	Me/Of	50/3.125	0.39/0.195	0.007/0.06	0.07	Synergistic
	Me/Cip	50/1.56	1.56/0.78	0.03/0.5	0.5	Synergistic
	Me/Mox	50/0.78	12.5/6.25	0.12/8	8.12	Antagonistic
***E. coli* (ATCC)**	Me/Ctx	100/0.78	0.78/0.39	0.007/0.5	0.5	Synergistic
	Me/R	100/6.25	1.56/0.78	0.01/0.12	0.1	Synergistic
	Me/Of	100/3.125	0.097/0.048	0.0009/0.01	0.01	Synergistic
	Me/Cip	100/3.125	0.78/0.39	0.007/0.12	0.1	Synergistic
	Me/Mox	100/0.39	12.5/6.25	0.12/16	16	Antagonistic

MIC(a); minimal inhibitory concentration for methanolic extract when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

MIC of *J. curcas* seed oil and de-oiled seed cake extracts and each of the selected antibiotics against the selected clinical, MDR and reference strains are given in Tables 3.1-3.8. When screened individually, methanolic extract exhibited higher antibacterial potential with lower concentration compared to seed oil, *n*-hexane and aqueous extracts against various clinical, MDR and reference bacterial strains.

Table 3.2 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of *n*-hexane extract, each antibiotic and their combination against clinical pathogenic and reference bacterial isolates

Strain	Compound	MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		FIC	FICI	Output
		Alone	Combined			
A. baumannii	n-hex/Ctx	100/0.19	1.56/0.78	0.01/4.11	4.1	Antagonistic
	n-hex/R	100/6.25	12.5/6.25	0.1/1	1.12	Indifference
	n-hex /Of	100/12.5	1.56/0.78	0.01/0.06	0.07	Synergistic
	n-hex /Cip	100/6.25	1.56/0.78	0.01/0.1	0.1	Synergistic
	n-hex /Mox	100/6.25	12.5/6.25	0.1/1	1.12	Indifference
E. coli	n-hex/Ctx	100/100	3.125/1.56	0.03/0.01	0.04	Synergistic
	n-hex/R	100/100	0.78/0.39	0.007/0.003	0.01	Synergistic
	n-hex /Of	100/50	100/50	1/1	2.0	Indifference
	n-hex /Cip	100/100	1.56/0.78	0.01/0.007	0.02	Synergistic
	n-hex /Mox	100/3.125	25/12.5	0.25/4	4.25	Antagonistic
E. faecalis	n-hex/Ctx	-/50	100/50	-/1	ND	ND
	n-hex/R	-/100	100/50	-/0.5	ND	ND
	n-hex /Of	-/12.5	100/50	-/4	ND	ND
	n-hex /Cip	-/50	6.25/3.125	-/0.002	ND	ND
	n-hex /Mox	-/1.56	25/12.5	-/8.01	ND	ND
P. vulgaris	n-hex/Ctx	100/0.19	1.56/0.78	0.01/0.1	0.1	Synergistic
	n-hex/R	100/6.25	0.78/0.39	0.007/0.06	0.07	Synergistic
	n-hex /Of	100/12.5	0.78/0.39	0.007/0.03	0.03	Synergistic
	n-hex /Cip	100/6.25	0.78/0.39	0.007/0.06	0.07	Synergistic
	n-hex /Mox	100/6.25	100/50	1/8	9.0	Antagonistic
S. aureus	n-hex/Ctx	-/50	-/-	-/-	ND	ND
	n-hex/R	-/6.25	100/50	-/8	ND	ND
	n-hex /Of	-/50	100/50	-/1	ND	ND
	n-hex /Cip	-/12.5	-/-	-/-	ND	ND
	n-hex /Mox	-/3.125	12.5/6.25	-/2	ND	ND
P. aeruginosa	n-hex/Ctx	200/50	12.5/6.25	0.06/0.1	0.1	Synergistic
	n-hex/R	200/6.25	3.125/1.56	0.01/0.2	0.2	Synergistic
	n-hex /Of	200/12.5	6.25/3.125	0.03/0.25	0.2	Synergistic
	n-hex /Cip	200/3.125	3.125/1.56	0.03/0.4	0.5	Synergistic
	n-hex /Mox	200/0.39	6.25/3.125	0.03/8.01	8.04	Antagonistic
S. aureus	n-hex/Ctx	100/100	25/12.5	0.25/8	8.25	Antagonistic
	n-hex/R	100/6.25	6.25/3.125	0.06/0.5	0.5	Synergistic
	n-hex /Of	100/3.125	100/50	1/16	17	Antagonistic
	n-hex /Cip	100/1.56	0.097/0.048	0.0009/0.03	0.03	Synergistic
	n-hex /Mox	100/0.78	6.25/3.125	0.06/4	4.06	Antagonistic
E. coli (ATCC)	n-hex/Ctx	100/0.78	3.125/1.56	0.03/0.5	0.5	Synergistic
	n-hex/R	100/6.25	0.39/0.195	0.003/0.3	0.03	Synergistic
	n-hex /Of	100/3.12	0.39/0.195	0.003/0.06	0.06	Synergistic
	n-hex /Cip	100/3.12	0.78/0.39	0.007/0.1	0.1	Synergistic
	n-hex /Mox	100/0.39	6.25/3.125	0.06/8	8.0	Antagonistic

MIC(a); minimal inhibitory concentration for *n*-hexane extract when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

Individually, methanolic extract was found to be the most potent with the least MIC 50 mg/mL against methicillin resistant *S. aureus* (MRSA1, MRSA2 and MRSA3) and *S. aureus* ATCC25923 strains (Table 3.5). A similar activity (least MIC ~50 mg/mL) was observed for seed oil only against MRSA1 strain (Table 3.8).

Table 3.3 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of aqueous extract, each antibiotic and their combination against clinical pathogenic and reference bacterial isolates

		MIC[(a)] (µg/mL)	**(mg/mL)/MIC[(b)]**	**FIC**	**FICI**	**Output**
Strain	Compound	Alone	Combined			
A. baumannii	Aq/Ctx	200/0.19	0.195/0.097	0.0009/0.5	0.5	Synergistic
	Aq /R	200/6.25	1.56/0.78	0.007/0.12	0.1	Synergistic
	Aq /Of	200/12.5	3.125/1.56	0.03/0.12	0.1	Synergistic
	Aq /Cip	200/6.25	1.56/0.78	0.007/0.12	0.1	Synergistic
	Aq /Mox	200/6.25	12.5/6.25	0.12/1	1.125	Indifference
E. coli	Aq /Ctx	-/100	100/50	-/0.5	ND	ND
	Aq /R	-/100	3.125/1.56	-/0.01	ND	ND
	Aq /Of	-/50	6.25/3.125	-0.06	ND	ND
	Aq /Cip	-/100	6.25/3.125	-/0.03	ND	ND
	Aq /Mox	-/3.125	25/12.5	-/4	ND	ND
E. faecalis	Aq /Ctx	-/50	100/50	-/1	ND	ND
	Aq/R	-/100	1.56/0.78	-/0.007	ND	ND
	Aq /Of	-/12.5	1.56/0.78	-/0.06	ND	ND
	Aq /Cip	-/50	3.125/1.56	-/0.03	ND	ND
	Aq /Mox	-/1.56	50/25	-/16	ND	ND
P. vulgaris	Aq/Ctx	-/0.19	6.25/3.125	-/0.5	ND	ND
	Aq/R	-/6.25	6.25/3.125	-/0.5	ND	ND
	Aq /Of	-/12.5	0.78/0.39	-/0.03	ND	ND
	Aq /Cip	-/6.25	0.78/0.39	-/0.06	ND	ND
	Aq /Mox	-/6.25	-/-	-/-	ND	ND
S. aureus	Aq/Ctx	-/50	100/50	-/1	ND	ND
	Aq/R	-/6.25	100/50	-/8	ND	ND
	Aq /Of	-/100	6.25/3.125	-/0.25	ND	ND
	Aq /Cip	-/12.5	50/25	-/2	ND	ND
	Aq /Mox	-/3.125	50/25	-/8	ND	ND
P. aeruginosa	Aq/Ctx	200/50	100/50	0.5/1	1.5	Indifference
	Aq/R	200/6.25	12.5/6.25	0.06/1	1.06	Indifference
	Aq /Of	200/12.5	12.5/6.25	0.06/0.5	0.5	Synergistic
	Aq /Cip	200/3.125	25/12.5	0.12/4	4.1	Antagonistic
	Aq /Mox	200/0.39	6.25/3.125	0.03/8	8.0	Antagonistic
S. aureus (ATCC)	Aq/Ctx	200/100	100/50	0.5/0.5	1.0	Indifference
	Aq/R	200/6.25	100/50	0.5/8	8.5	Antagonistic
	Aq /Of	200/3.12	3.125/1.56	0.5/0.4	0.9	Additive
	Aq /Cip	200/1.56	12.5/6.25	0.06/4	4.06	Antagonistic
	Aq /Mox	200/0.78	12.5/6.25	0.06/8	8.0	Antagonistic
E. coli (ATCC)	Aq/Ctx	-/0.78	1.56/0.78	-/1	ND	ND
	Aq/R	-/6.25	1.56/0.78	-/0.1	ND	ND
	Aq /Of	-/3.12	3.125/1.56	-/0.4	ND	ND
	Aq /Cip	-/3.12	1.56/0.78	-/0.2	ND	ND
	Aq /Mox	-/0.39	12.5/6.25	-/16	ND	ND

MIC(a); minimal inhibitory concentration for aqueous extract when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

On the other hand, seed oil and aqueous extract were determined as mostly inactive at any concentration against clinical, MDR bacterial strains shown in (Table 3.3, 3.4, 3.7 and 3.8).

Even the aqueous extract did not exhibit any activity against *E. coli* ATCC25922 reference strain. The MICs for *J. curcas* seed oil, methanolic, *n*-hexane and aqueous extracts were found in 50 to 200 mg/mL range against clinical, MDR and reference bacterial strains (Tables 3.1-3.8).

Table 3.4 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of seed oil each antibiotic and their combination against clinical pathogenic and reference bacterial isolates

		MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		**FIC**	**FICI**	**Output**
Strain	Compound	Alone	Combined			
A. baumannii	Oil/Ctx	100/0.19	0.39/0.195	0.0039/1.026	1.03	Synergistic
	Oil/R	100/6.25	0.78/0.39	0.0078/0.062	0.07	Antagonistic
	Oil /Of	100/12.5	6.25/3.125	0.25/0.25	0.5	Synergistic
	Oil /Cip	100/6.25	6.25/3.125	0.031/0.5	0.5	Synergistic
	Oil /Mox	100/6.25	50/25	0.5/2	4.5	Antagonistic
E. coli	Oil/Ctx	200/100	6.25/3.125	0.031/0.031	0.06	Synergistic
	Oil/R	200/100	0.78/0.39	0.0039/0.0039	0.0078	Synergistic
	Oil /Of	200/50	0.78/12.5	0.125/0.25	0.3	Synergistic
	Oil /Cip	200/100	25/0.195	0.0078/0.0039	0.01	Synergistic
	Oil /Mox	200/3.125	12.5/6.25	0.125/2	2.12	Indifference
E. faecalis	Oil/Ctx	100/50	1.56/0.78	0.015/0.015	0.03	Synergistic
	Oil/R	100/100	1.56/0.78	0.015/0.0078	0.02	Synergistic
	Oil /Of	100/12.5	1.56/1.56	0.015/0.124	0.1	Synergistic
	Oil /Cip	100/50	1.56/0.78	0.015/0.015	0.03	Synergistic
	Oil /Mox	100/1.56	1.56/3.125	0.06/2	2.0	Indifference
P. vulgaris	Oil/Ctx	100/0.19	0.39/0.195	0.0039/0.03	0.03	Synergistic
	Oil/R	100/6.25	3.125/1.56	0.03/0.24	0.2	Synergistic
	Oil /Of	100/12.5	3.125/0.195	0.03/0.015	0.04	Synergistic
	Oil /Cip	100/6.25	0.78/1.56	-/0.24	ND	ND
	Oil /Mox	100/6.25	100/50	-/16	ND	ND
S. aureus	Oil/Ctx	-/50	3.125/1.56	-/0.03	ND	ND
	Oil/R	-/6.25	1.56/0.78	-/0.12	ND	ND
	Oil /Of	-/100	1.56/0.78	-/0.06	ND	ND
	Oil /Cip	-/12.5	0.195/0.097	0.0009/0.0077	0.008	Synergistic
	Oil /Mox	-/3.125	12.5/6.25	0.03/2	2.0	Indifference
P. aeruginosa	Oil/Ctx	200/50	0.39/0.195	0.001/0.0039	0.005	Synergistic
	Oil/R	200/6.25	12.5/6.25	0.06/1	1.0	Indifference
	Oil /Of	200/12.5	6.25/3.125	0.03/0.1	0.1	Synergistic
	Oil /Cip	200/3.125	3.125/3.125	2/1	3.0	Indifference
	Oil /Mox	200/0.39	3.125/1.56	0.25/4	4.2	Antagonistic
S. aureus(ATCC)	Oil/Ctx	-/100	0.78/0.39	-/0.003	ND	ND
	Oil/R	-/6.25	0.195/0.097	-/0.01	ND	ND
	Oil /Of	-/3.125	0.78/0.39	-/0.12	ND	ND
	Oil /Cip	-/1.56	0.195/0.0485	-/0.03	ND	ND
	Oil /Mox	-/0.781	3.125/1.56	-/1.99	ND	ND
E. coli (ATCC)	Oil/Ctx	100/0.781	0.78/0.39	0.0078/0.5	0.5	Synergistic
	Oil/R	100/6.25	0.78/0.39	0.0078/0.06	0.07	Synergistic
	Oil /Of	100/3.125	0.095/0.0485	0.00097/0.015	0.01	Synergistic
	Oil /Cip	100/3.125	0.095/0.0485	0.0097/0.01	0.01	Synergistic
	Oil /Mox	100/0.39	6.25/3.125	0.06/8.01	8.0	Antagonistic

MIC(a); minimal inhibitory concentration for seed oil when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

Among all the antibiotics, when evaluated for their antibacterial activity individually, cefotaxime was found to be the most potent, with MIC value of 0.195 µg/mL against clinical

isolates, including *A. baumannii* and *P. vulgaris* (Tables 3.1-3.4). On the other hand, rifampicin was found to be the least potent drug, exhibiting no antibacterial activity at MIC of less than 3.125 µg/mL against any bacterial strain (Tables 3.1-3.8).

Table 3.5 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of methanolic extract, each antibiotic alone and their combination against MDR pathogenic bacterial isolates

Strain	Compound	MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		FIC	FICI	Output
		Alone	combined			
MRSA1	Me/Ctx	50/100	50/25	0.5/2	2.5	Indifference
	Me/R	50/100	100/50	0.5/2	2.5	Indifference
	Me/Of	50/100	100/50	0.5/2	2.5	Indifference
	Me/Cip	50/50	25/12.5	0.5/0.25	0.75	Additive
	Me/Mox	50/1.56	25/12.5	0.5/8.01	8.51	Antagonistic
MRSA2	Me/Ctx	50/100	50/25	1/4	5.0	Antagonistic
	Me/R	50/3.125	1.56/0.78	0.03/0.24	0.28	Synergistic
	Me/Of	50/25	100/50	2/2	4.0	Indifference
	Me/Cip	50/50	50/25	1/0.5	1.5	Indifference
	Me/Mox	50/0.781	12.5/6.25	0.25/8	8.25	Antagonistic
MRSA3	Me/Ctx	50/50	50/25	1/0.5	1.5	Indifference
	Me/R	50/3.125	0.19/0.09	0.003/0.006	0.01	Synergistic
	Me/Of	50/50	50/25	1/0.5	1.5	Indifference
	Me/Cip	50/12.5	25/12.5	0.5/1	1.5	Indifference
	Me/Mox	50/0.39	3.125/1.56	0.06/4	4.06	Antagonistic
MRSA4	Me/Ctx	100/50	1.56/0.78	0.01/0.01	0.03	Synergistic
	Me/R	100/3.125	0.78/0.39	0.007/0.12	0.13	Synergistic
	Me/Of	100/1.56	0.78/0.78	0.007/0.5	0.5	Synergistic
	Me/Cip	100/3.125	1.56/0.39	0.01/0.12	0.14	Synergistic
	Me/Mox	100/0.781	25/12.5	0.25/16	16.25	Antagonistic
MRSA5	Me/Ctx	100/3.125	100/50	1/16	17	Antagonistic
	Me/R	100/3.125	0.78/0.39	0.007/0.12	0.13	Synergistic
	Me/Of	100/100	50/25	0.5/4	4.5	Antagonistic
	Me/Cip	100/50	50/25	0.5/0.5	1.0	Indifference
	Me/Mox	100/1.56	6.25/3.125	0.06/2	2.06	Indifference
***A. baumannii* MDR**	Me/Ctx	100/6.25	3.12/1.56	0.03/0.24	0.2	Synergistic
	Me/R	100/3.125	0.39/0.195	0.003/0.06	0.06	Synergistic
	Me/Of	100/0.78	1.56/0.78	0.01/1	1.01	Indifference
	Me/Cip	100/6.25	0.39/0.195	0.003/0.03	0.03	Synergistic
	Me/Mox	100/1.56	6.25/3.125	0.06/2	2.0	Indifference
K. pneumoniae	Me/Ctx	100/100	100/50	1/2	3.0	Indifference
	Me/R	100/100	50/25	0.5/0.25	0.75	Additive
	Me/Of	100/25	50/25	0.5/0.03	0.5	Synergistic
	Me/Cip	100/50	50/25	0.5/0.003	0.5	Synergistic
	Me/Mox	100/1.56	12.5/6.25	0.12/4	4.13	Antagonistic
P. chlororaphis	Me/Ctx	100/-	25/12.5	0.25/-	ND	ND
	Me/R	100/100	25/12.5	0.25/0.125	0.38	Synergistic
	Me/Of	100/6.25	100/50	1/0.12	1.12	Indifference
	Me/Cip	100/3.125	12.5/6.25	0.12/0.06	0.18	Synergistic
	Me/Mox	100/1.56	3.125/1.56	0.03/1	1.03	Indifference
P. monteilii	Me/Ctx	-/100	0.39/0.195	-/512	ND	ND
	Me/R	-/-	0.39/0.195	-/-	ND	ND
	Me/Of	-/100	0.78/0.39	-/256	ND	ND
	Me/Cip	-/50	0.39/0.195	-/0.003	ND	ND
	Me/Mox	-/0.78	100/50	-/64	ND	ND

MIC(a); minimal inhibitory concentration for de-oiled seed extract when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

Overall, for antibiotics when applied individually, MICs were found to be in the range of 0.19 to 100 µg/mL against various bacterial strains (Tables 3.1-3.8).

Table 3.6 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of *n*-hexane extract, each antibiotic alone and their combination against MDR pathogenic bacterial isolates

		MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		FIC	FICI	Output
Strain	Compound	Alone	combined			
MRSA1	n-hex/Ctx	100/100	12.5/6.25	0.1/0.06	0.1	Synergistic
	n-hex/R	100/100	100/50	1/0.5	1.5	Indifference
	n-hex /Of	100/100	100/50	1/0.5	1.5	Indifference
	n-hex /Cip	100/50	100/50	2/1	3	Indifference
	n-hex	100/1.56	100/50	1/32	33.05	Antagonistic
MRSA2	n-hex/Ctx	-/100	6.25/3.125	-/0.03	ND	ND
	n-hex/R	-/3.125	12.5/6.25	-/2	ND	ND
	n-hex /Of	-/25	1.56/0.78	-/0.03	ND	ND
	n-hex /Cip	-/50	0.39/0.195	0.007/0.003	0.01	Synergistic
	n-hex	-/0.78	25/12.5	-/16	ND	ND
MRSA3	n-hex/Ctx	100/50	25/12.5	0.25/0.25	0.5	Synergistic
	n-hex/R	100/3.125	1.56/0.78	0.01/0.2	0.2	Synergistic
	n-hex /Of	100/50	100/50	0.01/1	1.01	Indifference
	n-hex /Cip	100/12.5	100/50	2/4	6.0	Antagonistic
	n-hex	100/0.39	6.25/3.125	0.1/8	8.13	Antagonistic
MRSA4	n-hex/Ctx	100/50	12.5/6.25	0.1/0.1	0.25	Synergistic
	n-hex/R	100/3.125	3.125/1.56	0.03/0.4	0.5	Synergistic
	n-hex /Of	100/1.56	1.56/0.78	1/0.5	1.5	Indifference
	n-hex /Cip	100/3.125	1.56/0.78	0.01/0.2	0.2	Synergistic
	n-hex	100/0.78	100/50	1/64	65	Antagonistic
MRSA5	n-hex/Ctx	100/3.125	6.25/3.125	0.06/1	1.06	Indifference
	n-hex/R	100/3.125	0.39/0.195	0.003/0.06	0.06	Synergistic
	n-hex /Of	100/100	100/50	0.01/0.5	0.5	Synergistic
	n-hex /Cip	100/50	100/50	1/1	2.0	Indifference
	n-hex	100/1.56	6.253.125	0.6/2	2.0	Indifference
***baumannii* MDR**	n-hex/Ctx	-/6.25	3.125/1.56	-/0.2	ND	ND
	n-hex/R	-/3.125	0.39/0.195	-/0.06	ND	ND
	n-hex /Of	-/0.78	1.56/0.78	-/1	ND	ND
	n-hex /Cip	-/6.25	1.56/0.78	-/0.1	ND	ND
	n-hex	-/1.56	6.25/3.125	-/2	ND	ND
K. pneumoniae	n-hex/Ctx	-/100	100/50	-/0.5	ND	ND
	n-hex/R	-/100	100/50	-/0.5	ND	ND
	n-hex /Of	-/25	100/50	-/2	ND	ND
	n-hex /Cip	-/50	100/50	-/1	ND	ND
	n-hex	-/1.56	12.5/6.25	-/4	ND	ND
P. chlororaphis	n-hex/Ctx	-/-	-/50	-/-	ND	ND
	n-hex/R	-/100	100/50	-/0.5	ND	ND
	n-hex /Of	-/6.25	100/50	-/8	ND	ND
	n-hex /Cip	-/3.125	50/25	-/8	ND	ND
	n-hex	-/1.56	12.5/6.25	-/4	ND	ND
P. monteilii	n-hex/Ctx	-/100	0.78/0.39	-/-	ND	ND
	n-hex/R	-/-	0.78/0.39	-/-	ND	ND
	n-hex /Of	-/100	1.56/0.78	-/0.007	ND	ND
	n-hex /Cip	-/50	1.56/0.78	-/0.01	ND	ND
	n-hex	-/0.78	100/50	-/64	ND	ND

MIC(a); minimal inhibitory concentration for *n*-hexane extract when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

Table 3.7 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of aqueous extract, each antibiotic alone and their combination against MDR pathogenic bacterial isolates

		MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		FIC	FICI	Output
Strain	Compound	Alone	combined			
MRSA1	Aq/Ctx	-/100	50/25	-/0.25	ND	ND
	Aq/R	-/100	1.56/0.78	-/0.007	ND	ND
	Aq /Of	-/100	100/50	-/0.5	ND	ND
	Aq /Cip	-/50	100/50	-/1	ND	ND
	Aq /Mox	-/1.56	100/50	-/32	ND	ND
MRSA2	Aq/Ctx	-/100	100/50	-/0.5	ND	ND
	Aq/R	-/3.125	0.78/0.39	-/0.1	ND	ND
	Aq /Of	-/25	100/50	-/2	ND	ND
	Aq /Cip	-/50	100/50	-/1	ND	ND
	Aq /Mox	-/0.78	25/12.5	-/16	ND	ND
MRSA3	Aq/Ctx	200/50	100/50	0.5/1	1.5	Indifference
	Aq/R	200/3.13	0.39/0.195	0.001/0.06	0.06	Synergistic
	Aq /Of	200/50	100/50	0.5/1	1.5	Indifference
	Aq /Cip	200/12.5	100/50	2/4	6	Antagonistic
	Aq /Mox	200/0.39	12.5/6.25	0.25/16	16.2	Antagonistic
MRSA4	Aq/Ctx	-/50	100/50	-/1	ND	ND
	Aq/R	-/3.125	100/50	-/16	ND	ND
	Aq /Of	-/1.56	100/50	-/32	ND	ND
	Aq /Cip	-/3.125	100/50	-/16	ND	ND
	Aq /Mox	-/0.78	50/25	-/32	ND	ND
MRSA5	Aq/Ctx	200/3.13	100/50	0.5/16	16.5	Antagonistic
	Aq/R	200/3.13	6.25/3.125	0.03/1	1.03	Indifference
	Aq /Of	200/100	12.5/6.25	0.03/0.06	0.09	Synergistic
	Aq /Cip	200/50	12.5/6.25	0.06/0.1	0.18	Synergistic
	Aq /Mox	200/1.56	6.25/3.125	0.06/2	2.0	Indifference
***A. baumannii* MDR**	Aq/Ctx	-/6.25	100/50	-/8	ND	ND
	Aq/R	-/3.125	0.78/0.39	-/0.1	ND	ND
	Aq /Of	-/0.78	100/50	-/64	ND	ND
	Aq /Cip	-/6.25	100/50	-/8	ND	ND
	Aq /Mox	-/1.56	6.25/3.125	-/2	ND	ND
Klebsiella	Aq/Ctx	-/100	100/50	-/0.5	ND	ND
	Aq/R	-/100	100/50	-/0.5	ND	ND
	Aq /Of	-/25	100/50	-/2	ND	ND
	Aq /Cip	-/50	100/50	-/1	ND	ND
	Aq /Mox	-/1.56	12.5/6.25	-/4	ND	ND
P. chlororaphis	Aq/Ctx	-/-	-/-	-/-	ND	ND
	Aq/R	-/100	100/50	-/0.5	ND	ND
	Aq /Of	-/6.25	50/25	-/4	ND	ND
	Aq /Cip	-/3.125	50/25	-/8	ND	ND
	Aq /Mox	-/1.56	6.25/3.125	-/2	ND	ND
P. monteilii	Aq/Ctx	-/100	100/50	-/0.5	ND	ND
	Aq/R	-/-	1.56/0.78	-/-	ND	ND
	Aq /Of	-/100	1.56/0.78	-/0.007	ND	ND
	Aq /Cip	-/50	0.78/0.39	-/0.007	ND	ND
	Aq /Mox	-/0.78	100/50	-/64	ND	ND

MIC(a); minimal inhibitory concentration for aqueous extract when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

Table 3.8 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of seed oil, each antibiotic alone and their combination against MDR pathogenic bacterial isolates

		MIC[(a)] (mg/mL)/MIC[(b)] (µg/mL)		FIC	FICI	Output
Strain	Compound	Alone	combined			
MRSA1	Oil/Ctx	50/100	100/50	2/0.5	2.5	Indifference
	Oil/R	50/100	100/50	-/0.5	ND	ND
	Oil /Of	50/100	3.125/1.56	0.06/0.01	0.07	Synergistic
	Oil /Cip	50/50	100/50	-/1	ND	ND
	Oil /Mox	50/1.56	25/12.5	-/8.01	ND	ND
MRSA2	Oil/Ctx	-/100	25/12.5	-/0.1	ND	ND
	Oil/R	-/3.125	25/12.5	-/4	ND	ND
	Oil /Of	-/25	1.56/0.78	-/0.06	ND	ND
	Oil /Cip	-/50	1.56/0.78	-/0.03	ND	ND
	Oil /Mox	-/0.781	6.25/3.125	-/4	ND	ND
MRSA3	Oil/Ctx	-/50	3.125/1.56	-/0.03	ND	ND
	Oil/R	-/3.125	3.125/1.56	-/0.03	ND	ND
	Oil /Of	-/50	50/25	-/0.0078	ND	ND
	Oil /Cip	-/12.5	25/12.5	-/0.03	ND	ND
	Oil /Mox	-/0.39	3.125/1.56	-/8.01	ND	ND
MRSA4	Oil/Ctx	-/50	0.39/0.195	-/0.003	ND	ND
	Oil/R	-/3.125	3.125/1.56	-/0.49	ND	ND
	Oil /Of	-/1.56	1.56/0.78	-/0.5	ND	ND
	Oil /Cip	-/3.125	3.125/1.56	-/0.49	ND	ND
	Oil /Mox	-/0.781	100/50	-/1.99	ND	ND
MRSA5	Oil/Ctx	-/3.125	50/25	-/8	ND	ND
	Oil/R	-/3.125	0.78/0.39	-/0.12	ND	ND
	Oil /Of	-/100	12.5/6.25	-/0.06	ND	ND
	Oil /Cip	-/50	100/50	-/1	ND	ND
	Oil /Mox	-/1.56	6.25/3.125	-/32	ND	ND
***A. baumannii* MDR**	Oil/Ctx	-/6.25	3.125/1.56	-/0.24	ND	ND
	Oil/R	-/3.125	0.195/0.097	-/0.03	ND	ND
	Oil /Of	-/0.78	0.78/0.3	-/0.5	ND	ND
	Oil /Cip	-/6.25	0.78/0.39	-/0.06	ND	ND
	Oil /Mox	-/1.56	3.125/1.56	-/1	ND	ND
K. pneumoniae	Oil/Ctx	-/100	100/50	-/0.5	ND	ND
	Oil/R	-/100	100/50	-/0.5	ND	ND
	Oil /Of	-/25	100/50	-/2	ND	ND
	Oil /Cip	-/50	12.5/6.25	-/0.12	ND	ND
	Oil /Mox	-/1.56	6.25/3.125	-/2	ND	ND
P. chlororaphis	Oil/Ctx	-/-	-/-	-/-	ND	ND
	Oil/R	-/100	1.56/0.78	-/0.0078	ND	ND
	Oil /Of	-/6.25	100/50	-/8	ND	ND
	Oil /Cip	-/3.125	3.125/1.56	-/0.4	ND	ND
	Oil /Mox	-/1.56	6.253.125	-/2	ND	ND
P. monteilii	Oil/Ctx	-/100	6.25/3.125	-/0.03	ND	ND
	Oil/R	-/-	0.78/0.39	-/-	ND	ND
	Oil /Of	-/100	0.78/0.39	-/0.0039	ND	ND
	Oil /Cip	-/50	0.78/0.39	-/0.0078	ND	ND
	Oil /Mox	-/0.781	100/50	-/64	ND	ND

MIC(a); minimal inhibitory concentration for seed oil when applied alone; MIC(b); minimal inhibitory concentration for antibiotic when applied alone; Me: methanolic extract; Ctx: cefotaxime; R: rifampicin; Of: ofloxacin; Cip: ciprofloxacin; Mox: moxifloxacin; ND: Not determined;

When screened in combination, the antimicrobial potential of extracts was enhanced compared to individual extract's MICs. For extracts in combinations, the methanolic and *n*-

hexane extracts in combination with ofloxacin and ciprofloxacin were found highly potent with least MIC (0.097 mg/mL) against *E. coli* (ATCC25922) and *S. aureus* (ATCC25923), respectively. Overall, the MIC for extracts in combination with antibiotics ranged from 0.097 to 100 mg/mL against all clinical MDR and reference bacterial strains (Tables 3.1-3.8). Moreover, in case of antibiotics combinations, the most potent activities were exhibited with MIC 0.049 µg/mL against reference strains. Ciprofloxacin in combination with seed oil and *n*-hexane extract exhibited MIC 0.049 µg/mL against *S. aureus* (ATCC25923). Similarly, ofloxacin in combination with seed oil or methanolic extract and ciprofloxacin in combination with seed oil exhibited MIC (0.049 µg/mL) against *E. coli* (ATCC25922) strain. Other antibiotics in combinations such as rifampicin plus methanolic extract and cefotaxime plus aqueous extracts were found to be potent with MIC 0.097 µg/mL against MRSA3 and *A. baumannii*, respectively. Similarly, rifampicin plus seed oil also exhibited antibacterial activity with MIC 0.097 µg/mL against *A. baumannii* MDR and MRSA3 and the same activity was also exhibited by ciprofloxacin in combination with seed oil against *S. aureus*.

3.4.4. Fractional inhibitory concentration

The combinatorial drug effects were evaluated using fractional inhibitory concentration (FICI) according to the criteria reported earlier (Hossain et al. 2016), where the effect of combination therapy is considered as "synergistic" if the FICI is ≤ 0.5; "additive" if FICI is > 0.5 and ≤ 1, "indifference" if FICI is > 1 and ≤ 4 and antagonistic if FICI > 4. Among all the combinations, methanolic extract in combination with rifampicin exhibited the highest synergistic effect (FICI ≤ 0.5) against the isolates including *A. baumannii*, *E. coli, E. faecalis, S. aureus,* and *P. aeruginosa*, methicillin resistant *S. aureus* (MRSA2, MRSA3, MRSA4 and MRSA5), *A. baumannii* (MDR strain), *P. chlororaphis*, *E. coli* ATCC25922 and *S. aureus* ATCC25923 (Table 3.1 and 3.5). The rate of synergism against clinical, MDR and reference bacterial strains remained the highest for methanolic extract in combination with rifampicin (15.29 %), followed by ciprofloxacin plus methanolic (11.76%), ofloxacin plus methanolic/seed oil (8.24%), cefotaxime plus *n*-hexane (8.24%) and moxifloxacin plus methanolic extract (2.35%). Among all the isolates, *E. coli* (ATCC25922) was found to be the most susceptible strain in combinatorial therapy. Methanolic, *n*-hexane extracts and seed oil in combination with rifampicin, ciprofloxacin, ofloxacin and cefotaxime showed synergistic effects against *E. coli* (ATCC25922) (Tables 3.1, 3.2 and 3.4). Among the selected antibiotics, moxifloxacin in combination with all extracts was found to have least synergistic while having highest antagonistic effects (7.35%) against clinical, MDR and reference strains (Tables 3.1-

3.8) but its effect remained synergistic when applied in combination with methanolic extract against *A. baumannii* and *S. aureus* strains (Table 3.1). Aqueous extract among others, was the least synergistic rate in combination with any antibiotic (9.41%), showing no synergistic effects against *E. coli*, *E. faecalis*, *P. vulgaris and S. aureus* strains (Table 3.8). On the other hand, seed oil was comparatively better than aqueous extract and showed 25.88% synergism rate. The seed oil showed strong synergistic effects in combination with cefotaxime against *A. baumannii*, *E. coli*, *P. vulgaris*, *E. faecalis*, *P. aeruginosa*, and *E. coli* ATCC25922. It was the least potent in combinatorial activities against MDR strains, determining none of the interactions but exhibited synergistic and indifferent interactions in combination with ofloxacin and cefotaxime only against MRSA1 (Table 3.8). Overall, the methanolic, *n*-hexane, aqueous extracts and seed oil in combination with antibiotics against all bacterial strains showed 44.71, 32.94, 9.41 and 25.88% synergism, respectively. In general, the synergistic, indifferent, antagonistic and additive effects by all extracts in combination with all antibiotics against various clinical strains were 28.24, 13.82, 11.76 and 1.76%, respectively (Tables 3.1-3.8).

3.4.5. Molecular docking

The MurF ligase enzyme was selected as a receptor for unveiling the binding conformation of methanolic extract compounds. MurF ligase enzyme has been an attractive drug target against bacterial pathogens because of its high specificity selectivity and well determined crystal structure. MurF ligase is involved in the final stage of peptidoglycan synthesis and has been validated as an ideal target for therapeutic compounds. *J. curcas* is highly enriched in long chain fatty acids and other phytochemicals which target the bacterial membranes or cell wall. Therefore, in the present study, MurF ligase was selected as target for phytochemical's intervention. It was revealed that among all the compounds of the extract, compound beta-monolaurin has the highest affinity for the MurF ligase active pocket with binding energy of -7.3 kcal/mol (Appendix 1 Table S10). The compound formed multiple hydrogen and hydrophobic interactions with the active side residues of the MurF ligase enzyme that is the key for the formation of stable complex. The 2-(vinyloxy) propane-1,3-diol, in particular, is involved in three strong hydrogen bondings: each with Ser44, Arg45 and Gln69 that constitute the core active pocket of the enzyme (Ahmad et al. 2017). This compound was further found to be in a position that can antagonistically block the access of natural substrate for MurF active site. The binding interactions and conformation of the compound can be seen in Figure S5. The compound formed multiple hydrogen and hydrophobic interactions with the active side residues of the MurF ligase enzyme, that are key for the formation stable complex.

The acetic acid in particular is involved in two strong hydrogen bonds: each with Ser44 and Asp43 that constitute the core active pocket of the enzyme. This compound was also further found to be posed in a position that can antagonistically blocked the access of natural substrate for MurF active site. Similarly 9,12 octadecadienoic acid present in *n*-hexane extract also exhibited stronger binding affinity for MurF ligase active pocket with binding energy of -6.2 kcal/mol (Appendix 1 Table S11). The 9,12 octadecadienoic acid also formed hydrogen and hydrophobic interactions with active site residues of the target protein and bonded to Ser44, Ser43, Phe61 and Leu153 or Leu56. The binding interactions and conformation of the 9,12 octadecadienoic acid can be seen in (Appendix 1 Figure S6).

3.5. Discussion

J. curcas has traditionally been used in medicine and its biological properties extensively investigated. During the last few decades, numerous biologically and medicinally important phytochemicals including flavonoids, tannins, steroids, saponins, glycosides, cardiac glycosides, volatile oils have been reported in *J. curcas* seed, increasing its medicinal importance (Rachana et al. 2012). Some of the bioactive constituents of this plant have been used to cure various diseases such as coated tongue, dysentery, infertility, gonorrhea, hemorrhoids, skin infections and inflammation (Hassan et al. 2004). Moreover, the phytochemicals play vital roles in plant defense mechanism against different microbial infections (Yadav and Agarwala 2011).

The current study aims to develop a novel strategy towards the discovery of new antibiotics by combining *J. curcas* seed oil and de-oiled seed extracts with commercially available antibiotics against various clinical, MRSA and MDR bacterial strains to combat prevailing antibiotic resistance.

The study included FTIR spectroscopic analyses of *J. curcas* seed oil and methanolic, *n*-hexane and aqueous extracts of its de-oiled seed. Various absorption bands in the FTIR spectra indicated the presence of different biological compounds such as proteins, carbohydrates, lignin, aromatic compounds, alkaloids, esters (phorbol esters and fatty acids methyl esters), amides, cellulose, hemicellulose and fatty acids. Alkaloids, phorbol esters, fatty acids and its methyl esters might be the main antimicrobial components as their antimicrobial activity has been reported earlier (Abdelgadir and Van Staden 2013; Chandrasekaran et al. 2008).

The GC-MS analyses determined the presence of a broad range of bioactive compounds in *J. curcas* extracts. In seed oil and *n*-hexane extract, a number of long chain fatty acids were

detected. The antibacterial mechanism of long chain fatty acids is still unknown but the OH groups present in these fatty acids target the bacterial cell membrane (Wojtczak and Wie 1999). Due to their amphipathic nature, fatty acids can solubilize various membrane components such as lipid bilayer and proteins that may lead to cell lysis (Greenway and Dyke 1979). They also affect various cellular processes including electron transport chain, oxidative phosphorylation reaction, enzyme inhibition, production of peroxides and altering electron gradient resulting in the leakage of cellular components from cells and manifests various inhibitory and bactericidal effects (Desbois and Smith 2010).

In the present study, beta-monolaurin (ester of glycerol and lauric acid) and 9-hexadecenal and 1-docosanol, found in the methanolic extract of *J. curcas* de-oiled seed, has previously been reported to have antimicrobial potential that may damage extracellular membrane, denature proteins and DNA or inhibit various macromolecular biosynthesis processes (Mamza et al. 2012; Sheela and Uthayakumari 2013; Skřivanová et al. 2006). Another medicinally important compound, I-(+)-Ascorbic acid 2,6-dihexadecanoate, identified in the methanolic extract has strong antioxidant activities and has been used in wound healing (OKWU and IGHODARO 2009). In GC-MS analysis of aqueous extract, a number of bioactive compounds such as 1,4-dithiane, dodecanoic acid, methyl ester, methyl tetradecanoate, vitamin D3, palmitic acid, methyl ester, isopropyl linoleate and di-n-octyl phthalate were identified. The antimicrobial potential of the aqueous extract can be attributed to the presence of these compounds (Chandrasekaran et al. 2008). Vitamin D3, identified in aqueous extract, which has the capability to mediate innate immunity in humans and can be used as defense against various infections (Farazi et al. 2017).

In the present study, methanolic extracts among others was found comparatively more potent against clinical, MDR and ATCC bacterial strains (Figure 3.2). Individually, methanolic extract exhibited the highest activity against *S. aureus*, *S. aureus* ATCC and MRSA4 among the clinical isolates, reference or MDR strains, respectively. Methanolic extracts of a number of medicinal plants had previously been reported with higher antimicrobial potential compared to *n*-hexane and aqueous extracts (Haq et al. 2016; Tripathi et al. 2016), suggesting its higher biological significance. This study also affirmed the antimicrobial potency of methanolic extract by molecular docking studies that unveiled beta-monolaurin as the best conformation in the active pocket of potential antimicrobial MurF target. Similarly, 9,12 octadecadienoic acid present in *n*-hexane extract also showed strong interaction and affinity with MurF ligase active pocket. In contrast to the previously reported data (Nazzaro et al. 2013), methanolic extract exhibited greater activity against Gram negative than Gram positive clinical isolates.

However, some studies revealed that Gram positive strains were less susceptible to bioactive compounds than Gram negative ones because the outer membrane of the latter is not fully impermeable. In contrast, in case of MDR strains, the extracts and oil were more active against Gram positive than Gram negative bacteria, probably due to the impermeability of outer membrane of the latter. The results are in close coherence with previously reported data (Kaur and Arora 2009).

The selected antibiotics at initial concentration were found active against most of the clinical and MDR bacterial strains in the following order: moxifloxacin > ciprofloxacin > ofloxacin > cefotaxime > rifampicin.

Combinatorial therapy or synergistic interaction is recommended as an effective strategy to help resolve the issue of antibiotic resistance, cellular toxicity and long-term treatments of the available antibiotics. It can also add to find broad-spectrum antibiotics compared to monotherapies (Marr et al. 2004). In the current study, antibiotics were combined with potent bioactive compounds of the *J. curcas,* aiming to increase their antibacterial potential, overcome resistance and reduce the cost and duration of antimicrobial therapy. When evaluated in combination with *J. curcas* extracts or seed oil, the activity of the selected antibiotics increased (MICs range of 0.097 to 100 mg/mL) as compared to the activity of *J. curcas* extracts or seed oil when screened alone (MIC range of 50 to 200 mg/mL). Individually, methanolic extract remained the most active (MIC 50 mg/mL) amongst the de-oiled seed extracts against various methicillin resistant *S. aureus* (MRSA1, MRSA2 and MRSA3) strains. The MIC value of the plant extract below 0.1 mg/mL is considered significant, moderate below or equal to 0.625 mg/mL and weak above 0.625 mg/mL (Kuete 2010). Individually, cefotaxime was the most potent (MIC 0.19 µg/mL) against clinical isolates, *A. baumannii* and *P. vulgaris*. Methanolic or *n*-hexane extracts in combination with ofloxacin or ciprofloxacin against *E. coli* (ATCC25922) or *S. aureus* (ATCC25923) exhibited the highest antibacterial activity (MIC 0.097 mg/mL), respectively. Moreover, in combination with seed oil and *n*-hexane extract, ciprofloxacin exhibited highest activity against *S. aureus* ATCC25923 (MIC 0.045 µg/mL). Ofloxacin in combination with seed oil or methanolic extract and ciprofloxacin with seed oil exhibited a similar activity (MIC 0.049 µg/mL) against *E. coli* ATCC25922 strain, while rifampicin with all extracts exhibited high activity (MIC 0.097 µg/mL) against *A. baumannii* MDR and MRSA3 strains.

The antibiotic/extracts combinations screened as antibacterial agents in this study, were also studied to evaluate their synergistic, indifferent, additive or antagonistic effect that occurs

when the antibacterial activity of the drug combination exceeds the sum of the individual drug activities, the activity of both drugs (in combination or individually) remains equal, there is no obvious change in the activity of both drugs (in combination or individually) or the activity of one drug is reduced in the presence of other, respectively. (Borisy et al. 2003; Branen and Davidson 2004). In combinations, methanolic extract and rifampicin exhibited synergistic rates of 15.29% against selected pathogenic strains. These treatments exhibited the highest synergistic activities against *A. baumannii*, *E. coli, E. faecalis, S. aureus*, and *P. aeruginosa*, methicillin resistant *S. aureus* (MRSA2, MRSA3, MRSA4 and MRSA5), *A. baumannii* (MDR strain), *P. chlororaphis*, *E. coli* ATCC25922 and *S. aureus* ATCC25923 (Table 3.1 and 3.5). Earlier studies have reported strong synergism between rifampicin and other antimicrobial agents (Timurkaynak et al. 2006). This makes rifampicin a strong candidate for combination antimicrobial therapies. Among all the strains, *E. coli* ATCC25922 was the most susceptible to extracts (methanolic, *n*-hexane and seed oil) in combination with four commercial antibiotics (rifampicin, ciprofloxacin, cefotaxime and ofloxacin). Among antibiotics, highest antagonistic effects were shown by moxifloxacin in combination with all extracts with the only exception of synergistic activities with methanolic extract against *A. baumannii* and *S. aureus* strains (Table 3.1). Aqueous extract was least active in combinatorial treatments and exhibited no synergistic activities against *E. coli, E. faecalis, P. vulgaris and S. aureus*. Aqueous extracts are known to exhibit relatively lower antibacterial activities compared to methanolic or *n*-hexane extract (Matu and Van Staden 2003). The present study found that extracts were more potent in combination than they were individually, against selected MDR strains. It is suggested that plant extracts hypothetically increase the efficacy of antibiotics against MDR strains and inhibit their efflux pumps or change resistance properties by releasing their antimicrobial compounds (Stermitz et al. 2000). In combination treatments, seed oil was the least active against MDRs and exhibited synergism only in combination with ofloxacin against MRSA1. The seed oil did not exhibit any combined effect against remaining MDR strains. The possible reason may be the instability of long chain fatty acids (Loftsson et al. 2016), or their tendency to bind non-specifically to proteins and other target compounds (Desbois and Smith 2010). Against MDR strains, moxifloxacin exhibited highest antagonism in combination with extracts and seed oil. However, seed oil exhibited higher synergism compared to the aqueous extract against clinical and reference strains. Literature has reported strong antimicrobial activity of oils (Thormar 2010).

The selected plant extracts and oil exhibited strong synergism with nucleic acids targeting antibiotics such as rifampicin, ofloxacin and ciprofloxacin. Rifampicin targets the DNA-dependent RNA polymerase inhibiting DNA-dependent RNA synthesis. Ofloxacin and ciprofloxacin mainly target DNA gyrase enzymes and inhibit bacterial cell division (Bébéar et al. 1998). There could be some possible reasons for this. Firstly, this strong synergism could be the result of interaction of the extracts and oil with outer membrane, cell wall and cell membrane and of antibiotics to nucleic acids. Firstly, this suggests that the extracts/oil target outer membranes allowing antibiotics to enter the cells, inhibiting nucleic acids machinery thus inhibiting cell division or apoptosis. Secondly, the two compounds used in combination form a new bioactive compound which has stronger antimicrobial effects. (Vaara 1992). Thirdly, the phytochemicals present in *J. curcas* extracts may reduce inherited bacterial resistance. For instance, flavonoids and polyphenols; methanolic extract combined with antibiotics may have altered bacterial resistance thereby increasing combination treatment efficacy (Olajuyigbe and Afolayan 2012). Cefotaxime, a cell wall inhibitor, binds to penicillin-binding proteins inhibiting peptidoglycan synthesis. The present study has found relatively lower synergism exhibited by cefotaxime with extracts and oil. Moxifloxacin was an exception; although it is a broad-spectrum DNA gyrase inhibiting antibiotic but in the present study, it exhibited antagonism in most of the interactions. Further study is required to elucidate structural changes in compounds during interactions. Cefotaxime, a cell wall inhibitor, exhibited potent activities in combination with extracts and oil, indicating enhancement in its antibacterial potential. A study by (Zhao et al. 2001) strengthens this claim, stating that cell wall targeting antibiotics exhibit increased activity when combined with phytochemicals targeting the same site. Moreover, efflux pump, an important tool for microbial resistance to antibiotics, is also affected by combination of antibiotics and phytochemicals. (Coutinho et al. 2008). In view of the previous combination therapy studies, it is held that phytochemicals from *J. curcas* seed oil and de-oiled seed extracts combined with some antibiotics can make human pathogenic clinical bacterial and MDR strains more sensitive. Purification of these phytochemicals and their utilization in combination with commercially available antibiotics against pathogenic bacteria in nosocomial and other infections could prove to be the next step in the discovery of new antibiotics to combat antibiotic resistance in bacteria.

The combination of antimicrobial compounds showing *in vitro* synergistic activities against infectious agents are considered as ideal options for effective treatment of bacterial infections, especially in patients with hardly curable infections. Since the discovery and development of

new classes of potent antibiotics is the need of the day, the crude extracts and seed oil of *J. curcas* appear to be promising as these exhibited potent antibacterial activities against varied clinical pathogenic and multidrug resistant bacterial strains. Among all de-oiled seed cake extracts and seed oil of *J. curcas*, crude methanolic extracts exhibited comparatively more potent antibacterial activities both individually and in combination with selected commercial antibiotics. In the current study, methanolic extracts were found with higher synergism compared to the *n*-hexane, aqueous extracts and seed oil in combination with commercially available antibiotics against selected strains. Especially, rifampicin had strong synergistic effects in combination with methanolic extract against various bacterial strains and is strongly recommended for combination therapies. The extracts and antibiotics combinations with higher synergism are suggested for effective therapy of infectious diseases caused by clinical and multi drug resistant pathogenic strains. Hence, evaluating the therapeutic potential of *J. curcas* allows one to see how it could be used best in combination with commercial antibiotics for effective treatment of bacterial diseases, especially, when the synergistic competency between plants and commercially available antibiotics is required for effective therapy. In addition, the utilization of *J. curcas* seeds for antimicrobial activities along with biofuels production in biorefinery concept may help to boost the economic viability of biofuel technology. This study has indicated the potential of *J. curcas* as a source of resistance modulation and novel chemotherapeutic agents for the production of synthetically improved therapeutic agents that can be used in combination with antibiotics, enhancing their antibacterial potential. However, further research is required to extract potential phytochemicals in pure form from *J. curcas* pressed seed cake and seed oil and to evaluate their effects on pathogenic microorganisms. In addition, it would also be interesting if the mechanism of action of these extracts, on target microorganisms, is determined individually as well as in combination with other drugs of choice that are unable to treat these resistant pathogenic microorganisms individually.

Additional file 1 (Appendix 1**)**

Declarations

List of Abbreviations

DMSO: dimethyl sulfoxide; MHA: Mueller Hinton agar; ATCC: American type culture collection; MDR: multidrug resistant; MRSA: methicillin-resistant *Staphylococcus aureus*; CFU: colony forming units; OD: optical density; GC-MS: gas chromatography coupled to mass spectrometry; FTIR: fourier transform infrared radiation; MIC: minimum inhibitory

concentration; FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index; MurF: UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent of publication

Not applicable

Availability of data and materials

All relevant data are included in this manuscript

Competing interests

All authors declare that they have no competing interest.

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Authors' contribution

AH, MB, MS and SZB designed this study. AH, MS and SZB performed the experiments. AH, MS, SZB, HK and MB interpreted the results. SK, AAS, FH, AI, and SA supported the project by critical evaluation of experimental results and providing facilities required. MB, AK, SK, AAS, FH, AI, DK and SA participated substantially in writing the manuscript, critical evaluation and modifications. MB and SK supervised the study. All authors read and approved the final manuscript.

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CHAPTER 4

Chapter 4: Bioactivities of *Jatropha curcas* seed cake and oil

Paper 2

Title: Cytotoxic and α-amylase inhibitory potential of *Jatropha curcas* seed oil and de-oiled seed cake extracts and their antifungal activities against phytopathogenic fungi

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4.1. Abstract

In this study, the antifungal, antioxidant, enzyme inhibitory (α-amylase), phytochemical and cytotoxicity profile of seed oil and de-oiled seed cake extracts *of Jatropha curcas*, a multipurpose plant with huge potential of biofuel production and medicinal purposes, was determined. The antifungal activities were investigated against the phytopathogenic fungal strains including *Colletotrichum coccodes, Pythium ultimum, Phytophthora capsici, Rhizoctonia solani, Bipolaris oryzae* and *Fusarium fujikuroi*. Methanolic extract was found to be highly fungicidal as compared to aqueous and *n*-hexane extracts, and seed oil against *P. capsici* and *F. fujikuroi* strains. Methanolic extract was also found to possess significantly higher antioxidant potential ($p<0.05$) than others. Conversely, seed oil was found to be rich in cytotoxic phytochemicals and exhibited significantly higher ($p<0.05$) α-amylase inhibitory activity while remained more cytotoxic against brine shrimps than *n*-hexane, methanolic and aqueous extracts. Methanolic extract was found to possess significantly higher content of total phenolics and flavonoids ($p<0.05$) than the seed oil and other extracts. These results indicated that *J. curcas* seed oil and its various extracts can be further investigated for their antimicrobial potential and as competent bio-pesticides.

Keywords: *Jatropha curcas*; phytopathogens; antifungal; antioxidant; cytotoxic; seed cake.

4.2. Introduction

Due to their adverse side effects, gradual decrease in efficacy and the emergence of resistance by the target cells towards synthetic drugs, they are currently being replaced by natural medicines. Drugs obtained from natural sources can be the best alternatives due to their negligible side effects and vast therapeutic applications (Ahmad et al., 2020). Herbal medicines obtained from more than 35,000 species of medicinal plants have been in use as traditional medicine for the treatment of different ailments since centuries (Shukla and Kumari, 2019). *Jatropha curcas,* an important plant with medicinal properties, is cultivated in various tropical and sub-tropical regions of the world (Abou-Arab et al., 2019). *J. curcas* shrub, belonging to the family *Euphorbiacea*, can reach to a height of 5-10 meters with a life span of about 50 years and it usually gives fruits in the third year after cultivations (Carels, 2009). Each fruit consists of about 3 seed containing seed kernels that are rich in lipids (55-58%) and crude proteins (31-35%) (Martinez-Herrera et al., 2006). The presence of various phytochemicals such as phenolic compounds, flavonoids, saponins and alkaloids have been reported in different parts of *J. curcas* plant (Thomas et al., 2008). It can grow in saline soil, barren lands, drought environment and at a wide range of pH and temperature ranging from 15 to 40 °C (Behera et al., 2010, Dada et al., 2014).

J. curcas possesses great potential for sustainable production of biofuels (biogas and biodiesel) and medicinally important bioactive phytochemicals. *J. curcas* seed oil has been reported for biodiesel production while the de-oiled seed cake, having high organic content, for biogas production and extraction of medicinally important phytochemicals. The multipurpose utilization of de-oiled seed cake may increase its economic viability and environmental sustainability (Kumar and Sharma, 2008). Traditionally, different parts of *J. curcas* plant have been used for the treatments of skin infections, dysentery, fever, coated tongue, gonorrhea, inflammation and rheumatoid arthritis (Hassan et al., 2004). Its stem bark, latex, leaves and de-oiled seed cake have vastly been reported for their antibacterial, antifungal, antiviral, cytotoxic, antioxidant and anti-molluscidal activities *in vitro* (Srinivasan et al., 2019). The seeds possess certain compounds such as phorbol esters, curcin and trypsin inhibitors, which make this plant a potential candidate for medicinal uses (Srinivasan et al., 2019). However, the antifungal activities of *J. curcas* plant have mostly been carried out against human pathogenic fungal strains. On the other hand, plant fungi are the main pathogens that cause a considerable loss to yields in agricultural crops. The plant pathogenic fungi such as *Colletotrichum coccodes, Pythium ultimum, Phytophthora capsici, Rhizoctonia solani,*

Bipolaris oryzae and Fusarium fujikuroi could develop resistance towards some synthetic fungicides and, therefore, seeking for effective alternatives is highly recommended. *C. coccodes* is one of the most pathogenic strains causing black rot on roots and anthracnose on fruits of plants (Alananbeh and Gudmestad, 2016). *P. ultimum*, which causes seed decay, root rots and damping off, is a threat to the agricultural economy (Broders et al., 2007). *P. capsici*, one of the highly pathogenic fungus, causes wilting, damping off, root, fruit and stem rot diseases in a range of important plants (Parada-Rojas and Quesada-Ocampo, 2018). *R. solani* generally targets roots and stems producing necrotic lesions from which they obtain nutrients (Sneh et al., 1991). *B. oryzae* infects the rice crops and is the second most pathogenic fungal strain that causes brown spot disease in rice (Ou, 1985). Similarly, *F. fujikuroi* causes bakanae (foolish seedling) disease resulting in hyper elongation of seedlings due to gibberellic acid production (Niehaus et al., 2017). *J. curcas* seed kernel has also been reported for alpha-amylase inhibitory activities using pig as experimental animal (Chivandi et al., 2006). To date, there are no findings on the *in vitro* antifungal activities of *J. curcas* seed oil and de-oiled seed cake extracts against these plant pathogenic fungal strains.

In the current study, *J. curcas* seed oil and de-oiled seed cake extracts were investigated for the presence of different phytochemicals. Moreover, their alpha amylase inhibition potential, antioxidant and cytotoxic activities and antifungal activities against different plant pathogenic fungal strains including *Colletotrichum coccodes, Pythium ultimum, Phytophthora capsici, Rhizoctonia solani, Bipolaris oryzae* and *Fusarium fujikuroi* were investigated.

4.3. Materials and methods

4.3.1. *J. curcas* seed oil extraction

J. curcas seeds were obtained from a local dealer and identified at herbarium of Department of Plant Sciences, Quaid-i-Azam University, Islamabad. The oil from *J. curcas* seed was extracted using mechanical oil expeller and stored in dark. The de-oiled seed cake was preserved in zip-lock bags at 4 °C for further use.

4.3.2. Preparation of extracts

Extracts from the de-oiled seed cake of *J. curcas* were prepared as previously described (Basri and Fan, 2005). The de-oiled seed cake was further pretreated in a grinder (Deuron Blender, Pakistan) to convert all cake to powder form. 100 g of the ground powder of the de-oiled seed cake was dissolved into 0.5 L of water, methanol or *n*-hexane and incubated at 30 °C for 48 h on a shaker at 100 rpm. The resulting solution was filtered with Whatman paper and the filtrate was concentrated at 45 °C under reduced pressure using rotary evaporator (Rotary Evaporator RE300 Stuart®). The concentrated filtrate was allowed to dry at room temperature to a constant weight. The yield of the dried methanolic, *n*-hexane and aqueous extracts were 15.0, 9.39 and 8.04%, respectively. The *J. curcas* seed oil, de-oiled seed cake extracts and the amphotericin B were filtered using sterile syringe filter (0.2 µm pore size). 100 µL of seed oil, each extract and amphotericin B (AmB®) were individually spread on Sabouraud dextrose agar (SDA) plates and allowed to incubate at 30 °C for 48 h to observe the sterility.

4.3.3. Fourier transform infrared analysis

J. curcas de-oiled seed cake extracts (encapsulated in KBr pellet) and seed oil were analyzed by Fourier transform infrared (FTIR) spectroscopy (Bruker Tensor 27) with a scan range of 400-4000 cm^{-1}.

4.3.4. Phytochemical screening

Phytochemical screening of *J. curcas* seed oil and de-oiled seed cake extracts was carried out using qualitative and quantitative phytochemical analyses as described below.

4.3.4.1. Qualitative phytochemical screening

J. curcas seed oil and de-oiled seed cake extracts were screened qualitatively for the identification of phytochemicals including flavonoids, glycosides, saponins, balsams, steroids, volatile oils and tannins by the methods as described previously (Amina et al., 2013, Arekemase et al., 2011, Haq et al., 2019). Flavonoids content was determined by the addition

of a 5% NaOH solution to the de-oiled seed cake extract or seed oil (3 mL). The presence of flavonoids was indicated by the appearance of yellow colour. Tannins and phenols were determined by the addition of 5% $FeCl_3$ solution to 3 mL of filtrate of the de-oiled seed cake extract or seed oil. The formation of bluish-black colour precipitate indicated the presence of tannins and phenols. For glycosides determination, a 2.5 mL of the de-oiled seed cake extract or seed oil was mixed with 50% H_2SO_4 (5 ml) in a test tube and heated for 15 min in boiling water. The mixture was allowed to cool and neutralized with 10% NaOH solution. 10 mL of the mixture of Fehling's solutions A and B in 1:1 ratio was added and boiled again for 5 min. The dense brick red precipitate formation indicated the presence of glycosides. The steroids were determined by filtration of 5 g de-oiled seed cake extract or seed oil dissolved in 5 mL of chloroform and the formation of reddish brown layer at the interface by the addition of concentrated H_2SO_4. The balsams were determined by the addition of 2 g de-oiled seed cake extract or seed oil in equal volume of 90% ethanol in test tube. The appearance of dark green colour by the addition of two drops of alcoholic ferric chloride indicated the presence of balsams.

4.3.4.2. Quantitative phytochemical analysis

The total content of phenolic compounds in *J. curcas* seed oil and de-oiled seed cake extracts was determined by Folin-Ciocalteu's method (Singleton and Rossi, 1965). Different concentrations of Gallic acid (6.25-50 µg/mL of methanol) as standard were prepared. 20 µL of the standard or seed oil or de-oiled seed cake extracts (4 mg/mL of DMSO) was mixed with 90 µL of Folin-Ciocalteu's phenol reagent (diluted 1:10 with deionized water). After 5 min, 90 µL sodium carbonate solution (6% w/v) was added to it. The plate was incubated for 30 min in dark with intermittent shaking at room temperature. DMSO was taken as a negative control. The absorbance of the test compounds was measured at 765 nm using automated microtiter plate reader (ELx800 BioTek, USA). The Gallic acid was used as standard to make the calibration curve and the results of total phenolics content was expressed as µg of Gallic acid equivalent (Chinnici et al., 2018) per mg of mass of sample on the basis of standard curve (6.25-50 µg GAE/mg of seed oil or de-oiled seed extracts, (R^2=0.986 and y = ax + c). All reactions was carried out in triplicate.

Total flavonoids content in *J. curcas* seed oil, de-oiled seed cake extract was determined using aluminium chloride colorimetric method (Lee et al., 2011). Quercetin (QU) was used as standard to make calibration curve. The stock solution of QU was two-fold diluted in methanol at concentrations of 2.5, 5, 10, 20 and 40 µg/mL. 20 µL of diluted QU standard solution or *J.*

curcas seed oil or de-oiled seed cake extract (4 mg/mL of DMSO) was separately mixed with 10 µL of 10% (w/v) $AlCl_3$, 10 µL of potassium acetate (1M) and 160 µL deionized water. Blank was prepared in similar way by replacing the 10% (w/v) $AlCl_3$ by the same amount of deionized water. The reaction mixtures of all samples were incubated at room temperature for 60 min. The absorbance of the reaction mixtures was measured at 415 nm against blank with an automated microtiter plate reader (ELx800 BioTek, USA). To calculate the flavonoids in each sample, a calibration curve was plotted using QU as standard. The total flavonoids content was expressed as µg of Quercetin equivalent (QUE)/mg of dried extract or seed oil from calibration curve of QU standard solution. All treatments were carried out in triplicate.

4.3.5. Antifungal activities

The plant pathogenic fungal strains including *C. coccodes, P. ultimum, P. capsici, R. solani, B. oryzae and F. fujikuroi* were received as a gift from Pakistan Agricultural Research Council (PARC), Islamabad, for research purposes only. All of the strains were grown on SDA growth medium at 30 °C for 72 h, routinely refreshed after each 10-15 days and stored at 4°C.

The antifungal activities of *J. curcas* de-oiled seed cake extracts and seed oil were carried out using microdilution method according to guidelines of National Clinical and Laboratory Standard Institute (Clinical and Institute, 2002) with some modifications. An aliquot (100 µL) of spore suspension (2×10^6 spores/mL in Sabouraud dextrose broth) of each fungal strain was dispensed in separate wells containing 100 µL of different dilutions of *J. curcas* seed oil or de-oiled seed cake extracts (aqueous, methanolic or *n*-hexane) with final concentrations of 25, 12.5, 6.25, 3.12 and 1.56 mg/mL of DMSO and incubated at 30 °C for 48 h. After the incubation period, the absorbance of each well was recorded at 630 nm with a microtiter plate reader (ELx800™ BioTek, USA). Amphotericin B (AmB®) with final concentration range of 1.56-50 µg/mL was used as positive control. DMSO was used as a negative control. The un-inoculated wells were used as blank. The percent growth was calculated by subtracting the absorbance of the blank from the absorbance of the test sample for each strain. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which 80% reduction in turbidity of the extract or seed oil occurred compared to that of the negative control. For determination of minimum fungicidal concentrations (MFCs), 20 µL of the extracts or seed oil was taken from the wells with no visual growth, spread on the SDA plates using sterile spreader and incubated at 30 °C for 48 h. The MFC was defined as the concentration of the extracts or seed oil at which no growth appeared at medium. The experiment was carried out in triplicates.

4.3.6. Antioxidant Activities

4.3.6.1. 2, 2-diphenyl 1-picrylhydrazyl (DPPH) free radical scavenging assay

The free radical scavenging capacity of *J. curcas* seed oil and de-oiled seed cake extracts against DPPH was evaluated as described previously (Bobo-García et al., 2015) with some modifications. Briefly, 20 µL of each serially diluted extract or seed oil with final concentration of 0.15-5.0 mg/mL of DMSO was mixed with 180 µL of methanolic solution of DPPH (0.95 mg/mL). The mixtures were incubated at room temperature in dark for 30 min. After incubation, the decrease in absorbance of the samples was measured at 517 nm using microplate reader (ELx800™ BioTek, USA). The antioxidant activity was also confirmed by the change in color from violet to yellow. Ascorbic acid (3.125-200 µg/mL of DMSO) was used as positive control. DMSO was used as a negative control. Percentage of radical scavenging was calculated using the following equation.

$$\text{DPPH Radical scavenging (\%)} = 100[1 - \frac{(C - T)}{C}]$$

Where T = absorbance of sample/standard and C= absorbance of negative control.

All the experiments were carried out in triplicate and the results were expressed as IC_{50} values (the concentration at which half of the free radical is scavenged as compared to the negative control).

4.3.6.2. Total ferric reducing power method

The total ferric reducing power of *J. curcas* seed oil and de-oiled seed extracts was evaluated as described previously (Ferreira et al., 2007) with some modifications. Briefly, 50 µL of *J. curcas* seed oil or deoiled seed extract (4 mg/mL of DMSO) was mixed with 50 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 50 µL of 1% (w/v) potassium ferricyanide ($K_3Fe(CN)_6$) solution. The reaction mixture was vortexed and incubated at 50 °C for 20 min. After incubation, 50 µL of 10% (w/v) tri-chloro acetic acid was added to the reaction mixture and centrifuged at 3000 rpm for 10 min. The supernatant (166.66 µL) was diluted with 166.66 µL of de-ionized water and 33.33 µL of 0.1% (w/v) ferric chloride ($FeCl_3$) was added to it. Ascorbic acid (2 mg/mL of DMSO) was used as positive control. DMSO was used as a negative control. The absorbance of all the samples was recorded at 630 nm with a microtiter plate reader (ELx800BioTek, USA). The reactions were carried out in triplicate and the total reducing power of the samples was expressed as ascorbic acid equivalent (Magda et al., 2015) per mg of dry weight (DW).

4.3.7. Alpha amylase inhibition

The alpha amylase inhibition potential of *J. curcas* seed oil and de-oiled seed cake extract was investigated *in vitro* as described previously (Ali et al., 2017). 10 µL of *J. curcas* seed oil or de-oiled seed cake extract (4 mg/mL) was mixed with 15 µL of phosphate buffer saline (20 mM), 25 µL of alpha amylase (0.5 mg/mL) and 40 µL 1% (w/v) starch solution. The reaction mixture was incubated at 50 °C for 30 min. After incubation, 20 µL of 1M HCl and 90 µL of iodine solution (iodine solution was prepared by adding 3.18 g iodine in a solution of 6.5% potassium iodide dissolved in de-ionized water. After shaking the solution was diluted up to 500 mL with deionized water) were added to the same mixture. Acarbose standard (200 µg/mL) was used as positive control and DMSO as negative control. Blank contained starch, phosphate buffer saline and deionized water. Absorbance was measured at 540 nm using microtiter plate reader (ELx800BioTek, USA). Amylase inhibition was calculated using the following equation.

$$\text{Amylase inhibition (\%)} = \left(\frac{(A_S - A)}{(A_B - A_N)}\right) \times 100$$

Where A_S = absorbance of test sample, A_N = absorbance of negative control and A_B = absorbance of blank.

4.3.8. Cytotoxicity on brine shrimp

Cytotoxic effect of *J. curcas* seed oil and de-oiled seed extracts was evaluated on brine shrimp (*Artemia salina*) as discussed previously (Meyer et al., 1982). Artificial sea water solution was prepared by dissolving 38 g of artificial sea salt in 1 L of distilled water, filtered through Whatman paper and sterilized in autoclave at 15 pounds per square inch (psi) and 121 °C for 20 min. About 1 g of *Artemia salina* cysts (eggs) were hatched in sterilized artificial sea water near light source at 37 °C for 24 h. Briefly, 100 µL of *J. curcas* seed oil or de-oiled seed cake extract were added separately to 2 mL artificial salt solution having 10 active nauplii and the volume was raised to 5 mL by adding further artificial sea salt water in test vials. Final concentrations of the extracts or seed oil in vials ranged 0.03-2 mg/mL. These vials were incubated at 37 °C for 24 h. Vincristine was used as positive control and DMSO as negative control. After 24 h incubation, alive and dead shrimps were counted and LD_{50} was calculated. The LD_{50} is defined as the concentration at which 50% nauplii are killed. All the reactions were carried out in triplicate. The percent mortality of nauplii was calculated using the following formula.

$$\text{Mortality (\%)} = \left(\frac{(C_N - S_T)}{C_N}\right) \times 100$$

Where (C_N) represents negative control and the (S_T) represents test sample.

4.3.9. Statistical analysis

Each experiment was performed in triplicate and data obtained are expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's test was used for multiple comparisons using GraphPad Prism software, version 5.0 (CA, USA), *p* value less than <0.05 was considered significant.

4.4. Results and discussion

4.4.1. Phytochemical analysis

A number of phytochemicals such as flavonoids, phenolic, steroids and tannins were detected in the *J. curcas* seed oil and the de-oiled seed cake extracts (methanolic, *n*-hexane and aqueous). The saponins and glycosides were found only in methanolic and aqueous extracts (Table 4.1). This is due to the fact that saponins bearing hydrophilic glycosides moieties are usually soluble in aqueous or sometimes in methanolic solvents but not in non-polar solvents (Cowan, 1999). Flavonoids are known for various biological activities such as antimicrobial, antioxidant, cytotoxic, anti-inflammatory and analgesic activities (Hodek et al., 2002). Tannins and flavonoids are also closely associated with antioxidant, anti-inflammatory and antimicrobial activities (de Sousa Araújo et al., 2008). These findings may partially justify the use of *J. curcas* as medicinal plant for the treatment of various ailments.

The FTIR absorption spectra obtained for *J. curcas* seed oil and de-oiled seed extracts **i**ndicated the presence of a broad range of phytochemicals including alcohol, esters, alkane, alkene, carboxylic acids, amines, aromatic amines, aliphatic ketones, amides, phenol, sulfone and ether (Appendix 2 Figures S1-S4). These functional groups play important role in the therapeutic efficacy of various medicinally important compounds (Oliveira et al., 2016).

Table 4.1. Phytochemical constituents in *J. curcas* seed oil and de-oiled seed extracts (methanolic, *n*-hexane and aqueous)

Extract	**Phytochemicals**						
	flavonoids	saponins	tannins	steroids	balsams	glycosides	phenol
methanolic	+	+	+	+	-	+	+
n-hexane	+	-	+	+	-	-	+
aqueous	+	+	+	+	-	+	+
Seed oil	+	-	+	+	-	-	+

+: Presence; -: absent

4.4.1.1. Total phenolics and flavonoids content

The presence of total phenolics and flavonoids contents can indicate the antioxidant activities of any medicinal drug/compound. Therefore, the quantitative analyses of these compounds were carried out to evaluate their quantity in *J. curcas* seed oil and de-oiled seed cake extracts (aqueous, methanolic and *n*-hexane).

Phenolic compounds possess effective hydrogen donating property and are known for their antioxidant activities as well as disruption of microbial cell walls, decrease in the ATP pool and enhancement of membrane permeability, ultimately causing microbial death (Oliveira et al., 2016). The total phenolics and flavonoids content present in *J. curcas* seed oil and de-oiled seed cake extracts are shown in Table 4.2. The total phenolics content determined in methanolic extract was 46.54±0.72 µg GAE/mg of de-oiled seed extract, and was significantly higher (p<0.05) than those in *n*-hexane (9.07±0.80 µg GAE/mg of de-oiled seed extract), aqueous extract (14.31±0.59 µg GAE/mg of de-oiled seed extract) and seed oil (26.02±0.7 µg GAE/mg of seed oil). It is due to the fact that methanolic solvents extract more phenolic compounds than the aqueous and *n*-hexane (Namuli et al., 2011). Comparatively, higher phenolics content in methanolic extract was due to the higher polarity index of methanol, which makes it capable to extract more phytochemicals than the *n*-hexane and aqueous extracts (Cowan, 1999). Moreover, flavonoids are highly important bioactive compounds due to their capability to inhibit microbial DNA/RNA and protein synthesis, and may alter the membrane permeability of microbial cells (Dzoyem et al., 2013). The flavonoids are also involved in the scavenging of free radicals, upregulate and protect antioxidant defense, and are actively involved in chelating free radical intermediate compounds (Ndhlala et al., 2010). Total flavonoids content found in methanolic extract (22.75±1.29 µg QUE/mg of de-oiled seed extract) was significantly higher (p<0.05) than those in seed oil (19.69±0.79 µg QUE/mg of seed oil), *n*-hexane (9.42±0.97 µg QUE/mg of de-oiled seed extract) and aqueous extracts (3.76±0.26 µg QUE/mg of de-oiled seed extract). Phenolic and flavonoid content, reported in various plants, are attributed to a variety of biological activities such as antioxidant, antimicrobial, cytotoxic, antiviral, anti-tumor, and anti-inflammatory activities (Muraoka and Miura, 2004, Barku et al., 2013, Özçelik et al., 2011, Zhang et al., 2011).

Table 4.2. Total phenolic and flavonoid content of *J. curcas* seed cake extracts and seed oil

Extract	Total phenolics content (µg GAE/mg DM ± SD)	Total flavonoids content (µg QUE/mg DM± SD)
Methanolic	46.54±0.72[a]	22.75±1.29[a]
n-hexane	9.07±0.80[b]	9.42±0.97[b]
Aqueous	14.31±0.59[c]	3.76±0.26[c]
seed oil	26.02±0.70[d]	19.69±0.79[d]

Results are expressed as mean ± SD; GAE: Gallic acid equivalent; QUE: Quercetin equivalent; Data marked with different letters in superscripts ([a], [b], [c], [d]) within the same column show significant difference according to Tukey's test (p<0.05). DM: represents dried mass of de-oiled seed cake extracts or seed oil.

4.4.2. Antifungal activities of de-oiled seed cake extracts and seed oil

Table 4.3 shows the MICs and MFCs of *J. curcas* seed oil or de-oiled seed cake extracts against various phytopathogenic fungal strains including *P. ultimum*, *P. capsici*, *R. solani*, *B. oryzae*, *F. fujikuroi* and *C. coccodes*. These strains cause various diseases in crops such as wilting, stem, fruit and root rot, anthracnose, brown spot, damping off and bakanae. The control of these infectious strains is a vital challenge to save the agricultural economy (Alananbeh and Gudmestad, 2016, Broders et al., 2007, Sneh et al., 1991, Ou, 1985, Parada-Rojas and Quesada-Ocampo, 2018). In this study, methanolic extract of de-oiled seed cake exhibited higher antifungal activities (MICs) as compared to seed oil and *n*-hexane and aqueous extracts against *P. ultimum*, *P. capsici*, *R. solani*, *B. oryzae*, *F. fujikuroi*. Generally, methanolic extract of medicinal plants have been reported to exhibit higher antifungal activity when compared to that of aqueous or *n*-hexane extract due to its higher antimicrobials extraction capability (Khan et al., 2018). The methanolic extract also exhibited MFCs at 3.1 and 6.3 mg/mL against *P. capsici* and *F. fujikuroi*, respectively. The seed oil only exhibited MFC at 12.5 mg/mL against *F. fujikuroi* strain, while the aqueous and *n*-hexane extracts did not exhibit any MFCs against any strain. The inefficiency of aqueous extract can be related to the fact that when plant materials are dissolved in water, some phenolases and hydrolases are released that may alter or reduce the activity of phytochemicals (El-Mahmood et al., 2008). The *n*-hexane extract also remained less active against fungal strains probably due to fact that the non-polar solvents have lower extraction capability of the phytochemicals than the polar solvents such as methanol

(Namuli et al., 2011). The high antifungal potential of methanolic extract strengthens its traditional use as antiseptic (Abdelgadir and Van Staden, 2013) and also as a drug of choice for the control of crop diseases. *P. ultimum* strain remained the most susceptible strain even at the lowest applied concentration of each extract and seed oil (Table 4.3), while *P. capsici* strain was found, however, to be the most susceptible against methanolic extract. The methanolic extract exhibited MIC and MFC against *P. capsici* strain at 1.5 and 3.1 mg/mL, respectively. There is no clear definition for the classification of plant extracts based on their antifungal potential, however, Aligiannis et al., (2001) (Aligiannis et al., 2001) proposed the plant extract classification based on MICs as follows: strong inhibitor (MIC≤ 0.5 mg/mL), moderate inhibitor (0.5≥MIC≤1.5 mg/mL) and weak inhibitor (MIC is > 1.5 mg/mL). Based on this classification, the highest efficacy of the extracts (at 1.5 mg/mL) in the current study comes under moderate class. After purification of active phytochemicals from these extracts, their activities may further be increased and hold a position as strong antifungal inhibitors according to the above classification. The use of methanolic extract against phytopathogens has an advantage that the residues left over after the methanolic extraction of phytochemicals can be used for biogas production. However, there is need to characterize the compounds present in methanolic extract and its use in field experiments. The antimicrobial activities of natural compounds might not be due to single active phytochemical, rather due to the synergistic combination of all phytochemicals present in it (Davicino et al., 2007). Plants do not have immune system and, thus, use other mechanisms in order to protect themselves from pathogenic fungal strains (Srinivasan et al., 2019). They produce certain bioactive compounds, proteins and peptides in order to combat fungal infection (Srinivasan et al., 2019).

Table 4.3. Minimum inhibitory concentration (MICs) and minimum fungicidal concentration (MFCs) of *J. curcas* seed oil and de-oiled seed cake extracts against selected plant pathogenic fungal strains

	MIC (mg/mL)						MFC (mg/mL)					
Extract/drug	*C. coccodes*	*P. ultimum*	*P. capsici*	*R. solani*	*B. oryzae*	*F. fujikuroi*	*C. coccodes*	*P. ultimum*	*P. capsici*	*R. solani*	*B. oryzae*	*F. fujikuroi*
methanolic	3.1	1.5	1.5	1.5	1.5	1.5	-	-	3.1	-	-	6.3
n-hexane	3.1	1.5	3.1	6.3	3.1	6.3	-	-	-	-	-	-
Aqueous	25	6.3	25	3.1	6.3	3.1	-	-	-	-	-	-
Jatropha oil	3.1	1.5	1.5	1.5	3.1	3.1	-	-	-	-	-	12.5
AmB®	1.5	0.8	0.8	1.5	3.1	0.8	-	1.5	3.1	-	-	3.1

AmB®: Amphotericin B as positive control (µg/mL), -: shows no MFC, results are presented as the mean of three replicates.

4.4.3. Antioxidant activities

The DPPH scavenging of free radicals is correlated to antioxidant potential of medicinal plants. The free radicals are the reactive oxygen species that are produced in response to an injury or after the digestion of certain foods (Lobo et al., 2010). The excess of free radicals in cellular environment may cause oxidative stress which sometime may lead to cell's damage/decay. The antioxidants are the compounds that scavenge free radicals, avoiding the cells from damage/decay (Poljsak et al., 2013). The de-oiled seed cake extracts and seed oil exhibited DPPH scavenging activities in concentration dependent manner (Table 4.4). The methanolic extract exhibited significantly higher scavenging activity as compared to the seed oil, *n*-hexane and aqueous extracts. The IC_{50} values are inversely proportional to the antioxidant activity of a compound. The lower IC_{50} value indicates a strong antioxidant activity of an extract or compound. Based on IC_{50} values, the antioxidant activity of methanolic extract was significantly higher ($p<0.05$) than the aqueous and seed oil and non-significantly higher than the *n*-hexane extract (Table 4.4).

Table 4.4. Free radical scavenging activities of *J. curcas* seed oil and de-oiled seed cake extracts using DPPH assay

Extracts	IC_{50} mg/mL	Percent radical scavenging activity of extracts and seed oil at final concentration						
		0.2 mg/mL	0.3 mg/mL	0.6 mg/mL	1.3 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL
Methanolic	4.29±0.01[a]	9±2.0	13±2.5	18±3.01	37±1.75	65±1.75	69±1.25	73±1.0
n-hexane	4.33±0.04[a]	12±3.0	21±3.0	35±2.0	42±1.0	48±1.75	68±2.0	69±2.0
aqueous	5.4±0.28[b]	6±0.5	14±1.0	23±1.5	30±3.0	58±2.0	60±1.0	62±1.7
Jatropha oil	5.5±0.27[b]	13±3.0	20±2.02	32±1.5	41±0.5	42±2.0	60±1.0	61±1.0
Ascorbic acid*	29.4±5.54[c]	25±1.0	44±1.0	49±2.0	58±0.7	62±3.0	74±1.0	75±2.0

*: Ascorbic acid was used at final concentration of 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL, values are the means of three replicates ± SD. Mean values with dissimilar letters in superscripts in IC_{50} column shows significant difference according to Tukey's test ($p<0.05$).

Antioxidant activities had been reported to be related to reductones which are actually the terminators of free radicals (Miliauskas et al., 2004). The presence of reductant substances in a sample indicates its ability to reduce the Fe^{+3} to Fe^{+2}. The electron transferring capability of a compound is considered its significant antioxidant attribute (Meir et al., 1995). *J. curcas* de-oiled seed cake extracts and seed oil have antioxidant potential by reducing the Fe^{+3} to Fe^{+2}. This was confirmed by changing the yellow colour of the extracts and seed oil into green and

blue shades. The reducing value of the *J. curcas* methanolic extract was significantly higher (p<0.001) than the seed oil, *n*-hexane and aqueous extracts (Figure 4.1). The reducing potential of *J. curcas* de-oiled seed cake extracts and seed oil indicated a positive relation between the DPPH scavenging, phenolics and flavonoids content. These results show that *J. curcas* de-oiled seed cake extracts and seed oil can be used to reduce the oxidized intermediates in lipid peroxidation process and can act as primary or secondary antioxidant compounds (Ordonez et al., 2006).

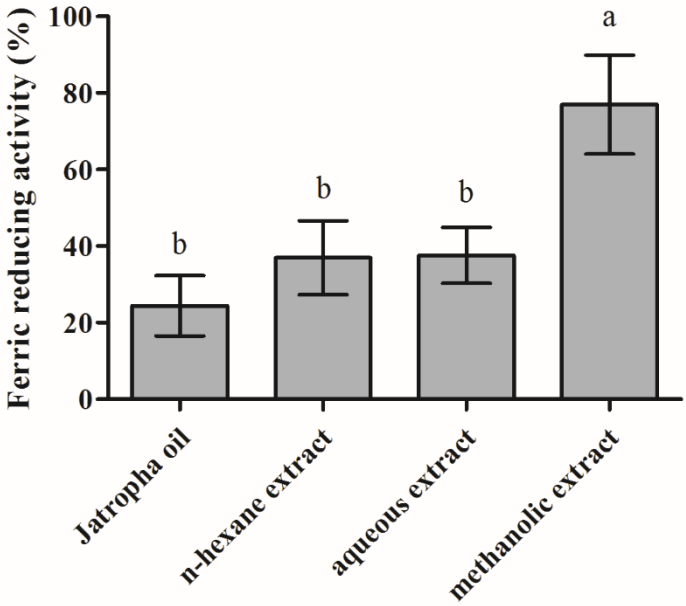

Figure 4.1. Total ferric reducing potential of *J. curcas* de-oiled seed cake extracts and seed oil. The data is presented as mean ± SD. One-way ANOVA was used followed by Tukey's test for multiple comparisons. Dissimilar letters on adjacent bars show significant difference according to Tukey's test (p<0.05, p<0.001, p<0.001).

4.4.4. Alpha amylase inhibition

Alpha amylase is an important enzyme used in the conversion of carbohydrates into glucose during metabolism. Therefore, it is considered as the main agent for liberation and increase in the after-meal blood glucose level in diabetic patients (Des Gachons and Breslin, 2016). If the alpha amylase enzyme is inhibited it can stop the release of high-level glucose in blood to avoid accumulation of keto-acids in the body (Kazeem et al., 2013). Currently, inhibition of amylases is considered an area of high interest in diabetes research (Dineshkumar et al., 2010). The highest percent amylase inhibitory activity was exhibited by *J. curcas* seed

oil followed by methanolic, *n*-hexane and aqueous extract. The amylase inhibitory activity of *J. curcas* seed oil was found significantly higher than methanolic (p<0.01), *n*-hexane (p<0.001) and aqueous (p<0.001) extracts.

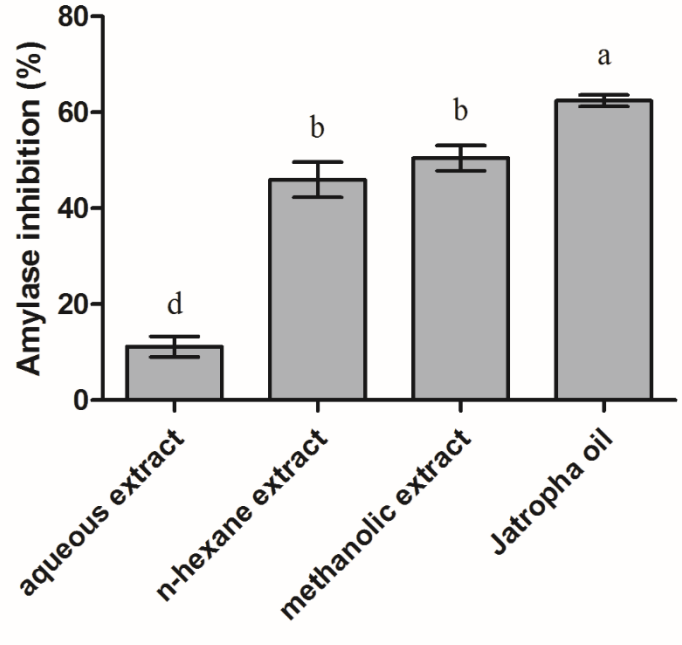

Figure 4.2. Alpha amylase inhibitory activity of *J. curcas* de-oiled seed cake extract and seed oil. The data is presented as mean ± SD. One-way ANOVA was used followed by Tukey's test for multiple comparisons. Dissimilar letters on adjacent bars show significant difference according to Tukey's test (p<0.05, p<0.001, p<0.001).

4.4.5. Cytotoxicity on Brine shrimp

Cytotoxic activity using Brine shrimp lethality is a simple assay which can be used to assess the cytotoxic activity of a compound (Apu et al., 2013). *J. curcas* de-oiled seed cake extracts and seed oil were used at different concentrations in cytotoxic assay (Table 4.5). The results indicated that the cytotoxic activities of *J. curcas* de-oiled seed cake extract and seed oil against brine shrimps were concentration dependent. The cytotoxic activity of *J. curcas* seed oil was significantly higher (p<0.05) than *n*-hexane, aqueous and methanolic extract. Usually, the cytotoxic activity decreases with increasing polarity. As methanolic and aqueous solvents are polar, while *n*-hexane and seed oil are non-polar. The non-polar nature of seed oil and *n*-hexane extract can be the reason of their higher cytotoxic activity compared to those with polar nature (methanolic and aqueous extract) (Apu et al., 2013). The higher cytotoxicity of seed oil than the other extracts make it an ideal choice for cancer and tumor related studies. While the methanolic extract with the least cytotoxicity can further be evaluated as drug of

choice for inhibition of plant pathogenic fungal strains and also as an antioxidant reduce free radicals produced inside the human body. The LD_{50} values show that seed oil (LD_{50}: 0.08 mg/mL) had exhibited significantly ($p<0.05$) higher mean cytotoxic activities than *n*-hexane (LD_{50}: 0.14 mg/mL), aqueous (LD_{50}: 2.83 mg/mL) and methanolic (LD_{50}: 5.69 mg/mL) extracts against brine shrimps. The compound with lowered LD_{50} value can be used as an alternative potent source of anti-cancer and pesticide drugs.

Table 4.5. Cytotoxic activities of *J. curcas* seed oil and de-oiled seed cake extracts against Brine Shrimps

Extracts	LD_{50} mg/mL	Percent mortality of Brine shrimps after 24 h						
		0.03 mg/mL	0.063 mg/mL	0.13 mg/mL	0.25 mg/mL	0.5 mg/mL	1 mg/mL	2 mg/mL
Methanolic	4.8±2.4[a]	30.3±4.9	31.6±4.7	32.0±4.3	33.3±4.9	34.6±3.0	36.0±5.2	40.3±2.5
n-hexane	0.9±0.2[b]	27.0±4.3	30.6±1.5	43.0±2.6	43.3±3.5	51.6±2.5	54.0±4.0	63.0±4.0
aqueous	2.8±0.2[ab]	23.0±2.6	27.0±2.0	31.0±2.6	33.6±2.08	34.6±4.04	38.3±2.08	41.6±1.5
Jatropha oil	0.1±0.02[b]	26.6±3.7	43.3±1.1	52.3±2.5	65.3±3.05	73.6±1.5	76.0±2.0	78.3±2.08
Vincristine sulfate	0.09±0.04[c]	59.6±1.6	75.3±1.0	78.3±2.9	80.6±2.5	81.3±1.9	88.6±1.9	92.0±2.0

LD_{50}: Lethal dose (the concentration at which half of the nauplii are killed). values are the mean of three replicates ± SD. Mean values with dissimilar letters in superscripts in LD_{50} column show significant difference according to Tukey's test ($p<0.05$).

The qualitative and quantitative phytochemical analyses, and bioactivities of *J. curcas* seed oil and de-oiled seed cake extracts show that the methanol is the most appropriate solvent for extracting bioactive compounds (phenolics and flavonoids), and for antifungal, antioxidant and alpha amylase inhibitory activities. The seed oil and *n*-hexane extract can be used as the drugs of choice for cytotoxic/anti-cancer activities. Moreover, the bioactivities of *J. curcas* seed oil and de-oiled seed cake extracts are not only attributed to these phytochemicals, but can also be associated to the other non-quantifiable phytochemicals, which needs further investigation to elucidate their individual potential.

4.5. Conclusions

The current study showed that *J. curcas* de-oiled seed cake extract and seed oil were rich in various medicinally important phytochemicals such as flavonoids, phenolics, tannins, saponins, steroids and glycosides. The methanolic extract was significantly rich in flavonoids and phenolics. All the extracts and seed oils exhibited antioxidant and antifungal activities at the concentrations studied, with the activities of methanolic extract being the highest. The methanolic extracts are highly recommended as bio-pesticides to control fungal infections in fields. The seed oil was significantly rich in cytotoxic and amylase inhibitory compounds and can be used as a potent source of therapeutic medicine for cancer and diabetes in future. The significances of the present study represent a broad range of applications of *J. curcas* seed.

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Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER 5

Chapter 5: Codigestion and pretreatment of different parts of *Jatropha curcas* fruit

Paper 3

Title: Enhancement of biogas yield during anaerobic co-digestion of different parts of *Jatropha curcas*

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5.1. Abstract

The sustainable use of non-edible feedstocks and waste for the production of biofuels is a potential means to reduce dependency on fossil fuels and mitigate environmental pollution. In the current study, the effects of carbon to nitrogen ratios on biomethane yield during anaerobic co-digestion of *Jatropha curcas* de-oiled seed kernel and mango peels were evaluated in continuous reactors. The biogas potential and effects of acid pretreatment on *Jatropha curcas* fruit were also evaluated during anaerobic digestion in batch setup. The biomethane yields of co-digested mango peel and seed kernel (1:4 weight ratio based on volatile solids) were 61 and 50% higher than the biomethane yields of mono-digestion of mango peel and seed kernel, respectively. The co-digestion of mango peel and seed kernel at 1:4 ratio resulted in the highest actual biomethane yield, followed by 1:1 ratio (25% lower yield) and 2:1 ratio (36% lower yield). The yields of 1:4, 1:1, and 2:1 ratios were 52, 39 and 32% of the theoretical yields, respectively, illustrating the importance of adjusting C/N ratio by co-digestion with the right ratios of co-substrate. The biogas yield of pretreated fruit coat was 7, 22, 34, 50, and 74% higher than that of the seed kernel, fruit coat (non-pretreated), de-oiled kernel plus seed coat (pretreated) (1.7:1, by weight), seed coat (pretreated), and seed coat (non-pretreated), respectively. Additionally, pretreatment of fruit coat and seed coat resulted in 22 and 47% higher biogas yields compared to their non-pretreated counterparts. These findings impact how *J. curcas* de-oiled seeds can be optimally treated to maximize energy production through anaerobic digestion.

Key words: Jatropha curcas, anaerobic digestion, dilute acid pretreatment, carbon to nitrogen ratio, biogas.

5.2. Introduction

Lignocellulosic biomass is a potentially sustainable renewable resource for the production of biogas through anaerobic conversion processes. However, lignocellulosic biomass has a high resistance to biodegradation due to a variety of structural and compositional properties such as accessible surface area, cellulose crystallinity, degree of polymerization of cellulose, degree of hemicellulose acetylation, and presence of lignin and hemicellulose (Cui et al., 2014, Xu et al., 2012, Kim et al., 2013, Li et al., 2020, Usmani et al., 2020). A possible solution is pretreatment before anaerobic digestion to increase the biodegradability. The main goal of pretreatment is to affect the lignocellulosic structure of biomass to decrease crystallinity as well as increase enzymatic accessibility. These structural changes will make the cellulose and hemicellulose accessible to bacterial enzymes resulting in increased production of biogas (Hendriks and Zeeman, 2009, Kim et al., 2013, Abraham et al., 2020, Dong et al., 2020).

Jatropha curcas is a drought and pest resistant shrub of the *Euphorbiaceae* family that typically grows to a height of 5 m and can reach up to 10 m under favorable conditions (Kumar and Sharma, 2008). After planting, the shrub produces fruit from the second year onward, and if properly managed, can produce 4-5 kg of seeds after the fifth year and over its life span of 40-50 years (Singh et al., 2008). During processing of seeds, de-oiled cake is produced. The *Jatropha curcas* de-oiled cake is a potential environmental pollutant, and anaerobic digestion has been identified as a possible solution to this problem (Raheman and Mondal, 2012). While it has been documented that the biogas yield of *J. curcas* seed cake is 60% higher than that of cattle dung (Hessami et al., 1996), mono-digestion of *J. curcas* seed cake still results in a low biogas yield, because of the relatively low carbon to nitrogen (C/N) ratio of about 9:1 (Raheman and Mondal, 2012). This suggests that co-digestion with other substrates having a high C/N ratio (e.g., 47:1 for fruit and vegetable wastes) (Saidi et al., 2018) is needed to adjust normal C/N ratio (20:1-30:1) (Ngan et al., 2020). A possible co-digestion substrate is fruit waste, such as mango peels, with a reported C/N ratio of 45.4 (Suryawanshi et al., 2013).

A number of studies have been conducted on biogas potential of different parts of *J. curcas* shrub, but to date not much attention has been paid to improve the biogas yield of *J. curcas* fruit and seed kernel using acid pretreatment and optimization of the C/N ratio. The objectives of the present study were to: (1) evaluate the biogas potential by anaerobically digesting different parts of *J. curcas* fruit, (2) determine the effect of dilute phosphoric acid pretreatment on biogas yield of different parts of *J. curcas* fruit, and (3) determine the impact

of manipulating the C/N ratio on biogas yield by co-digestion of *Jatropha curcas* seed kernel and mango peels

5.3. Materials and Methods

Jatropha curcas fruit was divided into three parts (seed kernel, seed coat, and fruit coat) to evaluate their biogas potential and the effects of acid pretreatment. The oil was extracted from seed kernel using an oil expeller. The seed kernels were pressed in the oil expeller using continuous friction and pressure of 30 MPa from the screw at about 120 rpm. This pressure heated the kernels to an approximate temperature range of 60-80 °C causing release of kernel oil into the reservoir. The pressed cake was moved towards exit and the cycle was repeated three times to obtain the maximum yield of oil. In all experiments, the fruit parts (de-oiled seed kernel, seed coat, and fruit coat) were mechanically pretreated in a fruit blender (Deuron Blender, Pakistan) to convert all substrates to powder form. To determine the effects of C/N ratios on methane yield, mono-digestion and co-digestion of mango peels and seed kernel were carried out at various C/N ratios, as described below.

5.3.1. Inocula

Effluent samples from a continuous-flow anaerobic digester at the National Agriculture Research Council (NARC), Islamabad, Pakistan (retention time of 60 days) treating cattle manure slurry, and operated at ambient temperatures, were collected twice and were used as inocula in both batch and continuous anaerobic digestion. The inocula were incubated at 37 °C for two weeks to minimize endogenous methane production (Holliger et al., 2016) and to acclimatize the microbial consortia at 37 °C. After incubation, total solids (TS) and volatile solids (Niehaus et al.) of the inocula were measured as described previously (Sluiter et al., 2008) (Appendix 3 Table S1).

5.3.2. Feed stock characterization

Jatropha curcas fruit parts (de-oiled seed kernel, seed coat, and fruit coat) and fruit waste (mango peels) were used as substrates. The *Jatropha curcas* seeds and fruits were purchased from a local merchant (pansari) in Peshawar Khyber Pakhtunkhwa, Pakistan. The different parts of *Jatropha curcas* fruits were transferred in ziploc bags and kept at 4 °C. Mango peels were taken from local market of Quaid-i-Azam University Islamabad and stored at -20 °C until use. Carbon, hydrogen, nitrogen and sulphur (CHNS) analysis of de-oiled seed kernel and mango peels was performed using a CHNS analyzer (EAS superuser elemental analyzer system GmbH (a suffix used after a private limited company in Germany) access: varioEL cube V1.2.1 2009-1-27).

5.3.3. Anaerobic digestion

Anaerobic digestion of *J. curcas* fruit was carried out in continuous mode to assess the effect of C/N ratio and in batch reactor to evaluate biogas potential. For continuous experiments for mono- and co-digestion, 2.5 L reactors were used with a working volume of 2 L. In the batch setup, the biogas potentials of de-oiled seed kernel, seed coat, fruit coat, and de-oiled seed kernel + seed coat (seed coat pretreated with 1% H_3PO_4) were determined. For batch experiments, 0.5 L reactors were used with working volumes of 0.4 L. The reactors were kept at 37 °C in both batch and continuous experiments.

5.3.3.1. Continuous anaerobic digesters setup to evaluate the effect of C/N ratio

In continuous mode setup, mono-digestion and co-digestion of de-oiled seed kernel and mango peels were investigated. The substrates were crushed to a fine size to avoid any hindrance during feeding, and stored at -20 °C. During co-digestion, mango peels and de-oiled seed kernel were fed in 2:1, 1:1 and 1:4 ratios (by VS weight). The hydraulic retention time of all reactors was 20 days, and were daily fed until the reactor reached quasi-steady-state conditions, i.e., when the differences in biogas production during three consecutive readings were less than 5%. The effluent flow rate was 0.1 L /day, and the organic loading rate was 1.5 g VS/L- day. The biogas produced was collected at room temperature (26 °C) and measured using a water displacement method. The obtained biogas values were converted into normalized L (at standard temperature and pressure). To quantify methane content during steady state, a scrubbing solution (3 M NaOH) was used for the removal of CO_2 in the last few days of all continuous reactors. The volatile fatty acids (VFAs) and alkalinity of the effluent from the continuous anaerobic digester were measured at regular intervals (APHA, 1995). The actual biogas and biomethane yields obtained during co-digestion of mango peel plus *J. curcas* de-oiled seed kernel (MP+SK) at different ratios were compared to calculated yields in the same ratios. For calculated yield of co-digestion, the mean values of actual yield of mono-digestion by each substrate (mango peel and de-oiled seed kernel) was taken in the same ratios as used in co-digestion and were summed up. The calculated yield obtained was compared to the actual yield of co-digestion in their respective ratios.

5.3.3.2. Dilute acid pretreatment

Fruit coat and seed coat were separately pretreated with 1% dilute H_3PO_4 to compare their biogas potentials to those of non-pretreated fruit coat and seed coat, respectively. Moreover, the de-oiled seed kernel was combined with seed coat (treated with H_3PO_4) (naturally present in the weight ratio 1.7:1) and abbreviated as SK+SC(H_3PO_4). On the basis

of pre-determined VS, a specific amount of each substrate (Table 5.1) was added to 1 % dilute H_3PO_4 solution in triplicate and autoclaved at 121 °C and 15 Pa for 20 minutes. The pH was adjusted to 7 by the addition (as needed) of 6 M NaOH and 1 M HCl. The non-pretreated samples along with controls were added to a volume of 50 mL distilled water.

5.3.3.3. Biogas potential and acid pretreatment setup

For batch anaerobic digestion, the substrates were incubated in 0.5 L bottles in triplicate at a 4:1 ratio (VS basis) of inoculum to substrate to a working volume of 0.4 L (Table 5.1). The reactors were incubated at 37 °C in a water bath and biogas was collected in air-tight bags and measured with a syringe. The biogas measurements were converted into normalized mL (at standard temperature 273.15 K and pressure 1.013 bar). After filling the reactors with inoculum and the respective substrates, the reactors were flushed with N_2 gas, sealed with a rubber stopper, and incubated for 60 days.

Table 5.1. Summary of batch biogas potential tests of different parts of *Jatropha curcas*

	Inoculum only	Inoculum+cellulose	Inoculum+Fats	Seed kernel	Seed Coat (without pretreatment)	Seed Coat (pretreated)	Fruit Coat (without pretreatment)	Fruit Coat (pretreated)	Deoiled Seed Kernel + Seed Coat (pretreated) *
Inoculum (g)	400	397.1	397.1	396.8	395.3	395.3	396.3	396.3	396.03
Seed Kernel (g)	0	0	0	3.13	0	0	0	0	2.53
Seed Coat (g)	0	0	0	0	4.68	4.68	0	0	1.44
Fruit Coat (g)	0	0	0	0	0	0	3.72	3.72	0
Cellulose (g)	0	2.90	0	0	0	0	0	0	0
Fats (g)	0	0	2.90	0	0	0	0	0	0
H_3PO_4 (1% w/v) (mL)	0	0	0	0	0	50	0	50	25
Water (mL)	50	50	50	50	50	0	50	0	25
Working volume (mL)	400	400	400	400	400	400	400	400	400
Total adjusted volume (mL)	450	450	450	450	450	450	450	450	450
Inoculum:Substrate (VS:VS)	4:1	4:1	4:1	4:1	4:1	4:1	4:1	4:1	4:1

* De-oiled seed kernel was mixed with seed coat (pretreated) in 1.7:1 ratio to mimic the actual composition of *Jatropha curcas* seed, when deoiled.

5.3.4. Statistical analyses

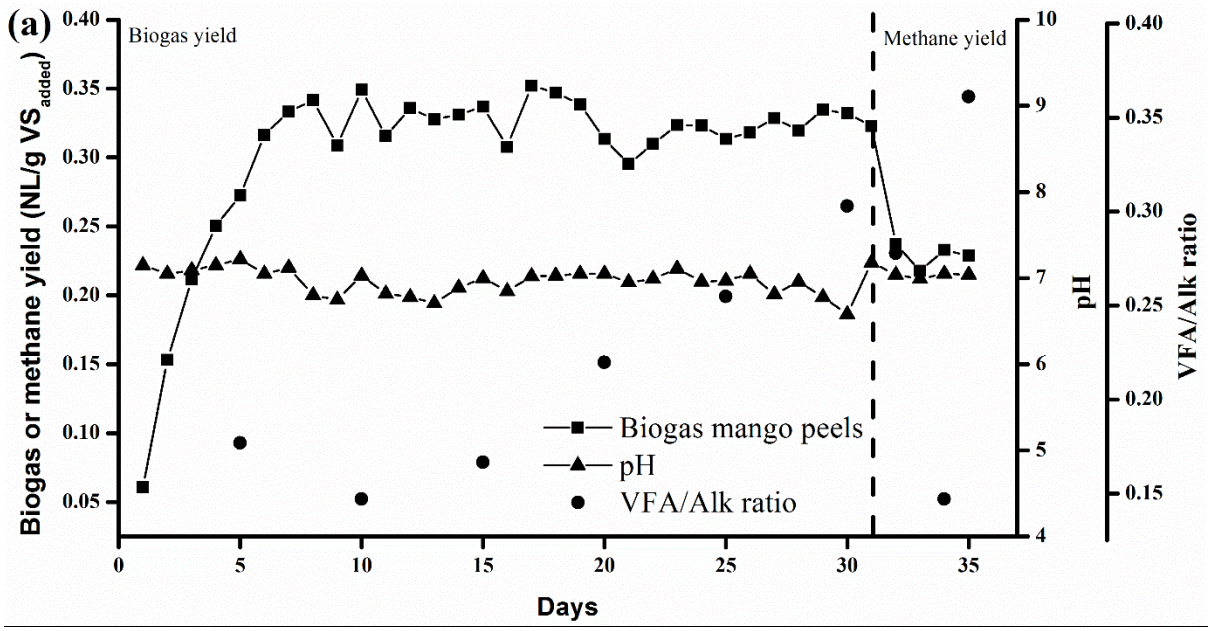
The yields in batch and continuous modes were presented as mean values ± standard errors. Multiple mean comparisons using Scheffe's test were used to check significant differences both in batch and continuous modes. The level of significance was set at $p<0.05$.

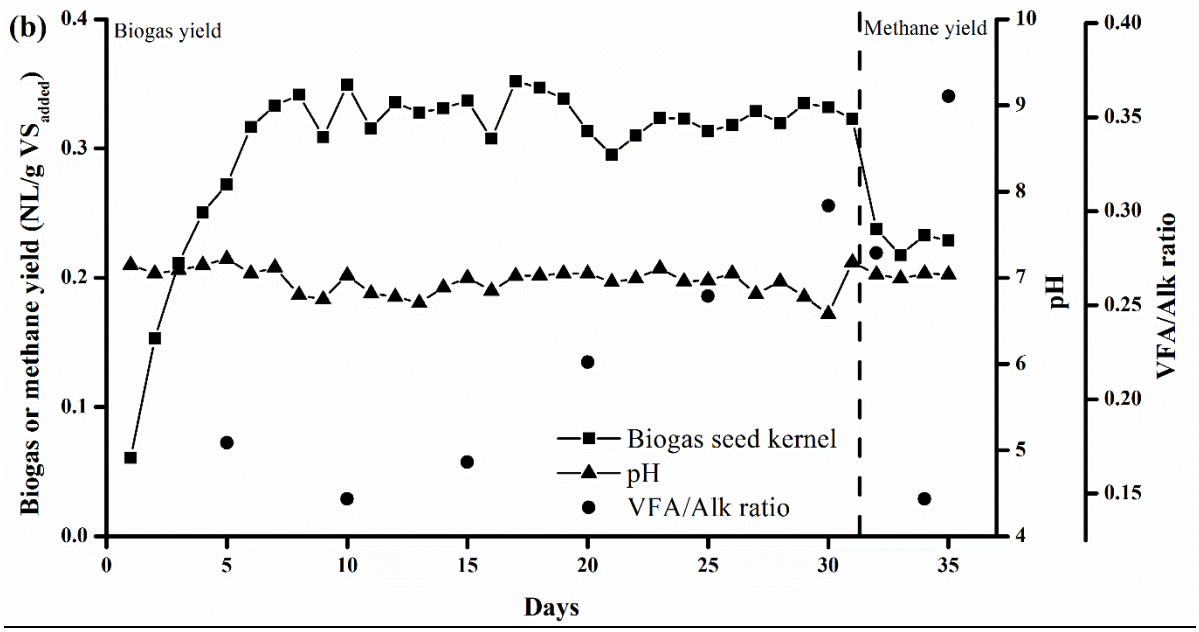
5.4. Results and Discussion

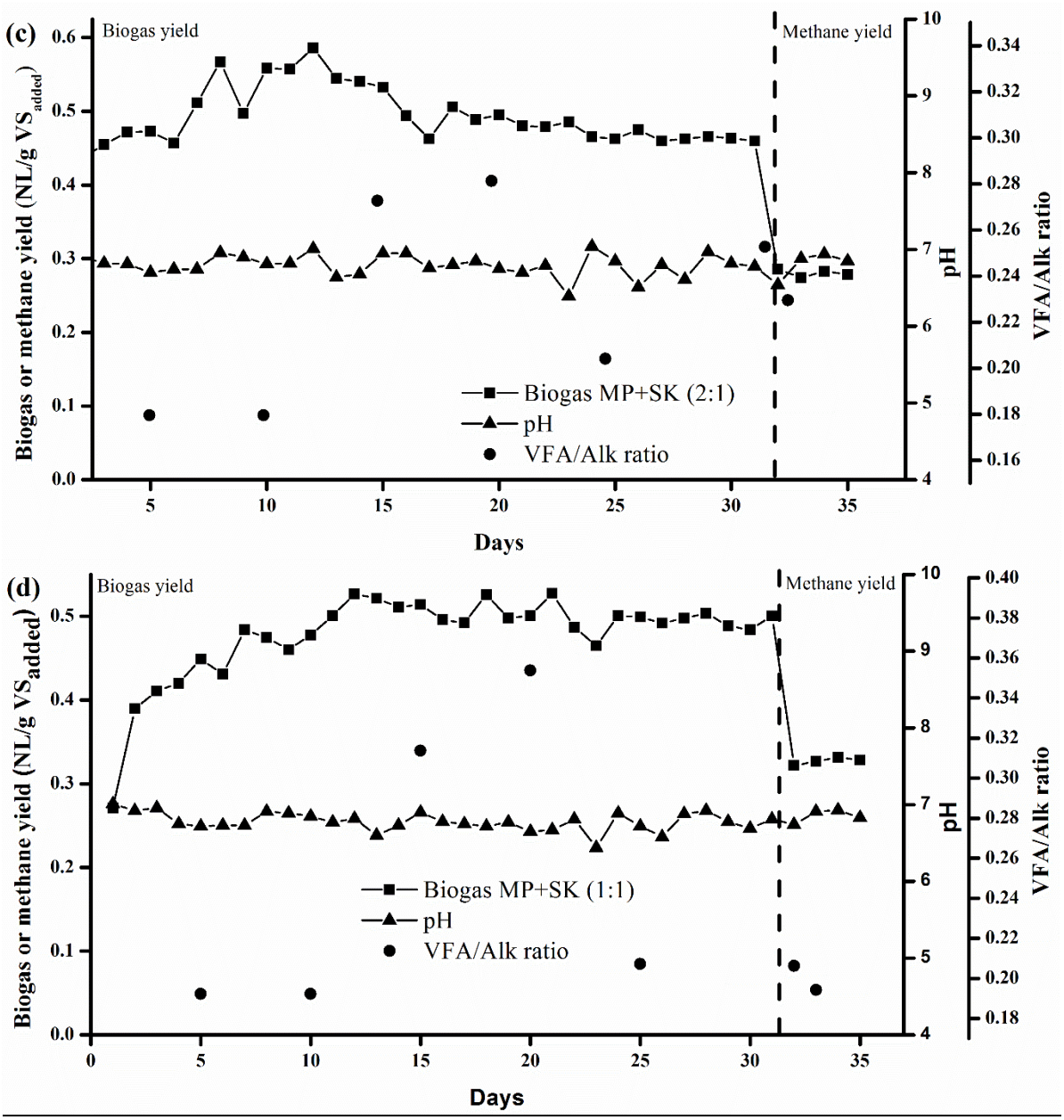
5.4.1. Effects of carbon to nitrogen (C/N) ratio on biogas yield of seed kernel

Nitrogen present in organic substrate is used for synthesis of amino acids, proteins and nucleic acids. The nitrogen is required for microbial growth and a low amount or absence of nitrogen may cause the washout of microbial communities during anaerobic digestion, ultimately resulting in lower biogas yield or reactor failure. Organic nitrogen is converted to ammonia which is a strong base, neutralizing the volatile acids produced by fermentative bacteria, thus maintaining pH conditions essential for growth of microorganisms. However, an increase in total ammonia nitrogen (free ammonia nitrogen plus ammonium nitrogen) concentration above 3 g/L will have toxic effects on methanogens and cause reactor failure (Nielsen and Angelidaki, 2008, Yenigün and Demirel, 2013). Thus, an appropriate concentration of nitrogen in the feedstock is needed to simultaneously avoid nutrient limitations and ammonia toxicity. Hence, an imbalance in C/N ratios would have significant effects on biogas yield and microbial activity (Kigozi et al., 2014). The optimum range of C/N ratio for biogas production has been reported to be 20:1 to 30:1 (Hessami et al., 1996, Raheman and Mondal, 2012). Since de-oiled seed kernel of *J. curcas* has a low C/N ratio of 11, addition of a substrate with high C/N ratio (mango peel with a C/N ratio of 53) was investigated.

During mono-digestion, the steady state biogas and biomethane yields of seed kernel in (Table 5.2) were 21.9 and 22.7% higher than those of mango peels. The biogas yield of seed kernels was significantly higher ($p<0.05$) than that of mango peels (Figure 5.1a and 5.1b). During mono-digestion of de-oiled seed kernel, the process reached pseudo-steady state on Day 23, with less than 5% variation in biogas production for the next eight days (Figure 5.1b).


(a)
Biogas yield
Methane yield
Biogas or methane yield (NL/g VS added)
pH
VFA/Alk ratio
Biogas mango peels
pH
VFA/Alk ratio
Days


(b)
Biogas yield
Methane yield
Biogas or methane yield (NL/g VS added)
pH
VFA/Alk ratio
Biogas seed kernel
pH
VFA/Alk ratio
Days


(c)
Biogas yield
Methane yield
Biogas or methane yield (NL/g VS added)
pH
VFA/Alk ratio
Biogas MP+SK (2:1)
pH
VFA/Alk ratio
Days
(d)
Biogas yield
Methane yield
Biogas or methane yield (NL/g VS added)
pH
VFA/Alk ratio
Biogas MP+SK (1:1)
pH
VFA/Alk ratio
Days

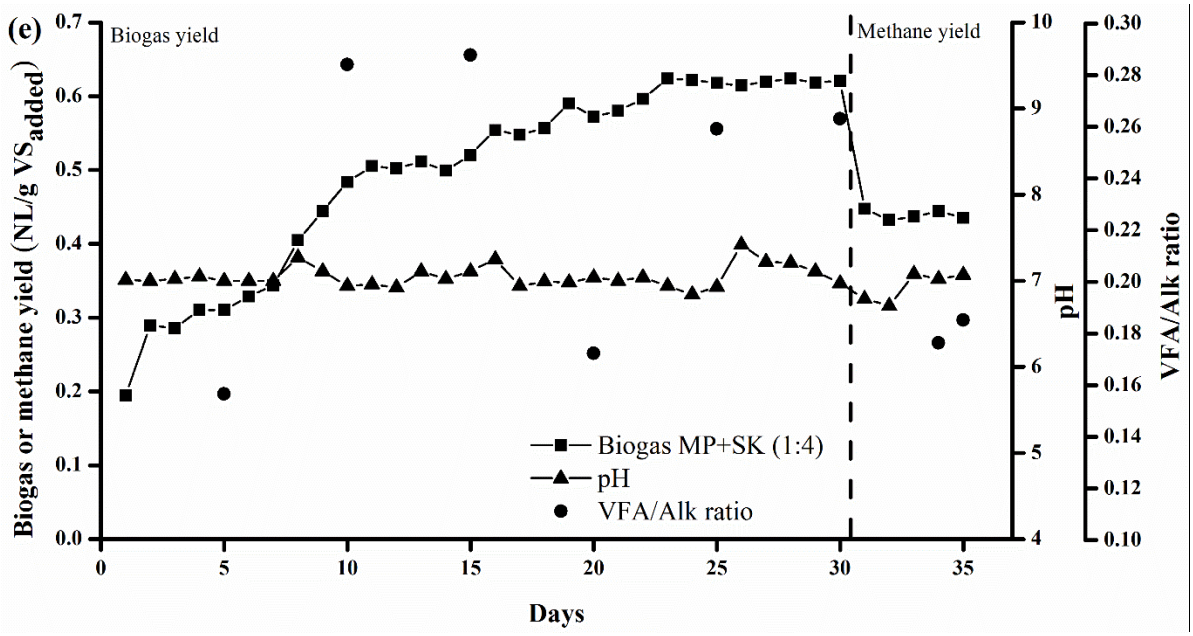


Figure 5.1. Biogas and methane yield, normalized liter per gram VS, during continuous anaerobic mono- and co-digestion mango peel and seed kernel. **(a)** mango peels, (b) seed kernel, **(c)** co-digestion of mango peel and seed kernel (MP+SK) in 2:1 ratio, **(d)** co-digestion of mango peel and seed kernel in 1:1 ratio, **(e)** co-digestion of mango peel and seed kernel in 1:4 ratio.The methane yields are shown only during pseudo steady state (Day 31-35).

For mango peels, steady state was reached on Day 26 and continued for the next 10 days (Figure 5.1a). The biodegradability of de-oiled seed kernel in continuous mode was higher than that of mango peels, indicated by the higher VS reduction. There were higher variations in the reactor pH of mango peels than for the de-oiled seed kernel during anaerobic digestion. The pH range (Table 5.2) during de-oiled seed kernel digestion (6.58-7.22) was closer to the normal range of 6.5-7.6 (Labatut and Gooch, 2012) than for the digestion of mango peels (5.91-7.04), indicating the relative stability of de-oiled seed kernel treating reactor. The lower pH of mango peels affected digestion, resulting in more fluctuations in biogas yield compared to that of the seed kernel. The pH of the mango peels treating reactor dropped below normal range at day 26 and 32, and a 2-3 g $NaHCO_3$ was added to bring the pH to normal range on these days. De-oiled seed kernel was found to have higher biomethane production than mango peels (Table 5.2). The VFA to alkalinity ratios of de-oiled seed kernel (Figure 5.1b) were closer to the normal range of 0.1-0.2 (Gerardi, 2003). The VFA to alkalinity ratios during MP+SK co-digestion 1:4 and 2:1 were in the normal range (0.1-0.2) (Figure 5.1c and 5.1e). On the other hand, the VFA to alkalinity ratios during co-digestion of MP+SK at 1:1 fluctuated from the normal range and ranged between 0.1-0.3 (Figure 5.1d). The pH during co-digestion of MP+SK

at 1:4 was in the normal range (Table 5.2), while the pH was below the normal range during co-digestion of MP+SK at 1:1 and 2:1 (Figures 5.1c and 5.1d).

Table 5.2. Mono-digestion and co-digestion results of mango peels and de-oiled seed kernel during anaerobic digestion in continous mode

Conditions	Average Daily biogas production rate (NL/L.day)	CH_4 %	Average Daily CH_4 production rate (NL/L.day)	VS Reduced (in %)	pH range	C/N ratio
Mango peel	0.37	67	0.25	52	5.91-7.04	53
Seed kernel	0.49	70	0.34	69	6.58-7.22	11
Mango peel + seed kernel (2:1)	0.70	61	0.42	56	6.40-7.0	39
Mango peel + seed kernel (1:1)	0.74	66	0.49	65	6.44-7.0	32
Mango peel + seed kernel (1:4)	0.93	71	0.66	70	6.84-7.42	20

The calculated C/N ratios in co-digestion of mango peel plus de-oiled seed kernel in 1:4, 1:1, 2:1 ratios were 20, 32, and 39, respectively (Table 5.3). In co-digestion, the average biogas yield of mango peel plus de-oiled seed kernel (MP+SK) co-digested in 1:4 was 25.8 and 30.3% higher than the 1:1 and 2:1 ratios respectively. The average biogas yield of MP+SK in 1:4 was significantly higher (p<0.05) than the co-digestion in 1:1 and 2:1 (Figure 5.2a). Similarly, the average biomethane yield of MP+SK in 1:4 was also significantly higher (p<0.05) than the co-digestion in 1:1 and 2:1 (Figure 5.2b). The average biomethane yield of MP+SK (1:4) was 25 and 36.4% higher than MP+SK co-digested in 1:1 and 2:1, respectively (Table 5.3).

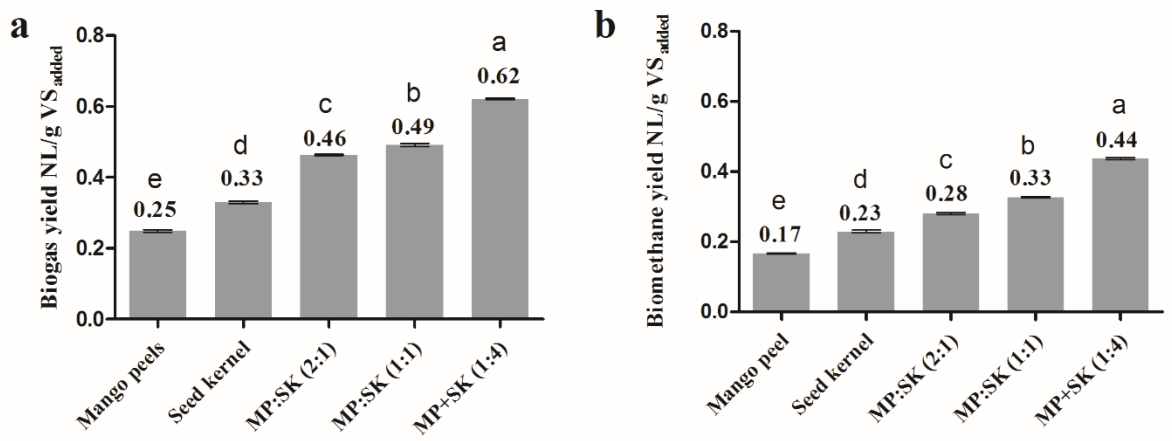


Figure 5.2. Comparison of biogas and biomethane yield of mango peels and de-oiled seed kernel in mono-digestion and co-digestion in continuous setup. **(a)** Biogas yield of mango peels and de-oiled seed kernel mono-digestion and co-digestion (MP:SK; 2:1, 1:1 and 1:4), **(b)** biomethane yield of mango peels and de-oiled seed kernel mono-digestion and co-digestion (MP:SK; 2:1, 1:1 and 1:4), MP: mango peels, SK; seed kernel. Data are means ± SE. For comparisons of all treatments one-way ANOVA, followed by Scheffe post test were used. Different letters on the bars show significant differences (p<0.05) between treatments.

The actual biogas yield of MP+SK in 1:4 (Figure 5.3a) was significantly higher p<0.05 (50% increase) than the calculated yield in same ratio (1:4). Similarly, the actual biogas yield of MP+SK in 2:1 was significantly higher with 41.7% increase than that calculated biogas yield for the same ratio (2:1). However, the actual biogas yield of MP+SK in 1:1 was 6.5% lower than the calculated yield for same ratio (Figure 5.3a). On the other hand, the actual biomethane yields of MP+SK in 1:4, 1:1, and 2:1 ratios were significantly (p<0.05) higher (50, 39.4, and 32.1% increase) than the calculated corresponding biomethane yields, respectively (Figure 5.3b). Biomethane production has been reported to be highly efficient at C/N ratios in the 20:1 to 30:1 range (Hessami et al., 1996, Raheman and Mondal, 2012). In the present study, the same trend was found, with higher biogas yields achieved at calculated C/N ratio of 20:1 and decreased biogas yields were observed as the C/N ratios moved away this ratio (e.g., at 32:1 and 39:1). The lowest biomethane yield was found at calculated C/N ratio of 39:1 with MP+SK (2:1). Similar effects of C/N ratio were also shown during co-digestion of Jatropha seed cake and bagasse (Sen et al., 2013).

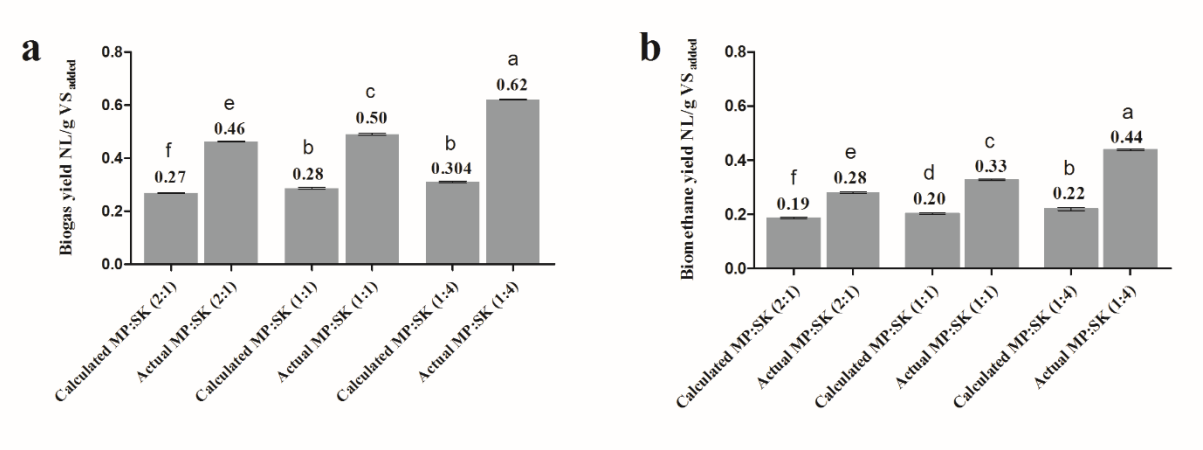


Figure 5.3. Comparison of actual and calculated biogas and biomethane yield of mango peels and de-oiled seed kernel co-digestion in different ratios. **(a)** Mango peels and de-oiled seed kernel co-digestion at different ratios, **(b)** mango peels and de-oiled seed kernel co-digestion at different ratios. Data are means ± SE. For comparisons of all treatments one-way ANOVA, followed by Scheffe post test were used. Different letters on the bars show significant differences (p<0.05) between treatments.

The VS reduction was higher during the anaerobic co-digestion of MP+SK in 1:4 compared to those at 1:1 and 2:1. This higher VS reduction results in higher biomethane production (Haider et al., 2015). The results showed that the biogas yields of MP+SK co-digestion in 1:4 ratio were 25.8, 30.3, 51.5, and 62.1 % higher than biogas yields of 1:1, 2:1, seed kernel (mono-digestion) and mango peel (mono-digestion), respectively (Figure 5.2a). The biogas yield during co-digestion of MP+SK in 1:4 was significantly ($p<0.05$) higher than all other treatments in continuous mode (Figure 5.2a). The biomethane yield also showed the same pattern; the biomethane yields during co-digestion at 1:4 were 25, 36.4, 50, and 61.4 % higher than the 1:1, 2:1, seed kernel (mono-digestion) and mango peel (mono-digestion) biomethane yields, respectively (Figure 5.3b). These results clearly indicate that for a stable biogas reactor with efficient biogas and biomethane yield the C/N ratio must always be in the range of 20-30.

5.4.2. Batch anaerobic digestion of different parts of *Jatropha curcas* fruit

The biogas yield of fruit coat (pretreated with 1% phosphoric acid) was 7, 22, 34, 50, and 74% higher than those of de-oiled seed kernel, fruit coat (without pretreatment), de-oiled seed kernel plus seed coat (pretreated with 1% phosphoric acid), seed coat (pretreated with 1% phosphoric acid) and seed coat (without pretreatment), respectively (Figure 5.4a). The biogas yield of fruit coat (pretreated with 1% phosphoric acid) was significantly (p<0.05) higher than

de-oiled seed kernel plus seed coat (pretreated with 1% phosphoric acid), seed coat (pretreated with 1% phosphoric acid) and seed coat (without pretreatment). This clearly indicated that the fruit coat was more easily biodegradable compared to the rest of the substrates. Moreover the pretreated fruit coat gave 22% higher biogas yield than the non-pretreated fruit coat. Similarly, the pretreated seed coat exhibited a tremendous increase of about 47% in the biogas yield as compared to the non-pretreated seed coat. Phosphoric acid hydrolysis of hemicellulose and cellulose containing organic biomass causes release of higher sugars compared to other acids (Gámez et al., 2006) that are directly available for anaerobic microbial communities resulting in enhanced biogas yield. Dilute sulfuric acid pretreatments has been found to be effective. However, sulfuric acid pretreatment was not used because of concerns with competition between sulfate reducing bacteria and methanogens (Badshah et al., 2012). The biogas yields (Figure 5.4a) show that fats and cellulose had the highest yields (normalized per g VS added) indicating that the inoculum used contained the necessary consortia required for methanogenesis. The biogas yield of fats and cellulose (Figure 5.4a) was below the theoretical methane yield of carbohydrates and lipids, which have been reported to be 0.415 L/g VS_{added} and 1.014 L/g VS_{added}, respectively (Angelidaki and Sanders, 2004).

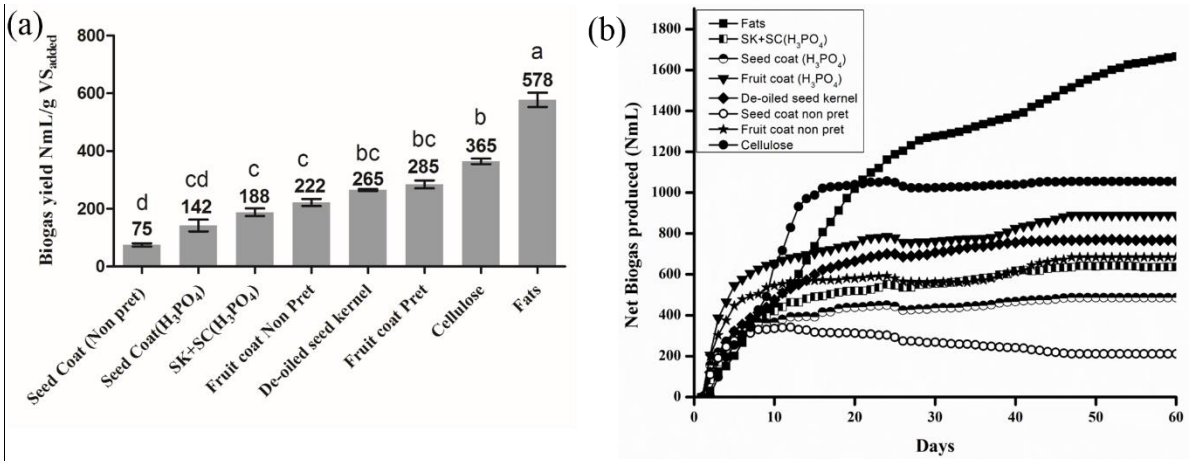


Figure 5.4. Evaluation of biogas potential of different treatments of *J. curcas* fruit during batch anaerobic digestion setup. **(a)** Biogas yield presented as NmL/g VS_{added}. SK + SC (H_3PO_4): (co-digestion of *J. curcas* de-oiled seed kernel and seed coat (pretreated) mixed in 1.7:1 ratio and the seed coat was pretreated with 1% phosphoric acid), H_3PO_4 means pretreated samples. Data are means ± SE. For comparisons of all treatments one-way ANOVA, followed by Scheffe post test were used. Different letters on the bars show significant differences (p<0.05) between treatments. **(b)** Net biogas produced is presented as normalized mL (at standard temperature 273.15 K and pressure 1.013 bar) by different parts and combinations of parts of *J. curcas* fruit.

The calculated biogas yield of SK + SC (H_3PO_4) was 219.4 NmL/g VS_{added} while the actual yield was 188 NmL/g VS_{added}, indicating that the actual yield is 16.7% less than the calculated one. It is evident that the seed coat (either pretreated or non-pretreated) reduces the co-digestion biogas yield potential of the seed kernel. The seed kernel is rich in fibrous proteins and lipids and the seed coat in seed cake mainly comprises of high percentages of hemicellulose followed by cellulose and lignin (Kumar et al., 2016, Liang et al., 2010). The hemicellulose on acid hydrolysis releases sugars which are sometimes subsequently converted into inhibitory products such as furfural, hydroxymethyl furfural, and acetic acid, resulting in microbial inhibition. In addition, the subsequent conversion of sugars to these inhibitors also causes sugar loss which ultimately lead to decrease in biogas yield (Gámez et al., 2006, Larsson et al., 1999, Lawford and Rousseau, 1998). The high concentration of lignin (49.4% content in the seed coat) may also be the reason for the low biogas yield, since lignin prevents microbial access to cellulose and sugars (Yamamura et al., 2012).

Initially, all of the different parts of *J. curcas* fruit exhibited an increase in biogas production rate (Figure 5.4b). The pretreated fruit coat followed by non-pretreated fruit coat were highest in biogas production in the initial phases during batch anaerobic digestion. The de-oiled seed kernel gained momentum after the initial phase and was second highest after the fruit coat in terms of biogas production. On the other hand, the biogas production rate of non-pretreated seed coat decreased in later stages of anaerobic digestion and exhibited the lowest biogas production rate. The pretreated seed coat was more stable and exhibited an even trend of biogas production rate in later stages compared to non-pretreated seed coat. Biodegradation of fats usually starts later than the sugar or protein containing compounds and the current study also showed a similar trend in biogas production by fats (Association et al.). There was a notable increase in the biogas yield of fats in later stages and had the highest biogas production rate (Figure 5.4b).

5.5. Conclusions

To allow higher biogas and biomethane yields compared to the calculated yield, the co-digestion of mango peel with de-oiled seed kernel of *J. curcas* is suggested in large scale reactors. A C/N ratio of 20:1 is needed for enhanced biogas yield and stability of reactor. This can be achieved by a 1:4 ratio of mango peel to de-oiled seed kernel. Moreover, dilute phosphoric acid (1% H_3PO_4) pretreatment of lignocellulosic materials enhanced biogas yield compared to non-pretreated biomass during anaerobic digestion. An optimized C/N ratio and dilute H_3PO_4 pretreatment are highly recommended for a stable and efficient anaerobic digestion process.

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CHAPTER 6

Chapter 6: Effect of *Jatropha curcas* seed on biogas and microbial communities

Paper 4

Title: Effect of *Jatropha curcas* seed on biogas production and microbial communities under different operational parameters during anaerobic digestion

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6.1. Abstract

The different parts of the seed of *Jatropha curcas*, a pest- and drought-resistant plant, were investigated as potential feedstocks for biogas production. *J. curcas* seed pressed cake, a by-product of the biodiesel industry, was subjected to methanolic extraction. The residues of seedcake after methanol extraction are referred as methanolic residues. The inhibitory effects of methanolic extracts of pressed cake on different steps of anaerobic digestion (AD) were evaluated in batch anaerobic digestion experiments. The effect of methanolic extract on biogas yield was evaluated under different operational parameters coupled with microbial communities' analyses in continuous reactors using 16S rRNA gene sequencing. In the batch experiments, higher biogas yield was obtained from methanolic residues (pressed cake after methanolic extraction) compared to pressed cake, aqueous, and *n*-hexane residues. It was also observed that methanolic extract exhibited inhibitory effect on hydrolysis step of AD, which resulted in reduction of biogas production by 35.5%. In the continuous reactors, the relative abundance of fermentative bacteria was higher in reactors fed with methanolic residues than in those fed with Jatropha pressed cake, seed oil and whole seed. However, Jatropha seed oil and whole seed did not exhibit inhibitory effects on methanogens. A higher relative abundance of methanogenic and a lower abundance of fermentative bacterial communities were observed in all reactors at a hydraulic retention time of 20 days compared to 15 and 10 days. The results of this study can be used to maximize biogas production from anaerobic digestion of *J. curcas* seed components, while providing an alternative route for production of value added compounds and biodiesel production.

Keywords: Anaerobic digestion, *Jatropha curcas*, microbial abundance, biogas, amplicon sequencing

6.2. Introduction

Biogas production is promising due to relatively lower energy consumption, reduction of biosolids, and reduced environmental impact (Kurade et al., 2019). There are many potential biogas feedstocks, but a large issue with many is that their production leads to a food-vs-fuel trade-off. Non-edible crops grown on land not suitable for food production do not suffer from that disadvantage, and are considered ideal for biogas production (Scarlat et al., 2015). So *Jatropha curcas,* an oil-rich, pest- and drought-resistant shrub, is considered a strong candidate for biodiesel and biogas production. The pressed cake after oil extraction cannot be used as animal fodder due to considerable toxicity and is an unused resource. Producing biogas from the unused cake is a potential option. However, *J. curcas* inhibits microbial activities (potentially including methanogenesis) due to the presence of compounds such as phorbol esters, curcin, and long chain fatty acids (Devappa et al., 2012). To increase the efficiency of anaerobic digestion, the antimicrobial phytochemicals can be extracted from *J. curcas* pressed cake using organic solvents such as methanol. The resulting residues of pressed cake are called methanolic residues can then be anaerobically digested, potentially enhancing the microbial diversity in the reactor and ultimately biogas yield. The use of *J. curcas* seed in a sustainable way for biogas, biodiesel, and other value added compounds in biorefinery context is shown in (Appendix 4 Figure S1).

Even with an acceptable substrate, biogas yield is highly affected by operational parameters. The main reason for microbial inhibition in a digester is the increase in volatile fatty acids at higher organic loading rates (OLRs). An appropriate hydraulic retention time (HRT) is required for sustaining biodegradability of organic matter during mesophilic anaerobic digestion. Thus studying operational parameters (OLR and HRT) to increase the efficiency of methanogenesis is needed. To date, a number of studies had been conducted on the biogas potential of *J. curcas* seed (Gavilanes et al., 2019, Jabłoński et al., 2017, Narra et al., 2016) but none has evaluated the effect of toxicity of seeds on microbial communities and biogas production during anaerobic digestion.

The goals of this study were to (1) evaluate the biogas potential of *Jatropha curcas* seed, (2) assess the inhibitory effects of *Jatropha curcas* methanolic extracts on different steps of anaerobic digestion, (3) microbial profiling during anaerobic digestion of different parts of *J. curcas* seed at different operational parameters (organic loading rates and hydraulic retention time).

6.3. Materials and methods

6.3.1. Substrate preparation, and characterization of inocula and susbstates

Inocula used in batch and continuous reactor experiments were collected from a running bioreactor (fed with fruit and vegetable wastes and cattle manure) at the Sustainable Bioenergy and Biorefinery Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad and was incubated at 37°C for 2-3 weeks for degassing. Specific methanogenic activities of inocula showed that the microbial communities were active (Appendix 4 Figure S2). The *J. curcas* seeds were obtained from a local dealer in Lahore and was identified at the National Herbarium of Pakistan, Quaid-i-Azam University. Oil was extracted from *J. curcas* whole seeds (JWS) using a mechanical oil expeller in Rawalpindi, Pakistan. After oil extraction, the remaining de-oiled Jatropha pressed cake (JPC) was further ground to a powder form and preserved in sterile zip-lock bags at -20°C until further use. Measurement of total solids (TS), volatile solids (VS) and pH of substrates and inocula were performed according to Standard Methods (APHA, 2005).

Preliminary qualitative tests of *J. curcas* seed oil (JO) and de-oiled pressed cake extracts were carried out for identification of balsams, flavonoids, saponins, glycosides, steroids, phenol, and tannins as previously described (Amina et al., 2013, Haq et al., 2019). The phytochemicals were removed from *Jatropha* pressed cake using aqueous, methanolic, and *n*-hexane extraction.

The aqueous, methanolic, and *n*-hexane extracts of de-oiled Jatropha pressed cake were prepared as described elsewhere (Basri and Fan, 2005). Fine powdered *J. curcas* de-oiled pressed cake (100 g) was dissolved in 500 mL of the individual solvents and incubated at 30°C for 48 hrs. The extracts were filtered and the solvents evaporated using a rotary evaporator (Rotary Evaporator RE300 Stuart®) at reduced pressure. The resulting crude extract was allowed to dry at room temperature to a constant weight. The residues obtained from aqueous, *n*-hexane, and methanolic extraction were termed as aqueous residues (AR i.e., Jatropha pressed cake after aqueous extraction), *n*-hexane residues (NR i.e., Jatropha pressed cake after *n*-hexane extraction) and methanolic residues (MR i.e., Jatropha pressed cake after methanolic extraction), respectively, and stored at 4°C for further use in anaerobic digestion. Fourier transform infrared (FTIR) spectroscopic analyses of *J. curcas* seed oil and JPC extracts were carried out using standard procedures (Haq et al., 2019). The chemical composition of *J. curcas* JPC extracts and seed oil was determined using gas chromatography coupled with mass

spectrometry (GC-MS) technique (GC-MS – QP5050A, Shimadzu, Europe) according to previously described methods (Mu'azu et al., 2013, Oskoueian et al., 2011), with the following modifications. A 2 µL aliquot of each sample was injected separately into the column using an automated injector split ratio 1/48 (for extracts) and 1/25 (for seed oil). The column (DB-5) had a length 30 m, internal diameter 0.25 mm, and thickness 0.25 µm with flow rates of 1 and 1.8 mL min^{-1} for extracts and seed oil, respectively. The analytes were detected using a thermal conductivity detector (TCD). The National Institute of Standards and Technology library (NIST 27 and NIST 147) was used for peak identification based on mass spectra.

6.3.2. Anaerobic digestion

Anaerobic digestion of different parts of *J. curcas* seed was investigated both in batch and continuous mode. The biogas potential of *J. curcas* seed and the effect of methanolic extracts of JPC on a number of substrates representing different degradation steps in anaerobic digestion (i.e., hydrolysis, acidogenesis, and acetoclastic methanogenesis) were evaluated in batch mode. The effect of methanolic extraction from JPC on microbial communities during anaerobic digestion in continuous setup was evaluated (see below).

6.3.3. Biogas potential and inhibitory effects of methanolic extracts of pressed cake on different stages of anaerobic digestion in batch mode

Batch anaerobic digestion was carried out in triplicate in 500 mL reactors with a working volume of 400 mL at 37°C. The inocula and substrate were added in 4:1 ratio (4 g VS of inocula and 1 g VS of substrate). The amounts of substrates added to their respective reactors were as follows; JWS (2.22 g), JO (1.89 g), JPC (2.5 g) MR (2.76 g), NR (2.68 g), and AR (7.05 g). Reactors with cooking oil (1.76 g) and cellulose (1.9 g) as substrates were used as positive controls (Astals et al., 2014). The background biogas produced by the inocula (negative control) was subtracted from the biogas yield of each substrate. To evaluate the inhibitory effect of methanolic extract of JPC on the different degradation steps in anaerobic digestion, cellulose (2.45 g, to investigate hydrolysis), glucose (2.9 g, to investigate acidogenesis), and sodium acetate (10.3 g, to investigate acetoclastic methanogenesis) were added to reactors with methanolic extract (2 $mg.mL^{-1}$) and without methanolic extract of JPC. Methanolic extract of JPC plus inocula was used as negative control and the biogas produced was subtracted from the biogas yield of each substrate representing different steps of anaerobic digestion. The pH of all reactors was adjusted to neutral using 1 M solutions of HCl and NaOH. Before incubation, the reactors were flushed with nitrogen gas and were sealed with butyl

rubber cork. The biogas was collected in air tight bags (UNOGUARD, China) attached to each reactor and measured using syringe, and the biogas volumes of each substrate were normalized at standard temperature and pressure (273.15 K and 101325 pascal).

6.3.4. Effect of operational parameters on biogas yield in continuous mode

In continuous mode, the anaerobic digestion of JWS, JO, JPC and MR was carried out at different organic loading rates (OLRs) and hydraulic retention times (HRTs) in 2.5 L reactors with working volumes of 2 L at 37°C in an incubator. Substrate was fed every 24 hrs. Depending upon the efficiency of each reactor, the reactors were operated at different OLRs and HRT of 20 days in single stage anaerobic digestion setup. The reactor treating MR was operated at OLRs 1-7 g VS L^{-1} day^{-1}. The MR reactor was initially operated at OLR of 1-3 g VS L^{-1} day^{-1} and HRT of 20 days in single reactor with a an influent/effluent flow of 100 mL day^{-1}, but at OLR 3 g VS L^{-1} day^{-1}, a sudden decrease in the biogas production occurred due to rapid biodegradability of MR. Therefore, the digestion of MR was further tested in two stage anaerobic digestion to address the issue of rapid biodegradability. In two stage anaerobic digestion, the MR treating reactors were operated at OLRs of 3, 4, 5, 6 and 7 g VS L^{-1} day^{-1}. To maintain equal volume as in one stage anaerobic digestion of MR, the reactors R_1 and R_2 were operated each at an HRT of 10 days with a flow rate of 200 mL day^{-1} for each reactor. In two stage setup, both reactors in combination had the same total volume as in single stage digestion. The JPC treating reactor was operated at OLRs starting from 1-6 g VS L^{-1} day^{-1}; reactor treating JWS was operated at 1, 1.5, 2, and 3 g VS L^{-1} day^{-1} and the reactor treating JO was operated at an OLR of 1 g VS L^{-1} day^{-1}, and HRT of 20 days with influent/effluent flow of 100 mL day^{-1}.

To determine the optimum HRT, the reactors were operated at HRTs of 10, 15 and 20 days with a flow rate of 200 mL, 130 mL and 100 mL, respectively, and the reactors were fed at the respective optimum OLRs determined in the previous experiment. During the startup phase, 1.5 L of acclimatized inocula was added to each reactor, flushed with nitrogen gas and plugged with butyl rubber cork attached to pipes for biogas collection, substrate feeding and effluent removal. Each reactor was fed with their respective substrates at the specific HRT without taking any effluent until it reached its working volume (2 L). The biogas was collected in air tight bags (UNOGUARD, China) attached to reactors and was measured using a syringe. When the reactor reached a pseudo-steady state (defined as less than 5% variation in consecutive biogas volume measurements), the biomethane produced was measured at the end

of each pseudo-steady state for a few days by passing the biogas produced through a scrubbing system containing 3M NaOH solution. The volume of biogas and biomethane were normalized at standard temperature and pressure (273.15 K and 101325 pascal). The effluent pH was measured on daily basis. The volatile fatty acids (VFAs) and alkalinity were measured at specific intervals using standard titrimetric procedures as described elsewhere (Clesceri et al., 1998).

6.3.5. Microbial community analysis using high throughput sequencing

Samples (50 mL) for microbial DNA analyses were collected at different intervals from each reactor operating at optimized OLR and different HRTs (20, 15 and 10 days). The samples were preserved at -20°C until further use.

6.3.5.1. DNA extraction, 16S rRNA gene amplification, sequencing, and data processing

Microbial DNA was extracted using DNeasy Powerlyzer Powersoil microbial DNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Quantity and purity of extracted DNA were assessed using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Wilmington, USA). DNA fragments of approximately 460 bp length flanking the V3 and V4 regions of the 16S ribosomal RNA (rRNA) gene of bacteria and archaea were amplified using forward and reverse primer pairs, modified 341F and modified 806R, respectively (Sundberg et al., 2013, Yu et al., 2005). Library preparation, quantification, normalization, and pooling were conducted following the Illumina 16S metagenomics protocol (Illumina, 2013). Library quantity and quality were assessed using a D1000 ScreenTape® Tapestation (Agilent technologies, Santa Clara, CA). Prepared libraries were pooled and run on an Illumina MiSeq platform for 300 bp paired-end read sequencing at the Genomic Sciences Laboratory, North Carolina State University, NC. Raw sequences were deposited to the National Centre for Biotechnology Information Sequence Read Archive (BioProject ID PRJNA557512).

6.3.5.2. Sequence analyses

QIIME version 1.9.1 (Caporaso et al., 2010b) was used for qualitative screening of genomic sequences. Forward and reverse sequences were merged, de-barcoded and trimmed (Bolger et al., 2014). *Multiple_split_libraries_fastq.py* script was used for demultiplexing and quality filtering. Chimeric sequences were identified by *parallel_identify_chimeric_seqs.py* script using ChimeraSlayer and filtered using *filter_fasta.py* script (Haas et al., 2011). The

screened sequences were then clustered into operational taxonomic units (OTUs) by *pick_open_reference_otus.py* script with UCLUST algorithm using 97% similarity index along with Greengenes database version (13_8) (Caporaso et al., 2010b). Taxonomic classification was done based on the latest Greengenes database version (13_8 and RDP classifier 2.2 with a confidence value of 0.8 using UCLUST (Edgar, 2010). Alignment was performed using PyNAST (Caporaso et al., 2010a) followed by generating phylogenetic trees using *make_phylogeny.py* script. The OTUs having less than 0.05 % of the total sequence reads were filtered out for further analysis.

6.3.6. Statistical analyses

Alpha and beta diversities of microbial communities in reactors treating JWS, JO, JPC and MR were analyzed using R software (McMurdie and Holmes, 2013). For alpha diversity, the observed and Shannon indexes were calculated. The observed index represents the species richness and the Shannon index includes both richness and evenness. Beta diversity was analyzed using Bray-Curtis two dimensional ordination plots in R software. Canonical correspondence analysis (CCA) was used to correlate microbial communities and the following parameters: VFA, Alkalinity, VFAs/Alkalinity ratio, pH, and biogas yield. One-way ANOVA followed by Tukey's post-test was used for multiple comparisons of biogas yield in batch and continuous setups using Prism Graphpad. Relative abundance of microbial communities was analyzed using R software.

6.4. Results and Discussion

Initially, *J. curcas* seeds were subjected to mechanical oil extraction and yielded 32.5 % oil. The TS and VS contents of the substrates are shown in (Appendix 4 Table S1).

J. curcas seeds have a number of compounds such as phorbol esters, curcin, and long chain fatty acids (LCFAs) that have been reported to have antimicrobial activities (Mendonça et al., 2019, Haq et al., 2019). Preliminary phytochemical analyses of *J. curcas* seed oil and de-oiled JPC extracts (aqueous, methanolic, and *n*-hexane) detected flavonoids, steroids, tannins, and phenol. Saponins and glycosides were only detected in the aqueous and methanolic extracts and were absent in seed oil and *n*-hexane extract. No balsams were found in any extract or seed oil. A broad range of phytochemicals were detected during GC-MS (Appendix 4 Tables S2-S5) and FTIR analyses of JO and JPC extracts (Appendix 4 Figures S3-S6). A number of LCFAs were identified in JO and *n*-hexane extracts (Appendix 4 Table S4 and S5). The LCFAs by itself have inhibitory effects on microbial cells, however, the exact mechanism of inhibition of LCFAs is unknown. The LCFAs target microbial cell membrane where a number of different processes occur, and interact with cells either biochemically or physiologically in nature. Biochemical inhibition caused by LCFAs is due to its amphipathic nature that act like detergent and solubilize microbial cell lipid bilayer or membrane proteins resulting in cell lysis, inhibit enzyme activities and disrupt the electron transport chain. On physical attachment, the LCFAs cover the microbial cells hindering the transport of substrate and subsequent biogas release (Ma et al., 2015). The methanolic extract is highly rich in medicinally important compounds such as beta-monolaurin, I-(+)-ascorbic acid 2,6-dihexadecanoate, 9-hexadecenal, bis (tridecyl) phthalate, 1-docosanol and diacetone alcohol (Appendix 4 Table S3). Beta-monolaurin had been reported as having antimicrobial properties and may damage microbial cell membranes, targeting various proteins and nucleic acids and macromolecular synthesis processes resulting in cell damage (Skřivanová et al., 2006). Similarly, ascorbic acid 2,6-dihexadecanoate had been reported as having antioxidant activities, and 9-hexadecenal and 1-docosanol for antimicrobial activities (Bhardwaj, 2018) . Methanolic solvent was a better extraction solvent than aqueous and *n*-hexane (Haq et al., 2019). The effects of methanolic extract of de-oiled JPC on different steps of the anaerobic process are discussed below.

6.4.1. Biogas potential of *J. curcas* seed and inhibitory effect of methanolic extract of pressed cake on different steps in anaerobic digestion

The biogas potential of *J. curcas* seed and the effect of methanolic extract of de-oiled JPC was evaluated on different steps of anaerobic digestion in batch process. Net biogas produced by methanolic residues was 32, 39, and 62% higher than Jatropha pressed cake, *n*-hexane residues, and aqueous residues, respectively (Figure 6.1C). This suggests that antimicrobial extraction with methanol of de-oiled JPC increases its anaerobic biodegradability or improves the response of anaerobic microbial communities. The higher biogas yield of methanolic residues (Figure 6.1A) also indicates more antimicrobials were extracted by methanol compared to *n*-hexane and aqueous solvents. This is likely because methanol has the capability to dissolve both polar and non-polar compounds as its polarity lies in the middle of polarity index (Haq et al., 2016).

When the methanolic extract was added to various substrates, the biogas production was not inhibited in reactors fed with sodium acetate and glucose (Figure 6.1B). However, methanolic extract addition to the reactor fed with cellulose significantly decreased ($p<0.05$) the biogas production (35.5%). These results show that methanolic extract of JPC affects hydrolysis and has no apparent adverse effects on the acetoclastic methanogenesis or acidogenesis steps (Figure 6.1B). The phytochemicals present in *J. curcas* seeds might affect hydrolytic bacterial communities or their enzymes, including hydrolases, amylases, proteases, and lipases. The effect of methanolic extract on microbial communities is explained in Section 6.4.2.

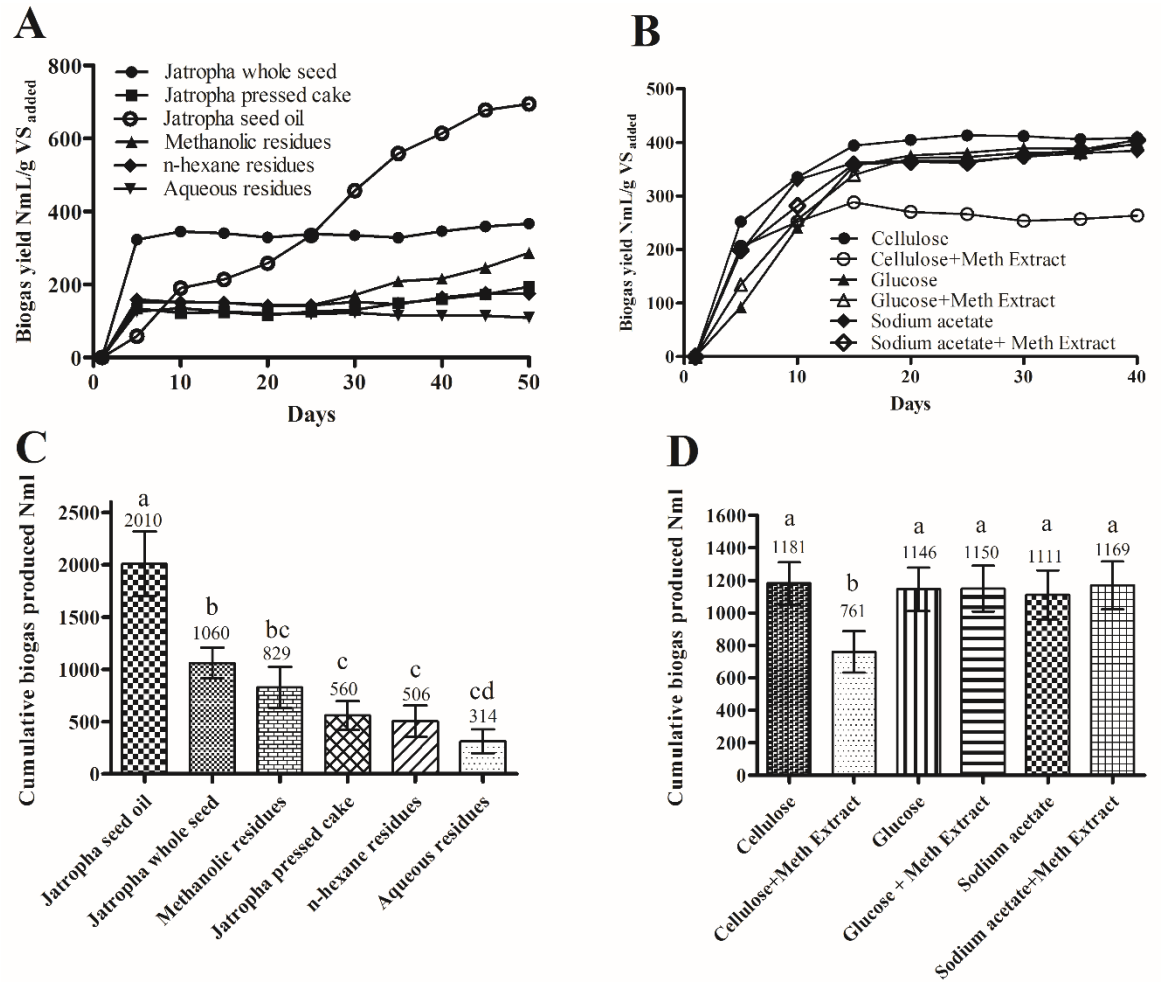


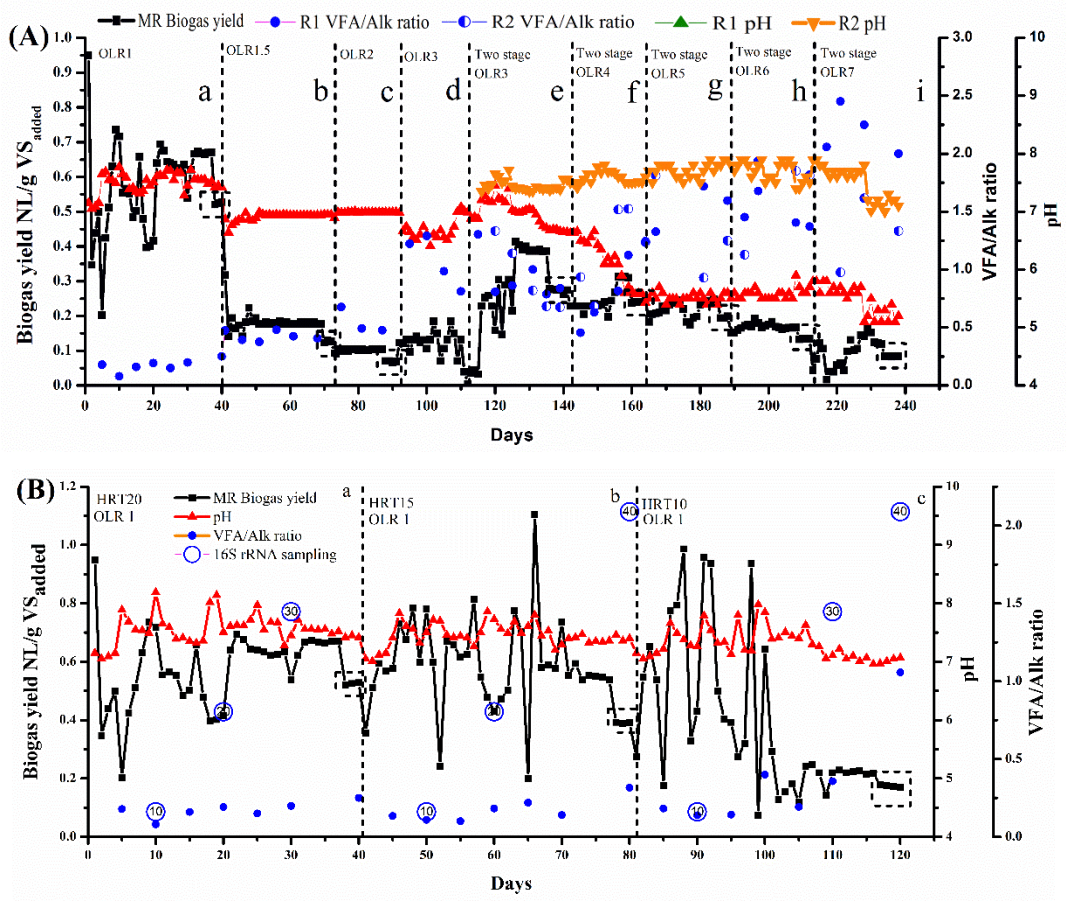
Figure 6.1. Net cumulative biogas yields and production of different treatments of *J. curcas* seed and the effect of methanolic extracts of de-oiled Jatropha pressed cake (JPC) on different steps of anaerobic digestion. (A) Biogas yield (in NmL/g VS_{added}) of different treatments of *J. curcas* seeds (B) Inhibitory effects of methanolic extracts of de-oiled JPC on net biogas yield (in NmL/g VS_{added}) of cellulose, glucose and acetate representing different steps of anaerobic digestion. (C) Biogas potential of *J. curcas* seed. Inocula was used as negative control that was subtracted from biogas yield of substrates (D) Inhibitory effects of methanolic extract of de-oiled JPC on net biogas production of cellulose, glucose and acetate representing different steps of anaerobic digestion. The background biogas produced by negative control (inocula plus methanolic extract) was subtracted from biogas produced by each substrate. The experiments were carried out in triplicates and the data was presented as mean ± standard deviation. One way ANOVA followed by Tukey's pot-test for multiple comparison was used. The same lowercase letters on the adjacent bars indicate no significant differences; different letters indicate significant differences. Level of significance was $p < 0.05$.

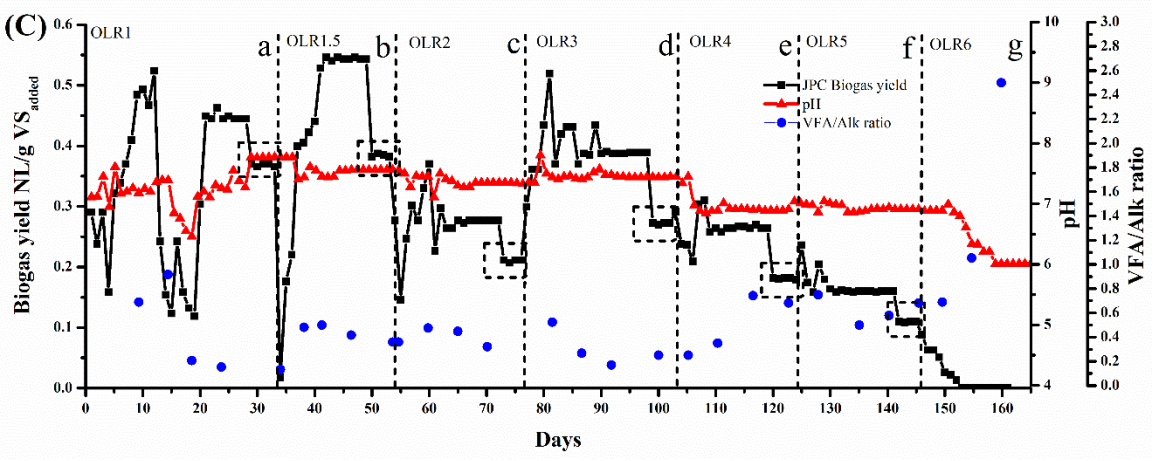
6.4.2. Effect of *J. curcas* seed's phytochemicals and operational parameters on biogas yield during anaerobic digestion

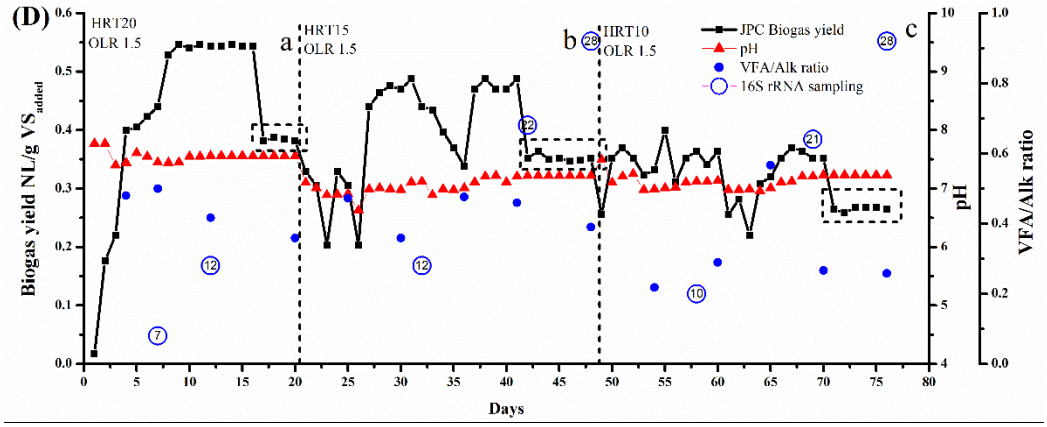
The inhibitors/phytochemicals present in *J. curcas* seed and the operational parameters are the key factors that affect the process of anaerobic digestion. These factors must be addressed in a way to increase the biogas yield and economic value of *J. curcas* seed for the process of anaerobic digestion. In the current study, the reactor treating MR (Jatropha pressed cake after methanolic extraction) was the most stable and efficient continuous mode reactor compared to those fed with JPC, JO and JWS (Figure 6.2). Optimum biogas and biomethane yield for MR was obtained at OLR of 1 g VS L^{-1} d^{-1} and HRT of 20 days and was significantly higher (p<0.05) than that obtained at OLRs (1.5, 2, 3, 4, 5, 6, and 7) and HRT (15 and 10 days (Figure 6.2A and 6.2B). In MR treating reactor, the biogas yield was decreased after 1 g VS L^{-1} day^{-1} due to its high biodegradability and increase in OLR that resulted in continuous accumulation of VFAs, subsequently not converted into methane in the same pace. The VFAs/alkalinity ratios (Supplemental Information, Table S6) was increased at OLR of 3 g VS L^{-1} day^{-1} and HRT 20 days; to address the issue of high VFAs/alkalinity ratio, the reactor was transferred at two stage anaerobic digestion. The MR treating reactor was stopped at OLR of 7 g VS L^{-1} day^{-1} as the small diameter pipes began to be clogged due to higher overload of MR during feeding. The JPC treating reactor gave optimum biogas and biomethane yield at an OLR of 1.5 g VS L^{-1} d^{-1} and HRT of 20 days (Figure 6.2C and 6.2D). It was sustained until OLR of 6 g VS L^{-1} d^{-1} (Figure 6.2C). The JO was rich in LCFAs and the reactor treating it was inhibited in early stages at OLR of 1 g VS L^{-1} day^{-1} at HRTs of 20, 15 and 10 days (Figure 6.2E). The LCFAs causes decrease in biogas yield and reactor failure (Xu et al., 2015). The JWS treating reactor was fluctuated and not sustained for longer. The optimum biogas yield for JWS was obtained at 1.5 g VS L^{-1} d^{-1} and a HRT of 20 days and the reactor was failed at OLR of 3 g VS L^{-1} d^{-1}. The possible reason of this early failure of JWS might be the higher antimicrobial compounds than JPC and MR and the increase in VFAs/alkalinity ratios (Appendix 4 Table S6) beyond the acceptable limit (<0.4) leading to over acidification of the reactor. The JWS is rich in proteins and relatively poor biodegradation of proteins during anaerobic digestion had been observed compared to the lipids and carbohydrates (Lee et al., 2016). To sum up, the MR treating reactor was sustained for longer time and exhibited significantly higher (p<0.05) biogas yield compared to JPC, JO and JWS treating reactors at optimized OLR and HRT. The biodegradability of MR in terms of VS reduction was higher at optimized condition (OLR and HRT) compared to that of JPC, JO and JWS (Appendix 4 Table S6). So it means using JWS

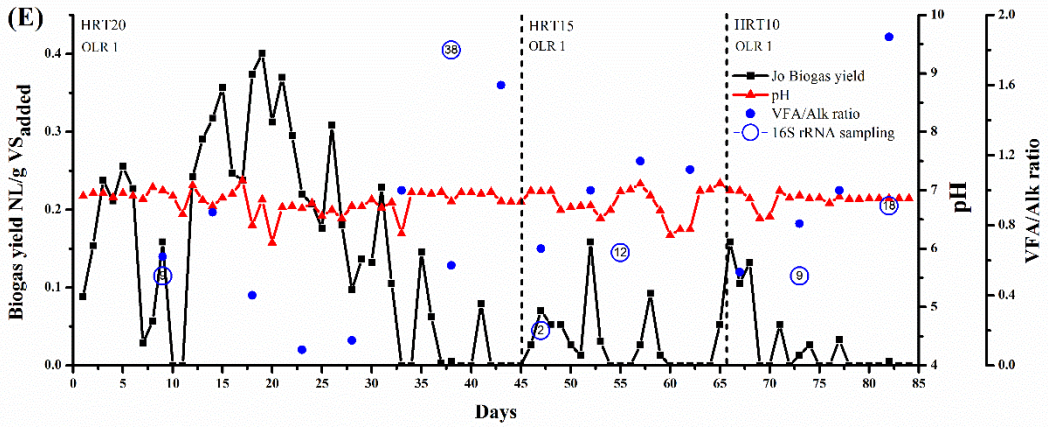
for biogas production is not a better option. But if oil is extracted, can be used for biodiesel (ul ain Rana et al., 2019).

The biomethane/biogas yield of all substrates was affected by feed composition and operational parameters (HRTs and OLRs). The substrates rich in antimicrobial compounds has shown low efficiency and reactor stability in terms of biogas yield. The main cause of reactors failure was substrate's toxicity due to long chain fatty acids and other antimicrobials present in *J. curcas* seeds and oil. Secondly, the operational parameters has also affected the reactor stability and efficiency in terms of biogas yield. At lower OLRs and longer HRTs, the reactors were more stable, and yielded higher biogas compared to that at higher OLRs and shorter HRTs.




(C)
OLR1
a
OLR1.5
b
OLR2
c
OLR3
d
OLR4
e
OLR5
f
OLR6
g
JPC Biogas yield
pH
VFA/Alk ratio
Biogas yield NL/g VS added
pH
VFA/Alk ratio
Days


(D)
HRT20
OLR 1.5
a
HRT15
OLR 1.5
b
IIRT10
OLR 1.5
c
JPC Biogas yield
pH
VFA/Alk ratio
16S rRNA sampling
Biogas yield NL/g VS added
pH
VFA/Alk ratio
Days


(E)
HRT20
OLR 1
HRT15
OLR 1
IIRT10
OLR 1
Jo Biogas yield
pH
VFA/Alk ratio
16S rRNA sampling
Biogas yield NL/g VS added
pH
VFA/Alk ratio
Days

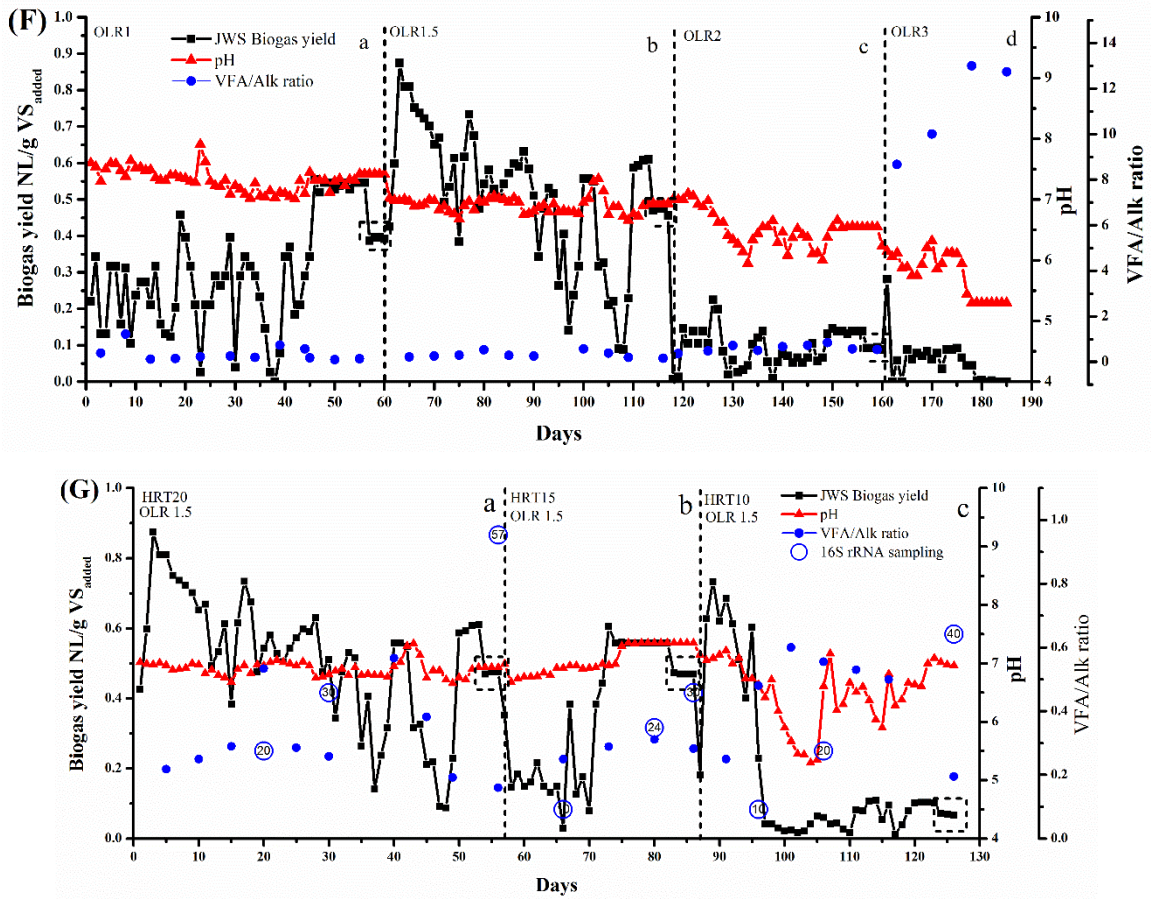


Figure 6.2. Biogas yield of different treatments of *Jatropha curcas* seed in NL/g VS_{added}. (**A**) Biogas yield of MR at different OLRs, (**B**) Biogas yield of MR at different HRTs at an OLR of 1 g VS L^{-1} day^{-1}, **(C)** Biogas yield of Jatropha pressed cake (JPC) at different OLRs, **(D)** Biogas yield of JPC at different HRTs at an OLR of 1.5 g VS L^{-1} Day^{-1}, **(E)** Biogas yield of Jatropha seed oil (JO) at different HRTs at an OLR of 1 g VS L^{-1} Day^{-1}, **(F)** Biogas yield of Jatropha whole seed (JWS) at different HRTs at an OLR of 1.5 g VS L^{-1} Day^{-1}, **(G)** Biogas yield of JWS at different HRTs, The Biomethane yield is covered by dashed squares. The same lowercase letter show no significant differences and the different letters show significant difference in biogas yields at steady state among different OLRs and HRTs. One-way ANOVA followed by Tukey's post test for multiple comparisons was used. The level of significance was (p<0.05). The days at which 16S rRNA samples were taken from reactors are shown by labelled blue circles.

6.4.3. Effects of methanolic extract of *J. curcas* pressed cake and hydraulic retention time on microbial diversity

The total count of raw reads were 7,786,706 sequences, with 97,334 average raw reads per sample.

There were no obvious differences in the observed index in all four substrates treating reactors. However, there were marked differences in richness and evenness (Shannon index) between the MR (JPC after methanolic extraction), JPC, JO and JWS treating reactors. According to the calculated indexes, the MR treating reactor had higher microbial diversity compared to that within the JPC, JO and JWS reactors (Figure 6.3A). The lowest Shannon index was shown by JO followed by JWS and JPC, suggesting that the presence of long chain fatty acids and other toxic compounds probably had an inhibitory effect on microbial communities. Higher richness and evenness are signs of functional stability of a reactor and the lower richness or evenness are usually considered as a warning indicator for reactor instability (Carballa et al., 2015).

The HRT also affected the microbial diversity during anaerobic digestion. Methanogenic consortia present in anaerobic digesters are mostly slow growers and require a longer retention time (Schnurer and Jarvis, 2010). It is assumed that the shorter HRT mostly have problems of microbial wash out leading to the reactor instability and failure (Couras et al., 2014). The observed index and Shannon index of all samples at HRT 20 was higher than at HRT of 15 and 10 days (Figure 6.3B).

The Bray-Curtis plots indicated the effects of substrate composition and HRTs on diversity of microbial communities during anaerobic digestion of *J. curcas* seed. The JWS and JO had similar microbial community composition and were clustered together. On the other hand, MR and JPC clustered close to each other especially at HRT of 20 days, indicating similar microbial communities and interestingly they clustered close to the inocula. Moreover, the microbial communities in all reactors at HRT of 20 days were dissimilar than that of HRT 10 and HRT 15, indicating the effect of HRT on the microbial shifts between the reactors.

The canonical correspondence analysis (CCA) plot shows that the microbial communities present in MR and JPC treating reactors exhibited positive correlation with increase in biogas and operating pH (Figure 6.3 D). Especially, MR20 (Microbial communities consuming methanolic residues at HRT 20 days) and JPC20 (Microbial communities in Jatropha pressed cake treating reactor at HRT 20 days) had a strong positive correlation with increased biogas yield and pH and negatively correlated with increased VFAs/Alkalinity ratios. This indicates that the feed's toxicity level and HRTs influenced the microbial community composition. On the other hand, the microbial communities in JO and JWS reactors were positively correlated with increased VFAs/Alkalinity ratios and negatively correlated with

increased biogas yield. In addition, the MR and JPC had positive correlation with VFA and alkalinity but this increase had no obvious negative effects on the biogas yield due to higher alkalinity (Eduok et al., 2017).

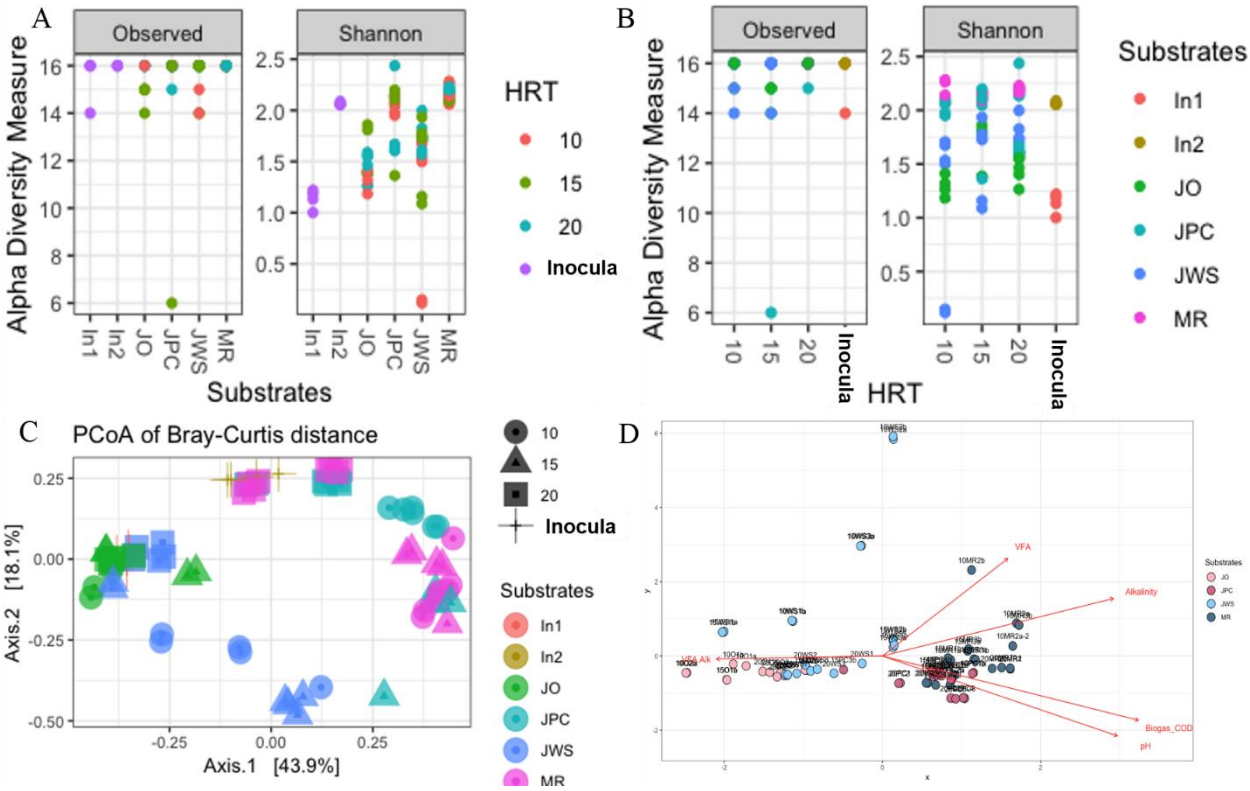


Figure 6.3. 16S rRNA analysis of microbial communities during anaerobic digestion of *J. curcas* seeds. (**A**) Observed and Shannon indexes of alpha diversity (within sample diversity) of microbial communities within different anaerobic reactors treating JO, JWS, JPC and MR substrates. The right side Y-axis shows different HRTs at which these substrates were treated in continuous anaerobic digesters. (**B**) Observed and Shannon indexes of alpha diversity of microbial communities at different HRTs in JO, JWS, JPC and MR treating anaerobic digesters, (**C**) Principal coordinate analysis (PCoA) plot using Bray Curtis distances (dissimilarity) between *J. curcas* substrates (JO, JWS, JPC, and MR), inocula (In1 and In2) at different HRTs (20, 15 and 10 days). The shaded circle, triangle, square and plus signs represents HRT 10, 15, 20 and inocula, respectively in continuous anaerobic digestion setup. The substrates are represented by different colors. (**D**) Canonical correspondence analysis to identify the relationship between operating parameters and microbial communities during anaerobic digestion of different setups of *J. curcas* seed. All of the substrates used in continuous anaerobic digesters are represented as; In1 (Inocula used for JO, JWS and JPC), In2 (Inocula used for

MR), JO (Jatropha oil), JWS (Jatropha whole seed), JPC (Jatropha pressed cake) and MR (methanolic residues).

The MR had higher relative abundance of fermentative bacteria than the JPC, JO and JWS treating reactors. *Clostridium* and *Syntrophomonas* were found in higher abundance in the MR compared to the JPC, JO and JWS treating reactors and are known for their broader range of substrate utilization and higher abundance in well operating and stable anaerobic digesters (Papp et al., 2016). Both of them are syntrophic acetate oxidizers and are in general involved in proteolytic and saccharolytic conversions (Vanwonterghem et al., 2014). *Syntrophomonas* is a facultative bacterium that consumes acetate and converts it into H_2 and CO_2 via acetate oxidation and the resultant products are further utilized by hydrogenotrophic methanogens to produce CH_4 (Treu et al., 2019). *Syntrophomonas* is highly resilient bacterium. However, it can be out-competed by acetoclastic methanogens, and is negatively correlated with higher VFAs (Treu et al., 2019). The taxa Bacteroidales with unidentified genera was found in higher abundance in MR compared to JPC, JO and JWS reactors (Table 6.1). The fermentative bacterial communities were dominated by methanogenic communities in JO and JWS treating reactors. Genus *Methanosaeta* was found relatively more abundant in JWS, JO and JPC compared to MR treating reactor (Table 6.1). *Methanosaeta* has higher affinity for acetate but can only sustain at lower concentration of acetate (Liu and Whitman, 2008). The *Actinobacteria* with unidentified genera were dominant in JO and JWS compared to JPC and MR reactors (Table 6.1), and are reported for propionate consumption; they can sustain in higher VFAs/Alkalinity ratios during anaerobic digestion (Cabezas et al., 2015). *Sedimentibacter* is a non-carbohydrates dependent bacterium, supported by pyruvate or amino acids fermentation (Gulhane et al., 2017). They were found in higher abundance in MR and JPC compared to JO and JWS treating reactors, indirectly suggesting that the metabolic processes in MR and JPC were probably diverse and methanogenesis occurred by different pathways. The Cloacimonetes (WWE1) with genus W22 are thought to play role in protein and cellulose fermentation and oxidizes VFAs during anaerobic digestion (Chojnacka et al., 2015). They were found in higher abundance in MR followed by JPC, JWS and JO (Table 6.1). Interestingly, the relative abundance indicated that the *J. curcas* seed long chain fatty acids and phytochemicals inhibited the hydrolytic and acidogenic bacterial groups. Conversely to the previous literature (Ma et al., 2015), no obvious decrease in methanogenic abundance was observed.

At shorter HRTs, the hydrolytic bacterial communities such as *Bacteroidetes* replaced the *Methanosaeta* to a high level resulting reactor instability and lower biogas yields (Table 6.1). The *Methanosaeta* being slow growing methanogens are linked to washout problems and the hydrolytic communities have chances of high abundance at lower HRTs. Conversely, the *Methanosaeta* and *Bacteroidetes* were found in adequate number to maintain the process of anaerobic digestion stable at HRT 20 compared to HRT of 15 and 10 days. Genera *Pediococcus*, *Proteiniclasticum*, *Clostridium*, *Sedimentibacter* and *Sporanaerobacter* within *Firmicutes* were found highly abundant at shorter HRTs compared to longer HRTs. The *Sporanaerobacter* is a sulphur reducing acetogen and is considered fast grower compared to methanogens (Hernandez-Eugenio et al., 2002). The *J. curcas* seed being rich in oil have a low level of sulphur contents (Islam et al., 2015), which can be used to nourish the sulphur reducing acetogens during anaerobic digestion process, and ultimately might out-compete the methanogens leading to reactor perturbations and failure. On the other hand, some of the *Firmicutes* genera including *Syntrophomonas* and *Ruminococcus* were found in higher abundance at longer HRTs compared to shorter HRTs (Table 6.1). Synergistetes with genera HA73 and vadinCA02 had higher relative abundance at longer HRT compared to shorter HRT. The HA73 and vadinCA02 have capability to convert protein into acetic acids during anaerobic digestion (Yamashita et al., 2016).

These observations show that antimicrobial compounds present in *J. curcas* seeds and oil had adverse effects on the efficiency of biogas yield, reactor stability and microbial profiling during anaerobic digestion. They must be extracted from *J. curcas* seed in order to increase the biogas yield. The extracted antimicrobial compounds and oil from *J. curcas* seeds can be respectively used for pharmaceutical purposes and biodiesel production. This approach will increase the economic value of *J. curcas* plant for biofuel sector.

Table 6.1. Relative abundance of microbial communities at Genus level in anaerobic digestion of Jatropha substrates in continuous mode

Genus level	Percent relative abundance in Jatropha				Percent relative abundance in Jatropha				Percent relative abundance in Jatropha			
	JWS	**JO**	**JPC**	**MR**	**JWS**	**JO**	**JPC**	**MR**	**JWS**	**JO**	**JPC**	**MR**
Methanomicrobiale	3.2	1.2	0.4	0.4	0.2	0.8	0	0.1	1.4	0.6	0.3	0
Methanobrevibacte	0	0	0	0	0	0	0	0	2.5	2.3	0	1.2
Methanospirillum	1.6	6.0	0.9	0.8	0.1	0.4	0	0	0.2	0.6	0	0.1
Methanosaeta	44.4	37.9	17.7	12.5	18.3	29.5	2.1	2.4	16.2	41.0	5.0	1.4
Propionibacteriacea	12.4	9.2	5.3	3.8	9.5	23.4	1.0	1.0	6.4	14.4	2.9	0.3
Propionibacteriacea	0.2	0.7	0.1	1.3	0	6.1	0	0.1	0.1	8.2	0.3	0
Coriobacteriaceae	0	0	0	0	0	0.1	0.3	0.2	5.1	0.9	0	2.0
Bacteroidales	2.6	0	2.3	10.9	0.1	0	1.0	0.9	0	0	15.4	1.1
Bacteroides	2.1	0	0.1	0.5	21.2	0	9.5	2.2	1.2	0.1	8.2	8.0
Marinilabiaceae	0	0.1	0	2.4	0	0	0	0.7	0	0	0.2	0
Porphyromonadace	1.3	0.3	7.1	5.4	13.1	10.0	40.2	29.8	12.8	0.4	11.4	18.6
Dysgonomonas	0	0	0	0	0.1	0	1.0	4.9	0.7	0	8.8	4.8
Prevotella	0	0	0	0	0	0	0	0	3.5	0	0.1	4.2
Carnobacteriaceae	3.3	2.4	0.8	3.1	0.5	3.4	1.2	1.7	0	0.2	0.6	0.3
Pediococcus	0	0	0	0	8.4	0	0	0	3.7	0	0	0
Enterococcus	0	0	0	0	0.4	0.4	0.2	0.9	3.5	0	0.4	0.6
Proteiniclasticum	0	1.0	0	0	0	4.9	2.2	4.5	0	0	4.5	1.3
Ruminococcus	0	1.0	8.3	2.0	4.6	3.3	1.2	0.6	0	0	0	1.5
Clostridium	0	0	0.4	0.7	0.1	1.8	7.7	9.0	0.1	0.1	1.6	7.0
Syntrophomonas	2.5	4.0	9.6	12.1	1.0	0.2	0.3	0.2	0	0	0	0
Sedimentibacter	1.9	1.4	8.5	4.5	0.1	0.8	18.9	16.4	0	0.4	18.0	7.4
Sporanaerobacter	0	0	0	0	0.7	0.7	0	0	1.2	7.6	0	0.1
Acrobacter	0	0	0	0	0	0	0	3.0	0	0	2.2	3.0
Escherichia	1.4	0.1	0.3	0.3	13.8	0	4.9	5.0	18.0	0.1	5.3	9.5
Trabulsiella	0	0	0	0	3.2	0	1.4	0.6	0.2	0.1	1.4	0.2
Acinetobacter	0.1	0	0	0.1	0	0	2.4	8.8	0.4	0	1.9	2.5
Pseudomonas	0	0	0	0	0	6.8	0	0	0	0	0	0
Stenotrophomonas	0	0	0	0	0	0	0	0	4.8	0	0	0
OPB54	0	0.1	0.7	7.3	0	0.3	0.1	0.8	0	0.3	0.3	0.7
SHA-98	0.1	0	0	2.6	0	0	0	0	0	0.1	1.8	0
OP8	0.9	0	2.7	8.3	0	0	0	0.8	0	0	0.4	11.7
SHA-1	0.9	2.1	1.3	0.5	0	0.2	0	0.3	0	0	0.4	0.1
Synergistales	0.2	1.4	2.7	2.0	0	0.2	0	0.1	0	1.0	0.1	2.2
HA73	9.8	6.0	3.1	1.8	0.6	0.6	0.4	0.3	0.2	1.8	0.3	0.2
vadinCA02	11.5	24.0	19.5	13	4.1	7.4	2.8	4.4	4.0	10.2	5.6	4.4
WCHB1-15	2.7	1.1	4.9	0.1	0.1	0.2	0.9	0.3	0	0.1	4.4	0.2
W22	0.3	0.1	4.0	5.2	0	0	0	0.1	0	0	0.3	0.4

JO: Jatropha seed oil, JWS: Jatropha whole seed, JPC: Jatropha pressed cake, MR: Methanolic residues.

6.5. Conclusions

Jatropha curcas seed is potent feedstock for biogas production. However, phytochemicals in its seeds affect methane production, specifically by inhibiting hydrolytic and acidogenic bacterial communities. Residues from methanolic extraction appear to be non-inhibitory to microbial communities and produced higher methane yields than other seed preparations. This was associated with richer, more even microbial communities. Enhanced biogas yield was achieved at longer HRTs and lower OLRs. The extracted phytochemicals are themselves also potentially valuable to the pharmaceutical industry, increasing the economic value of *Jatropha curcas* seeds beyond biogas production.

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Author Contributions are **"**Conceptualization: A.H., M.B; Experimental Works: A.H., A.M., L.W., J.W; Supervision, M.B., F.L; Results analysis, revision and improving the manuscript by all authors".

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Conflict of interest

The authors declare no conflict associated with this research work.

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CHAPTER 7

Chapter 7: Two-step transesterification of *Jatropha curcas* seed oil

Paper 5

Title: Two-Step process optimization for biodiesel production from non-edible *Jatropha curcas* seed oil with high free fatty acids

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7.1. Abstract

The demand for vegetable oil as feed is increasing with the increase in world population. Therefore, interest is increasing in the alternative non-edible feedstocks such as *J. curcas* seed oil for biodiesel production. The quality and efficiency of biodiesel produced is affected by a various parameters. In the current study, various physicochemical properties of *J. curcas* methyl esters have been analyzed to investigate its suitability as biodiesel fuel. The initial free fatty acid contents of raw oil was 13.11% and required two-step transesterification. Acid number of *J. curcas* seed oil was brought down to 1.2% by acid pretreatment using sulfuric acid as catalyst and keeping oil to methanol molar ratio 1:12. Afterwards, alkaline transesterification of purified acid pretreated seed oil resulted in 96% biodiesel yield at oil to methanol molar ratio 1:6, agitation 600 revolution per minute (rpm), temperature 60 °C, and time 2 hours. Moreover, factors such as oil to methanol molar ratio, amount of catalyst, time and temperature were optimized and the highest volumetric biodiesel yield obtained was 98% at optimized conditions. The optimized conditions for maximum biodiesel yield were oil to methanol molar ratio 1:6, KOH 1%, time 90 minutes and temperature 60 °C. The fuel properties of *J. curcas* seed biodiesel are closely related to standard values specified by ASTM D6751.

Keywords: Acid pretreatment; alkaline transesterification; biodiesel; *Jatropha curcas*; two-step biodiesel production.

7.2. Introduction

The consecutive decline in fossil fuels reserves and elevation of global warming caused by the extensive combustion of petro-diesel has turned the focus of related industries and scientific community to the utilization of renewable bioenergy resources such as biodiesel (Lippke et al. 2011; Srithar et al. 2017). Biodiesels are mono-alkyl esters that are biodegradable, non-toxic and environmental friendly green energy resource. It is acquired from vegetable or microalgae oils and animal fats (Qian et al. 2010; Xue et al. 2009). It can be effectively used as substitute to petro-diesel alone as well as in blended form with petro-diesel. The biodiesel blend possesses characteristics similar to the petro-diesel but with comparatively less hazardous emissions of particulate matter such as hydrocarbons and oxides of nitrogen, sulfur and carbon NO_x, SO_x, CO, respectively. The blend is evidenced to have lower toxicity, higher safety and reduced CO_2 emissions (Koh and Ghazi 2011; Smith et al. 2010).

Both edible and non-edible oils are used for biodiesel. The use of edibles for biodiesel production is controversial due to the risk of diverting farmland for biofuel production and the detriment of the food supply, while non-edible oils have no food versus fuel competition and therefore, their use for biodiesel production is increasing worldwide (Balat 2011; Ong et al. 2014). To date, a number of non-edible oil feedstocks have been used for biodiesel production but *Jatropha curcas* being a non-edible feedstock is considered an ideal option for biodiesel production due to its ability to thrive both in tropical and subtropical climates in marginal and non-agricultural wastelands. It has been originated from North America, Central America and now also inhabited in South Africa, South East Asia, India and China. *J. curcas* belongs to *Euphorbiacea* family, has a life span of 50 years and contains 27-40% oil in its seeds (Nahar and Ozores-Hampton 2011; Reddy et al. 2015). However, *J. curcas* seeds are also toxic to human and animal consumption due to toxic components such as phorbol esters and curcin (Leung et al. 2010; Mofijur et al. 2012).

Biodiesel can be produced using different methods but transesterification using methanol has remained the most widely used method as compared to the direct use of oil or biodiesel blending, pyrolysis and micro-emulsion (Meher et al. 2013). In transesterification, the triglycerides react with an alcohol to form fatty acid alkyl esters. However, it has been investigated that alkaline transesterification efficiency rely on free fatty acids (FFA) composition of feedstocks. It has been reported that alkaline transesterification is not recommended for feedstocks having 3-40 % FFA. So the alternate option is to use both acidic and alkaline catalysts for higher FFA. Acid pretreatment can esterify FFA and after decreasing

FFA to acceptable range i.e. 1-2%, its product can further be used for alkaline transesterification to obtain a higher biodiesel yield (Chung et al. 2008). The homogenous alkaline catalyst produces soap on reaction with FFA and hence the quantity of catalyst required for transesterification is reduced resulting into incomplete reaction and complicated downstream processing (Gübitz et al. 1999; Marchetti et al. 2007).

A number of studies have been conducted to increase the yield of biodiesel and improve its fuel properties (Berchmans and Hirata 2008; Smith et al. 2010; Xue et al. 2009). Various factors have been considered during biodiesel optimization such as oil to methanol molar ratio, agitation, temperature and time. Optimization of these factors is necessary to decrease the process cost and increase the biodiesel yield (Micic et al. 2015). According to our knowledge, no such comprehensive study has been conducted on the quality of the oil and biodiesel produced by Pakistani variety of *J. curcas* seed oil (JCO). In the present study, the JCO was characterized and acid pretreatment of the oil was carried out followed by its alkaline transesterification. The optimization of various factors including oil to methanol molar ratio, time, amount of catalyst and temperature was investigated.

7.3. Materials and Methods

In the current study, various physicochemical properties of raw oils and its methyl esters have been analyzed to investigate its suitability as biodiesel fuel. The initial free fatty acid (FFA) contents of raw oil were 13.11%, which were reduced by acid pretreatment. Afterwards, alkaline transesterification of purified acid pretreated seed oil was carried out. Moreover, oil to methanol molar ratio, Amount of KOH, temperature and time were optimized for alkaline transesterification to achieve high biodiesel yield.

Sample collection

J. curcas seeds were supplied by local suppliers from Lahore, Pakistan. Seeds were sun-dried and subjected to mechanical oil extraction using oil expeller. Oil was filtered to remove solid impurities. The crude oil was then heated at 105 °C for 2-3 hrs to remove moisture and stored in dark for further use. The oil yield was calculated using the following formula.

$$\text{Oil yield } (\%) = \left(\frac{\text{Oil extracted in Litres}}{\text{Total weight of seeds in kg}}\right) \times 100$$

7.3.1. Two step transesterification of *J. curcas* seed oil

The crude JCO was brown in colour. The amount of FFA in JCO (Table 7.1) was determined as previously described (Ghadge and Raheman 2006). *J. curcas* seed oil had an initial acid number 26±0.02 milli gram KOH/gram seed oil corresponding to FFA level 13.11%, which is exceedingly above the acceptable limit (1%) limit satisfactory for alkaline transesterification reaction. The main goal was to complete conversion of FFA and triacylglycerides (TAG) into biodiesel using two-step process. Therefore, FFA were converted to esters by esterification as discussed below.

7.3.2. Acid catalyzed esterification of *J. curcas* seed oil

Esterification was used to convert seed oil FFA to esters using sulfuric acid with methanol as according to Veljković et al., (2006) with little modifications. In esterification step, JCO and methanol were used in a 1:12 molar ratio. Initially, 100 mL JCO was added to three necked round bottom flasks (250 mL) equipped with a reflux condenser, thermometer and rubber corks and heated up to 60 °C. Absolute methanol and sulfuric acid (2% in accordance to final volume of seed oil) were pre-mixed together, heated and then added to the seed oil in flasks and continuously mixed at 600 revolution per minute (rpm) to homogenize the mixture. The ACE batch reaction was maintained at 60 °C for 2 hrs on a heating mantle. After the completion of ACE process, the mixture was allowed to get completely separated into different

layers using separating funnel. After separation of oil layer, sulfuric acid catalyst was washed out and removed by neutralization with calcium oxide (CaO). About 20 mL of hot 1% CaO solution was added to pretreated oil mixture. The mixture was centrifuged at 1744 x g for 10 min resulting in $CaSO_4$ formation that was precipitated out and settled down. This washing was repeated twice to properly remove the acid catalyst. The supernatant containing catalyst-free pretreated oil was decanted and centrifuged again at aforementioned conditions to separate water. The product obtained after ACE was again dried in oven for 2-3 hrs and cooled down at 25 °C. Finally, the pretreated *JCO* was treated with anhydrous sodium sulfate to absorb any remaining moisture completely. The acid number of acid pretreated oil was determined and was found 2.4±0.01 mg KOH per gram of pretreated oil that was corresponding to FFA level 1.2%, which is within the acceptable limits for alkaline transesterification. This product was further used in alkaline transesterification reaction.

7.3.3. Alkaline transesterification of *J. curcas* seed oil

Alkaline transesterification of acid pretreated JCO was carried out as described by Berchmans and Hirata (2008) and Veljković et al., (2006) with slight modifications. KOH, 2% w/w (2% in accordance to final volume of seed oil) was dissolved in methanol (1:6 oil to methanol molar ratio) and added to same reactor as described earlier. The mixture was heated to 60 °C stirred at 600 rpm for 2 hrs, afterwards, it was allowed to get completely separated into different layers using separating funnel.

After the completion of reaction, the product (biodiesel) was poured in the round bottom flask of distillation apparatus and its temperature was adjusted to 65°C. Distillation apparatus was stopped when methanol collection in the flask on the other side of distillation tube was stopped. Removal of KOH was done by neutralization and washing with acidified distilled water. For this purpose, a 100 mL of 1% dilute sulfuric acid hot solution was then added to the transesterified mixture and centrifuged at aforementioned conditions, the resultant product (potassium sulfate) was precipitated out, settled down and removed from the mixture. This step was performed twice to remove any remaining catalyst. The catalyst-free product was again centrifuged at aforementioned conditions to separate water from the final product. The water-free product was passed through anhydrous Na_2SO_4 on a whatman filter paper to remove any remaining moisture. Volume of fatty acids methyl esters (FAMEs) was noted. FAMES yield was calculated using the following equation:

$$\text{FAME yield (\%)} = \left(\frac{\text{weight of FAME in grams}}{\text{total weight of oil in grams}}\right) \times 100$$

7.3.4. Optimization of alkaline transesterification

Parameter affecting the alkali catalyzed transesterification reaction were optimized using the method of Patil and Deng (2009) with little modifications. An alkali catalyzed transesterification reaction setup was used to determine optimum oil to methanol molar ratio, amount of catalyst and time at 600 rpm and 60 °C. For batch setup, an amount of 25 mL preheated JCO was used in each batch reaction. The catalyst along with methanol was preheated at a desired temperature, added to oil in batch setup and stirred at 600 rpm for 2 hrs. The reactions were conducted at different molar ratios of oil to methanol (1:6, 1:9, 1:12), amount of catalyst (1, 1.5, and 2%) and reaction time (30, 60, 90 and 120 min). After confirming the optimum parameters, the effect of different temperatures (50, 55 and 60 °C) on biodiesel yield were evaluated at optimized molar ratio, Amount of KOH and reaction time.

7.3.5. Physicochemical properties of oil and biodiesel

Fuel properties of oil and biodiesel samples, obtained from chemical transesterification reactions, was carried out using standard procedures according to Mehta et al., (2015) and Onukwuli et al., (2017) with some modifications, as discussed below.

7.3.5.1. Acid value and free fatty Acid content

The acid number (AN) and of JCO, acid pretreated oil and biodiesel was determined by adding 5 g of sample in a flask, and mixing with 25 mL of absolute ethanol and 2-3 drops of phenolphthalein. They were heated at 65 °C in water bath for 10 min under continuous gentle shaking, allowed to cool and titrated against 0.1 Normal KOH solution until a permanent pink color appeared. Amount of KOH solution needed for the reaction was calculated. The acid number (AN) was determined using the following formula.

$$\text{AN} = \left(\frac{\text{mL of KOH} \times \text{Normality of KOH} \times 56.1}{\text{Weight of sample}}\right) = \text{mg of KOH/g}$$

where, 56.1 = molecular weight of KOH (g/mol)

The FFA content of JCO and acid pretreated oil were determined by the following formula. The percent of FFA in a sample is always approximately half of the acid number.

% FFA = AN x 0.503

7.3.5.2. Saponification number

The saponification number was determined by adding 2 g of JCO in a 100 mL Erlenmeyer flask, followed by the addition of 25 mL of 0.5 Normal alcoholic KOH solution. The same procedure was carried out for the B (Blank) without addition of oil sample. The flask was connected to the reflux condenser, placed in water bath and saponification was completed by gently and steady boiling for an hour with random shaking. After 1 hour, few drops of phenolphthalein were added to it and the solution was titrated against 0.5 Normal Hydrochloric acid until the disappearance of pink color (end point). The saponification number of JCO was determined using the following formula:

$$\text{SP Number} = \left(\frac{56.1\ (\text{H}° - \text{S}°) \times \text{Normality of of hydrochloric acids}}{\text{Weight of sample}}\right)$$

where, "H°" is the amount of hydrochloric acids used for blank in mL; "S°" is the amount of hydrochloric acids used for sample in mL and N: normalized.

Ester Value and percent glycerine

Ester value is the number of milli grams of KOH to saponify the esters present in 1 gram of substance and is determined by subtracting acid number from saponification number.

$$\text{Ester value (EV)} = (\text{Saponification number (SN)} - \text{Acid number (AN)})$$

During saponification reaction, the triacylglycerol are hydrolyzed in the presence of KOH to produced potassium salts of soap and glycerine. The percent glycerine can be obtained using the following formula.

$$\%\ \text{Glycerine} = (\text{Ester value} \times 0.054664)$$

7.3.5.3. Peroxide value

It was determined as described by Association of Official Analytical Chemists (AOAC) method (Society 1988). *J. curcas* seed oil (5 g) was added in flask and 30 mL of acetic acid and chloroform mixture (at a molar ratio of 3:2), was added to it with swirling to dissolve oil in it. A 0.5 mL solution of potassium iodide (saturated) was added to the mixture and swirled for 1 min in dark until its color turned into a light brown color. Afterwards, 30 mL of distilled water was added to it and titrated against 0.1 Normal $Na_2S_2O_3$ until the solution turned pale yellow. Then, 1 mL of 1% starch indicator was added and further titrated against 0.1 N $Na_2S_2O_3$ until the disappearance of blue color. For blank, the same procedure was used without adding sample. Peroxide value is expressed as milli equivalent of peroxide oxygen per kg of the sample, as given below

$$\text{Peroxide value} = \left(\frac{(S° - B°) \times N \times 100}{\text{Weight of sample (g)}}\right)$$

where, S° = test sample titrated; B° = blank sample titrated; and N = Normality of $Na_2S_2O_3$.

7.3.5.4. Specific gravity

A 25 mL dried, clean bottle was weighed either empty or with water. The oil and biodiesel samples were added separately in it and weighed. All the steps were carried out at similar temperature (30°C). Specific gravity was calculated using the following equation:

$$\text{Specific gravity} = \left(\frac{A° - B°}{C° - B°}\right)$$

where, A° = weight (in grams) of bottle along with oil; B° = weight (in grams) of empty bottle; and C° = weight (in grams) of bottle plus water.

7.3.5.5. Boiling point determination

A 10 mL biodiesel sample was taken in test tube and thermometer was inserted into it. After that the temperature of magnetic plate was gradually increased. The point at which biodiesel started bubble formation was recorded as a boiling point.

7.3.5.6. Cloud point and pour point

About 30 mL oil and biodiesel samples were poured into separate jars and placed in cooling jacket in water bath. Thermometer was fixed in each jar and the jars were observed at different intervals without disturbing the liquids. The temperature at which clouds of crystals appeared was recorded as cloud point. For pour point about 20 mL of each sample was placed in beaker, sealed with corks and incubated in refrigerator to solidify. After solidification, the samples were kept in open, the temperature at which each sample was melted and started flowing was recorded as pour point.

7.3.6. Chemical Characterization of *J. curcas* seed oil and biodiesel

J. curcas seed oil and biodiesel produced by chemical transesterification method were analyzed using FTIR spectroscopy (Bruker, model Tensor27 with software version Opus65, equipped with Zn-Se ATR). A 5 µL of seed oil or its FAMEs were loaded at sample injector and FTIR spectra for all samples ranged in 4000-400 cm^{-1}. Gas chromatography and mass spectrophotometry (GC-MS; Model GCMS-QP5050A; Shimadzu-Europe) with thermal conductivity detector (TCD) was used for analysis of both oil and biodiesel samples with following conditions: A 2 µL sample was injected in GC column using split mode. Separation was performed on a capillary column DB-5 (Column length: 30 m; internal diameter 0.25 mm

and thickness 0.25 μm). Initial temperature was 50 °C, ramped at 5 °C/min to 80 °C, 10 °C to 300 °C and kept at 300 °C for 18 min. Injector was set at 250 °C with column flow rate of 1.8 mL/min for seed oil and 1 mL/min for biodiesel. Helium gas was used as mobile phase with flow rate of 1 mL/min and a split ratio for oil 1/25 and biodiesel 1/48, while TCD was set at 300 °C. The *m/z* scanning of MS was in the range of 50-650 with electron impact (EI) at 70 eV.

7.3.7. Statistical analyses

Each batch was conducted in triplicate and the fatty acids methyl esters obtained from each setup were analysed individually in triplicates to calculate the average values of experimental data. One-way ANOVA and multiple comparisons was carried out by Tukey's posttest, (Significance: $p < 0.05$).

7.4. Results and Discussions

The mechanical extraction resulted in 32.5 % crude oil, which is in close proximity with previously reported extraction yields (de Oliveira et al. 2009).

7.4.1. Acid pre-treatment and alkaline transesterification of *J. curcas* seed oil

The JCO had high FFA content of 13.11% (Table 7.1), which is higher than the acceptable value for alkaline transesterification. The alkaline transesterification of high FFA containing JCO is considered complicated due to higher soap formation, loss of catalyst, and loss in product yield. The higher FFA content causes saponification of oil and requires extra addition of alkali catalyst for transesterification (Canakci and Van Gerpen 2001). So, in order to decrease the FFA prior to alkaline transesterification, acid pretreatment was used to reduce the FFA acids present in JCO. The FFA was significantly reduced (p <0.05) from 13.11 % to 1.2 % after 120 min of pretreatment (Figure 7.1).

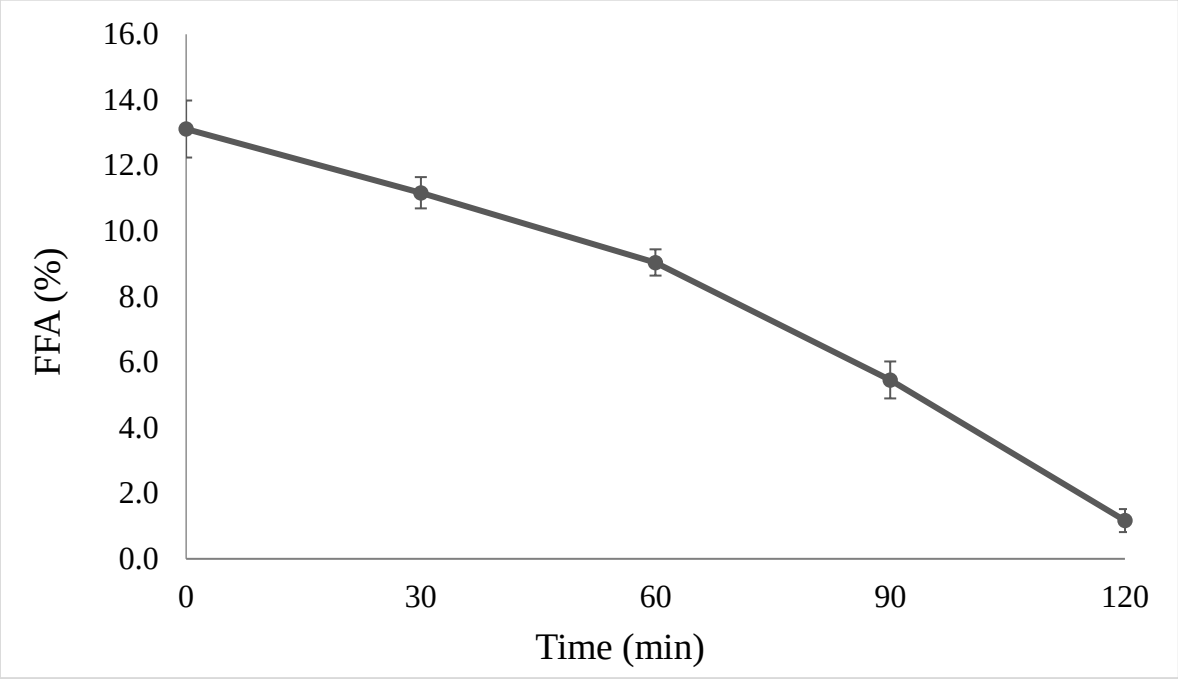

Figure 7.1. Acid catalyzed pretreatment of *J. curcas* seed oil FFA with concentrated sulfuric acid. The data is presented as mean ± SD. The acid pretreatment was carried out in triplicate independently and the conditions were 2% sulfuric acid, oil to methanol molar ratio 1:12, temperature 60 °C, time of 120 min and agitation at 600 rpm. The FFA percentage was investigated after each 30 min. The free fatty acid reduction was statistically analyzed using one-way ANOVA and multiple comparisons was carried out by Tukey's posttest, (Significance: p <0.05).

After acid pretreatment, the purified product was subjected to alkaline transesterification and processed at 2% KOH (w/v), oil to methanol molar ratio of 1:6, time of 2 hrs, 60 °C and agitation at 600 rpm. The resultant amount of methyl esters of JCO were determined at different reaction time but the highest biodiesel yield (96%) was obtained after 2 hrs of reaction with no significant increase compared to the methyl esters obtained at 30, 60 and 90 min (Figure 7.2). Meher et al., (2006) also reported similar findings, in which the Karanja gave the highest biodiesel yield of 97% at oil to methanol molar ratio 1:6 using KOH catalyst in the alkaline transesterification.

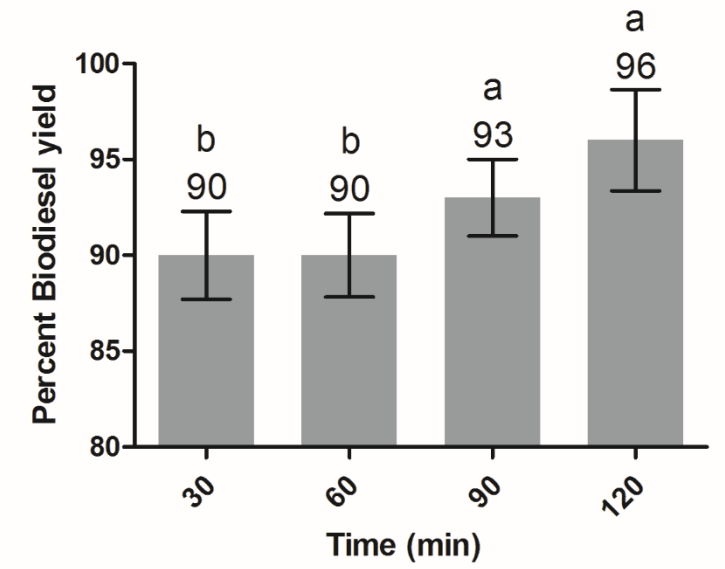

Figure 7.2. Alkaline transesterification of *J. curcas* seed oil carried out at a 1:6 oil to methanol molar ratio, 2 % KOH (alkaline catalyst), 600 rpm and 60 °C. All of the setups were carried out as independent experiments in triplicates. The data presented as mean ± SD. One-way ANOVA, and multiple comparisons was carried out by Tukey's posttest. The lowercase alphabets on adjacent bars show significant and non-significant differences. Similar alphabets on adjacent bars show non-significant whereas different alphabets show significant difference. (Significance: p <0.05).

7.4.2. Optimization of Alkaline Transesterification of *J. curcas* seed oil

The alkaline transesterification process depends upon certain factors such as oil to methanol molar ratio, amount of catalyst, temperature and time. Therefore, in order to enhance biodiesel yield, different oil to methanol molar ratios, Amount of KOHs and time were investigated at 60 °C as mentioned earlier. Stoichiometrically one mole of oil requires three moles of alcohol for transesterification reaction completion. In practice higher amount of methanol are needed for higher conversion efficiency of oil (to ensure that the reaction goes to

completion). The conversion efficiency is defined as increased yield of biodiesel in terms of percentages. Therefore, biodiesel optimization setup was started at a molar ratio of 1:6 (oil:methanol) (Freedman et al. 1984; Fukuda et al. 2001). Optimum catalyst concentration and oil to methanol molar ratio are highly necessary for complete and efficient transesterification process and a slight variation in these parameters can result either in incomplete reaction or may produce unnecessary products leading to lower biodiesel yield. The highest biodiesel yield was obtained at 1% KOH, molar ratio of 1:6 of oil to methanol at 90 as well as 120 min reaction time incubated at 60 °C (Figure 7.3). Initially, there were no significant difference in biodiesel yield obtained at 30 and 60 min. But at 90 and 120 min, the 1:6 molar (oil to methanol) ratio gave significantly higher (p<0.05) yields than that obtained at 30 and 60 min. The biodiesel yield recorded for 1:6 molar ratio was significantly higher (p<0.05) than 1:9 at 90 min and 60 °C (Figure 7.3).

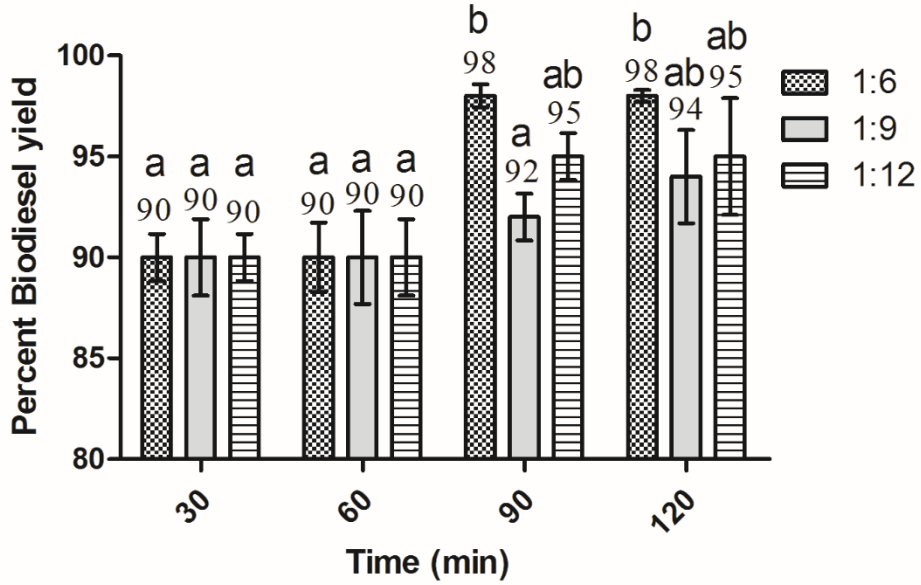

Figure 7.3. Effect of molar ratios and reaction time on biodiesel yield of *J. curcas* seed oil maintained at 1 % KOH, 600 rpm and 60 °C. All the setups were carried out as independent experiments in triplicate. The data presented as mean ± SD. One-way ANOVA and multiple comparisons was carried out by Tukey's posttest. The alphabets on bars show significant and non-significant differences. Similar alphabets on different bars show non-significant whereas different alphabets show significant difference. The alphabets "ab" on bars represent non-significant difference between bars annotated by "a" and "b". (Significance: p <0.05)

A similar pattern was also observed for alkaline transesterification at 1.5% KOH, the biodiesel yield of 1:6 was higher than 1:9 and 1:12 at 90 as well as 120 min reaction time

incubated at 60 °C (Figure 7.4). Here at 1.5% KOH, the biodiesel yield of 1:6 molar ratio was significantly higher (p<0.05) than 1:12 at 90 as well as 120 min reaction time.

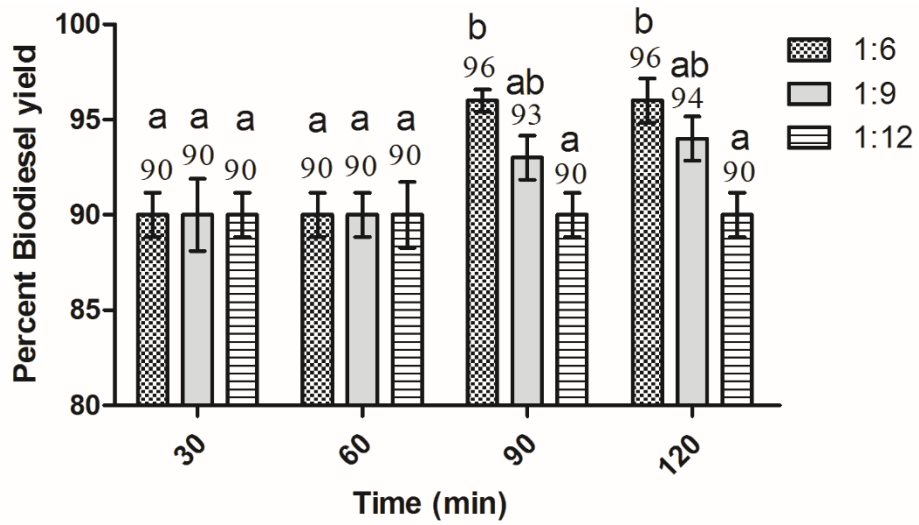

Figure 7.4. Effect of molar ratios and reaction time on biodiesel yield of *J. curcas* seed oil maintained at 1.5% KOH, 600 rpm and 60 °C. All of the setups were carried out as independent experiments in triplicate. The data presented as mean ± SD. One-way ANOVA and multiple comparisons was carried out by Tukey's posttest. The alphabets on bars show significant and non-significant differences. Similar lowercase alphabets on different bars show non-significant while different alphabets show significant difference between them. The alphabets "ab" on bars represent non-significant difference between bars annotated by "a" and "b". (Significance: p <0.05)

Moreover, at 2% KOH, the same biodiesel yield was obtained at 1:6 and 1:12 molar ratios of oil to methanol (Figure 7.5) and found slightly higher compared to the 1:9 with no significant increase. The methyl esters yield for all molar ratios incubated at 60 °C and 2% KOH were found higher at 120 min compared to other reaction times with no significant increase (p>0.05).

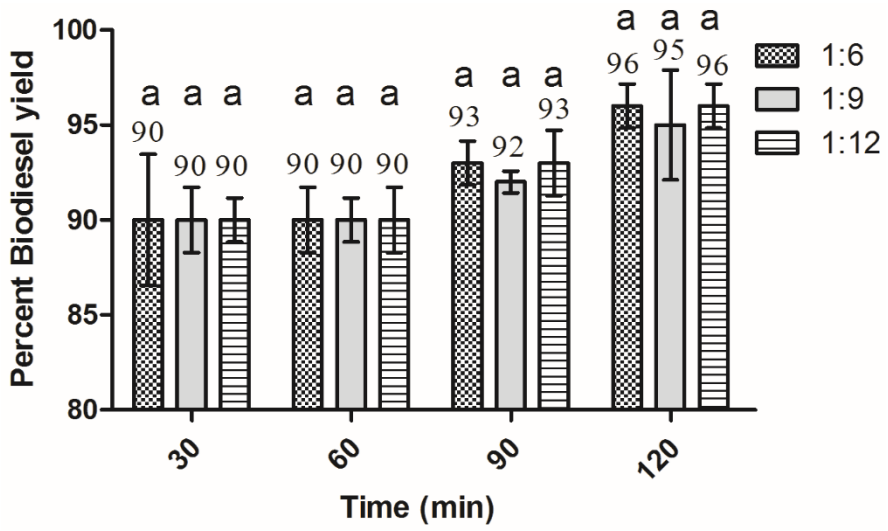

Figure 7.5. Effects of oil to methanol molar ratios and reaction time on biodiesel yield of *J. curcas* seed oil maintained at 2% KOH, 600 rpm and 60°C. All of the setups were carried out as independent experiments in triplicate. The data presented as mean ± SD. One-way ANOVA and multiple comparisons was carried out by Tukey's posttest. The alphabets on bars show significant and non-significant differences. Similar alphabets on different bars show non-significant whereas different alphabets show significant difference. (Significance: p <0.05).

It is evident from the results presented above that the highest biodiesel yield was achieved at 1% KOH, oil to methanol molar ratio 1:6 and reaction time 90 min. Furthermore, it was also noted that increase or decrease in the Amount of KOH from 1% resulted in lower biodiesel yield (Figure 7.6). The lower concentration than the optimum limit causes incomplete transesterification reaction ultimately producing lower methyl esters. Therefore, catalyst concentration must be enough to complete the reaction. While the excess amount of catalyst beyond its optimum limit, reacts with triglycerides and free fatty acid producing emulsion, which leads to the formation of viscous gel. It also makes the glycerol layer separation difficult, ultimately resulting in the loss of ester yields (Dorado et al. 2004). Similar findings were reported by Berchmans et al., (2010); Rashid et al., (2008), achieving a biodiesel yield of 97% and 96% from the transesterification of JCO and rapeseed oil, respectively, with 1% KOH catalyst.

From cost and process time perspectives, the ideal and highest biodiesel yield was obtained at molar ratio 1:6 at 1% KOH catalyst, temperature 60 °C and 90 min of reaction time with an optimum biodiesel yield of 98%. For standard biodiesel, the permissible level of glycerol is 0.24% in the final product, to achieve this standard the biodiesel yield must be ≥ 97.7% (Van Gerpen et al. 2004). The current study assumed optimal molar ratio of oil to methanol as 1:6 in order to obtain higher ester yield (Figure 7.6). No significant change in the

biodiesel yield was observed when oil to methanol molar ratio higher than 1:6 was used. The results showed similarities with the reports of Meher et al., (2010) and Usta, (2005), where highest yield of ester from methanolysis of *P. pinnata* and tobacco seed oil at 6:1 alcohol to oil molar ratio, respectively, was reported. Similarly, 96% yield was obtained from the transesterification of rapeseed oil using molar ratio of methanol to oil 6:1 and 1% KOH as catalyst (Rashid et al. 2008).

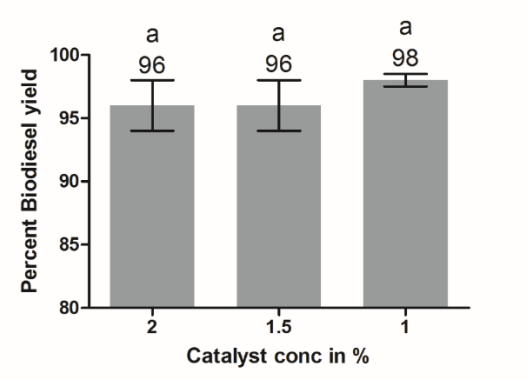

Figure 7.6. Effects of catalyst concentrations on biodiesel yield maintained at 1:6 molar ratios, 600 rpm, 60 °C and 90 min. All of the setups were carried out as independent experiments in triplicate. The data presented as mean ± SD. One-way ANOVA and multiple comparisons was carried out by Tukey's posttest. The alphabets on bars show significant and non-significant differences. Similar alphabets on different bars show non-significant whereas different alphabets show significant difference. (Significance: p <0.05).

Similarly, the effect of different temperatures (50, 55 and 60 °C) on biodiesel yield was evaluated at the aforementioned optimized conditions (1:6 molar ratio, reaction time 90 min and 1% KOH). The reaction temperature above the boiling point of methanol was ignored just because of the fact that at higher temperature saponification rate for glycerides of oil remains faster than that of the transesterification. Also, temperature above the boiling point of methanol causes excessive evaporation, loss of methanol from reaction mixture that leads to incomplete reaction and loss of esters formation. As shown in Figure 7.7, the highest yield for biodiesel was achieved at 60 °C, followed by 55 °C and 50 °C with no significant difference. The increase in temperature until optimum limit (60 °C) increased the conversion efficiency of oil into its methyl esters and any decrease from optimum temperature caused decrease in the methyl esters yield. Similar effect were shown by P. Chitra et al., (2005) and U. Rashid et al., (2008) during the transesterification of JCO and rapeseed oil, respectively.

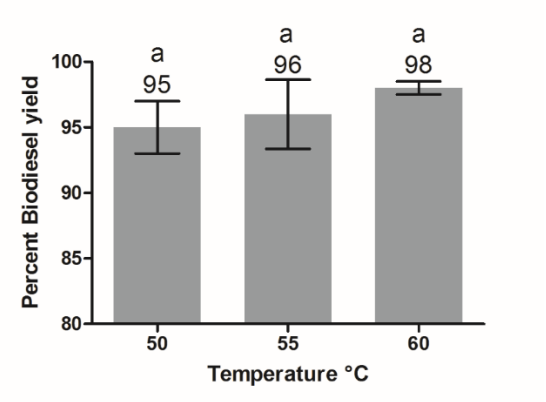

Figure 7.7. Effect of different temperatures on biodiesel yield of *J. curcas* seed oil maintained at 1:6 oil to methanol ratio, 1% KOH, 600 rpm and reaction time 90 min. All of the setups were carried out as independent experiments in triplicate. The data presented as mean ± SD. One-way ANOVA and multiple comparisons was carried out by Tukey's posttest. The alphabets on bars show significant and non-significant differences. Similar alphabets on different bars show non-significant whereas different alphabets show significant difference. (Significance: $p < 0.05$).

7.4.3. Physicochemical properties of oil and biodiesel

The quality of seed oil and its methyl esters is expressed in terms of physicochemical properties such as acid number, saponification number, peroxide value, specific gravity, pour point and cloud point that were analyzed in the present study (Table 7.1). The least temperature at which biofuels can flow is called pour point where as the temperature at which the wax first appeared when fuels become cooled is called cloud point (Coutinho et al. 2002). The determined values of pour point and cloud point of *J. curcas* biodiesels are closely in line with ASTM D6757 (Table 7.1). The acid number shows the amount of FFAs present in fuel oil. If the acid number for biodiesel is higher than 0.5 mg KOH g^{-1} FAME, it causes corrosion of the fuel engine. In the present study, the acid pretreatment of the oil reduced the acid number of oil from 26±1.7 mg KOH per g of oil to 2.4±0.01 mg KOH per gram of pretreated oil. The acid number of biodiesel was 0.45 mg KOH g^{-1} FAME, which is below the critical value and is considered safe for fuel engine operation (Table 7.1). Saponification value is an index of the mean molecular mass of fatty acids present in seed oil. The FFA, saponification, ester and acid numbers of JCO were 13.11 %, 213 mg/g, 186.6 mg/g and 26 mg/ KOH g, respectively. Similarly, the peroxide value, specific gravity, cloud point and pour point of JCO FAMEs were 1.43 Meq O_2/kg, 0.88 kg/m^3, 3.8 °C and 2 °C, respectively. The present study showed that transesterification improved the fuel quality of oil with respect to the above-mentioned

properties. All of the physicochemical characteristic determined in the present study may slightly vary from other studies due to difference in habitats of feedstock, geography and soil conditions but are in close coherence with standards for biodiesel specified by ASTM D6751 and EN 14214 (Atadashi et al. 2010; Demirbas 2009; Murugesan et al. 2009).

Table 7.1. Fuel properties of *J. curcas* seed oil and biodiesel (Ong et al. 2011; Patil and Deng 2009)

Property	**Unit**	***J. curcas* oil**	**Biodiesel**	**Biodiesel standard ASTM D 6751-02/ ASTM D 6751-06**
Acid number[a]	mg KOH/g	26±1.7	0.45±0.1	0.80 max
FFA[a]	%	13.11±0.9	-	-
Saponification number[a]	mg/g	213±5.1	-	-
Ester value[a]	mg/g	186.6±3.7	-	-
Glycerin[a]	%	10.1	-	0.240 max
Cloud point[a]	°C	5±1.0	3.8±0.4	−3 to 12
Pour point[a]	°C	4±0.2	2±0.1	−15 to 10
Peroxide value[a]	Meq O_2/kg	N.D	1.43±0.1	N.D
Boiling point[a]	°C	390±1.5	261±1.0	>201.85
Specific gravity[a]	kg/m^3	0.916±0.1	0.88±0.02	0.87–0.90
Odour	N.D	Agreeable	Agreeable	-
Colour	N.D	Brown	Light Brown	-
Percent Oil yield	%	32.5	N.D	-

[a]: values presented as mean ± SD.

7.4.4. Chemical composition of *J. curcas* seed oil and Biodiesel produced

The FTIR spectra for JCO and its FAME obtained at conditions of 1% KOH, molar ratio of methanol to oil 1:6, reaction time 90 min and temperature 60 °C are presented in Figure 7.8. The FAMEs were confirmed as described previously (ul ain Rana et al. 2018). One major and two minor stretches of ester bands observed at 1742.07, 1198.16 and 1034.90, were only present in the biodiesel confirming FAMES (Figure 7.8). The peaks at 1163.55 and 1198.16

corresponds to C-O stretches for esters, are only present in FAME confirming the biodiesel produced from JCO.

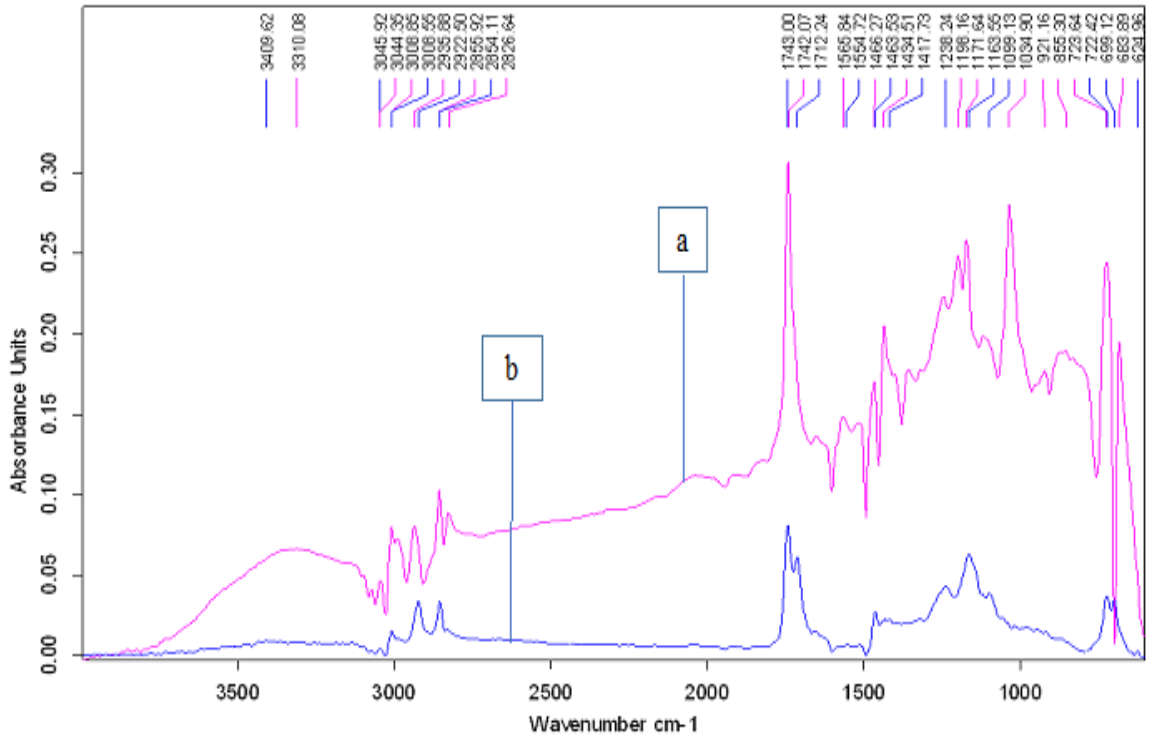

Figure 7.8. FTIR spectra of crude *J. curcas* oil and its alkaline catalyzed FAMEs, the upper peaks denoted by letter "a" shows biodiesel and the lower peaks labeled with letter "b" represents JCO spectra.

The chemical composition of JCO and its FAMEs was further analyzed by GC-MS. The GC-MS analysis showed 13 different saturated and unsaturated fatty acids in JCO (Table 7.2). Additionally, about 8 different kinds of saturated and unsaturated FAMES were observed in the total ion chromatogram (Table 7.3). All of these fatty acids and fatty acids methyl esters were confirmed using library match software provided by National Institute of Standards and Technology (NIST). The GC-MS analysis also confirmed the presence of five saturated, two mono unsaturated and four poly-unsaturated FAMEs in the alkali catalyzed biodiesel of JCO (Table 7.3). Usually saturated and mono-unsaturated fatty acids or its esters are considered ideal for increasing energy yield and oxidative stability. However, oil or biodiesel containing mono-unsaturated fatty acids or its esters are prone to solidification at low temperature. While the fuels or oils containing polyunsaturated fatty acids or its esters have very good cold flow properties but are vulnerable to oxidation. This tendency of fuels oil or biodiesel causes negative impact on fuel storage and combustion (Imahara et al. 2006). The determined components were in accordance to the reported composition of JCO (Akbar et al. 2009). Here

in this case, the composition of fatty acids and its FAMEs may vary from other reports due to different geological variations, soil conditions and seed varieties. De Oliveira et al., (2009) also supporting the same statement.

Table 7.2. Fatty acids composition of *J. curcas* seed oil identified by GC-MS

Systemic Name	Common Name	Molecular formula
Octanoic acid	Caprylic Acid	$C_6H_{16}O_2$
Nonanoic acid	Pelargonic acid	$C_9H_{18}O_2$
n-Decanoic acid	Capric Acid	$C_{10}H_{20}O_2$
Dodecanoic acid	Lauric Acid	$C_{12}H_{24}O_2$
Undecanoic acid	Undecylic Acid	$C_{11}H_{22}O_2$
Tridecanoic acid	Tridecylic Acid	$C_{13}H_{26}O_2$
Tetradecanoic Acid	Myristic acid	$C_{14}H_{28}O_2$
n-Hexadecanoic acid	Palmitic Acid	$C_{16}H_{32}O_2$
Pentadecanoic acid	Pentadecylic Acid	$C_{15}H_{30}O_2$
Eicosanoic Acid	Arachidic acid	$C_{20}H_{40}O_2$
Octadecanoic Acid	Stearic acid	$C_{18}H_{36}O_2$
11-Eicosenoic acid	Gondoic Acid	$C_{21}H_{40}O_2$
13-Docosenoic Acid	Erucic acid	$C_{22}H_{42}O_2$

Table 7.3. FAMEs composition of biodiesel produced from *J. curcas* seed oil identified by GC-MS

Systemic Name	Common Name	Molecular Formula
Dodecanoic acid methyl ester	Methyl laurate	$C_{13}H_{26}O_2$
Methyl tetradecanoate	Methyl myristate	$C_{15}H_{30}O_2$
Palmitic acid, methyl ester	Methyl palmitate	$C_{17}H_{34}O_2$
Octadecenoic acid, methyl ester	Methyl linoleate	$C_{19}H_{34}O_2$
Palmitic acid, methyl ester	Methyl palmitate	$C_{17}H_{34}O_2$
Methyl octadecanoate	Methyl stearate	$C_{19}H_{38}O_2$
11-Eicosenoic acid, methyl ester	Stearyl acrylate	$C_{21}H_{40}O_2$
Arachidic acid methyl ester	Methyl icosanoate	$C_{21}H_{42}O_2$

7.5. Conclusions

The study conducted concludes that the seeds of *J. curcas* contains high amount of oil that can be used for production of high quality biodiesel. However, *Jatropha curcas* seed oil contains high free fatty acid content of about 13.11% that hinders the process of biodiesel production using alkali catalyst. This study depicts successful implementation of two-step transesterification process in which at first step the acid pretreatment with sulfuric acid reduced the FFA amount in oil to 1%. In the second step the low FFA containing pretreated oil was subjected to alkali based transesterification to yield biodiesel. The study conducted was successful in optimizing the alkali transesterification process to achieve 98% biodiesel yield. The properties of the produced biodiesel satisfied the standards required for good quality biodiesel. All the results obtained confirmed that *J. curcas* seed oil and two-step transesterification process can find successful applications in the field of biofuel production.

ACKNOWLEDGMENTS

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CHAPTER 8

Chapter 8: Lipase mediated biodiesel production from *Jatropha curcas* seed oil

Paper 6

Title: Lipase mediated biodiesel production from *Jatropha curcas* seed oil using whole cell approach

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8.1. Abstract

Biodiesel production from non-edible feedstocks, such as *Jatropha curcas,* by lipase producing bacteria is considered a sustainable measure to reduce food versus fuel competition and dependency on fossil fuels. In the current study, lipase producing bacterial strains were isolated from oil-contaminated soil, followed by their biochemical & molecular identification and determination of their capacity to produce biodiesel from *J. curcas* seed oil. Plackett-Burman and central composite designs were used to optimize various factors during whole cell based transesterification of *J. curcas* seed oil. The highest volumetric yield of biodiesel (~97%) was obtained by using *Brevibacterium* SB11 MH715025 and *Pseudomonas* SB15 MH715026, strains. With the most optimum biodiesel yield at 37°C, oil to methanol molar ratio of 1:9 and agitation (100 rpm); *Pseudomonas* SB15 MH715026 was identified as the most potent strain. Confirmation of fatty acid methyl esters was done through fourier transform infrared spectroscopy having infrared spectra in ranges 1735-1750 at cm^{-1} and 1300-1000 cm^{-1} that corresponds to C=O and C-O functional groups present in biodiesel esters, respectively. The quality of biodiesel was evaluated and the qualitative fuel properties determined for acid value, pour point, cloud point, peroxide value, boiling point and specific gravity were 0.44±0.1 mg potassium hydroxide (KOH)/g, 3±0.1 °C, 4.1±0.4 °C, 1.41±0.1 milli equivalent (Meq) O_2/kg, 260±1 °C and 0.87±0.02 kg/m^3, respectively. The fuel properties of biodiesel produced by the selected strains were found in line with quality standards specified by ASTM D6751 and EN-14103.

Key words: Fuel properties; Transesterification; Plackett-Burman; central composite; Fatty acids methyl esters.

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8.2. Introduction

Biodiesel, a mixture of mono-alkyl esters of fatty acids, has gained a global attention as a renewable energy fuel. A number of edible and non-edible feedstocks have been used for biodiesel production. Although the edible feedstocks, especially, vegetable oils such as palm, sweet basil, soybean and rapeseed oils have been extensively used for biodiesel production and dominated the global biodiesel market, but the food versus fuel competition due to the use of edible feedstocks has raised questions that alternatively strengthened. This promoted the focus on non-edible feedstocks for biodiesel production.

Use of a number of non-edible feedstocks have been reported for biofuel production such as *Brucea javanica*, Moringa, Neem, Karanja, Rubber seed tree, Castor and Jatropha (Kumar and Purushothaman, 2012, Arumugam and Ponnusami, 2019, Hasni et al., 2017, Amini et al., 2017b). Most of the non-edible crops may only be grown on the arable land which indirectly lead to the dilemma "Food versus fuel competition". The cultivation of non-edible feedstocks replace the food producing crops, which still questions their feasibility as sustainable crops for biodiesel production. The *Jatropha curcas* however, requires less intensive attention, can grow well in both tropical and subtropical climates of barren/wasteland, acidic, sandy and drought areas which are usually not considered appropriate for the cultivation of food producing crops, and thus eliminate competition for food. These fascinating features of this plant may increase its economic viability to be used as a biofuel producing feedstock. The cultivation of *J. curcas* seeds does not have any direct or indirect effect on the food producing crops until they are grown on the arable land to replace the food bearing crops (Nahar and Ozores-Hampton, 2011). *J. curcas* is a member of *Euphorbiacea* family, contains 27 to 40% oil, and originated from North America (Kamel et al., 2018, Reddy et al., 2015). Some studies have reported *J. curcas* amongst the top oil producing non-edible plants for biodiesel production. Even some studies have estimated its seed's oil yield up to 60% which may be considered enough for an efficient biodiesel production rate (No, 2011).

The fatty acid composition of the non-edible feedstocks varies from species to species. The nature of fatty acids greatly influences the properties of biodiesel. Fatty acids may be long chain, short chain/medium, saturated or unsaturated, branched and unbranched. The level of mono-unsaturated fatty acid esters in biodiesel are positively correlated to the ignition quality, fuel stability and flow properties at low temperature (Keneni and Marchetti, 2017). The oxidative stability of biodiesel increases as the degree of poly-unsaturation of fatty acid methyl

esters decreases. The cold properties of the fuels are negatively affected by increase in the chain length of saturated fatty acids. However, no such effects have been observed for the unsaturated fatty acid alkyl esters as they have lower melting point than the saturated ones. The unsaturated fatty acids tend to act as solvent at low temperature to dissolve the other saturated fatty acid esters (Ramos et al., 2009). Moreover, the cetane number, combustion heat, melting point and viscosity of the fuel increases with increase in chain length and decreased with increase in unsaturation of fatty acids. Similarly, heating higher unsaturated fatty acid containing oil causes polymerization of glycerides, which can lead to deposits or to deterioration of lubricating properties of fuel (Mittelbach, 1996, Ramos et al., 2009). Interestingly, the *J. curcas* seed is rich in short/medium chain saturated and mono-unsaturated fatty acids which increases its economic viability for biodiesel production (Haq et al., 2019).

Biodiesel can be produced by a number of methods. The most common method are homogenous and heterogeneous catalyzed chemical mediated transesterification reactions in which the triglycerides (TGs) react reversibly with alcohol producing glycerol and mono-alkyl esters (Mohamad et al., 2017, Sai et al., 2020). In transesterification, TGs react with an alcohol to form fatty acid alkyl esters. However, in conventional chemical mediated biodiesel production processes it has been observed that free fatty acids present in oil could lead to soap formation and incomplete reaction (Sai et al.). The conventional alkaline transesterification is usually not recommended for 3 to 40% FFA as its downstream processing is highly cumbersome (Marchetti et al., 2007). In this scenario, lipases are gaining more attention for biodiesel production. Lipases (Triacylglycerol lipases, EC 3.1.1.3) are water-soluble enzymes that hydrolyze triacylglycerol releasing FFA and glycerol. Lipases are insensitive to FFA and have enormous biotechnological applications (Kumar et al., 2019). A number of lipase producing bacterial strains have been reported for biodiesel production such as *Pseudomonas aeruginosa* BUP2 and *Bacillus subtilis* strain Q1 KX712301, etc. (ul ain Rana et al., 2019, Unni et al., 2016, Onoji et al., 2016). Bacterial lipases are more economical and stable (Snellman et al., 2002). In addition, bacterial lipases have been extensively used for biodiesel production and can catalyze both esterification of FFA and transesterification of TGs simultaneously, resulting into a cleaner product with no hazardous wastes. The biodiesel could be produced using purified lipases or whole cell approach (Amini et al., 2017a, Amini et al., 2017b, ul ain Rana et al., 2019). The lipase producing bacterial strains living in the oil rich environment of oil expellers are considered ideal candidates for whole cell approach because they are equipped with already developed mechanisms to utilize these oils as carbon and energy

sources. Hypothetically, they may efficiently synthesize biodiesel from *J. curcas* seed oil and tolerate seed oil toxicity due their indigenous adaptabilities to cope with such challenges in the open oil rich environment.

A number of studies have been conducted to optimize different factors to increase the process efficiency and yield of biodiesel. These factors include methanol to oil molar ratio, temperature, reaction time and agitation (Micic et al., 2015). Different methods have been reported for biodiesel optimization from *J. curcas* seed oil such as response surface methodology, Box–Behnken design (BBD), Placket-Burman design and central composite design (ul ain Rana et al., 2019, Kashyap et al., 2019, Hasni et al., 2017).

In the present study, indigenous lipase producing bacterial strains were used for biodiesel production from *J. curcas* seed oil. The objectives of the study are: (1) Isolation and screening of lipase producing bacteria from oil expeller units in Rawalpindi district of Pakistan and utilization of these indigenous strains for biodiesel production. (2) The optimization of biodiesel from *J. curcas* seed oil using indigenous lipase producing strains. (3) Determination of fuel properties of *J. curcas* seed oil and its fatty acid methyl esters.

8.3. Materials and Methods

8.3.1. Sample collection and Isolation of strains

Jatropha curcas seeds were purchased from a local supplier in Lahore, Punjab. Seeds were sun dried for a week and the oil was extracted using oil expeller. The crude oil was filtered and stored. Oil contaminated soil samples from four different expeller units were collected in zip-lock bags and stored at 4°C till further processing. The samples were serially diluted and spread on plates containing nutrient agar with composition: 0.5% peptone, 0.3% yeast extract, 1.5% agar and 0.5% NaCl and incubated at 37°C for 24 h. A number of colonies obtained were sub-cultured on the nutrient agar and incubated again at aforementioned conditions to obtain the pure isolates.

8.3.2. Qualitative screening of isolates for lipase activity

A number of qualitative screening tests were carried out for determination of lipase activity of bacterial isolates. Only 30 isolates were selected randomly for qualitative screening of lipase activity after serial dilutions of the samples. Tributyrin agar containing (g/L): peptone, 5; yeast extract, 3; agar, 15; NaCl, 20 and 10 mL of tributyrin at pH 7.5±0.2 and rhodamine B agar containing (g/L): peptone, 5; yeast extract, 3; agar, 15; NaCl, 20; 2 mL of rhodamine B and 20 mL of olive oil were used as the screening media for the lipase producing microorganisms. The media were prepared as described previously (ul ain Rana et al., 2019). The cultures were incubated at 37°C for 48 h. The results for lipase activity on tributyrin agar were observed after 48 h by naked eyes and the lipolytic activity of lipase producing strains on rhodamine B agar was measured under UV light after 24 h.

8.3.3. Identification of bacterial isolates

Out of 30 bacterial isolates, only 8 best lipase producing strains were selected for Gram staining and biochemical tests including; catalase, nitrate reduction, oxidase, triple sugar iron, urease, citrate utilization, and Methyl Red Voges-Proskauer (MRVP) activities as described elsewhere (Goodfellow et al., 2012). For molecular identification, the genomic DNA of selected strains was extracted using cetyl trimethylammonium bromide (CTAB) method as described previously (Wilson, 2001). For 16S rRNA sequencing, only four best lipase and biodiesel producing isolates were selected. The quality of bands was observed by running the extracted DNA of the selected isolates on 1% agarose gel for 30 min. The isolated DNA samples were sequenced using Sanger method by Macrogen Standard Custom DNA Sequencing Services (Macrogen Inc., Seoul, Korea) for 16S rRNA. The phylogenetic

evolutionary correlation of the acquired sequences was carried out using Neighbor-Joining method in MEGA X as described previously (Saitou and Nei, 1987, Kumar et al., 2018).

8.3.4. Production of lipases through submerged fermentation

The selected bacterial strains were first enriched in nutrient broth with composition: 0.5% peptone, 0.3% yeast extract, and 0.5% NaCl at 37°C for 24 h with constant agitation at 150 revolution per minute (rpm). Then enzyme production media with composition previously described (ul ain Rana et al., 2019), was inoculated with 5% freshly enriched culture and incubated at 37°C for 48 h at 150 rpm. A 5 mL of 24 and 48 h fermented culture from each flask was centrifuged at 18,816 x g for 10 min. Pellet was discarded and supernatant further proceeded for lipase assay.

8.3.5. Lipase assay

Lipase activity of selected bacterial strains was assayed as previously described (ul ain Rana et al., 2019, Kumar et al., 2005). The unknown concentration of para nitrophenol (PNP) released from para-nitro phenyl Laurate (PNP-L) by lipase enzyme was measured from standard curve. One unit of enzyme activity was defined as enzyme catalysing the release of 1 µM of para-nitro phenol per minute per mL under standard assay conditions.

8.3.6. Protein estimation

Protein estimation was carried out according to standard Lowry method (Lowry et al., 1951). Peptides were reacted with copper under alkaline conditions to produce Cu^{++}, which further reacted with folin reagent forming a strong blue colour complex. The test sample was mixed well with alkaline copper sulfate reagent in (1:1) ratio and incubated at room temperature for 10 min. Then 0.1 mL Folin Ciocalteau solution was added to reaction tube in dark and shaken well. It was then incubated for 30 minutes in dark. Absorbance was measured at 650 nm.

8.3.7. Inocula preparation for methanol toxicity test

Lipase producing bacterial cultures were prepared for methanol toxicity as described by Gahlaut and co-workers (Gahlaut and Chhillar, 2013). The bacterial cultures were prepared in nutrient agar and incubated at 37°C for 24 h. The pellet was obtained by centrifuging the culture at 3011 x g for 5 min and washed with sterilized 0.9% normal saline. The pellet was re-suspended in normal saline and its optical density (OD) was measured at 630 nm. The OD of

all bacterial suspension was brought in range 0.5-1.0 that corresponds to 5×10^6 CFU/mL (Sarker et al., 2007).

8.3.8. Methanol toxicity

Methanol toxicity on the selected strains was evaluated using 96 well microtiter plate. The methanol was added to respective wells for each strain at concentrations of 11, 20 and 27 % that corresponds to oil to methanol molar ratios of 1:3, 1:6 and 1:9, respectively. The bacterial inocula (20 µL) was added in reaction wells for each strains and the total volume of the reaction was brought to 100 µL by adding nutrient broth medium. The plate was immediately covered with microseal film and incubated at 37°C for 48 h. The OD of reaction wells was measured at 630 nm using microplate reader (ELx800BioTek) and the results were recorded. Moreover, a 20 µL of sample from each well was spread on nutrient agar medium and incubated 37°C for 48 h to evaluate bactericidal effects on the selected bacterial strains. The experiment was conducted in triplicate.

8.3.9. Oil toxicity

J. curcas seeds are known for their antimicrobial activities (Haq et al., 2019). In order to determine its antimicrobial activities, the toxicity of seed oil was evaluated on the selected bacterial strains. Olive oil was used as reference oil and the *Staphylococcus aureus* ATCC25923 as standard reference strain. The media for oil toxicity was prepared with composition previously described (ul ain Rana et al., 2019) supplemented with 2% *J. curcas* seed oil. A 5% freshly enriched inocula of each strain was added separately in flasks, incubated at 37°C with constant agitation at 150 rpm. The well without inocula was used as negative control. After incubation, the OD was recorded for each strain at 650 nm.

8.3.10. Microbial synthesis of biodiesel

The top eight lipase producing bacterial isolates were selected for biodiesel production using whole cell approach. Initially, the selected lipase producing strains were refreshed in Luria broth (LB) with composition: Tryptone, 1% w/v; NaCl, 0.5% w/v and yeast extract, 0.5% w/v and incubated at 37°C for 48 h. The media was centrifuged at 1693 × g for 15 min and the cell pellet was used for biodiesel production. Initially, oil was added to 50 mL culture bottles followed by 1 mL of cell pellet and the cells were mobilized in the oil layer to protect them from direct methanol exposure which may lead to their inhibition. Afterwards for oil to

methanol ratio of 1:6, the methanol was added in two phases at an interval of 24 h in order to avoid sudden shock of methanol. A 300 µL *n*-hexane was added as solvent and emulsifier. The batch reaction was incubated at 37°C and 150 rpm for 48 h. The resultant mixture was transferred to separatory funnel, kept in static position overnight till layers formation. The yellow layer appeared at the top was removed and stored in glass vials. For removal of excess methanol and *n*-hexane, a rotary evaporator (Rotary Evaporator RE300 Stuart®) was used and its temperature was adjusted to 68°C. After removing the remaining methanol and *n*-hexane, the yield of fatty acid methyl ester (FAME) was calculated using the following equation.

$$FAME\ yield\ in\ \% = \left(\frac{Weight\ of\ FAME\ in\ grams}{Total\ weight\ of\ oil\ in\ grams}\right) \times 100.$$

8.3.11. Plackett-Burman design for optimization of biodiesel

Plackett-Burman design was used for optimization of parameters of four most potent biodiesel producing strains from batch process using Stat-Ease Design Expert Software version 7.0. Plackett-Burman design is very helpful in cases where complete knowledge of system is unavailable or in case of optimizing higher number of factors. Plackett-Burman design is the most popular design used for screening and optimization of enzymes in various studies. It is also one of the most efficient screening design when only the main effects are of interest. It is considered an economical screening for optimization of different factors, and usually main factors are estimated using two level interactions. This method has been used in a number of biological studies for the optimization of different factors (Giordano et al., 2011). Plackett-Burman design gives information about the effect of single factor on response i.e. percentage yield. For optimization five factor including; temperature, agitation, oil to methanol ratio, *n*-hexane percentage according to oil volume and inocula concentration were selected with 3 central points. The reaction batches were set up and incubated for a period of 48 h.

8.3.12. Response surface methodology to optimize the significant parameters using central composite design

The response surface model (central composite design) was used for optimization of significant factors determined by Plackett-Burman design. Two significant variables; molar ratio and agitation for strain SB11 and SB15 and three significant variables including

temperature with the former two for SB29 and SB30 were optimized by central composite design using the same software.

8.3.13. Physicochemical properties of *Jatropha curcas* seed oil and biodiesel

The *J. curcas* seed oil and biodiesel synthesized from it through bacterial lipases were analyzed for fuel properties analysis using standard procedures according to Mehta et al., (2015) and Onukwuli et al., (2017) with few modifications, as given below.

8.3.13.1. Acid value and percent free fatty Acid

For acid value (AV) determination, 5 g of Jatropha oil or biodiesel was mixed with 25 mL absolute ethanol, followed by addition of 2-3 drops of phenolphthalein. The mixture was heated at 65°C for 10 min with thorough gentle shaking. The reaction mixture was titrated against 0.1 N KOH solution and continued until the appearance of pink colour (end point). The KOH amount used was calculated. The equation for AV given below.

$$\mathrm{AV} = \left(\frac{\text{mL of KOH} \times \mathrm{N} \times 56.1}{\text{sample weight}}\right) = \text{mg of KOH/g}$$

where, N = Normality of KOH and 56.1 = molecular weight (g/mol)

Percent FFA are approximately half of the acid value of a sample and was found using the following equation.

% Free Fatty Acid (Sai et al.) = AV x 0.503

8.3.13.2. Saponification value

A 2 g *J. curcas* seed oil was mixed with 25 mL of 0.5 N alcoholic KOH solution in a flask. For blank 25 mL of 0.5 N alcoholic KOH solution was taken without seed oil. The flask was then fixed with a condenser, incubated in water bath at gentle and steady boiling with random shaking for an hour to complete the saponification reaction. Later on, few drops of phenolphthalein were added to the solution and titrated against 0.5 N HCl till the appearance of pink colour (end point). The saponification value against the blank was calculated using the following equation.

$$\text{SP Value} = \left(\frac{56.1\ (\mathrm{B}^\circ - \mathrm{S}^\circ) \times \text{N of HCl}}{\mathrm{W}}\right)$$

where, B°: represent the HCl in mL required by Blank control; S°: the HCl in mL required by sample and N: normalized, W: weight.

Ester Value

The amount of KOH in mg required to saponify esters in one g of a substance is called ester value. The ester value is determined by subtracting acid value from saponification value.

8.3.13.3. Peroxide value

Peroxide value was determined as described by Association of Official Analytical Chemists (AOAC) method (Society, 1988). *J. curcas* seed oil was mixed with 30 mL of acetic acid and chloroform (present in 3:2 molar ratio) and swirled to dissolve completely. Then 0.5 mL saturated potassium iodide (KI) solution was added to the reaction mixture and swirled until the appearance of a light brown colour. Later on, 30 mL distilled water was added to the reaction mixture and titrated against 0.1 N sodium thiosulphate ($Na_2S_2O_3$) till the appearance of pale yellow colour. Afterwards, 1% starch indicator (1 mL) was added and titrated against 0.1 N $Na_2S_2O_3$ again till the disappearance of blue colour. For blank, 30 mL of acetic acid and chloroform (molar ratio of 3:2) mixture was mixed with 0.5 mL saturated KI solution, followed by 30 mL distilled water addition and titrated against 0.1 N $Na_2S_2O_3$. The peroxide value is represented as milli equivalent of peroxide oxygen per kg of the sample. The formula for peroxide value is given below

$$\text{Peroxide value} = \left(\frac{(S° - B°) \times N \times 100}{W}\right)$$

where, S° = test sample titration; B° = blank titration; W = weight of sample in grams; and N = Normality of $Na_2S_2O_3$.

8.3.13.4. Specific gravity

A crystal clear specific gravity bottle (25 ml volume) weighed either empty or with water. The water was drained out and replaced by seed oil or biodiesel and weighed again. The whole process was carried out at 30 ˚C. The specific gravity is calculated as follows.

$$\text{Specific gravity} = \left(\frac{A° - B°}{C° - B°}\right)$$

where, A° = weight in grams of bottle along with oil or biodiesel; B° = weight in grams of empty bottle; and C° = weight in grams of specific gravity bottle with water.

8.3.13.5. Boiling point

For boiling point determined, 10 mL *J. curcas* oil or biodiesel was taken in a test tube and thermometer inserted into it. Later on, the temperature of magnetic stirrer was increased gradually. The point at which bubble formation started was recorded as boiling point.

8.3.13.6. Cloud point and pour point

J. curcas seed oil or biodiesel (30 mL) was taken in separate jars and placed in cooling jackets in water bath. Thermometer was fixed in each jar and observed at different intervals without disturbing the liquids. The temperature at which clouds of crystals appeared was recorded as cloud point. The pour point was determined, using 20 mL of seed oil or biodiesel in beakers sealed with corks and incubated in refrigerator for solidification. After solidifying, the samples were taken out and placed at room temperature. The temperature at which each sample was melted and started flowing was termed as pour point.

8.3.14. Analytical methods

J. curcas seed oil and biodiesel produced via bacterial lipases were analyzed using Fourier Transform Infrared Spectrometer (FTIR, Bruker, model Tensor27 with software version Opus65.equipped with ZnSe ATR). Each sample (5 µL) was loaded at the sample injector, and analyzed using infra-red spectrum in a range of 400-4000 cm^{-1}.

8.3.15. Statistical analyses

The statistical analyses were performed using Prism software (ver. 5.0). The values for statistical analysis are presented in triplicate to calculate the mean values of experiments. One-way ANOVA was used followed by Tukey's posttest for multiple comparisons. The level of significance was $p<0.05$.

8.4. Results and discussion

8.4.1. Isolation and screening of lipolytic bacteria from oil contaminated soil of expeller units

Oil contaminated soils are considered an ideal habitat for lipase producing bacteria due to abundant quantity of lipids. The dwelling microorganisms utilized these lipids as their nutrition for building their biomass and fulfilling energy requirements. The whole process of microbial lipid consumption occurs at surface layer under aerobic condition releasing CO_2 as their end product (Polyak et al., 2018). Countable range of lipase producing bacterial colonies were obtained from all four samples collected from different expellers at 10^{-6}, 10^{-6}, 10^{-7} and 10^{-5} serial dilutions corresponding to 5.2 $\times 10^{-8}$, 2.7$\times$ 10^{-8}, 3.5$\times 10^{-9}$ and 4.6$\times 10^{-7}$ CFU/mL, respectively. Initially, 30 lipase producing strains were confirmed using tributyrin agar and rhodamine B olive oil agar plate assays. Lipase producing strains gave a clear hollow zone on tributyrin agar plates (Appendix 5 Figure S1) and an orange fluorescent colour under UV light on rhodamine B olive oil agar plates (Sirisha et al., 2010). On the basis of these screenings, eight isolates with top most prominent hollow and large zones were selected for further identification. The selected lipase producing strains were abbreviated as SB3, SB6, SB11, SB15, SB21, SB25, SB29 and SB30.

8.4.2. Identification of isolates

A number of biochemical tests and Gram staining were performed for identification of the selected bacterial strains (Table 8.1).

Table. 8.1. Biochemical characterizations of isolated bacterial strains from oil expeller units

	Isolated bacterial lipase producing strains							
Tests	SB3	SB6	SB11	SB15	SB21	SB25	SB29	SB30
Catalase	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	+	-	-	-	-	-
Citrate utilization	+	+	-	-	+	+	+	+
Triple sugar iron test	K/K	K/A H_2S	K/A	K/K	A/A	A/A	A/A H_2S	K/A
Urease	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-
Vogues Proskauer	-	-	-	-	-	-	-	-
Gram Staining	-	+	+	-	+	+	-	-
Identified strains	*P*	*B*	*Br*	*P*	*B*	*B*	*A*	*A*

P: Pseudomonas sp., B: Bacillus sp., Br: Brevibacterium sp., A: Acinetobacter sp., A/A: acidic slope/acidic butt, K/A: alkaline slope/acidic butt , A/A: acidic slope/acidic butt.

Moreover, Appendix 5 Figure S2 shows the DNA bands of the four selected lipase producing strains for the optimization of biodiesel production. Phylogenetic tree (Figure 8.1) indicates that strain SB29 is in close resemblance with *Acinetobacter* species isolated from oil contaminated soil. Similarly, SB30 also shows resemblance to *Acinetobacter* species isolated from soil source. While SB15 have unique clades showing resemblance with *Pseudomonas* species isolated from thermal water sources. On the contrary, SB11 shows resemblance to multiple species such as *Brevibacterium luteolum*, *Bacillus sp*. and *Actinomycetales bacterium* but the highest resemblance was shown with *Brevibacterium sp*. isolated from algae associated habitats. The 16S rRNA gene sequences of these strains have been submitted in EMBL/GenBank database under the following accession numbers for species SB11: MH715025, SB15: MH715026, SB29: MH715027 and SB30: MH715028 (Table 8.2).

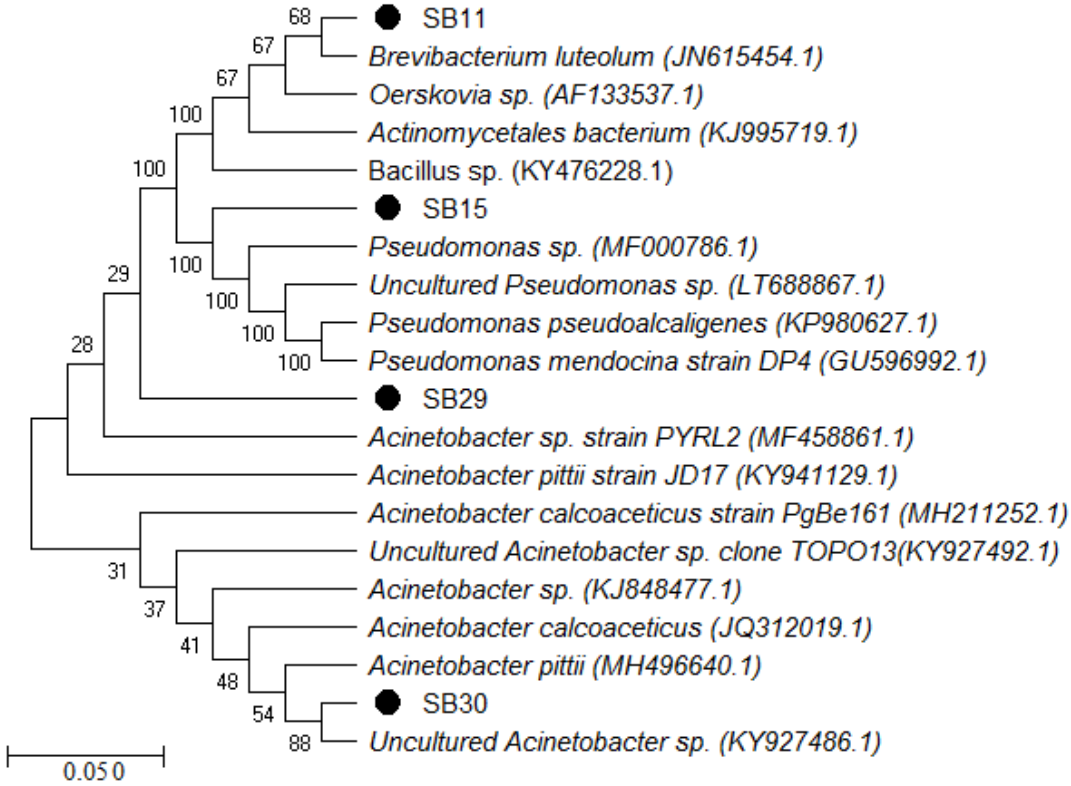

Figure 8.1. Phylogenetic tree was constructed using neighbor joining method of nucleotide sequences of 16S rRNA genes. The number in each branch represents bootstrap values in percent that are in multiple of 1000. Additionally, the scale represents one per 1000 substitutions of 16S rRNA gene nucleotide sequence.

Table 8.2. Isolated lipase producing bacterial strains with accession numbers and percent identity

Isolates	Accession number	Resemblance to	Percent identity
SB11	MH715025	*Brevibacterium sp.*	93
SB15	MH715026	*Pseudomonas*	100
SB29	MH715027	*Acinetobacter sp.*	100
SB30	MH715028	*Acinetobacter sp.*	95

8.4.3. Enzyme activity

The strain SB29 gave the highest lipase activity both at 24 and 48 h incubations (Figure 8.3). The lipase activity of SB29 was found significantly higher (p<0.05) than that of SB3, SB6 and SB25 at 24 h. Similarly, the lipase activity of SB29 was also found significantly higher (p<0.05) than that of SB21 and SB25 strains at 48 h incubation. *S. aureus* ATCC25923 (reference strain) was found the second most active bacterial strain with relatively higher lipase activity than the others (Figure 8.2). Overall, the ratio of lipase activity of the selected bacterial strains was higher at 48 h compared to 24 h. Similar findings were also observed by the Ul Ain Rana et al., 2019, where the isolated strains exhibited higher lipase activity at 48 h comapred to 24 h incubation (ul ain Rana et al., 2019). The lipase activity shows that the strains can further be utilized for biodiesel production process.

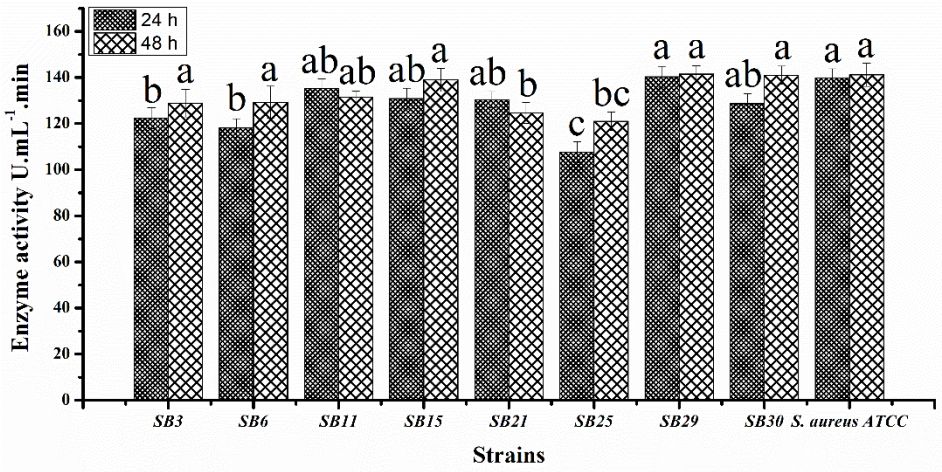

Figure 8.2. Crude enzyme activities of selected lipase producing bacterial strains at 24 and 48 hours incubation. Different lowercase letters on the adjacent bars represent significant

(p<0.05) difference. The data was presented as mean ± standard deviation (SD). One-way ANOVA was used followed by Tukey's posttest for multiple comaprsisons.

Moreover, the specific enzyme activity of all strains was higher at 24 h compared to 48 h except SB6 and SB11 (Figure 8.3). The highest specific enzyme activity was exhibited by strain SB30 followed by *S. aureus* ATCC25923, SB15, SB29, SB25, SB21, SB11, SB3 and SB6. The activity of SB30 was found significantly higher (p<0.05) than that of all other selected bacterial strains except *S. aureus* ATCC25923.

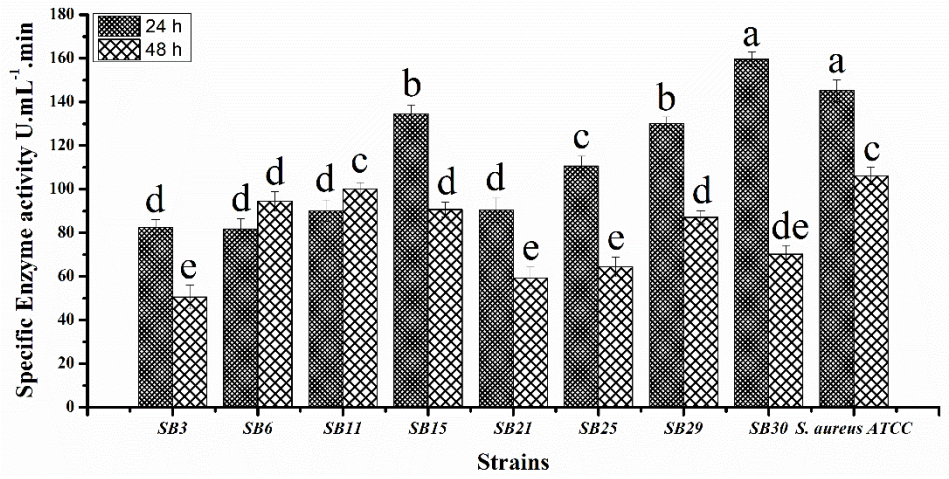

Figure 8.3. Specific enzyme activities of selected lipase producing strains at 24 and 48 hours. The data was presented as mean ± standard deviation (SD). Different lowercase letters on the adjacent bars represent significant (p<0.05) difference. One-way ANOVA was used followed by Tukey's posttest for multiple comaprsisons.

8.4.4. Methanol toxicity on the selected lipase producing strains

Short chain alcohols such as methanol are organic solvent with hydrophilic nature and have lower solibility in oil and interfere with water coating the enzymes resulting in their deactivation and decreased yield of esters (Royon et al., 2007). Methanol has also been reported to affect the integrity and permeability of bacterial cell membrane. Many bacterial cells are susceptible to methanol toxicity. However, some cells have adapted strategies to resist the toxicity of these solvents such as modification in cis/trans conformation of membrane fatty acids. Methanol usually, at higher concentration, promotes dehydration of lipases and their deactivation by changing the 3D conformation of the enzyme (Heipieper et al., 1994). Lipases, specifically deactivated by methanol during tranesterification, results in lower biodiesel yield (Kaieda et al., 2001, Lotti et al., 2018). In the present study, the selected bacterial strains were found tolerant to methanol toxicity. The indigenous lipase producing cells exhibited higher

tolerance to methanol exposure compared to reference strains; *S. aureus* ATCC25923 and *E. coli* ATCC25922 (Figure 8.4).

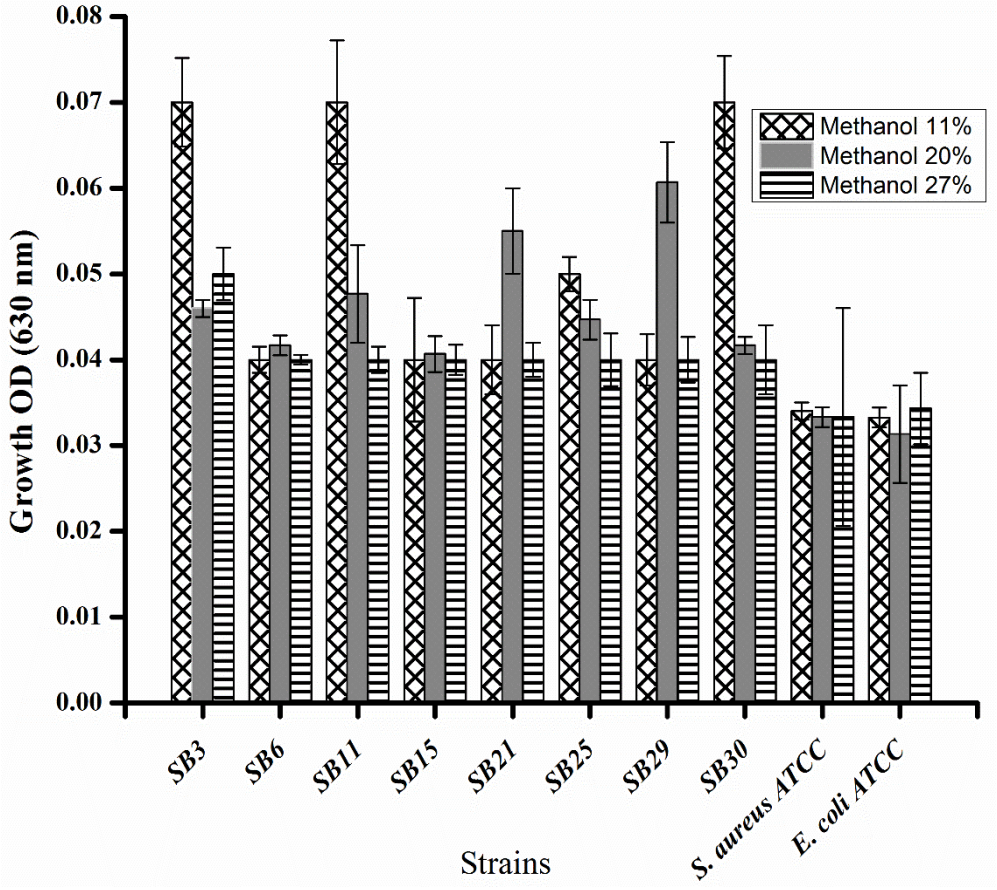

Figure 8.4. Methanol toxicity on the selected bacterial strains. All of the strains were incubated at 37°C for 48 h. The methanol toxicity on each strain was evaluated in triplicate. The data is presented as mean ± standard deviation.

In addition, they also exhibited higher growth rate on the nutrient agar medium after incubation at 37°C for 48 h in 11, 20 and 27% methanol that corresponds to 1:3, 1:6 and 1:9 oil to methanol ratios for biodiesel production. Strains SB15, SB29 and SB11 were found more tolerant to 11% methanol compared to the other strains. The strain SB3 was significantly inhibited at 27% methanol, compared to other selected bcaterial strains (Figure 8.5). The isolated lipase producing bacterial strains have exhibited more efficient growth on medium and higher tolerance than the bacterial strains isolated in the previous studies like the strains isolated by Ul Ain Rana et al., 2019, were only tested for methanol toxicity at 5%, 10% and 15%, and reasonable growth was shown only untill 10% of methanol exposure (ul ain Rana et al., 2019). In our study the all of the strains except SB3 were highly tolerant to methanol toxicity, which

make them ideal candidates for processing at higher methanol to oil ratios in biodiesel producing industries.

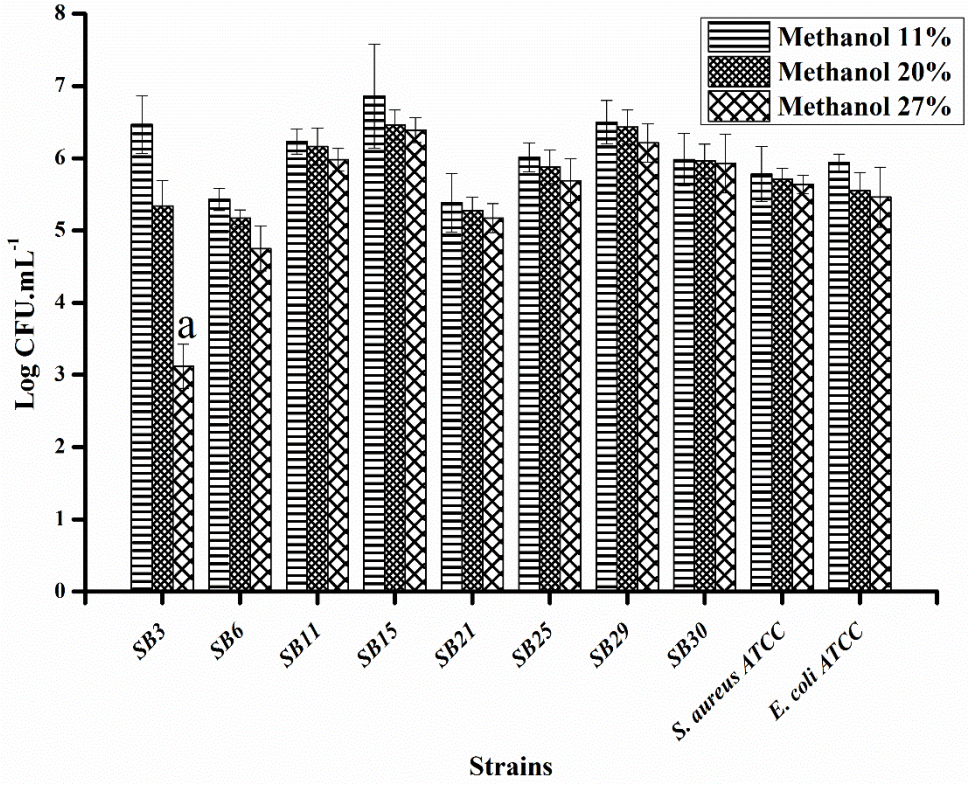

Figure 8.5. Methanol toxicity on the growth of selected bacterial strains. All of them were incubated at 37°C for 48 h and their growth was calculated based on colony forming units per mL (CFU/mL). The data is presented as mean ± standard deviation. One-way ANOVA was used followed by Tukey's posttest for multiple comaprsisons.

8.4.5. *Jatropha curcas* seed oil toxicity

Jatropha curcas seeds have toxic compounds that are known for their antimicrobial activities (Ohtani et al., 2017). Strains SB3, SB11, SB29 and *S. aureus* ATCC25923 were found highly active and unaffected by seed oil toxicity (Fig. 6). *J. curcas* seed oil significantly reduced (p<0.05) the growth of SB6 and SB25 strains. The rest of them were found highly active in the presence of Jatropha seed oil and showed similar growth pattern as in the presence of reference olive oil. The olive oil was used as reference oil for biotoxcity assay as prevoisly reported (ul ain Rana et al., 2019). All of the strains were found active in the presence of olive oil and exhibited sufficient growth (Figure 8.6). The olive oil has no toxic effects on the lipases and had been reported as an inducer of the lipases in previous studies. It is rich in unsaturated fatty acids which are used to enhance the extracellular and intracellular lipolitic activity of the lipase producing bacteria (Zarevúcka, 2012). The strains *Brevibacterium* SB11 MH715025,

Pseudomonas SB15 MH715026, *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 were found highly tolerant to the oil toxicity and non-significantly inhibited by *J. curcas* seed oil.

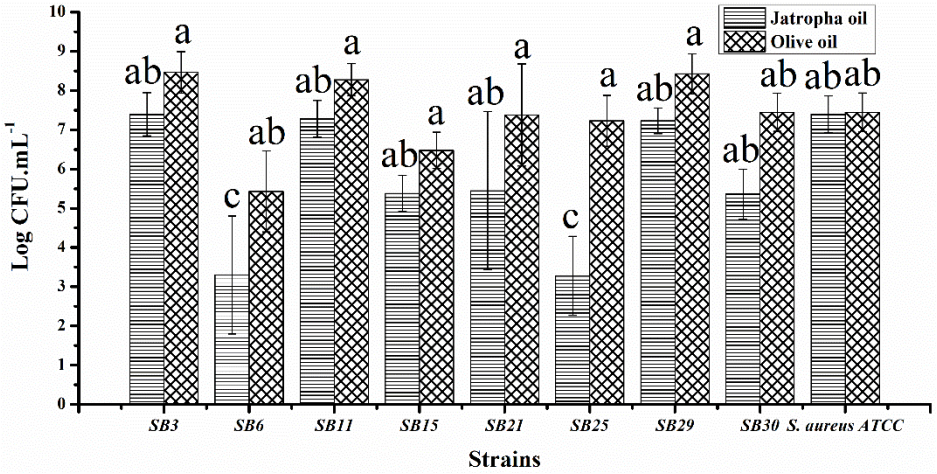

Figure 8.6. *Jatropha curcas* seed oil toxicity on the growth of selected bacterial strains. All of them were incubated at 37°C for 48 h and their growth was calculated based on colony forming units per mL (CFU/mL). The data was presented as mean ± standard deviation (SD). Different lowercase letters on the adjacent bars represent significant (p<0.05) difference. One-way ANOVA was used followed by Tukey's posttest for multiple comaprsisons.

Moreover, on the basis of OD, strain SB29 exhibited higher growth compared to all other strains. The SB29 growth in the presence of Jatropha seed oil was found significantly higher (p<0.05) compared to SB3, SB6, SB21, SB25 and SB30 strains. The *Acinetobacter* sp. has been reported as the sole consumer of diesel oil as a substrate (Lin et al., 2015). *Acinetobacter* sp. has been reported to possess higher degradation potential for hydrocarbon or oils (Czarny et al., 2020). *Pseudomonas*, *Acinetobacter* and *Brevibacterium* species have been reported with tremnedous capabilities of biodegradation of oils (Yang et al., 2019).

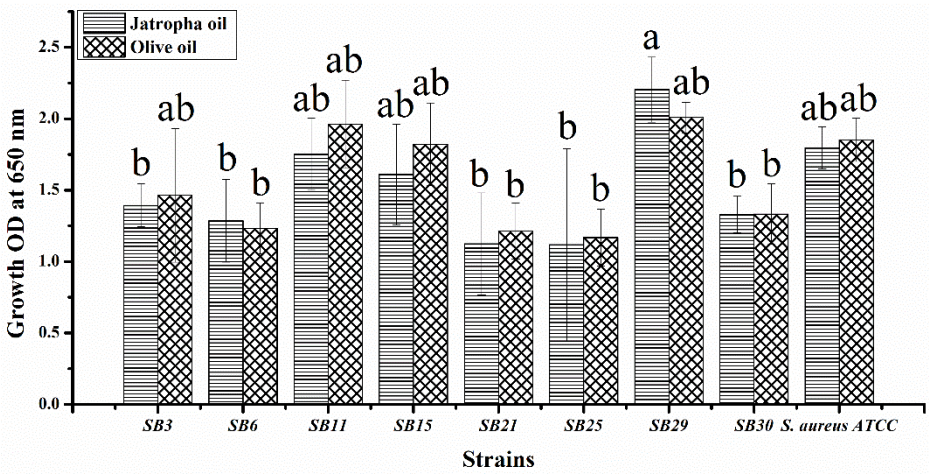

Figure 8.7. *Jatropha curcas* seed oil toxicity on the growth of selected bacterial strains using optical density (OD) at 650 nm. Different lowercase letters on the adjacent bars represent significant (p<0.05) difference. The data was presented as mean ± standard deviation (SD). One-way ANOVA was used followed by Tukey's postest for multiple comaprsisons.

8.4.6. Microbial Synthesis of Biodiesel

Based on the lipase activities, top eight lipase producing strains were selected for biodiesel production with conditions; oil to methanol ratio (1:6), solvent *n*-hexane 300 µL, incubation temperature 37°C, agitation of 150 rpm and incubation time of 48 h. The highest volumetric biodiesel yield was exhibited by strain SB29 followed by SB15, SB30 as well as SB11 with a yield of 93, 92 and 90%, respectively. These results are in correlation with the toxicity results of the methanol and seed oil as mentioned earlier. The strains that were more tolerant to methanol and seed oil have exhibited higher biodiesel yield compared to those strains who were susceptible and prone to inhibition by methanol and seed oil.

Table 8.3. Biodiesel yield of the selected lipase producing bacterial strains

Isolates	Biodiesel Yield (%)
SB29	93
SB15	92
SB11	90
SB30	90
SB21	89
SB6	81
SB3	80
SB25	77

8.4.7. Plackett-Burman design for optimization of biodiesel synthesis

Four strains *Brevibacterium* SB11 MH715025, *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027, and *Acinetobacter* SB30 MH715028 were selected for optimization for biodiesel synthesis. Five parameters, temperature, oil to methanol ratio, *n*-hexane percentage, agitation, and inocula size were optimized for biodiesel production. Experiments were performed according to the conditions given by 15 runs of Placket-Burman design and response in terms of percent volumetric biodiesel yield was measured (Table 8.4). The highest biodiesel yield was observed for strain *Pseudomonas* SB15 MH715026 followed by *Acinetobacter* SB29 MH715027 at run 14, with conditions temperature 37°C, molar ratio 9, *n*-hexane 10%, inoculum 30% and agitation 150 rpm. While *Brevibacterium* SB11 MH715025 exhibited higher biodiesel yield at temperature 43.5°C, molar ratio 6, *n*-hexane 8%, inoculum 20% and agitation at 225 rpm (Table 8.4).

Table. 8.4. Percent volumetric biodiesel production by selected bacterial strains in response to conditions specified by Plackett-Burman Design for each run.

F.1.A	F.2. B	F.3. C	F.4. D	F.5.E	SB11: Response biodiesel yield (%)	SB15: Response biodiesel yield (%)	SB29: Response biodiesel yield (%)	SB30: Response biodiesel yield (%)
37	3	30	6	300	20	10	30	20
50	3	10	6	300	0	10	0	0
50	3	30	10	300	10	0	10	20
50	9	30	6	150	84	87	63	67
50	9	10	10	300	80	88	72	75
50	9	10	6	150	73	88	75	81
50	3	30	10	150	55	60	54	57
43.5	6	20	8	225	93	85	84	82
37	9	10	10	300	89	87	86	88
37	9	30	6	300	88	87	81	87
37	3	10	6	150	72	63	79	75
43.5	6	20	8	225	93	82	83	82
37	3	10	10	150	84	79	82	87
37	9	30	10	150	88	95	94	89
43.5	6	20	8	225	92	83	84	81

F: factor, SB11: *Brevibacterium* SB11 MH715025, SB15: *Pseudomonas* SB15 MH715026, SB29: *Acinetobacter* SB29 MH715027, and SB30: *Acinetobacter* SB30 MH715028, F.1.A: Temperature (ºC), F.2. B: Molar ratio, F.3. C: Inoculum size (%), F.4. D: *n*-hexane (%), F.5.E: Agitation (rpm).

ANOVA analysis of design for *Brevibacterium* SB11 MH715025, *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027, and *Acinetobacter* SB30 MH715028 had shown F values 13.98, 11.38, 8.74 and 8.79, respectively, indicating that the experimental design of the factors was significant. For identification of significance, F-test was also applied on each factor. The factors having values of porb> F less than 0.05 are considered significant. In the present study the molar ratio and agitation were found signifcant factors with porb> F value less than 0.05 for all strains i.e. *Brevibacterium* SB11 MH715025, *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027, and *Acinetobacter* SB30 MH715028 (Appendix 5 Figure S3). Moreover, temperature was also found a significant factor only for strains

Acinetobacter SB29 MH715027, and *Acinetobacter* SB30 MH715028 by design (Appendix 5 Figure S3).

Increase of methanol to oil molar ratio had positive effects on the biodiesel yield (response), indicating that the increase in molar ratio will increase the biodiesel yield (Appendix 5 Figure S4). The stoichiometric ratio of alcohol to oil for biodiesel production is 3:1 and the reaction is reversible. Higher molar ratios are required to increase the miscibility and contact between the oil & alcohol and to continue the reaction in forward direction. The amount of alcohol in excess is required to break the glycerol-fatty acids linkages during transesterification of oil (Miao and Wu, 2006). Therefore, higher alcohol to oil molar ratios are required to speed up the reaction in forward direction and produce alkyl esters in shorter time. Moreover, the increase in the molar ratio of alcohol to oil also leads to the higher biodiesel yield and purity. The increase in alcohol to oil molar ratio beyond a certain level causes decrease in the ester yield due to the complications in separation of glycerol from the ester contents (Musa, 2016). Similar pattern was also shown in the present study by the molar ratio effect on the biodiesel yield, which has been described in the below sections for the respective strains. While in case of purified or immmobilized enzymes the biodiesel yield decreases with increase in molar ratios (Veljković et al., 2006). Similarly, Rodrigues et al., 2013 have also reported that the enzymatic biodiesel yield was decreased as the molar ratio of methanol to oil was increased (Rodrigues et al., 2013). This unusual behaviour of lipase producing bacterial strains can be possibly explained as they have high potential of adaptability to adverse conditions in open environment so they might have higher tolerance to methanol or oil compared to commercially available strains or enzymes. The higher microbial inocula concentration ranging from 10-30% according to *J. curcas* seed oil volume might also be the reason of higher efficiency at higher molar ratio because some of the cells if get inhibited the rest of the viable cells still would perform their activities. The microbial inocula were pre-mixed with the seed oil which mask the cells from sudden exposure of methanol. These findings are inline with previous study carried out on lipase mediated biodiesel production (ul ain Rana et al., 2019). The methanol were added in two phases at an interval of 24 h to reduce the sudden toxic shock of methanol. Similar findings were also observed by Shamida et al., 1999, in which the biodiesel yield was significantly increased by adding methanol in three phases (Shimada et al., 1999). The *n*-hexane solvent was added to increase the miscibility of methnaol in oil and to reduce the inactivation of lipase enzymes (Soumanou and Bornscheuer, 2003).

On the other hand, the agitation had negative effect on biodiesel yield, indicating that the increase in agitation will lead to decrease in the biodiesel yield (Appendix 5 Figure S5). Strains *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027, and *Acinetobacter* SB30 MH715028 exhibited higher biodiesel synthesis at lower agitation (150 rpm). While, the *Brevibacterium* SB11 MH715025 was relatively more tolerant to higher agitation and exhibited optimum biodiesel yield at 225 rpm. All of the strains exhibited lower potential of biodiesel synthesis at higher agitation (300 rpm). This might be due the lower contact of microbial cells with methanol and oil during higher agitation. Normally, agitation favors the microbial contact to their respective substrates but if the agitation is increased beyond a certain limit, it will lead to a decrease in the biodiesel yield due to shearing of lipases or foam formation (Muanruksa and Kaewkannetra, 2020). Therefore, adequate agitation must be provided to lipase producing strains in order to get higher biodiesel yield.

Morever, the increase in temperature had negative effects on the biodiesel yield of *Acinetobacter* SB29 MH715027, and *Acinetobacter* SB30 MH715028 strains (Appendix 5 Figure S6). The optimum biodiesel yield was given by *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 at 37ºC. Only *Brevibacterium* SB11 MH715025 exhibited optimum biodiesel yield at 43.5ºC. The higher enzymatic biodiesel production at lower temperature favors it over the chemical synthesis, as chemically mediated tranesterification is carried out at higher temperature which requires more energy resulting in higher cost. Final equations given by software for volumetric biodiesel yield in terms of significant factors for each strain are given in (Appendix 5 Table S1).

8.4.8. Optimization of significant factors using central composite design

The signifcant factors identified by Plackett-Burman design for all selected strains were further optimized using central composite design and their interactive effects on biodiesel yield were studied using response surface methodology. In case of *Brevibacterium* SB11 MH715025 and *Pseudomonas* SB15 MH715026, molar ratio (9-12 range) and agitation (ranging 100-150 rpm) were selected for optimization with 11 runs (Table 8.5). Conversely, for *Acinetobacter* SB29 MH715027, and *Acinetobacter* SB30 MH715028 strains, methanol to oil molar ratio (9-12), temperature (20-37ºC) and agitation (100-150 rpm) were selected for optimization with 17 runs using central composite design (Table 8.6). ANOVA analysis of design gave F-vlaue 62.20, 45.66, 13.10 and 8.09 for *Brevibacterium* SB11 MH715025, *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028, respectively. These values indicated that the generated model was significant. In case of central

composite design, the increase in molar ratio of methanol to oil has negative effect on biodiesel yield for all strains which implies that further increase in methanol could severely inhibit the bacterial enzymes as described elsewhere (Rodrigues et al., 2013). The increase in agitation has negative effect on biodiesel yield of all strains except *Brevibacterium* SB11 MH715025 whose biodiesel yield was positively correlated with increase in agitation. In case of *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028, the increase in temperature has positive effect on biodiesel yield, indicating that lower temperature affect the microbial efficiency of biodiesel production. The strain *Brevibacterium* SB11 MH715025 gave optimum biodiesel yield (97%) at oil to methanol molar ratio of 1:9, 150 rpm and 43.5ºC. The strain *Brevibacterium* SB11 MH715025 was however, better in response to the agitation than the previous studies, as Muanruksa et al., 2020 and Suwanno et al., 2017 had reported that the optimum enzymatic biodiesel yield was obtained at 200 rpm (Muanruksa and Kaewkannetra, 2020, Suwanno et al., 2017). The strain *Pseudomonas* SB15 MH715026 gave optimum yield (97%) at oil to methanol molar ratio of 1:9 at 100 rpm (Table 8.5). Modi et al., (2006) reported optimum biodiesel yield (91.3%) with 10% Novozyme 435, at ethyl acetate to oil molar ratio of 11:1 at 50ºC for a period of 12 h (Modi et al., 2007). Simiarly, Devanesan et al., (2007) reported that *Pseudomonas fluorescens* entrapped in sodium alginate exhibited optimum biodiesel yield (72%) at 50ºC with oil to methanol ratio 1:4, 3 g beads, reaction time 48 h (Devanesan et al., 2007). These findings indicate, that the biodiesel process using whole cell approach is more efficient and cost effective than that of the free or immobilized lipases. On the other hand, the strain *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 gave optimum yield of 96% and 92% at oil to methanol molar ratio of 1:9 at 37ºC and 100 rpm, respectively (Table 8.6). Interestingly, the lipase are stable at 30-45 ºC (Satti et al., 2019), and the optimum temperature in the current study lies in this range (30-45 ºC). All of the strains have shown optimum biodiesel yield at 1:9 molar ratio of oil to methanol which are in line with the previous findings, like in a study maximum biodiesel yield was obtained from rubber seed oil at molar ratio 1:9 (Sai et al., 2020). The optimum 1:9 molar ratio of oil to methanol The interactive effects of these factors on biodiesel yield are given in contour and 3D model plots for *Brevibacterium* SB11 MH715025 and *Pseudomonas* SB15 MH715026 (Figure 8.8), for *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 (Figure 8.9).

Table 8.5. Percent biodiesel yield in response to the conditions specified by central composite design for strain *Brevibacterium* SB11 MH715025 and *Pseudomonas* SB15 MH715026

Run	Factor 1A: Molar ratio	Factor 2. B: Agitation (rpm)	Response yield (%) for *Brevibacterium* SB11 MH715025	Response yield (%) for *Pseudomonas* SB15 MH715026
1	10.50	160.36	69	46
2	10.50	125.00	78	59
3	10.50	89.64	56	71
4	10.50	125.00	72	68
5	10.50	125.00	62	61
6	9	100.00	81	97
7	9	150.00	97	79
8	12	150.00	24	12
9	12	100.00	18	22
10	12.62	125.00	0	9
11	8.38	125.00	88	81

Final equations by central composite design in terms of significant factors for biodiesel yield of selected bacterial strains are given in (appendix 5 Table S2).

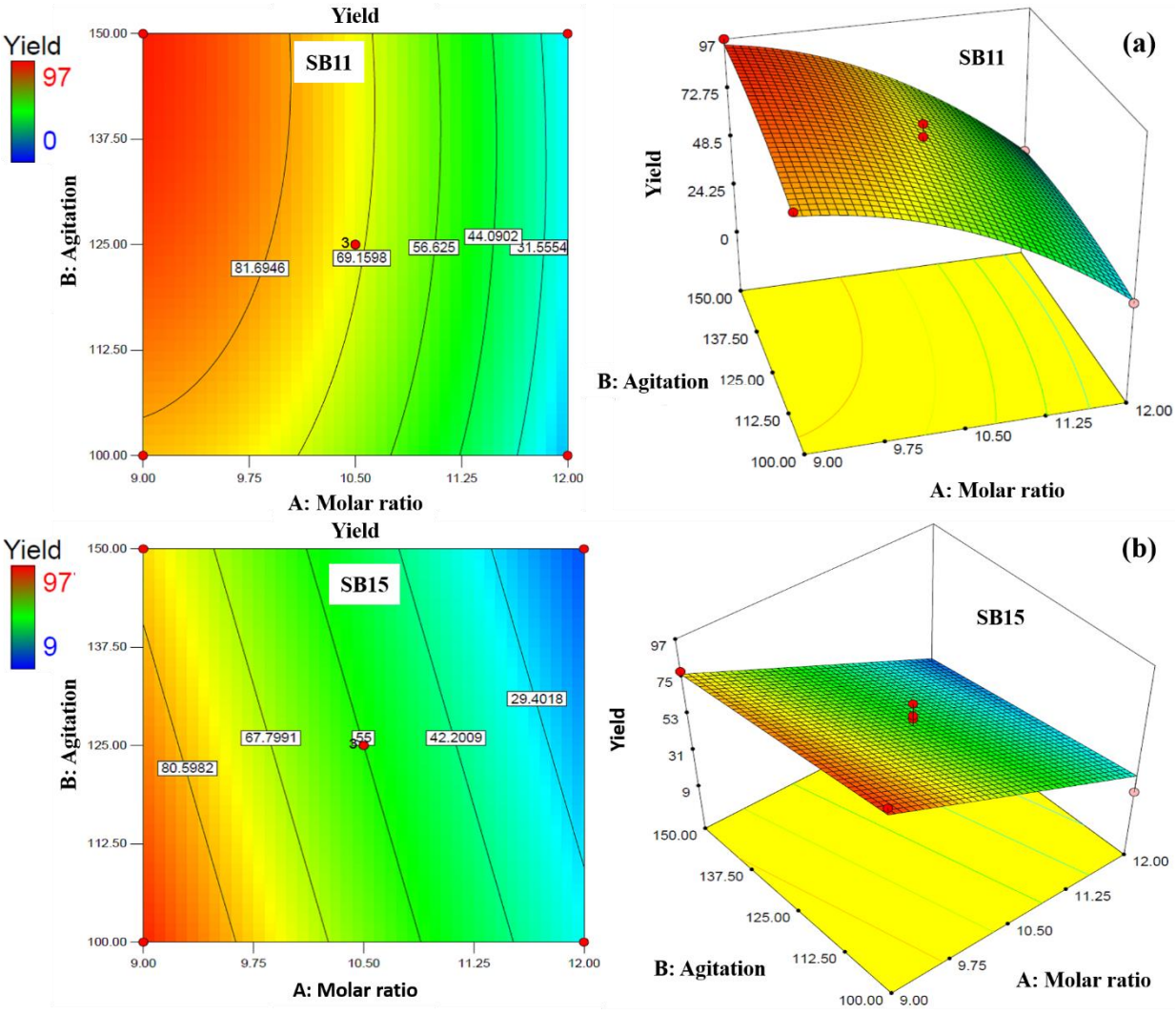

Figure 8.8. Contour and 3D surface plots showing interactions between molar ratio and agitation for strains *Brevibacterium* SB11 MH715025 and *Pseudomonas* SB15 MH715026. (a) Illustrates interactive plots for *Brevibacterium* SB11 MH715025 strain, (b) Illustrates interactive plots for *Pseudomonas* SB15 MH715026 strain. The colour variation in vertical colour bars represent the biodiesel yield level in contour and 3D surface model plots.

Table 8.6. Percent biodiesel yield in response to the conditions specified by central composite design for strain *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028

Run	Factor 1.A: Temperature (ºC)	Factor 2.B: Molar ratio	Factor 3.C: Agitation (rpm)	Response yield (%) for *Acinetobacter* SB29 MH715027	Response yield (%) for *Acinetobacter* SB30 MH715028
1	42.50	10.50	125.00	26	20
2	20.00	9	100.00	46	38
3	28.50	10.50	82.96	72	66
4	28.50	10.50	125.00	70	76
5	20.00	12	100.00	10	0
6	28.50	7..98	125.00	77	73
7	37.00	12	100.00	25	0
8	37.00	9	150.00	72	83
9	14.2	10.5	125.00	0	0
10	20.00	9	150.00	30	20
11	28.50	10.5	167.04	30	30
12	28.50	10.5	125.00	46	40
13	20.00	12	150.00	0	0
14	37.00	12	150.00	9	0
15	37.00	9	100.00	96	92
16	28.50	13.02	125.00	0	0
17	28.50	10.50	125.00	26	20

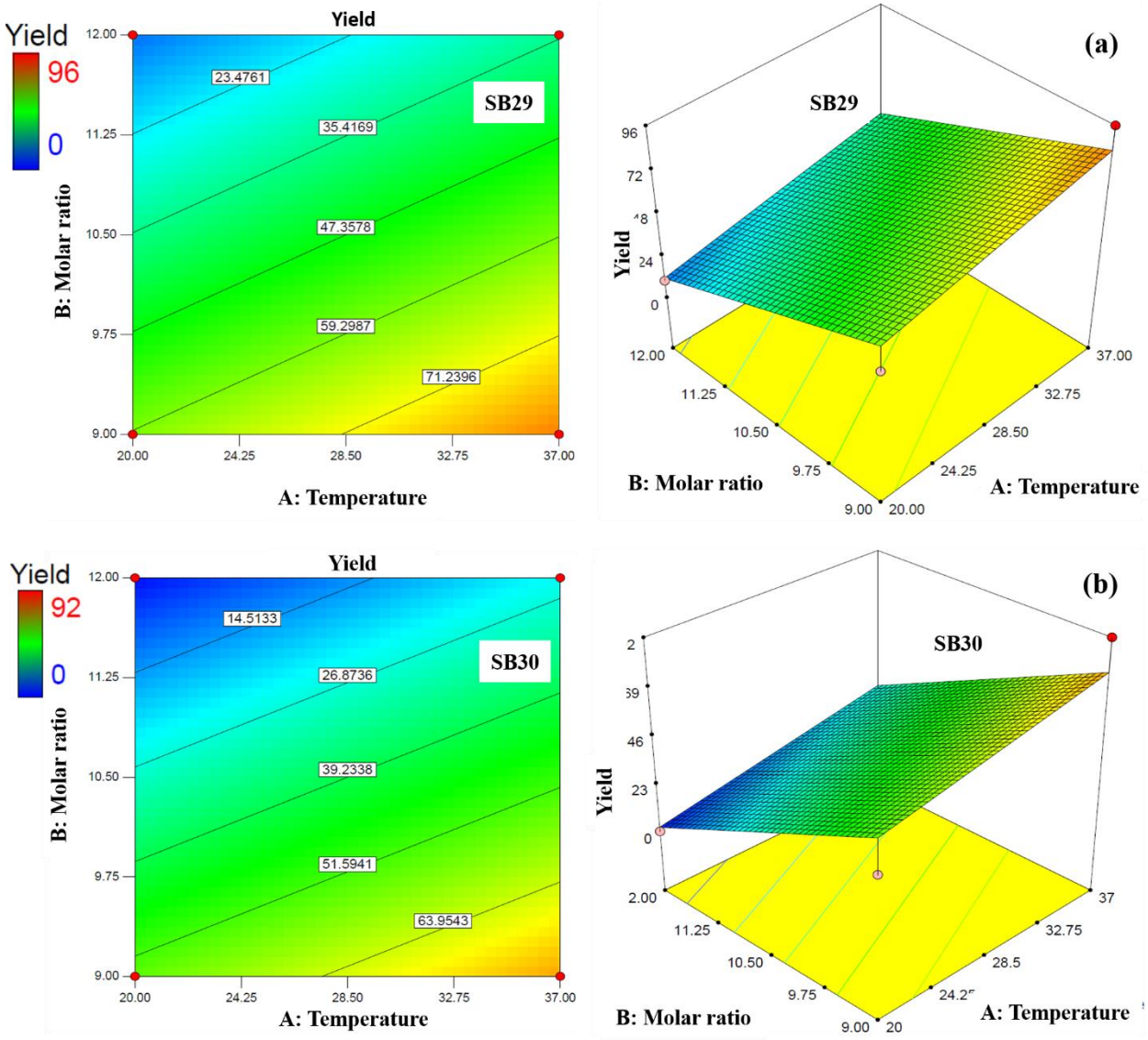

Figure 8.9. Contour and 3D surface plots showing interactions between agitation, molar ratio and temperature for strains *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028. (a) Illustrates interactive plots for *Acinetobacter* SB29 MH715027 strain, (b) Illustrates interactive plots for *Acinetobacter* SB30 MH715028 strain. The colour variation in vertical colour bars represent the biodiesel yield level in contour and 3D surface model plots.

8.4.9. Characterization and fuel properties of *Jatropha curcas* seed oil and biodiesel

The FTIR analysis of *J. curcas* biodiesel was carried out to confirm the fatty acid methyl esters (FAME). The IR spectra in a range 1735-1750 at cm^{-1} represents the biodiesel esters (Krishnamurthy et al., 2018). The infrared spectra of biodiesel samples (Appendix 5 Figure S7-S10) produced by each strain were in 1735-1750 range indicating fatty acids methyl esters (ul ain Rana et al., 2019). Table 8.7 shows the fuel properties of *J. curcas* seed oil and biodiesel. Acid value is a measure of FFA present in oil or biodiesel. The presence of FFA indicate the

incomplete biodiesel production and generally, oil containing FFA contents higher than 1% are not recommended for transesterification. However, lipases can convert both FFA and long chain fatty acids without affecting the quality of biodiesel. Acid value higher than 0.8 mg KOH per gram of fuel had been reported to associate with fuel system deposites and reduce shelf life of fuel pumps and filters (Rabu et al., 2013). In the current study, the acid value of lipase mediated biodiesel produced was inline with the ASTM D 6751-02/ ASTM D 6751-06 standard (Ong et al., 2011). Saponification value of oil indicate its suitability for soap and shampoo prodcution. The peroxide value is an indicater of oxidative stability of a fuel and the peroxide value of *J. curcas* biodiesel is lower (Table 8.7) indicating the oxidative stability of its biodiesel (Akbar et al., 2009). The other fuels proerties of *J. curcas* seed oil and its biodiesel are shown in Table 8.7.

Table 8.7. Physicochemical properties of *Jatropha curcas* seed oil and biodiesel

Property	Unit	*J. curcas* oil	Biodiesel	Biodiesel standard ASTM D 6751-02/ ASTM D 6751-06
Acid value[a]	mg KOH/g	26±1.7	0.44±0.1	0.80 max
FFA[a]	%	13.11±0.9	N.D	-
Saponification value[a]	mg/g	213±5.1	N.D	-
Ester value[a]	mg/g	186.6±3.7	N.D	-
Cloud point[a]	°C	5±1.0	4.1±0.4	−3 to 12
Pour point[a]	°C	4±0.2	3±0.1	−15 to 10
Peroxide value[a]	Meq O_2/kg	N.D	1.41±0.1	-
Boiling point[a]	°C	390±1.5	260±1	>201.85
Specific gravity[a]	kg/m^3	0.916±0.1	0.87±0.02	0.87–0.90
Odour	N.D	Agreeable	Agreeable	-
Colour	N.D	Brown	Light Brown	-
Percent Oil yield	%	32.5	-	-

N.D.: not determined, [a]: values presented as mean ± SD.

8.5. Conclusions

The current study concludes that all of the indegenous strains isolated from oil-contaminated soils had higher potential for biodiesel production. Especailly, *Brevibacterium* SB11 MH715025, *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 showed higher tolerance for methanol toxicity and possess higher potential of efficient biodiesel production at lower temperature, agitation and higher methanol to oil molar ratios. Strain *Pseudomonas* SB15 MH715026 was the most efficient in terms of biodiesel yield at moderate conditions. These moderate conditions for optimum lipase mediated biodiesel production provide an advantage over the conventional chemical biodiesel production which requires higher temperature and agitation resulting in increased energy demand and higher economic cost. Moreover, lipase mediated biodiesel production is also advantageous compared to chemical transesterification in terms of free fatty acids conversion. These optimized parameters required for lipase mediated production could help further in selecting the other variables to enhance the biodiesel yield efficiency of these strains. All of these strains could be used for potential applications in biofuel sector. Furthermore, immobilization of these cells or their enzymes could enhance their biodiesel efficiency, stability to protect them from severe conditions and make them re-usable for further biodiesel production. The genetic modification is also suggested as potential mean to increase their stability and efficiency in terms of biodiesel production.

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CHAPTER 9

CONCLUSIONS

Conclusions

The current study has shown that *Jatropha curcas* plant is an ideal feedstock for biorefinery. The results conclude that the *J. curcas* seed oil is suitable for biodiesel production using chemical (two-step process) or biological (lipase based) means. The Jatropha de-oiled pressed cake can be used as feedstock for bioactive compounds and biogas production. It was interesting that the residues of *J. curcas* pressed cake after the extraction of antimicrobial compounds had higher biogas production and positive effect on evenness/richness and relative abundance of microbial communities during anaerobic digestion. Additionally, the extract had potential to be used as antimicrobial and antioxidant. The methanolic extracts of pressed cake had significant inhibitory effects on the fermentative bacterial communities in batch process and was also confirmed by studying the inhibitory effects of methanolic extract on microbial abundance in continuous anaerobic digestion mode. Moreover, the methanolic extract was also found highly potent individually as well as in combination with commercial antibiotics against clinical and multidrug resistant bacterial strains. In addition, the methanolic extract of *J. curcas* pressed cake can be used as a potent drug against phytopathogenic fungi and as an antioxidant to treat various free radicals. In order to increase the biogas and biomethane yield compared to the calculated yield, the co-digestion of mango peel with de-oiled seed kernel of *J. curcas* is suggested. A C/N ratio of 20:1 is very important for enhanced biogas yield and stability of reactor. This was achieved by a 1:4 ratio of mango peel to de-oiled seed kernel. The operational parameters, organic loading rate and hydraulic retention time also greatly influence the reactor efficiency and stability in terms of biogas yield during anaerobic digestion. The *J. curcas* pressed cake are suggested for an efficient biogas production at hydraulic retention time 20 days. For low loading of reactors, the methanolic residues (Jatropha pressed cake after methanolic extraction) are suggested for a stable and efficient process of biogas production at organic loading rate 1 g VS L^{-1} day^{-1}, hydraulic retention time 20 days. The methanolic residues were easily biodegradable and therefore accumulation of VFAs was observed when OLR was increased. Therefore, at higher organic loading rates, methanolic residues were evaluated for biogas production in two stage continuous anaerobic digesters to ensure efficient and stable biogas production. The seeds of *J. curcas* contains high amount of oil that can be used for production of high quality biodiesel having 97-98% yield using chemical (two-step process) and biological methods (lipase mediated). The oil showed to have high biogas potential but biogas production in continuous reactor was not a feasible option. The properties of the produced biodiesel satisfied the standards required for good quality biodiesel. The indigenous

isolated lipase producing bacterial strains can be used for biodiesel production due to their higher biodiesel production efficiency and tolerance to seed oil and methanol toxicity**.**

The study concludes that whole seed is not considered an ideal feedstock for anaerobic digestion. The utilization of *J. curcas* in a biorefinery concept rather than simply for bioenergy carrier (biogas or biodiesel) production is an ideal solution to increase the economic value of *J. curcas* plant for biofuel pharmaceutical industrial sectors. *J. curcas* seed could be used for production of a number of products including antimicrobials, antioxidants, cytotoxic and bioenergy carriers (biogas and biodiesel). Therefore, using *J. curcas* in a biorefinery context rather than simply for biofuel production is an ideal solution to increase the economic value of *J. curcas* plant for biofuel and pharmaceutical industrial sectors. Moreover, by extracting the antimicrobials, the seeds toxicity is reduced, increasing the efficiency and economic value for biofuel production.

FUTURE PROSPECTS

Future prospects

- The effect of different pretreatments on methanolic residues (seed cake after methanolic extraction) such as ionic solvent, alkaline and enzymatic pretreatments on biogas yield can be studies. The structural and compositional study of cellulose, hemicellulose and lignin of the methanolic residues will be interesting.
- The crude phytochemical compounds obtained from *J. curcas* seed can further be purified using various techniques and can be used for individual as well as synergistic activities in combination with various antibiotics against various pathogenic strains
- Bioactive compounds after purification from *J. curcas* extracts can be used for antioxidant, cytotoxic and enzyme inhibition assay both *in vitro* and *in vivo*
- The mechanism of action of purified compounds obtained from *J. curcas* extracts can further be investigated on various pathogenic microbial cells
- The isolated lipase producing bacterial strains can further be genetically modified to enhance the biodiesel production from *J. curcas* seed oil
- The mechanism of action of long chain fatty acids present in *J. curcas* seed oil can be investigated on microbial communities during anaerobic digestion
- Solid state anaerobic digestion of *J. curcas* seed to evaluate its potential for biogas production
- Codigestion of seed kernel with other substrates (high carbon to nitrogen containing substrates) in continuous mode at different operational parameters to increase the efficiency of the reactor.
- *J. curcas* seed has low carbon to nitrogen ratio and it can be used in codigestion with other high carbon to nitrogen containing substrates to enhance the biogas yield

APPENDICES

Appendix 1: Additional file 1

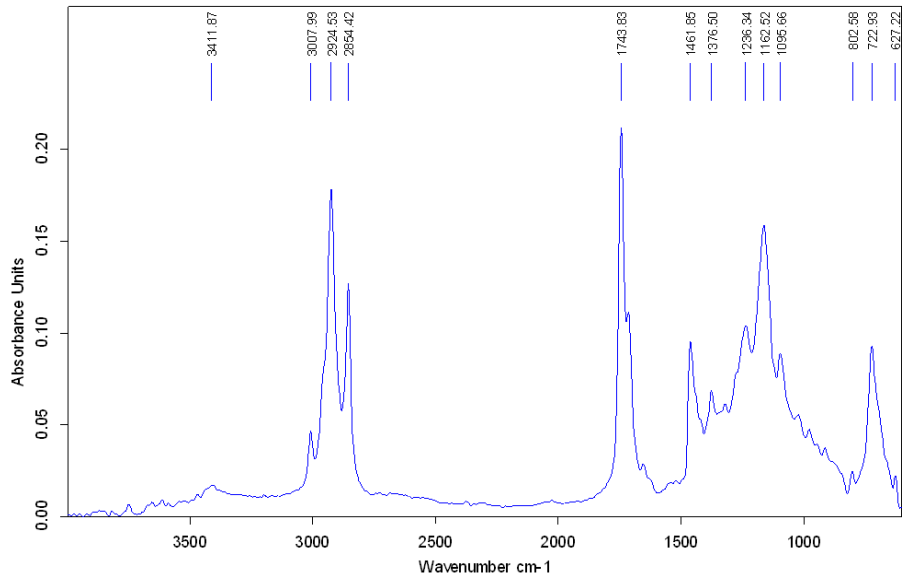


Fig. S1 FTIR absorption spectrum obtained for *J. curcas* seed oil in the range of 4000-400 cm^{-1}.

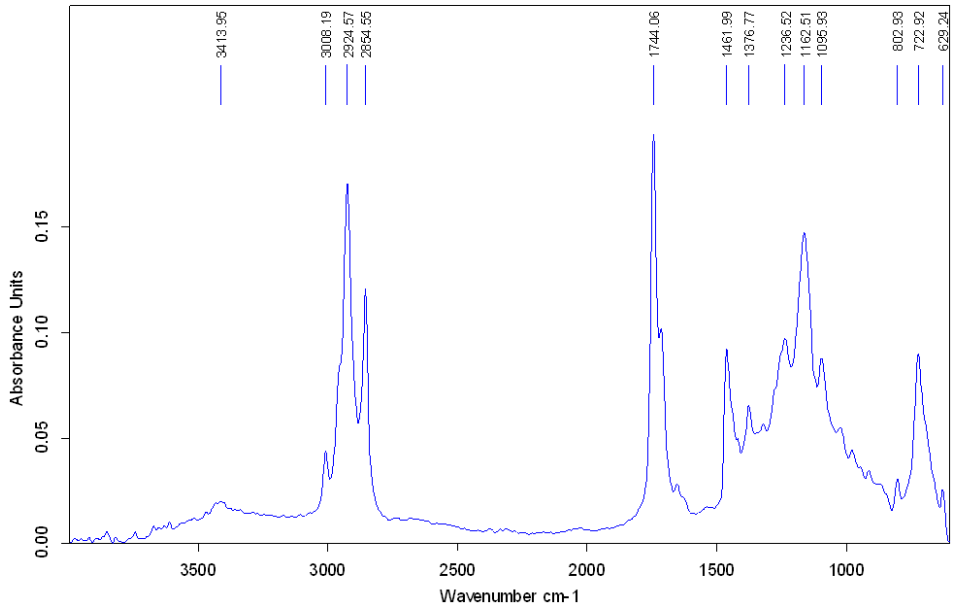


Fig. S2 FTIR absorption spectrum obtained for *n*-hexane extract of *J. curcas* de-oiled seed in the range of 4000-400 cm^{-1}.

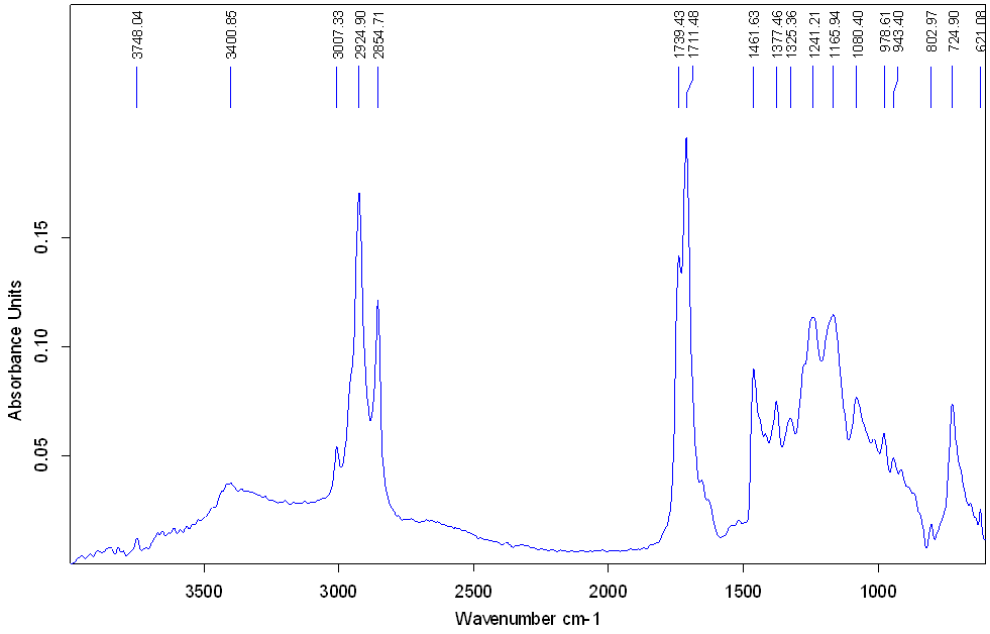


Fig. S3 FTIR absorption spectrum obtained for methanolic extract of *J. curcas* de-oiled seed in the range of 4000-400 cm^{-1}.

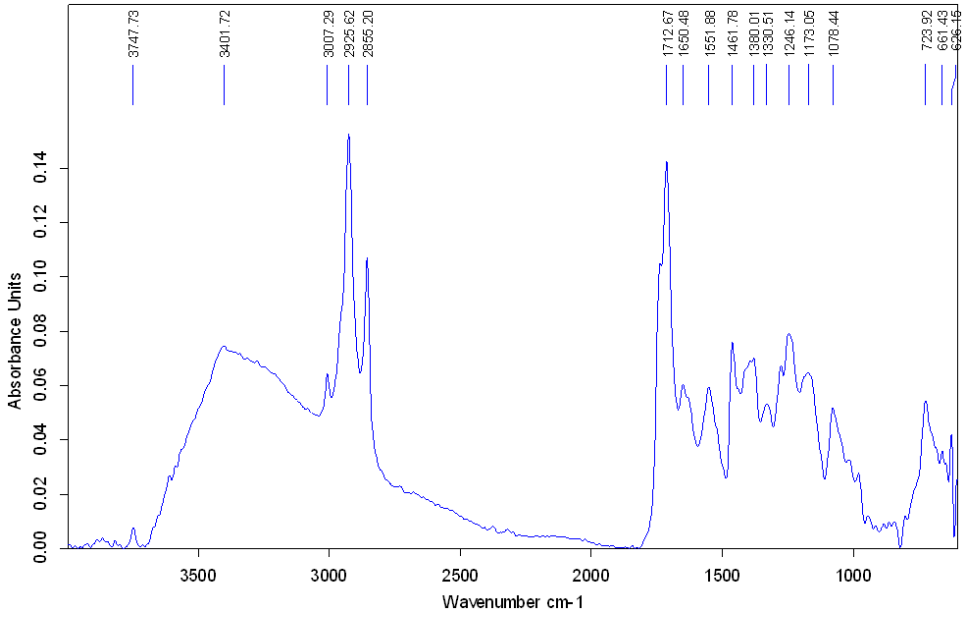


Fig. S4 FTIR absorption spectrum obtained for aqueous extract of *J. curcas* de-oiled seed in the range of 4000-400 cm^{-1}.

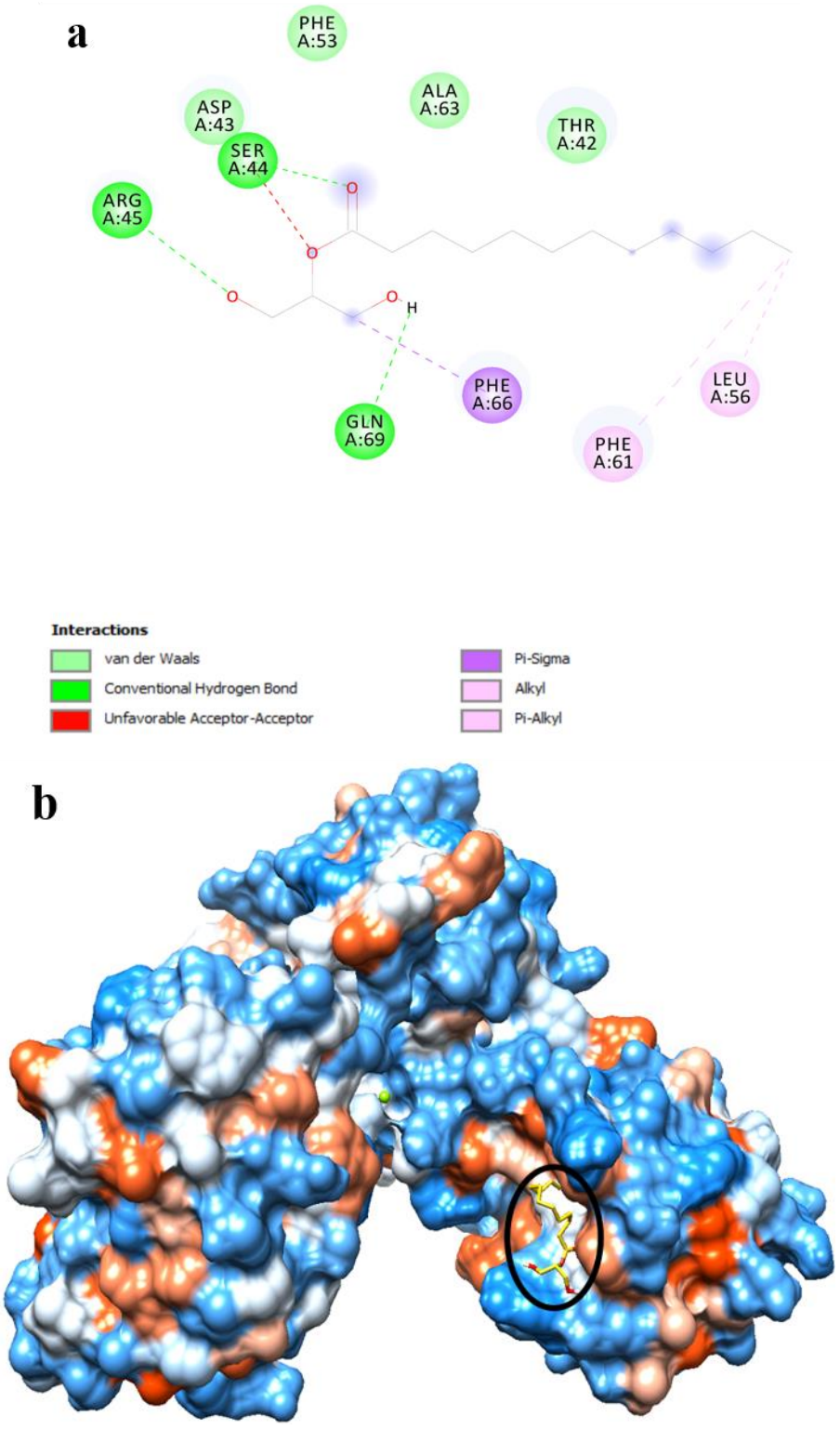


Fig. S5 Molecular docking of beta-monolaurin in MurF active pocket **(a)** Binding interaction of beta-monolaurin in MurF active pocket, **(b)** Binding conformation of beta-monolaurin in MurF active pocket.

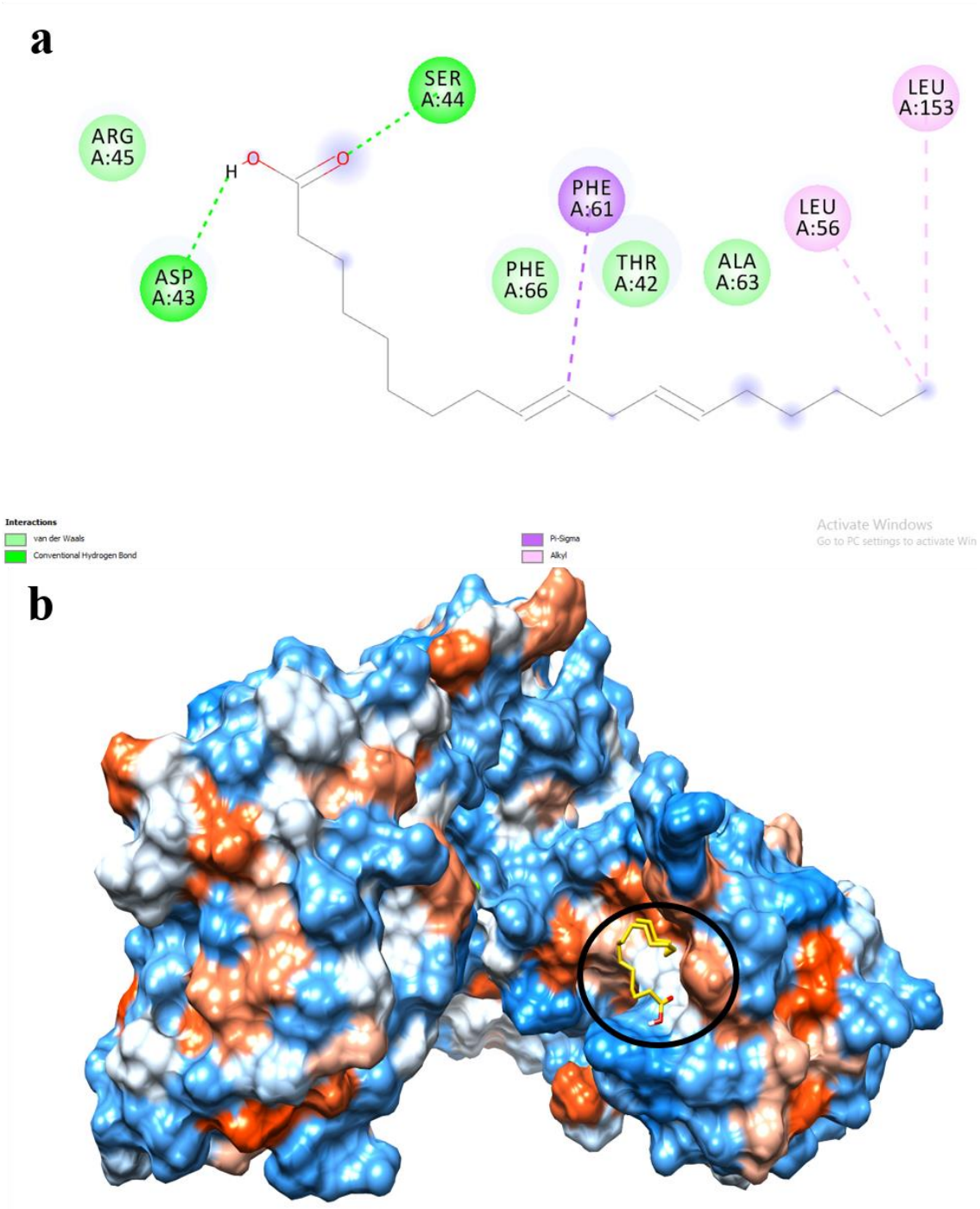


Fig. S6 Molecular docking of 9,12 octadecadienoic acid in MurF active pocket **(a)** Binding interaction of 9,12 octadecadienoic acid in MurF active pocket, **(b)** Binding conformation of 9,12 octadecadienoic acid in MurF active pocket.

Table S1 Antibiotic resistance profiling of MDR strains.

Bacterial strain	**Antibiotics the strain is resistant to**
K. pneumoniae	Amikacin, Fucidic acid, Quinupristin-dalfopristin, Piperacillin-tazobactam, Vancomycin, Ceftriaxone, Oxacillin, Amoxicillin-clavulanate (2:1), Erythromycin, Cefoxitin, Meropenem, Cefazolin, Trimethoprim-sulfamethoxazole,
P. chlororaphis	Cefoxitin, Meropenem, Erythromycin, Nobycin, Amoxicillin-clavulanate (2:1), Cefazolin , Amikacin, Trimethoprim-sulfamethoxazole
P. monteili	Erythromycin, Cefoxitin, Trimethoprim-sulfamethoxazole, Meropenem, Tetracycline, Ampicillin, Nobycin, Tigecycline, Cefazolin, Amoxicillin-clavulanate (2:1)
A. baumannii	Ampicillin, Erythromycin, Trimethoprim-sulfamethoxazole, Amoxicillin-clavulanate (2:1), Ceftriaxone
MRSA1	Tetracyclin, Trimethoprime Sulfamethoxazole, Ciprofloxacin, Gentamicin
MRSA2	Tetracyclin, ciprofloxacin, Gentamicin, Erythromycin, Cefoxitin
MRSA3	Cefoxitin, Ciprofloxacin, Gentamicin
MSSA	Chloramphenicol, Tetracyclin, ciprofloxacin,
MRSA5	Tetracyclin, Cefoxitin, Ciprofloxacin, Clindamycin, Fosfomycin

Table S2 Assignments of the important FTIR absorption bands to corresponding functional groups present in *J. curcas* seed oil

No.	Band positions (cm^{-1})	Inferences of FTIR Spectrum
1	1743 [s]	C=O Ester Stretch
2	3411 [s]	carboxylic acid OH stretch, N-H stretch, alcohol OH stretch
3	3007 [w]	=C-H stretch
4	2924 [w]	-C-H stretch
5	2854 [w]	-C-H stretch
6	1461 [m]	CH_3 , CH_2 stretches
7	1376 [m]	CH_3 stretches
8	1236 [s]	C-O-C stretch
9	1162, 1095 [s]	C-OH stretch

[s]: strong; [w]: weak; [m]: medium

Table S3 FTIR stretches with corresponding functional groups present in n-hexane extract of *J. curcas* seed

No	Band positions (cm^{-1})	Inferences of FTIR Spectrum
1	1744 [s]	C=O Ester Stretch
2	3413 [s]	carboxylic acid OH stretch, N-H stretch, alcohol OH stretch
3	3008 [w]	=C-H stretch
4	2924 [w]	-C-H stretch
5	2854 [w]	-C-H stretch
6	1461 [m]	CH_3 , CH_2 stretches
7	1376 [s]	CH_3 stretches
8	1236 [s]	C-O-C stretch
9	1162, 1096 [s]	C-OH stretch
10	802, 722, 629 [s]	CH out of plane bending (carbohydrate)

[s]: strong; [w]: weak; [m]: medium

Table S4 FTIR stretches with corresponding functional groups present in methanolic extract of *J. curcas* seed

No	Band positions (cm-1)	Inferences of FTIR Spectrum
1	1744 [s]	C=O Ester Stretch
2	3413 [s]	carboxylic acid OH stretch, N-H stretch, alcohol OH stretch
3	3008 [w]	=C-H stretch
4	2924 [w]	-C-H stretch
5	2854 [w]	-C-H stretch
6	1461 [m]	CH_3 , CH_2 stretches
7	1376 [m]	CH_3 stretches
8	1236 [s]	C-O-C stretch
9	1162, 1096 [s]	C-OH stretch
10	802, 724, 621 [s]	CH out of plane bending (carbohydrate)

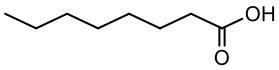
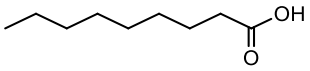
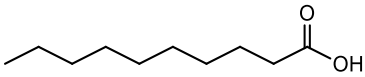
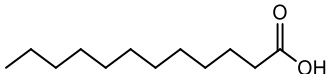
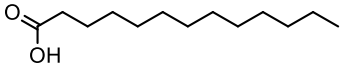
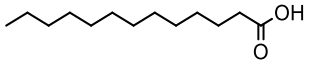
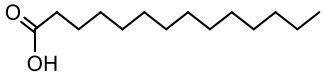
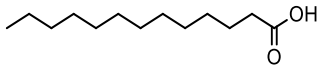
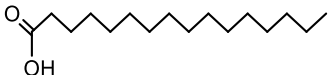
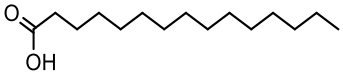
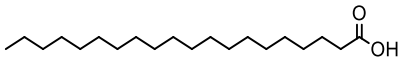
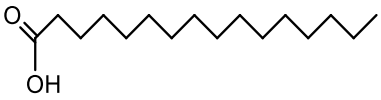
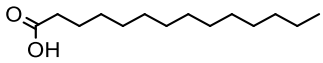
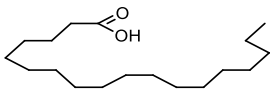
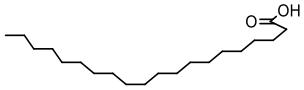
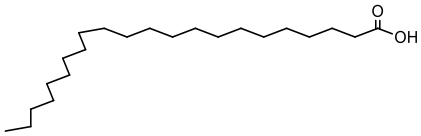
[s]: strong; [w]: weak; [m]: medium

Table S5 FTIR stretches with corresponding functional groups present in aqueous extract of *J. curcas* seed

No	Band positions (cm-1)	Inferences of FTIR Spectrum
1	1712 [s]	C=O ketone Stretch
2	3401 [s]	carboxylic acid OH stretch, N-H stretch, alcohol OH stretch
3	3007 [w]	=C-H stretch
4	2925 [w]	-C-H stretch
5	2855 [w]	-C-H stretch
6	1461 [m]	CH_3 , CH_2 stretches
7	1380 [m, s]	CH_3 stretches, NO_2 stretch
8	1246 [s]	C-O-C stretch
9	1173, 1078 [s]	C-OH stretch
10	723, 661, 626 [s]	CH out of plane bending (carbohydrate)
11	1650 [w]	C=C alkene stretches
12	1551 [s]	C=O amide, C=C aromatic stretches
13	1330 [s]	NO_2 stretch

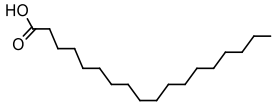
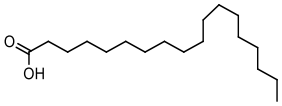
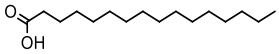
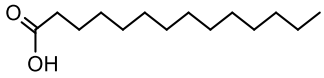
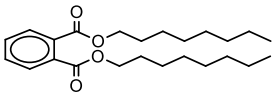
[s]: strong; [w]: weak; [m]: medium

Table S6 Types and structures of fatty acids present in *J. curcas* oil identified by GC-MS analysis

Compound Name	Molecular structure	MW	RT	Area%	Height %
Octanoic acid		144	8.90	0.24	0.77
Nonanoic acid		158	8.90	0.24	0.77
n-Decanoic acid		172	11.95	0.25	1.23
Dodecanoic acid		200	14.63	1.59	5.23
Undecanoic acid		186	14.63	1.59	5.23
Tridecanoic acid		214	14.63	1.59	5.23
Myristic acid		228	16.95	3.54	9.51
Tridecanoic acid		214	16.95	3.54	9.51
n-Hexadecanoic acid		256	19.083	6.62	10.35
Pentadecanoic acid		242	19.083	6.62	10.35
Arachidic acid		312	19.083	6.62	10.35
Palmitic acid		256	19.083	6.62	10.35
Tetradecanoic acid		228	19.083	6.62	10.35
Stearic acid		284	21.15	51.60	24.39
11-Eicosenoic acid		324	22.67	17.08	19.45
Erucic acid		338	24.33	12.58	19.46

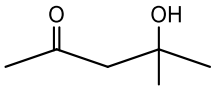
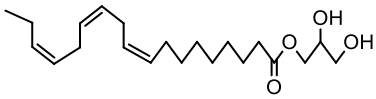
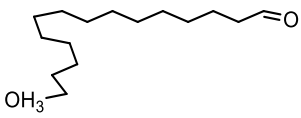
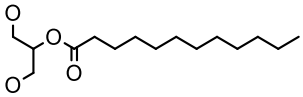
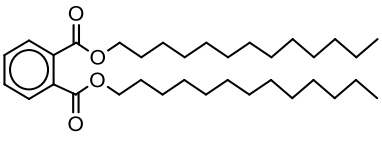
M.W.: molecular weight; RT: Retention time

Table S7 Structures of bioactive compounds present in *J. curcas* n-hexane extract identified by GC-MS analysis

Compound Name	Molecular structure	MW	RT	Area%	Height%
Oleic acid		282	27	5.72	4.24
9,12-Octadecadienoic acid (Z,Z)-		280	25.33	70.61	61.37
Palmitic acid		256	23.54	9.28	14.89
Myristic acid		228	21.47	5.08	9.19
Di-n-octyl phthalate		390	28.75	2.33	4.67

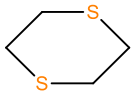
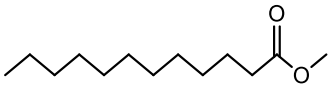
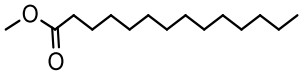
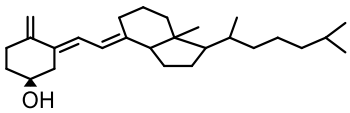
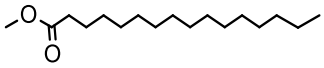
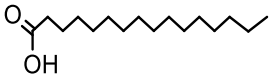
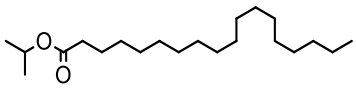
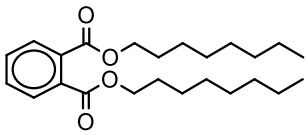
M.W.: molecular weight; RT: Retention time

Table S8 Structures of bioactive compounds present in *J. curcas* methanolic extract identified by GC-MS analysis

Compound Name	Molecular structure	MW	RT	Area%	Height%
Diacetone alcohol		116	4.892	38.42	5.52
I-(+)-Ascorbic acid 2,6-dihexadecanoate		652	23.51	0.19	0.15
9-hexadecenal		238	25.25	4.95	5.06
Beta-Monolaurin		274	28.53	0.27	0.32
bis (tridecyl) phthalate		530	28.75	1.61	4.78
1-docosanol		326	29.35	1.20	1.15

M.W.: molecular weight; RT: Retention time

Table S9 Structures of bioactive compounds present in *J. curcas* aqueous extract identified by GC-MS analysis

Compound Name	Molecular structure	MW	RT	Area%	Height%
1,4-Dithiane		120	14.53	2.95	4.38
Dodecanoic acid methyl ester		214	18.51	0.51	0.50
Methyl Tetradecanoate		242	20.90	1.42	0.19
Vitamin D3		384	21.45	1.89	3.12
Palmitic acid, methyl ester		270	23.03	1.07	3.16
Palmitic acid		256	23.52	2.96	5.11
Isopropyl linoleate		322	24.76		
Di-n-octyl phthalate		390	28.73	1.61	4.79

M.W: molecular weight; RT: Retention time

Table S10 Binding energy of the structures present in *J. curcas* methanolic extract found by using molecular docking

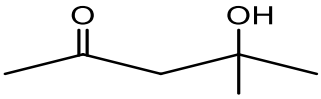
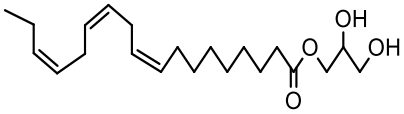
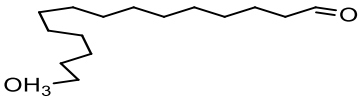
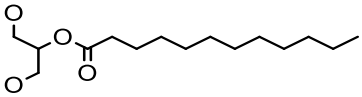
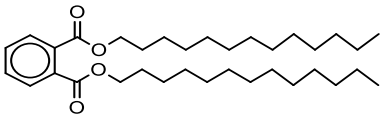
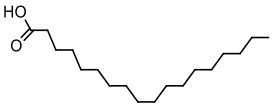
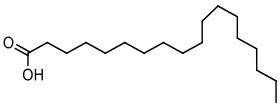
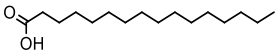
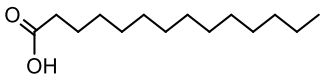
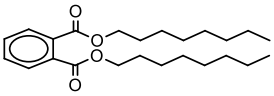
Compound Name	Molecular structure	Binding energy (kcal/mol)
Diacetone alcohol		-4.5
I-(+)-Ascorbic acid 2,6-dihexadecanoate		-2.3
9-hexadecenal		5.5
Beta-Monolaurin		-7.3
Bis-(tridecyl) phthalate		-6.8
1-docosanol		-1.1

Table S11 Binding energy of the structures present in *J. curcas* n-hexane extract found by using molecular docking

Compound Name	Molecular structure	Binding energy (kcal/mol)
Oleic acid		-2.1
9,12-Octadecadienoic acid (Z,Z)-		-6.2
Palmitic acid		-4.1
Myristic acid		-1.2
Di-n-octyl phthalate		-5.1

Appendix 2:

Supplementary file

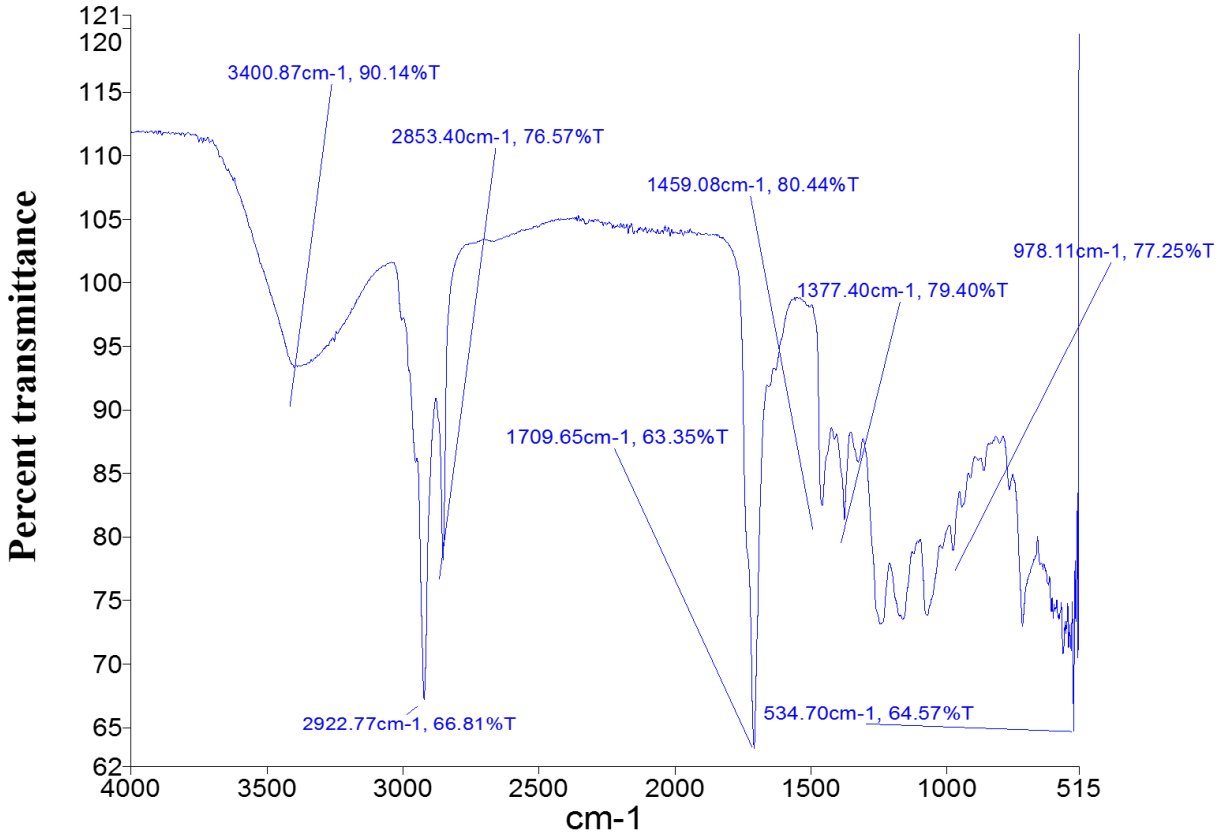


Figure S1. FTIR transmittance of *J. curcas* seed cake methanolic extract in 4000-400 cm^{-1} range.

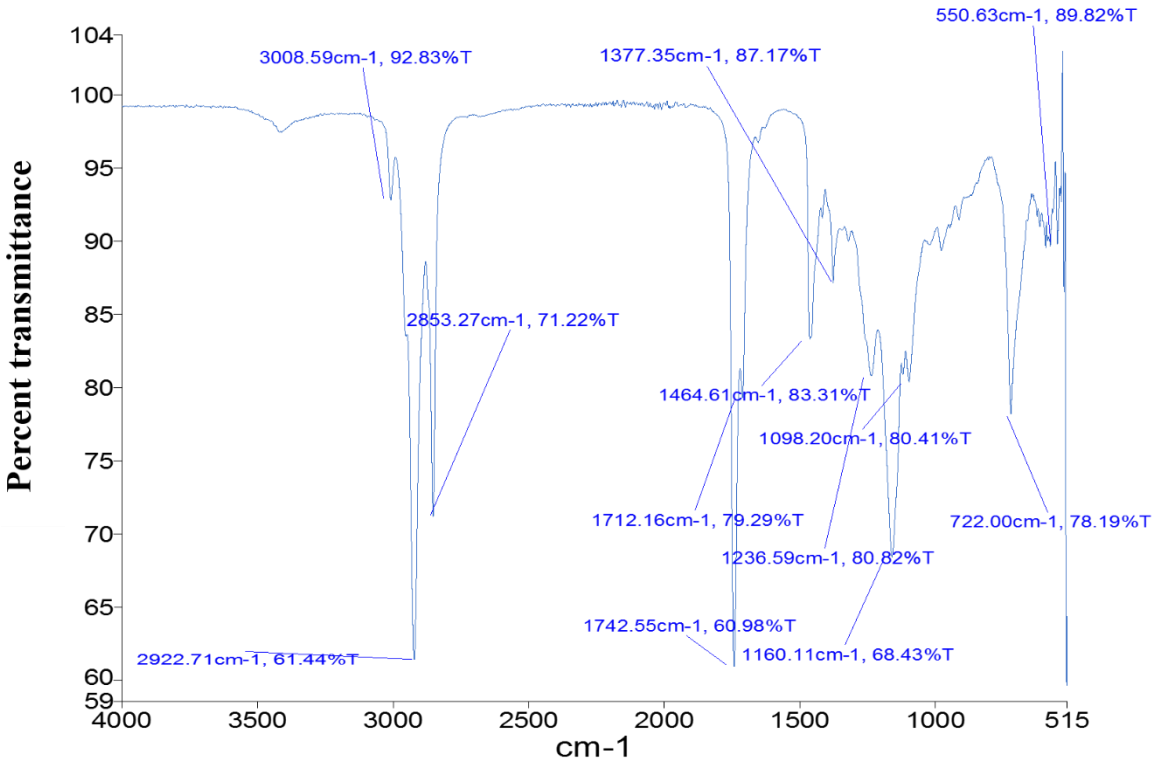


Figure S2. FTIR transmittance of *J. curcas* seed cake *n*-hexane extract in 4000-400 cm^{-1} range.

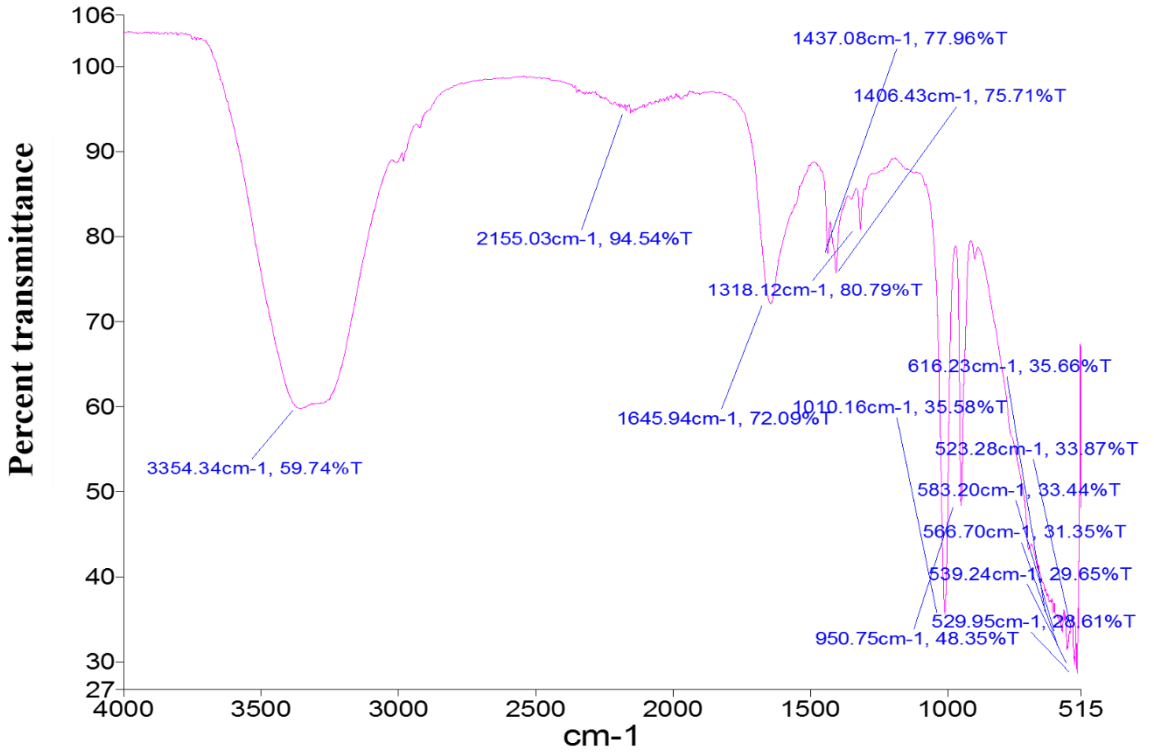


Figure S3. FTIR transmittance of *J. curcas* seed cake aqueous extract in 4000-400 cm^{-1} range.

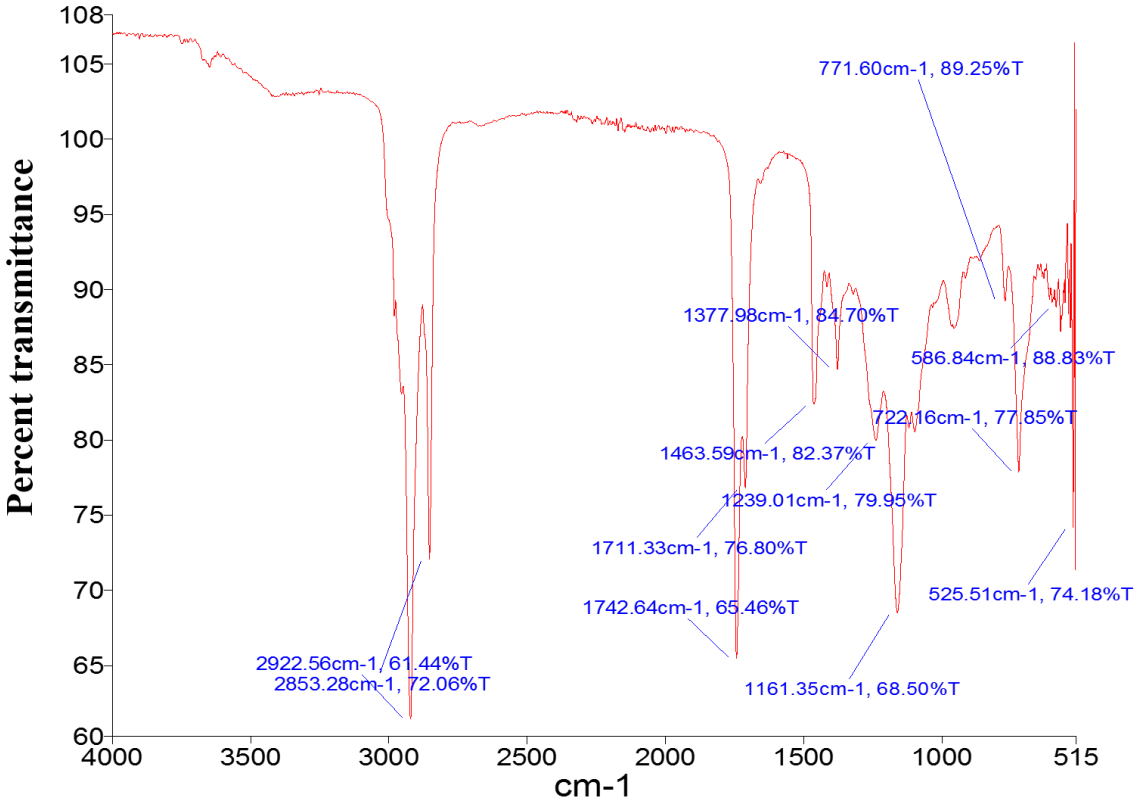


Figure S4. FTIR transmittance of *J. curcas* seed oil in 4000-400 cm^{-1} range.

Appendix 3:

Table. S1. Characteristics of *Jatropha curcas* fruit used in both batch and continuous anaerobic digesters

Biomass	TS%	VS (% of TS)	% VS
Seed kernel	98	95	93
Seed coat	91	68	62
Fruit coat	88	88	78
Cellulose[a]	100	100	100
Inoculum[b]	4.2	70	2.9
Inoculum[c]	4.8	68.4	3.3
Fats (oil)[d]	100	100	100
Mango peels	20.1	95.4	19.2

[a]: Cellulose powder was used as positive control and purchased from Sigma-Aldrich® (St. Louis, MO, USA), [b]: (Inoculum for batch process), [c]: (Inoculum for continuous process) and [d]: Cooking oil was used as positive control.

Appendix 4:

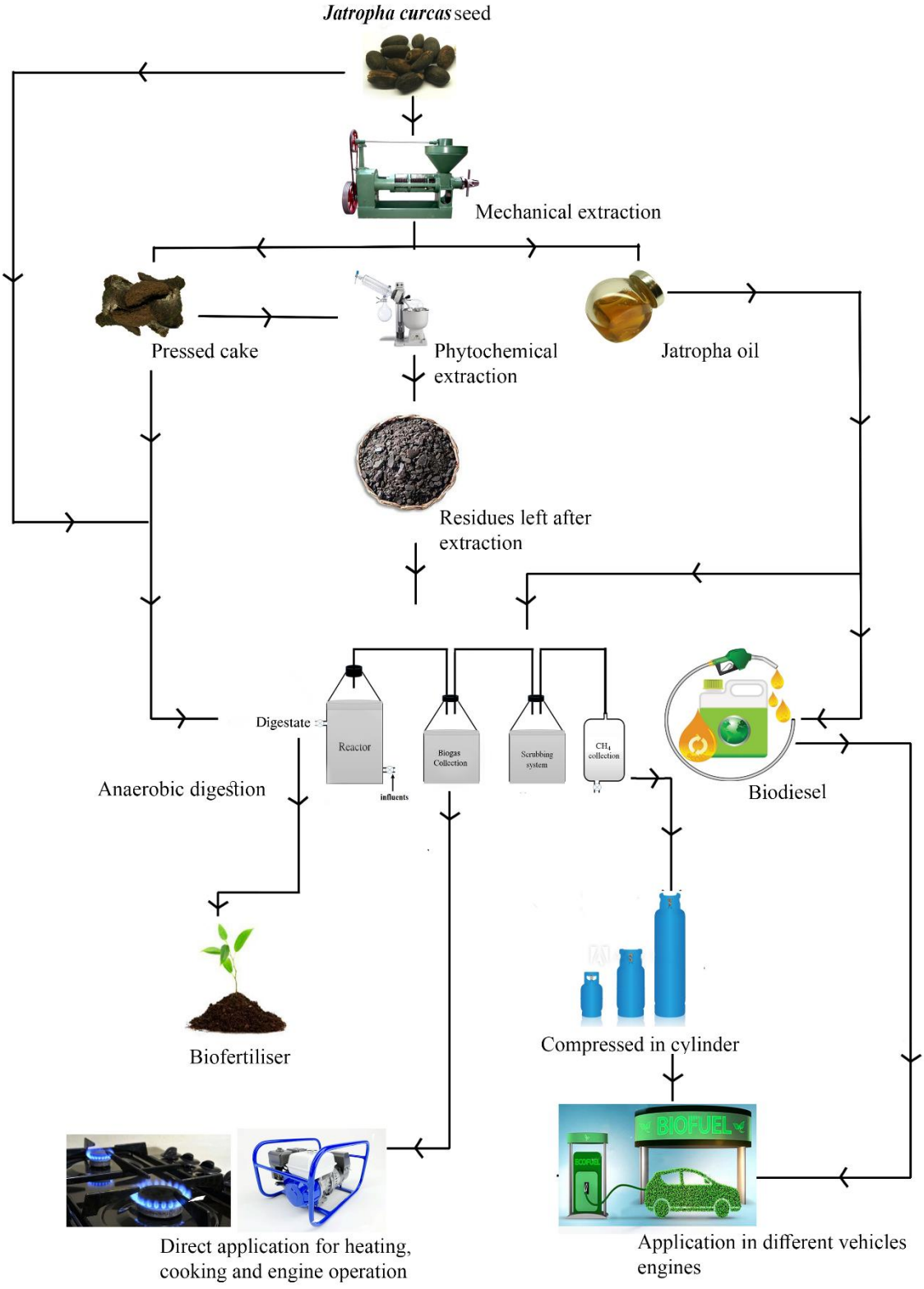


Figure S1. Sustainable utilization of *Jatropha curcas* seed for bioenergy and value added compounds in biorefinery context

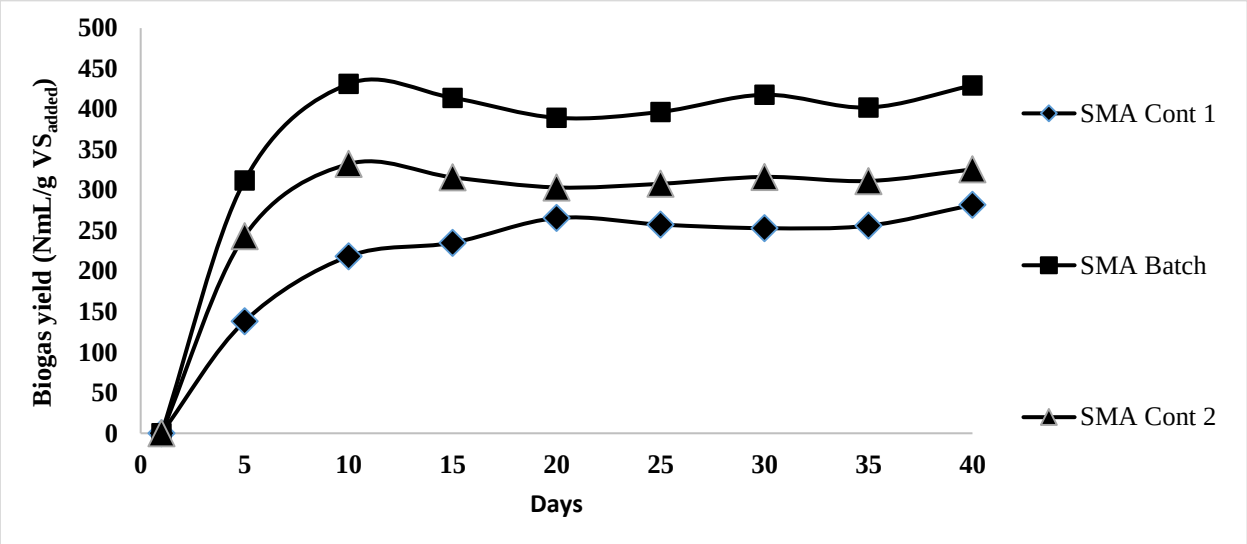


Figure S2. Specific methanogenic activities of inocula used in batch and continuous anaerobic digestion modes.

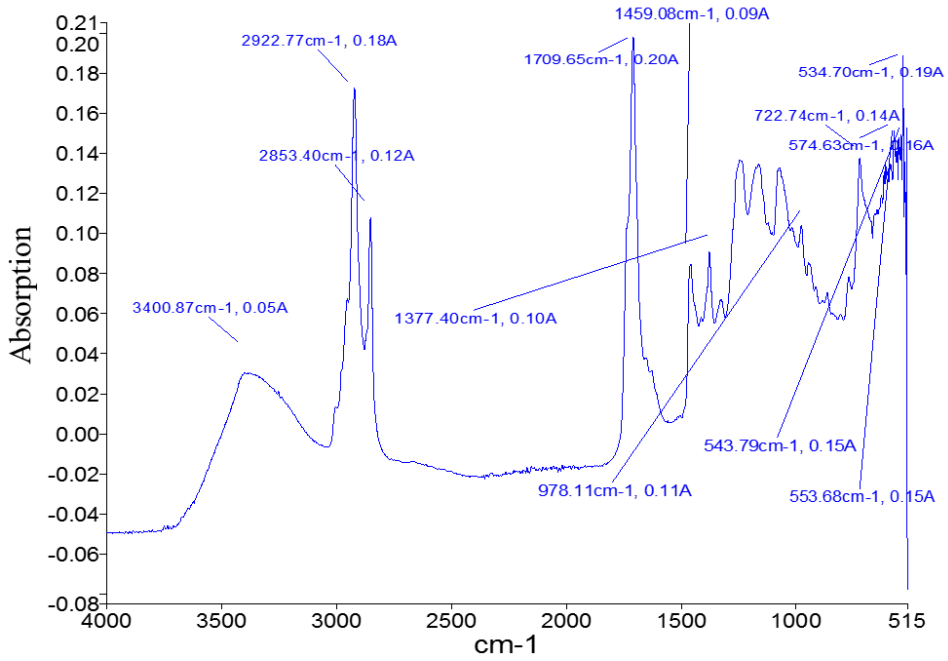


Figure S3. FTIR absorption spectrum of methanolic extract ranging from 4000-400 cm^{-1}.

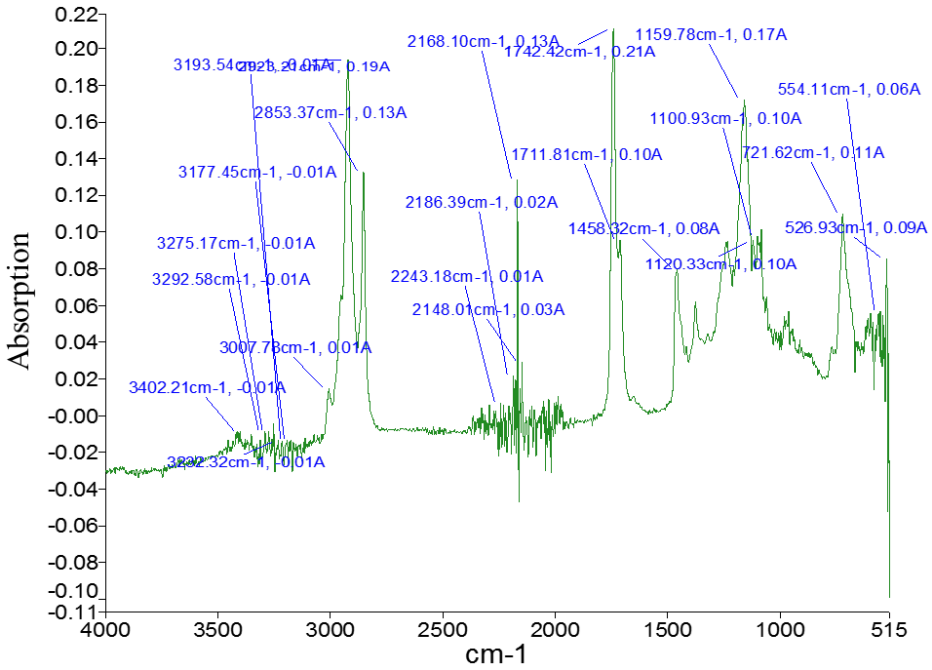


Figure S4. FTIR absorption spectrum of n-hexane extract ranging from 4000-400 cm^{-1}.

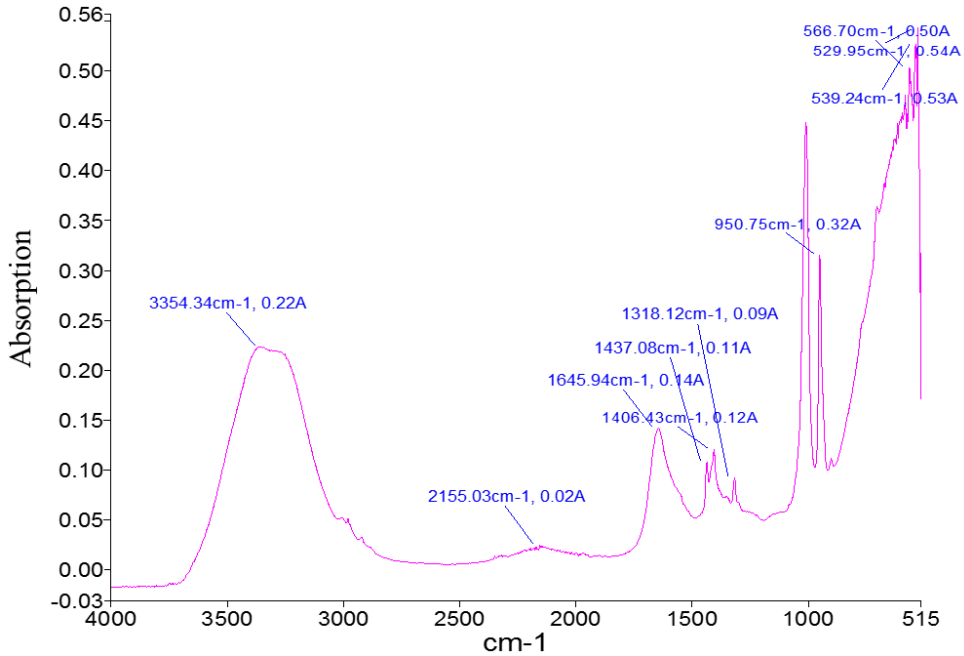


Figure S5. FTIR absorption spectrum of aqueous extract ranging from 4000-400 cm^{-1}.

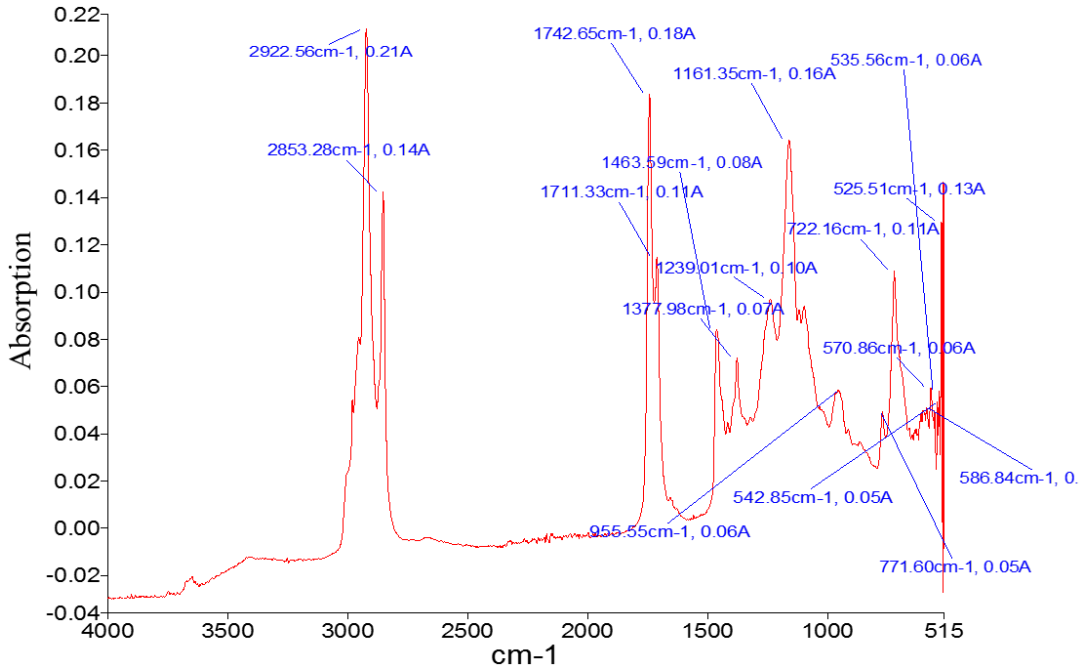


Figure S6. FTIR absorption spectrum of *J. curcas* oil ranging from 4000-400 cm^{-1}.

Table S1. Solids content analysis of reactor components

Biomass	Total Solids %	Volatile Solids %
Jatropha whole seed	91.1	79.2
Jatropha pressed cake	82.2	68.9
Jatropha oil	93.6	93.0
Methanolic residues	82.5	63.5
Aqueous residues	27.6	24.7
n-hexane residues	87.2	65.6
Cellulose	94.3	94.2
Fats*	99.9	99.9
Sodium acetate	60.8	21.9
Glucose	84.38	78.6
$Inoculum^{B1}$	2.7	2.3
$Inoculum^{B2}$	4.2	2.92
$Inoculum^{C1}$	4.8	3.28
$Inoculum^{C2}$	4.6	3.23

*: Cooking oil was used as fats control, B1: inoculum used in biogas potential experiment, B2: inoculum used for the effects of methanolic extracts on different steps in anaerobic digestion, C1: inoculum used in anaerobic

digester treating *Jatropha* whole seed, oil and pressed cake in continuous mode, C2: inoculum used in anaerobic digester treating methanolic residues in continuous mode.

Table S2. GC-MS analysis of *J. curcas* de-oiled seed aqueous extracts

Phytochemicals	Molecular Formula	Molecular weight	Retention time
1,4-Dithiane	$C_4H_8S_2$	120	14.53
Dodecanoic acid methyl ester	$C_{13}H_{26}O_2$	214	18.51
Methyl Tetradecanoate	$C_{15}H_{30}O_2$	242	20.90
Vitamin D3	$C_{24}H_{44}O$	384	21.45
Palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270	23.03
Palmitic acid	$C_{16}H_{32}O_2$	256	23.52
Isopropyl linoleate	$C_{21}H_{38}O_2$	322	24.76
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390	28.73

Table S3. GC-MS analysis of *J. curcas* de-oiled seed methanolic extracts

Phytochemicals	Molecular Formula	Molecular Weight	Retention Time
Diacetone alcohol	$C_6H_{12}O_2$	116	4.892
I-(+)-Ascorbic acid 2,6-	$C_{38}H_{68}O_8$	652	23.51
9-hexadecenal	$C_{16}H_{30}O$	238	25.25
Beta-Monolaurin	$C_{15}H_{30}O_4$	274	28.53
bis (tridecyl) phthalate	$C_{34}H_{58}O_4$	530	28.75
1-docosanol	$C_{22}H_{46}O$	326	29.35

Table S4. GC-MS analysis of *J. curcas* de-oiled seed *n*-hexane extracts

Phytochemicals	Molecular Formula	Molecular Weight	Retention Time
Oleic acid	$C_{18}H_{34}O_2$	282	27
9,12-Octadecadienoic	$C_{18}H_{32}O_2$	280	25.33
Palmitic acid	$C_6H_{32}O_2$	256	23.54
Myristic acid	$C_{14}H_{28}O_2$	228	21.47
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390	28.75

Table S5. GC-MS analysis of *J. curcas* seed oil

Phytochemicals	Molecular Formula	Molecular Weight	Retention Time
Octanoic acid	$C_6H_{16}O_2$	144	8.90
Nonanoic acid	$C_9H_{18}O_2$	158	8.90
n-Decanoic acid	$C_{10}H_{20}O_2$	172	11.95
Dodecanoic acid	$C_{12}H_{24}O_2$	200	14.63
Undecanoic acid	$C_{11}H_{22}O_2$	186	14.63
Tridecanoic acid	$C_{13}H_{26}O_2$	214	14.63
Myristic acid	$C_{14}H_{28}O_2$	228	16.95
Tridecanoic acid	$C_{13}H_{26}O_2$	214	16.95
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	19.083
Pentadecanoic acid	$C_{15}H_{30}O_2$	242	19.083
Arachidic acid	$C_{20}H_{40}O_2$	312	19.083
Palmitic acid	$C_{16}H_{32}O_2$	256	19.083
Tetradecanoic acid	$C_{14}H_{28}O_2$	228	19.083
Stearic acid	$C_{18}H_{36}O_2$	284	21.15
11-Eicosenoic acid	$C_{21}H_{40}O_2$	324	22.67
Erucic acid	$C_{22}H_{42}O_2$	338	24.33

Table S6: Volatile fatty acids and alkalinities different operational parameters during anaerobic digestion of *Jatropha curcas* whole seed, oil, pressed cake and methanolic residues in continuous mode

Substrate	OLR (g VS L^{-1} day^{-1})	HRT (days)	VFAs range	Alkalinity range	VFA/Alkalinity ratio range	pH range	% VS reduced
Methanolic residues (MR)	1	20	500-1250	5000-6500	0.07-0.3	7.05-7.69	70
	1.5	20	950-1200	2000-2800	0.37-0.48	6.64-6.98	53
	2	20	3100-4400	6500	0.47-0.67	6.89-7.01	48
	3	20	5500-15000	4500-11500	0.8-1.3	6.41-7.08	39
	3*	10	(2100-2850)^{R1}/ (1250-200)^{R2}	(2500-3500)^{R1}/ (4500-6400)^{R2}	(0.5-1.2)^{R1}/ (0.2-0.4)^{R2}	(6.6-7.5)^{R1}/ (7.4-7.7)^{R2}	56
	4*	10	(2500-4600)^{R1}/ (1200-3250)^{R2}	(3700-5500)^{R1}/ (3850-6400)^{R2}	(0.4-1.2)^{R1}/ (0.2-0.5)^{R2}	(5.4-6.6)^{R1}/ (7.4-7.7)^{R2}	50
	5*	10	(5500-6900)^{R1}/ (2200-4250)^{R2}	(3200-5200)^{R1}/ (7100-9600)^{R2}	(1.3-1.7)^{R1}/ (0.3-0.6)^{R2}	(5.4-5.7)^{R1}/ (7.5-7.9)^{R2}	42
	6*	10	(4500-4950)^{R1}/ (3100-4450)^{R2}	(2950-3500)^{R1}/ (6400-8250)^{R2}	(1.3-1.6)^{R1}/ (0.3-0.6)^{R2}	(5.5-5.9)$^{R1/}$ (7.4-7.9)^{R2}	33
	7*	10	(7200-10000)^{R1}/ (3400-9750)^{R2}	(3100-5000)^{R1}/ (5300-14400)^{R2}	(2-2.4)^{R1}/ (0.3-1.8)^{R2}	(5.1-5.8)^{R1}/ (7.0-7.1)^{R2}	26
	1	15	750-1500	4750-6000	0.1-0.3	7.01-8.27	47
	1	10	700-3750	3550-5600	0.13-1.05	6.96-7.98	37
Jatropha pressed cake (JPC)	1	20	350-1650	1800-3400	0.13-0.9	6.46-7.77	65
	1.5	20	700-1200	1500-2500	0.36-0.5	7.40-7.77	68
	2	20	800-1000	1900-2500	0.32-0.5	7.11-7.50	60
	3	20	1100-1500	2100-6500	0.17-0.5	7.41-7.8	58
	4	20	3500-6500	5000-10000	0.4-0.8	6.85-7.04	55
	5	20	5000-5850	8500-10000	0.5-0.68	6.86-7.04	48
	6	20	10000-15000	4500-9500	1.05-2.7	6.01-6.99	43
	1.5	15	700-1000	1800-2100	0.36-0.5	6.63-7.22	63
	1.5	10	400-850	1500-2750	0.2-0.6	6.96-7.49	60
Jatropha whole seed (JWS)	1	20	250-2000	1650-3700	0.09-1.2	7.01-7.64	58
	1.5	20	200-850	1250-2750	0.16-.56	6.72-7.3	60
	2	20	650-3800	1800-5300	0.4-0.84	5.95-7.10	54
	3	20	5000-7000	500-750	8.6-13	5.30-6.32	49
	1.5	15	500-700	2000-2300	0.3-0.31	6.68-7.35	59
	1.5	10	250-2000	3300-6400	0.25-0.6	5.3-7.17	44
Jatropha oil (JO)	1	20	50-800	250-3345	0.09-1.6	6.26-7.17	-
	1	15	800-1900	1200-1700	0.6-1.17	6.23-7.11	-
	1	10	350-850	350-1500	0.5-1.88	6.52-7.12	-

^{R1}: Reactor R_1 in two stage anaerobic digestion, ^{R2}: Reactor R_2 in two stage anaerobic digestion,

Appendix 5:

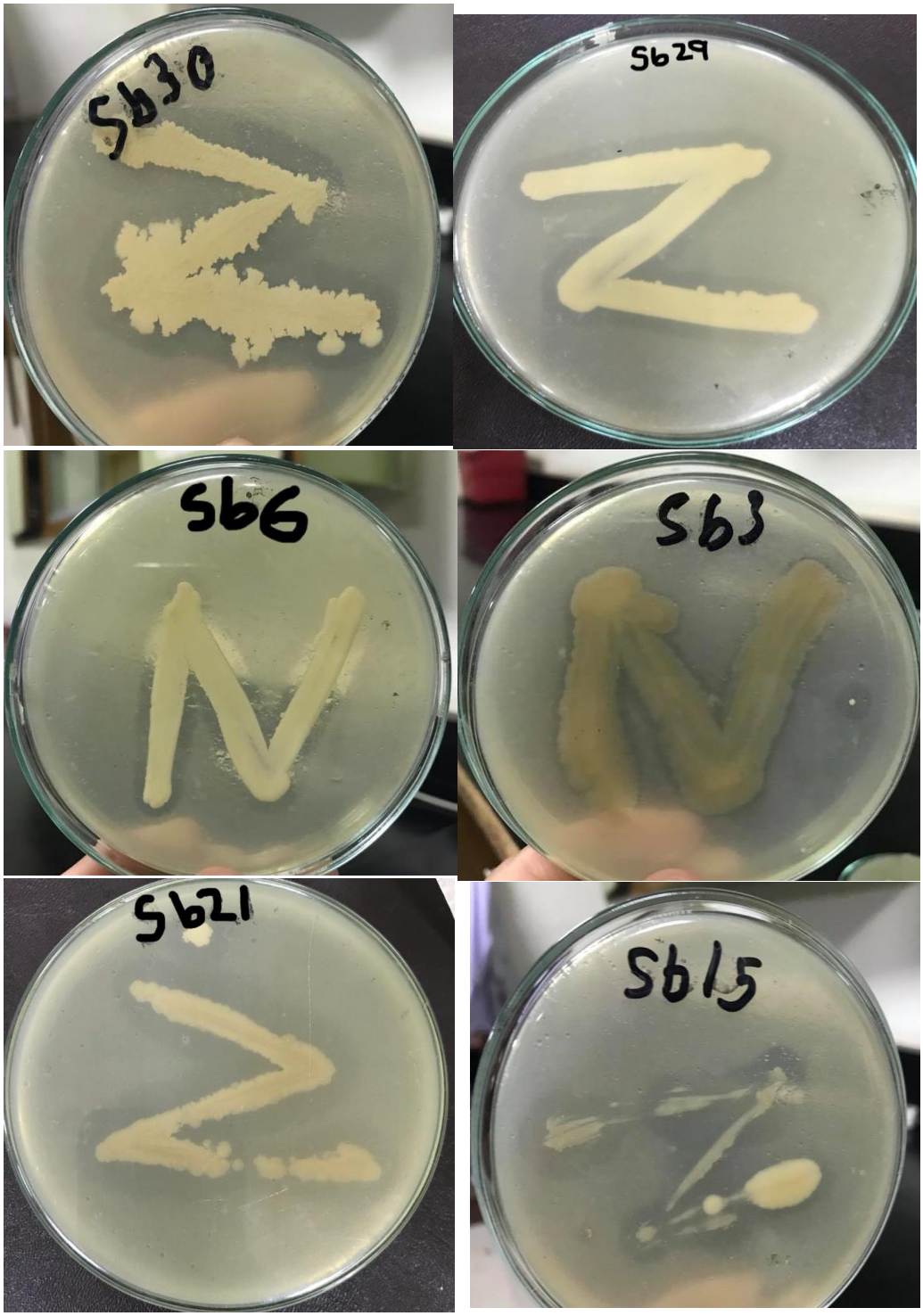


Figure S1. Hollow zones on tributyrine agar plates

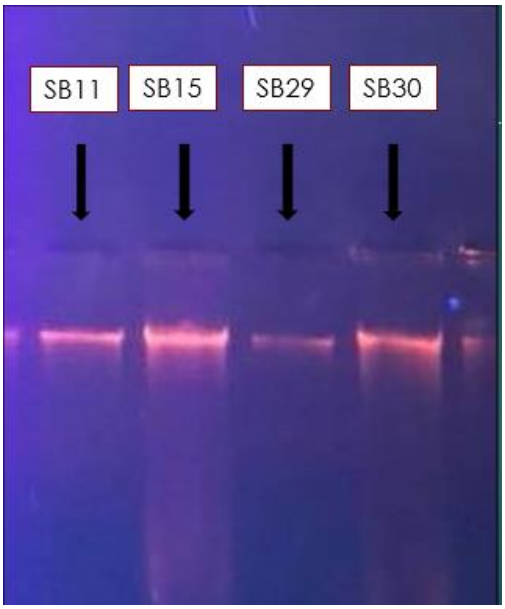


Figure S2. DNA bands of selected strains for biodiesel optimization on agarose gel.

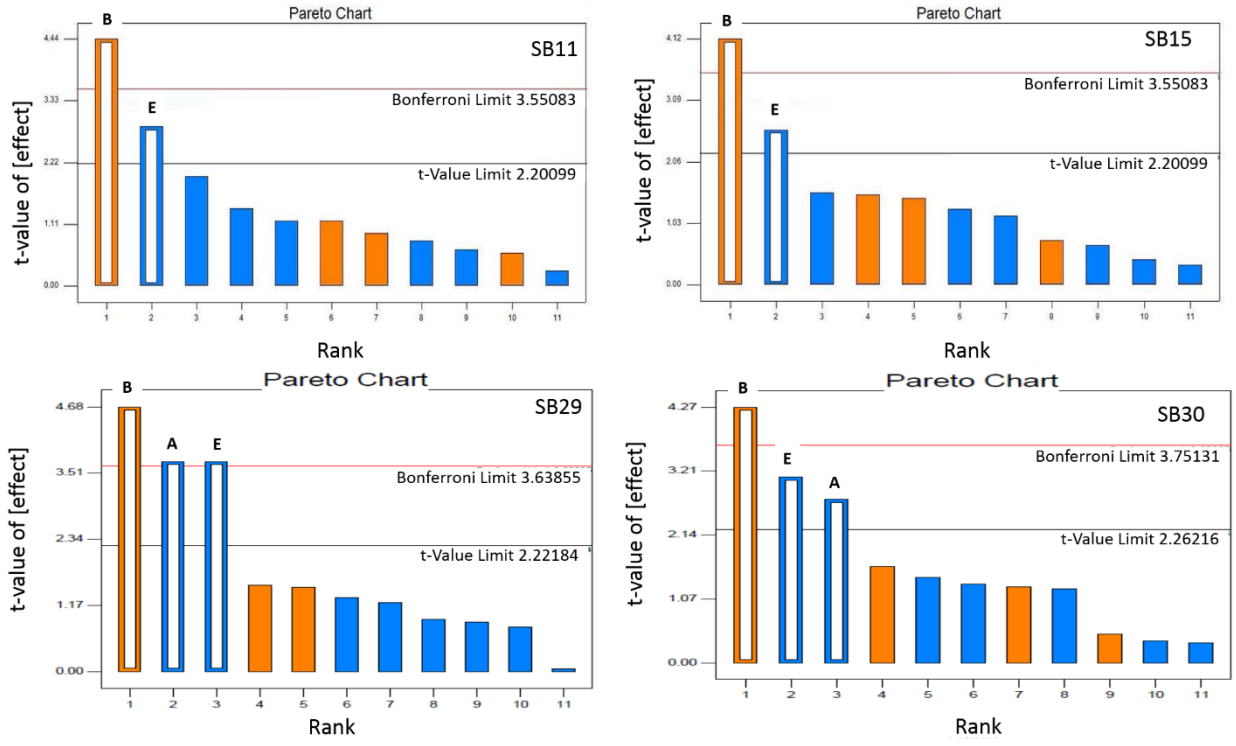


Figure S3. Representation of significant parameters for each selected strain via pareto chart, where A corresponds to temperature, B to molar ratio and E to agitation.

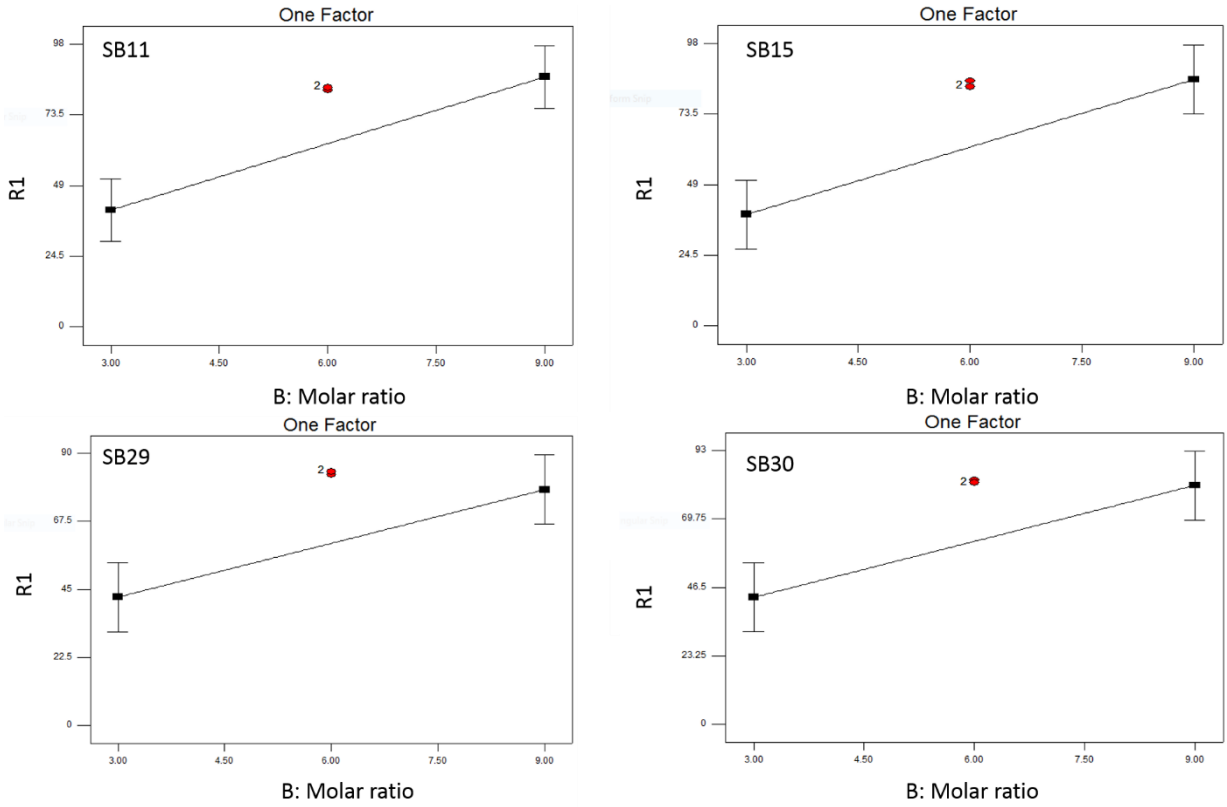


Figure S4. Response of volumetric biodiesel yield of *Brevibacterium* SB11 MH715025, *Pseudomonas* SB15 MH715026, *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 strains to molar ratio (significant factor). **R1** represents response of the respective strains biodiesel yield to molar ratio.

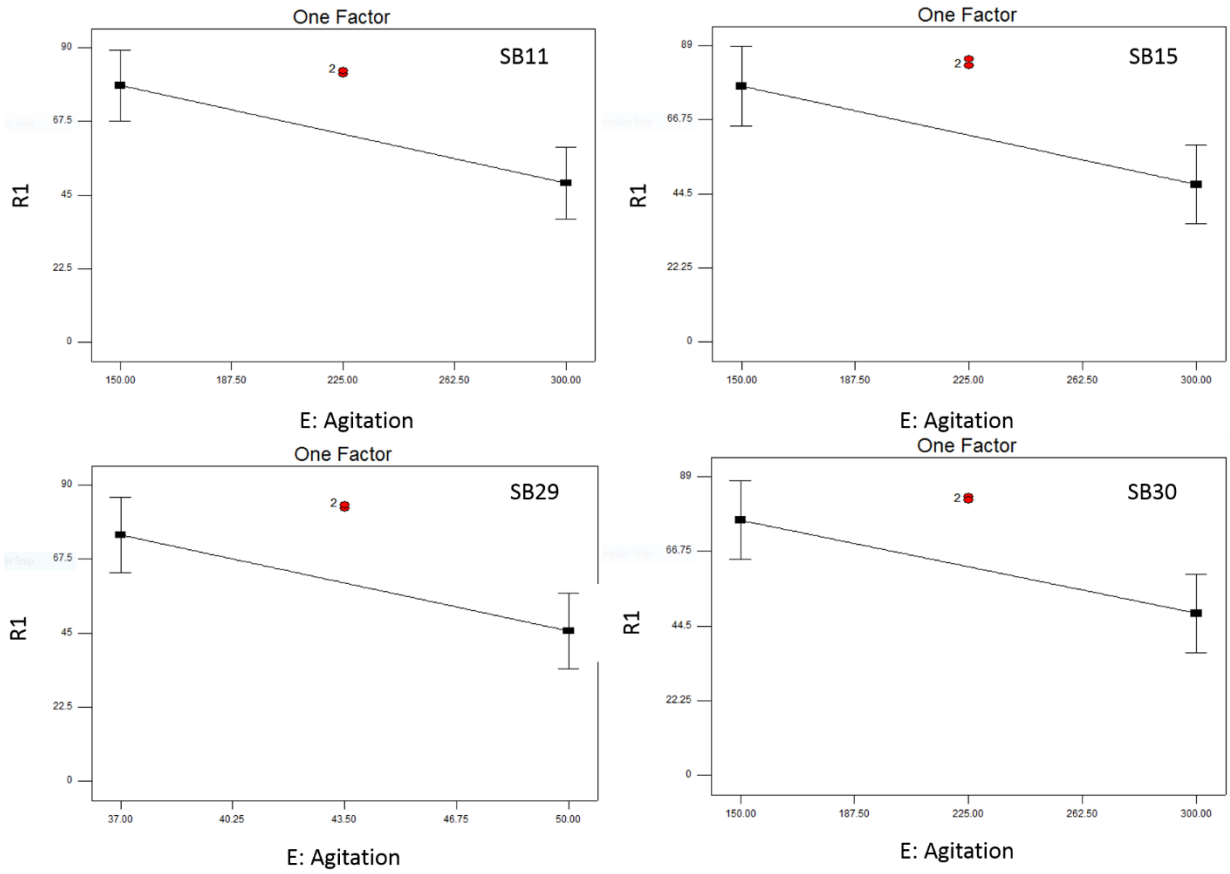


Figure S5. Volumetric biodiesel yield of each strain in response to agitation (significant factor). R1 represents biodiesel response of the respective strains to agitation.

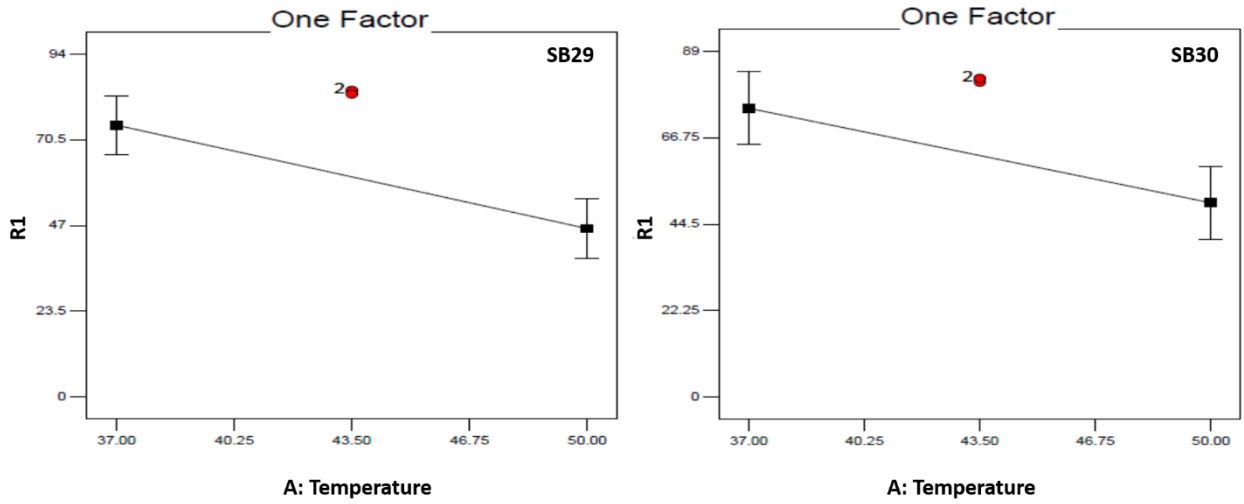


Figure S6. Volumetric biodiesel yield of *Acinetobacter* SB29 MH715027 and *Acinetobacter* SB30 MH715028 in response to temperature (significant factor). R1 represents biodiesel yield response of the respective strains to temperature.

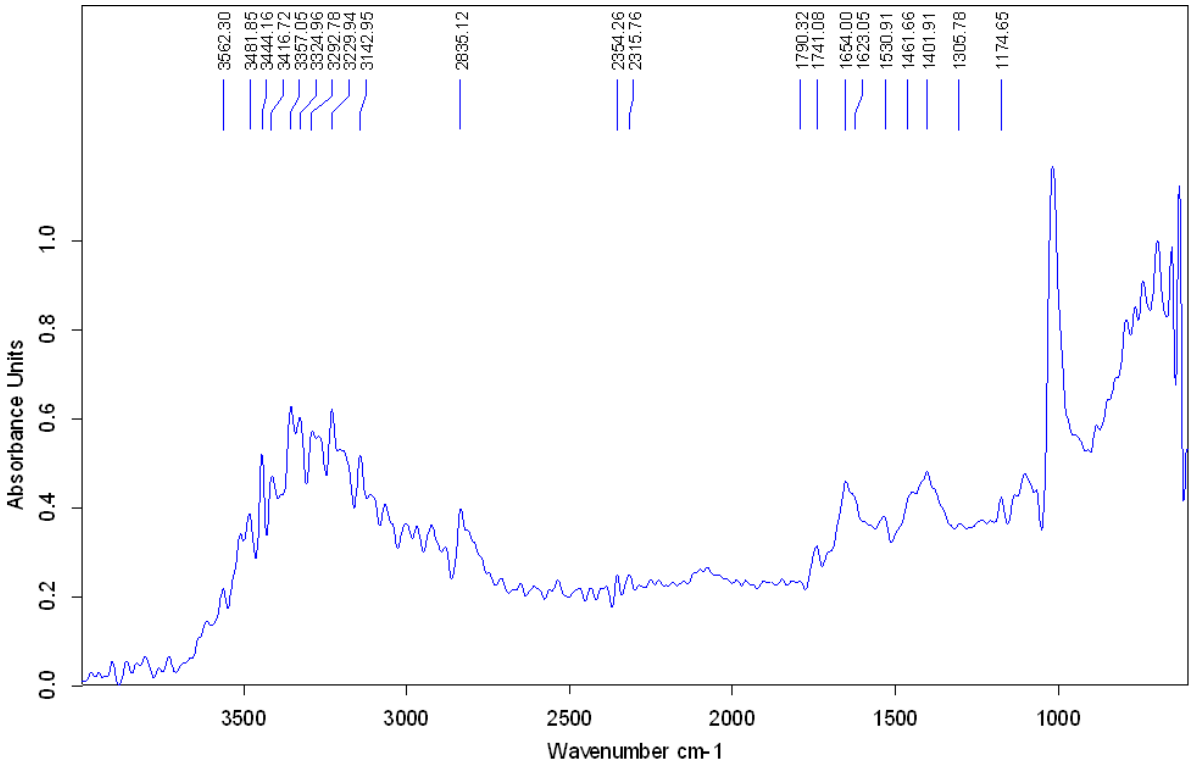


Figure S7. FTIR absorption spectrum of biodiesel produced by strain *Brevibacterium* SB11 MH715025 from *Jatropha curcas* oil.

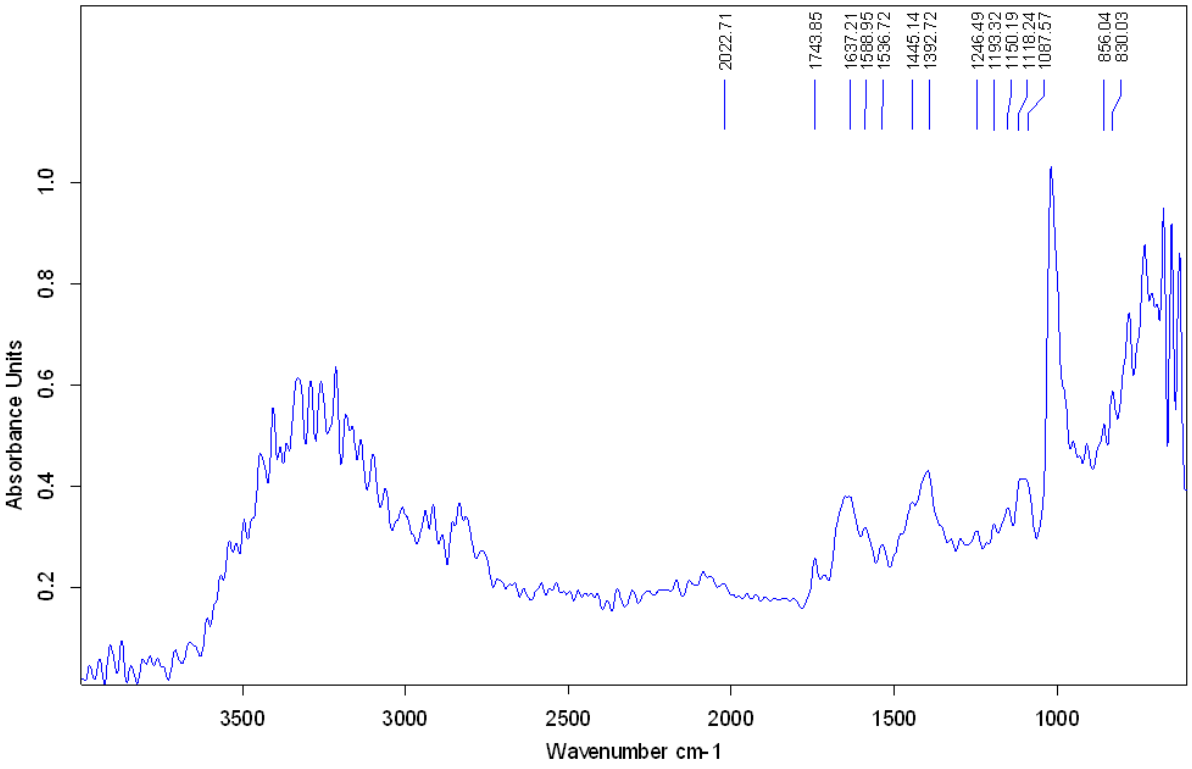


Figure S8. FTIR absorption spectrum of biodiesel produced by strain *Pseudomonas* SB15 MH715026 from *Jatropha curcas* oil.

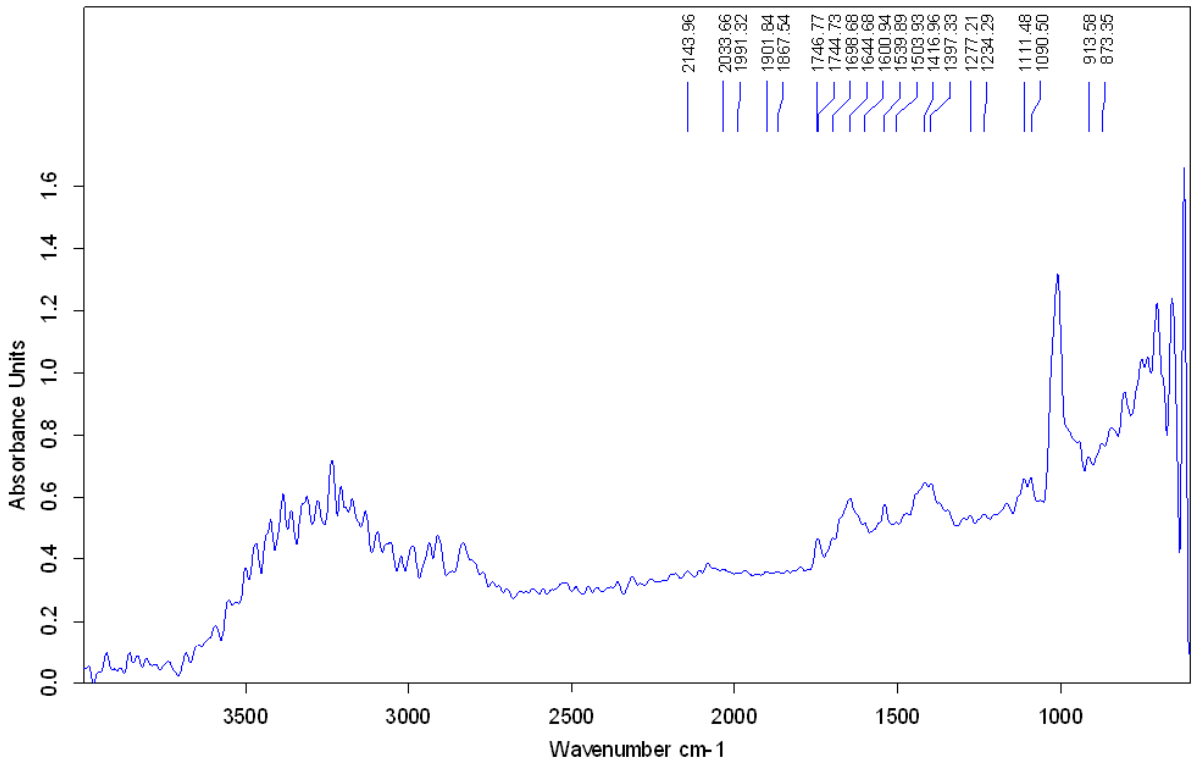


Figure S9. FTIR absorption spectrum of biodiesel produced by strain *Acinetobacter* SB29 MH715027 from *Jatropha curcas* oil.

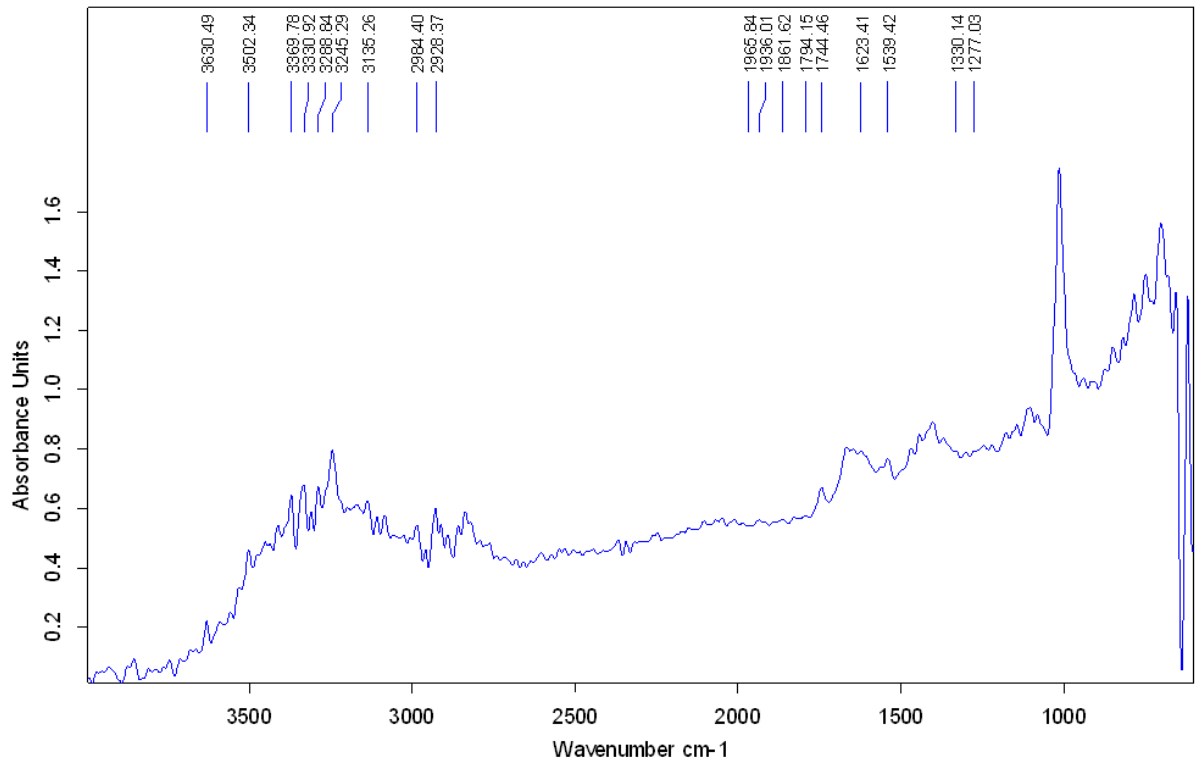


Figure S10. FTIR absorption spectrum of biodiesel produced by strain *Acinetobacter* SB30 MH715028 from *Jatropha curcas* oil.

Table S1. Final equations for significant coded and actual factors required by selected four strains during biodiesel optimization process using Plackett-Burman design

Coded factor equations	Actual factor equations	Strain
Yield = + 61.08 + 22.58 × B - 14.92 × E	Yield = + 60.66667 +7.52778 × molar ratio - 0.19889 × agitation	*Brevibacterium* SB11 MH715025
Yield = + 63.50 + 26.50 × B - 15.17 × E	yield = + 56.00000 + 8.83333 × molar ratio - 0.20222 × agitation	*Pseudomonas* SB15 MH715026
yield = + 60.08 - 14.42 × A + 17.58 × B - 14.42 × E	yield = + 164.6474 - 2.21795 × Temperature + 5.861 × molar ratio - 0.192 × agitation	*Acinetobacter* SB29 MH715027
yield = + 62.17 - 12.17 × A + 19.00 × B - 13.83 × E	yield = + 147.0897 - 1.8718 × Temperature + 6.333 × molar ratio - 0.184 × agitation	*Acinetobacter* SB30 MH715028

Table S2. Final equations for significant coded and actual factors required by selected four strains during biodiesel optimization process using central composite design

Coded factor equations	Actual factor equations	Strain
Yield = + 70.67 - 32.56 × A + 5.05 × B - 2.50 × A × B - 12.90 × A^2 - 3.65 × B^2	Yield = - 537.22104 + 106.99021 × Molar Ratio + 2.36026 × Agitation - 0.066667 × Molar Ratio × Agitation - 5.73148 × Molar $Ratio^2$ - 5.83333E-003 × $Agitation^2$	*Brevibacterium* SB11 MH715025
Yield = + 55.00 - 30.48 × A - 7.92 × B	Yield = + 307.94254 - 20.31861 × Molar Ratio - 0.31678 × Agitation	*Pseudomonas* SB15 MH715026
Yield = + 37.35 + 11.70 × A - 24.13 × B - 10.00 × C	Yield = + 217.051 + 1.376 × Temperature - 16.085 × Molar ratio - 0.40 × Agitation	*Acinetobacter* SB29 MH715027
Yield = + 32.82 + 11.03 × A - 26.05 × B - 6.41 × C	Yield = + 210.247 + 1.298 × Temperature - 17.367 × Molar ratio - 0.26 × Agitation	*Acinetobacter* SB30 MH715028