Biogas Production Potential of Castor Bean (*Ricinus Communis L.*) Cake

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FACULTY OF BIOLOGICAL SCIENCES

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A thesis submitted in partial fulfilment of the requirements for the Degree of

Master of Philosophy

In Microbiology

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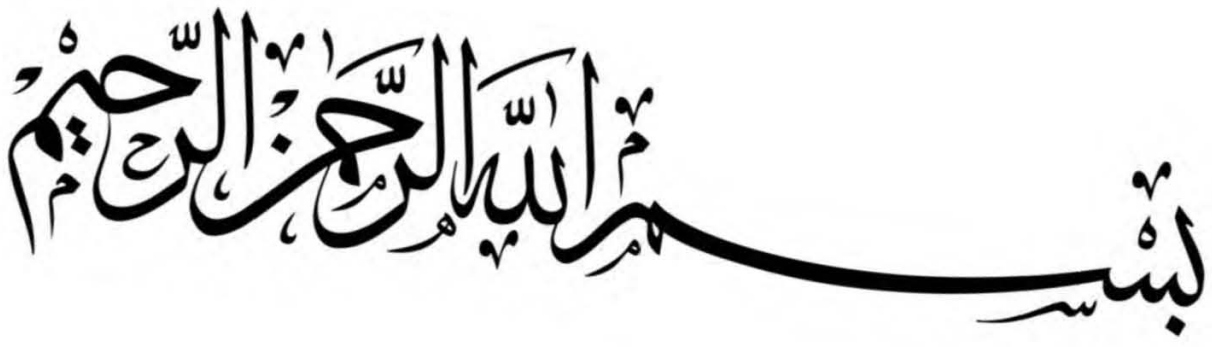
بسم الله الرحمن الرحيم

DEDICATION

To my Parents, family and friends who were standing by my side in every thick and thin during research.

DECLARATION

The information and content contained in this thesis is my original work. I have not previously presented any part ofthis work elsewhere for any degree.

Sobia Fatima

Certificate

This thesis submitted by ***Sobia Fatima*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Acronyms / Abbreviations

Acronyms	Full form
mg	Milli gram
g	Gram
NL/D	Normalize litre per day
TS	Total solids
VS	Volatile solids
AD	Anaerobic digestion
CH_4	Methane gas
CO_2	Carbon dioxide
PCRET	Pakistan Council of Renewable Energy Technologies
PCAT	Pakistan Council for Appropriate Technologies
PRES	Pakistan Renewable Energy Society
VFA	Volatile Fatty Acids

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ABSTRACT

ABSTRACT

Every passing day the world's population is increasing that means more and more energy demand and the resources fulfilling energy demand are depleting rapidly. Most commonly available resources in developing countries like Pakistan are fossil-fuel based energy sources. There are two major shortcomings of using fossil fuels, first is environmental hazard and second is depletion of fossil fuels. Both of above-mentioned reasons encourage researchers like us to search for renewable energy resources that will help us to make our environment greener and safer. In developing countries like Pakistan, the second most important issue is waste management. Waste management is not properly administered, and basic infra-structure is missing. So, utilization of waste material as a source of energy is an emerging concept. For example, utilization of biomass as a substrate for biogas production etc. In this study castor seeds were evaluated for their biogas production ability. Residues left after the solvent extraction process were utilized and their net biogas production was calculated. In the batch anaerobic digestion maximum. Biogas yield was exhibited by the oil which was 619 Nml/g of VS (0.619 NL/g VS). Methanol and Ethyl acetate residues also produced good amount of biogas, 499 Nml/g of VS and 564 Nml/g of VS respectively. During the continuous anaerobic digestion process, reactors were run at OLR 1g of VS per day and HRT was kept constant at 10 days. After achieving the steady state biogas production from the reactors was calculated. Maximum biogas yield was achieved by methyl acetate residues which was 312 Nml/ g of VS of substrate added per day, followed by chloroform 297 Nml/ g of VS of substrate per day. Minimum yield was produced by the whole seed 110 Nml/ g of VS of substrate per day. VFAs and alkalinity of the reactors were also measured after regular interval of time. Maximum VS reduction was achieved in the methyl acetate residues around 64% followed n-Hexane and ethyl acetate residues in which VS reduction was observed around 63 and 61% respectively. The current study signifies a direction to use the use of castor seeds and its residues for the use of biogas production. Leftover residues after the solvent extraction not only provide a renewable source for biogas production but also can also be managed and utilized in a more sustainable way.

Chapter 1

INTRODUCTION

INTRODUCTION

By mandating that by 2020 at least 20% of the Europian Union energy needs originate from renewable sources, the Renewable Energy Directive seeks to cut greenhouse gas emissions. The power of the sun is a promising renewable energy source since it is reliable and not greatly affected by seasonal weather patterns (Agarwal et al., 2022). Pakistan's coastal regions have an abundance of wind resources, while some areas are investigating geothermal energy. Along the country of Pakistan Arabian Sea coastline, biomass and tidal generation of energy are also possibilities (Hulio et al., 2022). By 2030, EU member states are required to provide assurances that no more than ten percent of their transportation fuel will be derived from renewable sources.

Pakistan's current use of fossil fuels is extremely problematic because it contributes to environmental problems, climate change, and a dearth of non-renewable resources (Yu et al., 2023). Pakistan must concentrate on obtaining its energy from ecologically benign sources, such as geothermal, biomass, solar, hydro, wind, and hydropower, in order to overcome these problems. The nation's dependence on imported crude oil and the emission of greenhouse gases (Raza et al., 2023).

First generation biofuels are produced from edible crops like corn, starch and edible oils but they can pose a great threat to food prices. While second-generation biofuels like ethanol made from cellulosic materials and fuels generated from biomass are more advanced, first-generation biofuels like bioethanol and biodiesel are still widely used. Algal biomass and other microorganisms are examples of third-generation biofuels that are also being produced. Several fourth-generation biofuels are still in the testing phase. The fermentation of sugar using sources of biomass like corn, sugarcane, or wheat results in bioethanol, which is an environmentally friendly alcohol fuel (Syahirah et al., 2020). Pakistan can address its economic and environmental problems and help create a future that is more ecologically friendly and sustainable by putting a greater emphasis on renewable energy sources.

A sustainable and efficient way to produce bioethanol that can reduce emissions of greenhouse gases and aid in the fight against climate change is through bioethanol. Recyclable oils for

cooking, fats from animals, or vegetable oils are used to make biodiesel, a cleaner substitute that burns more cleanly. In comparison to traditional fossil fuels, biohydrogen, which is a source of renewable energy, emits only water vapor when burned, making it a more beneficial to the environment fuel (Singh Deora et al., 2021).

Power engineering either electricity generation, or methanol manufacturing are just a few applications for biogas, which is created when organic matter is broken down by anaerobic bacteria. Biogas is a byproduct of anaerobic digestion, a method in which anaerobic bacteria digest organic matter. used in agriculture fields as a bio-fertilizer. A future with less environmental impact can be attained by incorporating biofuels into the current transportation and energy infrastructures (Zhang & Fujimori, 2020).

Anaerobic digestion is especially helpful for digesting organic matter, such as wastewater treatment sludge and green, grocer waste, and it creates anaerobic digestate, a source of high-nutrient food with antibacterial qualities. A sequence of metabolic processes called acidogenesis, acetogenesis, hydrolysis, and methanogenesis make up anaerobic digestion. Acetate-, acid-, hydrolytic-, and methane-forming bacteria are just a few examples of the specific microbes needed for these processes (Laiq Ur Rehman et al., 2019). The molecules known as mono amino acids, peptides, and fatty acids are among the soluble forms of insoluble lipids, proteins, and carbohydrates that are converted during the hydrolysis process. Particle size, pH, enzymes, and enzyme diffusion are only a few examples of the parameters that affect how effectively an enzyme hydrolyzes (Meegoda et al., 2018).

Acidogenesis results in soluble chemical byproducts, including such as alcohols, pentanoic acids, butyric acid, propionic acid, acetic acid, and other substances. For the microorganisms participating in the AD process, these degraded products serve as energy sources. The routes of hydrogenation and dehydrogenation can be used to categorize acidogenesis. Acetate, carbon dioxide, and hydrogen are the fundamental byproducts (Richard et al., 2019). While acetogens transform them into hydrogen, methanogens employ these byproducts as energy sources. Acetate-producing bacteria use the process of acetogenesis to transform the byproducts of acidogenesis into acetate and hydrogen. While *Methanobacterium propionicum* breaks down propionic acid into acetic acid, *Methanobacterium suboxydans* breaks down pentanoic acid into propionic acid (Parawira et al., n.d.). Acetogens and an autotrophic methane bacterium

must work together cooperatively in this process to use hydrogen. This stage serves to measure the efficiency of biogas generation because methanogens use acetate to produce over 70% of the gas. Substrates like acetate, hydrogen, carbon dioxide, a methanol methylamine, and dimethyl sulfide are used by methanogenic bacteria, which are substrate-specific microorganisms, to create methane (Koutrouli et al., n.d.). They can be split into two main categories as well as hydrogenotrophic methanogens. Methanogens that employ hydrogen and carbon dioxide as their substrates are known as hydrogenotrophs, as opposed to acetotrophs, which decrease methyl groups. The stability of methanogenic archaea, which are heavily reliant on temperature, is crucial for anaerobic digesters. The digester's pH and the substrate being utilized both have an impact on the composition of the biogas. Methane-forming bacteria prefer a pH range of 7-8, whereas acid-forming microorganisms have a lower pH range, with the mesophilic digesters that have a pH of 6.5 to 8. Ammonia, also in the mixture, has the ability to raise pH levels. The method of anaerobic digestion depends on temperature stability, and ammonia in a mixture can interfere with the process (Parawira et al., n.d.).

These variables affect a bicarbonate buffers system, that governs the pH level of an anaerobic digester based on the quantity of acidic or alkaline materials in the solution mixture and the relative pressure of CO_2 that is present. When acid or base concentrations are too high or too low, the solution's buffering ability prevents a sharp drop in pH that would otherwise prevent anaerobic digestion from taking place (Tao et al., 2020). The long-term viability of the process depends on the production of volatile fatty acids, which are intermediate intermediates. Products made from propionate, acetate, butyrate, and lactate are among them. Process stability may be impacted by the presence of various bacteria inside the reactor (Harirchi et al., 2022). As a source of ammonia during the process, ammonia is a required component for anaerobic digestion. The proportion of ammonia to macro nutrients is critical because anaerobic digesters with high ammonia concentrations run the risk of producing hazardous levels of the nitrogen. Instability and halting of the process can be brought on by inadequate supply of minerals and micronutrients as well as high substrate digestibility (Jiang et al., 2019). As a result of the introduction of toxic substances into the reactor, both of these scenarios are possible. In the construction and operation of a biogas plants, the rate of organic loading (OLR), with an ideal ratio of 600:15:5:1, is a crucial component. The time required for hydraulic retention (HRT), which must be equal or greater than the time it takes for doubling

of the microorganisms involved in the process, is a crucial parameter for the operation of anaerobic digesters (De Groof et al., n.d.). Hydrolytic, acidogenic, acetogenic, or methanogenic bacteria are only a few of the many kinds of microorganisms that can be found in anaerobic digesters. When animal dung was utilized as a substrate, *Methanosarcina sp.* was the predominant species in the chemical reaction mixture. *Methanosphaera stadtmanae* and *Methhanobrevibacterwolinii* were the predominant hydrogenotrophic and acetotrophic microbes, respectively, whereas *Methanosaeta conciliiwas* were the principal heterotrophic microbes (Sharma et al., 2023). Finally, it should be noted that a number of variables, such as the presence of microbes, the rate at which organic material is loaded, and the substrate being used, affect the stability and performance of anaerobic digestion.

AIM AND OBJECTIVES

Aim and Objectives

Aim:

To study the biogas production potential of Castor Bean (*Ricinus Communis L.*) Cake.

Objectives:

- Calculate the biogas potential of castor seeds in batch anaerobic digestion
- Calculate the biomethane possibility of de-oiled seed cake and leftover residues after solvent extraction.
- Evaluate the biogas potential of *Ricinus Communis* seed, seed cake, oil and residues in continuous reactors

Chapter 2

LITERATURE REVIEW

Literature Review:

Two major issues in Pakistan related to the green economy are the economic crisis and environmental destruction. In order to lessen the harmful effects of the greenhouse gases (GHC), which are mostly released through combustion of fossil fuels, renewable energy production is becoming increasingly crucial, in accordance with the European Union's (EU) Sustainable Energy Direction (Levihn, 2014). This directive stipulates that by the year 2020, the EU must satisfy a minimum of twenty percent of its overall energy requirements using sources that are renewable, which will be achieved by meeting specified state targets. This directive does this by setting a general plan for the generation and dissemination of energy from sources that are renewable in the EU (Orawski et al., 2019). Furthermore, by the year 2020, all EU nations must guarantee that a minimum of ten% of their fuel for transportation come from renewable sources. The Commission published an outline for a modified Renewable Energies Commission on November 30, 2016, having the goal to make the EU an international leader in the generation and utilization of energy from renewable sources by 2030, having an objective of a minimum of 27% sustainable Power Units in EU energy consumption. Pakistan is the sixth most climate change-vulnerable country in the world (Victor Bekun et al., n.d.).

2.1. Pakistan's present fossil fuel situation

The effects of climate change on Pakistan's population growth and urbanization would be catastrophic. Due to the consequences of global warming on agribusiness and lifestyles, Pakistan is most severely impacted by it, coming in at twelve on the list (Otto et al., 2023). Due to their impact on the preservation of heat in the earth's stratosphere, greenhouse gas (GHG) emissions that result from the widespread consumption of fossil fuels are thought to be the main cause. This increase in average global temperature hastened the process of rising temperatures and brought about significant climate change. Nearly 25 million jobs in Pakistan are supported by Argo based industry. Despite their benefits, such as their ability to monitor operations effectively in heat. Their use brings up a number of issues that have been studied

and addressed in several studies. Fossil fuels are a major factor in the transition to low-carbon economies because of issues like the harm they do for the power source environment, shortages, supply instability, price fluctuations and market volatility found a significant connection in Pakistan between natural capital rents, consumption of energy, air pollution, and the availability of water (Borowski, 2022). Fossil fuel consumption is a factor in a number of ecological issues, including climate change and contamination of the environment, which have adverse effects on people's health and way of living. Using them effectively is hampered by the markets' and prices' erratic nature, which has negative effects on the economy. So, it is the need of the time to shift our attention towards some renewable sources as renewable energy sources play a crucial part in new energy strategies, and European countries remain among the center of these innovations (Zaidi et al., 2018).

2.2. Pakistan's sources of sustainable or ecologically friendly energy

Since the dawn of civilization, human beings have also relied heavily on sources of renewable energy. For example, biomass is being used for cooking, heating, and producing steam, and wind has been used to propulsion ships; and both hydropower as well as the wind can be employed to power grain mills. It is possible to supply electrical power involving no or nearly zero emissions of greenhouse gases and air pollutants thanks to energy from renewable sources that utilize local resources. The world's energy needs may be more than satiated by renewable energy sources because of their ubiquitous naturally and are widely regarded as being important (Zafar Ilyas et al., 2021).

2.2.1. Solar power:

Among the most promising forms of renewable energy is solar power. It has a high degree of consistency and is not overly susceptible to fluctuations within seasonal weather patterns. Both of them rural and urban communities can use it to generate power when needed. Solar heat and solar PV (photovoltaic) energy can both be used to power a variety of devices. Solar photovoltaic technology allows for the simple transformation of sun towards power through semi-conductor devices known as solar cells, meanwhile solar thermal technologies make use of the warmth energy from the sun for various reasons. Pakistan is a prime prospect for solar energy use due to its geographic position, topography, and climatic conditions. There are often

more than 300 sunny days each year across practically the whole nation. Annual global irradiance fluctuates between 1900 and 2200 kWh/m^2 (Khalil & Zaidi, 2014).

2.2.2. Energy from wind:

Excellent wind resources can be found in Pakistan's coastal regions, particularly in Sindh and Baluchistan provinces. To capture wind energy and add to the national grid, wind turbines have been constructed (Raza et al., 2023).

2.2.3. Geothermal Energy:

Although the technology for producing geothermal power is still in its infancy, there has been considerable interest in analyzing its potential in specific regions in Pakistan (Shah et al., 2023).

2.2.4. Energy from the tidal:

Tidal energy production is a possibility along Pakistan's Arabian Sea coast. Utilizing tide energy to generate electricity is a goal of tidal power technologies (Rehman et al., 2023).

2.2.5. Energy from biomass:

Biogas and biomass-based energy can be generated from biomass, comprising organic materials, animal and agricultural waste, and waste of animals. The biomass as a source of renewable energy has been investigated in Pakistan (Saghir et al., 2019).

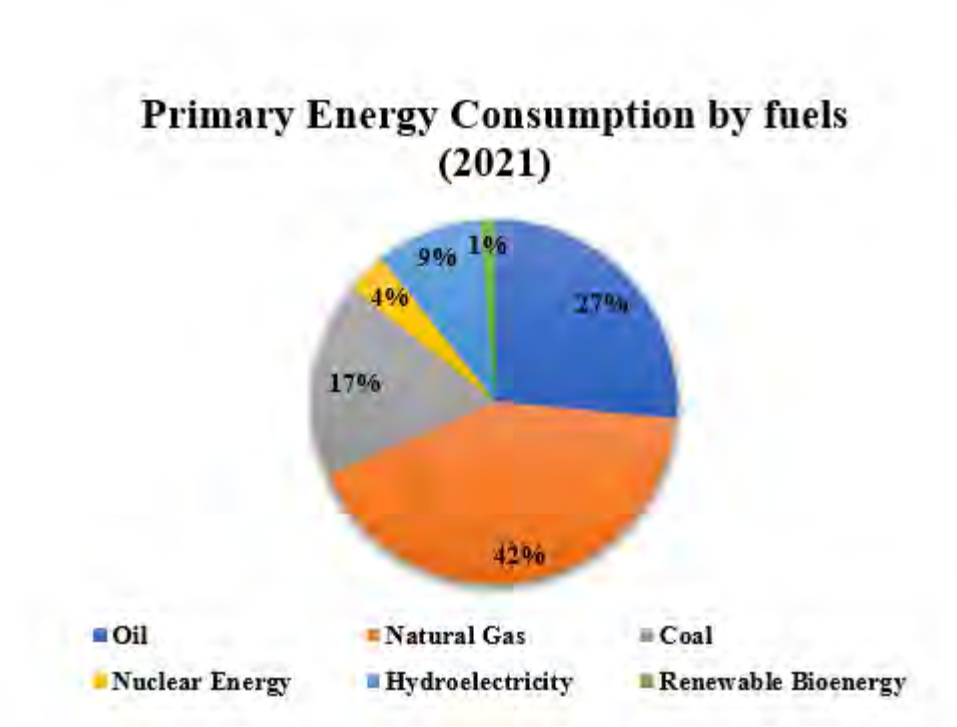

Figure 2.1. Primary energy consumption by fuels in Pakistan (Statistical Review of World Energy | Energy Economics, 2023)

2.3. Biofuels:

By promoting the use of biofuels such as ethanol and biodiesel, it is feasible to diminish the country's dependency on crude oil imports and lower greenhouse gas emissions in contrast to the transportation sector. By promoting the use of biofuels such as ethanol and biodiesel, it is feasible to diminish the country's dependency on crude oil imports and lower greenhouse gas emissions in contrast to the transportation sector (Vickram et al., 2023).

The initial and most popular type of biofuels are first-generation fuels. These are made from plant oils, sugarcane, and other food crops like corn. Bioethanol and biodiesel are the most popular first-generation biofuels (Alalwan et al., 2019).

The shortcomings of first-generation biofuels are somewhat overcome by second-generation fuels, which are better developed. Because they are made from waste biomass and non-food

crops, food production is less competitive with these biofuels. Cellulosic ethanol and fuels made from biomass are two examples (Mujtaba et al., 2023).

Algal biomass and other microbes are used to create third-generation biofuels. Algae are a viable source of biofuels since they contain a lot of oil and can be grown in several types of conditions (Chowdhury & Loganathan, 2019).

The development of fourth-generation biofuels, which concentrate on using synthetic biology to create organisms that produce biofuels effectively, remains in the experimental stage. The biofuel sector could be completely transformed by these cutting-edge fuels (Abdullah et al., 2019).

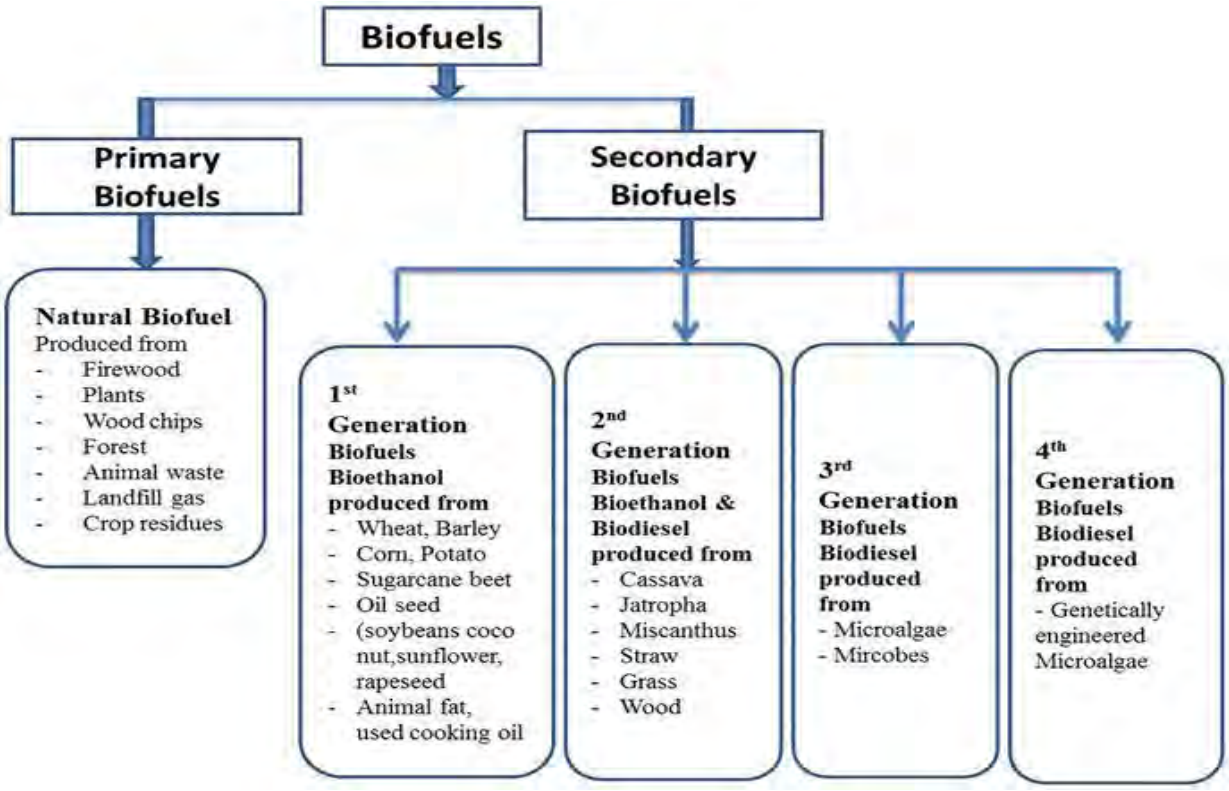

Figure 2.2. Generation of biofuels (Syahirah et al., 2020)

2.3.1. A sustainable Alcohol fuel called bioethanol:

Biomass-based production: Using sugars from sources of biomass like sugarcane, corn, or wheat, bioethanol is a kind of alcohol fuel that is created through fermentation. These sugars

are broken down by microbes like yeast, which release ethanol and CO_2 in the process. A sustainable and effective technique of producing bioethanol is provided by this procedure (Mahmud et al., 2022).

2.3.2. Benefits of bioethanol:

The ability to lower greenhouse gas emissions is one of the main environmental advantages of bioethanol. The process of absorbing of CO2 during the growth of plants results in significantly lower net CO2 emissions for bioethanol as compared to conventional petrol. This makes bioethanol a promising option for reducing climate change since it balances the CO2 emissions produced when it is consumed as fuel (Ayodele et al., 2019).

2.3.3. A cleaner substitute is biodiesel:

The important biofuel biodiesel is made from renewable sources. It is made from recycled cooking oils, animal fats, or vegetable oils. An alcohol, usually methanol, and an oil or fat are mixed throughout the production process to produce biodiesel and glycerol as the byproducts of the transesterification reaction (Rathore et al., 2022).

2.3.4. Benefits of biodiesel:

Biodiesel delivers environmental benefits by lowering harmful emissions, much like bioethanol does. Compared to regular diesel, biodiesel produces fewer pollutants during combustion, including Sulphur and particulate matter. Additionally, because the feedstock for biodiesel is carbon-neutral, the fuel's carbon emissions are substantially lower (Ahmad et al., 2015).

2.3.5. Utilizing Biohydrogen's Power:

Produced using biomass and biogas:

The process of fermentation of organic material or gasification of biomass can yield biohydrogen, a clean gas. Biomass is transformed into a combination of carbon and hydrogen dioxide during the gasification process, which can subsequently be separated to produce pure hydrogen (Nikhil et al., 2018).

2.3.6. Alternative Green Fuels:

In terms of renewable energy, biohydrogen has enormous promise. It is a more environmentally friendly fuel than conventional fossil fuels since when it is burned as fuel, all that is released is water vapors. Because of its adaptability, it can be utilized to generate power and in a variety of other industries without causing climate change (Kamaraj et al., 2020).

2.4. Waste Management: Its Importance

2.4.1. Taking Care of the Waste Issue:

The ability of biofuels to control waste is one of its many positive attributes. It is possible to use organic Making biofuels using garbage as a feedstock Making biofuels using garbage as a feedstock instead of allowing it to disintegrate and emit a powerful greenhouse gas called methane. Biofuels assist in reducing the negative environmental effects of waste disposal in this way (Olguin-Maciel et al., 2020).

2.4.2. Decreased Dependence on Landfills:

The amount of trash going into landfills is decreased by using organic waste to make biofuel. As a result, there is less chance of water and soil contamination, which helps to create a waste management system that is more sustainable. The circular economy, where trash is recycled and utilized is promoted by putting an emphasis on waste-to-energy practices (Mahmudul et al., 2022).

2.5. Biofuels have advantages:

2.5.1. Energy source that is Renewable:

The organic resources needed to produce biofuels can be replaced through trash recycling or agricultural output, making them a renewable resource. Biofuels offer a reliable and sustainable energy source, in contrast to petroleum and coal, which took thousands of years to develop (Rekleitis et al., 2020).

2.5.2. Decreased emissions of greenhouse gases:

Carbon dioxide (CO_2) is emitted into the environment when biofuels are burned. However, since the amount of CO_2 released during burning is roughly equivalent to the amount of CO_2

plants absorb during their growth, a closed carbon cycling is established. The use of biofuels as a weapon to combat climate change is therefore made possible (Mendiara et al., 2018).

2.6. Biofuels' Problems and Restrictions:

2.6.1. Utilization of Land and Water:

Large-scale production of biofuel feedstocks has the potential to cause habitat destruction, deforestation, and rivalry between food production for water and land (Barlow et al., 2007).

2.6.2. Food production and competition:

Concerns have been made concerning possible food shortages and rising food prices as a result of the usage of food agricultural products for the manufacture of biofuel (Ajanovic, 2010).

2.6.3. Constraints on technology and the economy:

The scalability and cost-effectiveness of advanced biofuel technologies, which are still in the development stage, could provide difficulties (Panoutsou et al., 2021).

2.6.4. Loss of Biodiversity and Deforestation:

Deforestation and other ecological disruptions can result from clearing land for the production of biofuel feedstocks, which will significantly reduce biodiversity (Barlow et al., 2007). **2.6.5. Affects the quality of the air and water:**

Although they are often lower than those produced by burning fossil fuels, the manufacturing and combustion of biofuels can nonetheless emit pollutants that have an impact on the quality of the air and water (Commane & Schiferl, 2022).

2.6.6. Biofuels' Potential in the Future:

2.6.7. Modern Research and Technology:

The potential to overcome current constraints and increase the viability of biofuels on a broader scale lies in the ongoing study and development of biofuel technology.

2.6.8. Including Existing Infrastructure in the Integration:

A more seamless shift to a more environmentally friendly future might be made possible by the effective incorporation of biofuels into the current energy and transportation infrastructures.

2.7. Biogas

Biogas is the gas produced as a result of anaerobic bacteria's activity and the breakdown of organic substances (Amjid et al., 2011). The biogas generated can be used in different fields mostly used to power engineering process and different technological process as follows:

- Biogas is used to produce thermal energy from gas boilers and can produce electrical energy in associated units
- Electricity production from spark-ignition or turbine engines
- Upgraded biogas can be used as fuel for car engines
- Use in production of methanol

2.7.1. Anerobic digestion:

Anaerobic digestion is a process where anaerobic bacteria carry out fermentation and the product produced at the end of one step as a substrate for the next step. These monomers are subsequently employed as a substrate by fermentative bacteria, which transform them into volatile fatty acids (Harirchi et al., 2022). Acetate and a combination that gases such as carbon dioxide and the hydrogen are produced when acetogenic microorganisms utilize these volatile fatty acids. Acetate, carbon dioxide, and hydrogen are ultimately used by methanogens to produce methane and carbon dioxide. Biogas, which contains methane (50–75%) and carbon dioxide (25–50%), is the primary byproduct of anaerobic digestion (Reith & Wijffels, 2003). Reaction carried out for biogas production is as follows

$$CH_3COOH \rightarrow CO_2 + CH_4$$

The carbon dioxide content of biogas decreases the calorific value so it must be removed or converted. It is called as enhancement and up gradation of biogas. And once it is upgraded it can be easily used for electricity generation and also fuel for vehicle transport (Q. Zhao et al., n.d.). Anaerobic digestion is used worldwide to treat organic waste especially sludge from

waste water treatment and green grocery waste because of its immense amount and for proper management this technique is very helpful and efficient. Biogas production yields two important products one is biogas used as energy and the other one is anaerobic digestate that is used as a bio-fertilizer in the crop field (Jin et al., 2022).

a mixture of carbon dioxide or methane gas is known as methanation.

Anaerobic digestion produces more than just biogas; it also produces anaerobic digestate. The composition of biogas is mostly determined by the substrate's composition. The gas methane and carbon dioxide gases comprise the majority of biogas, with some water vapors, nitrogen, and hydrogen sulphide thrown in for good measure. Anaerobic digestate is a high-nutrient source that can also be utilized as a bio-fertilizer and has antibacterial properties. Carbon is mostly converted to biogas, while digestate obtained is a rich source of N, P, S nutrients. So digestate derived biofertilizers have the potential to increase the crop yield (Koszel & Lorencowicz, 2015).

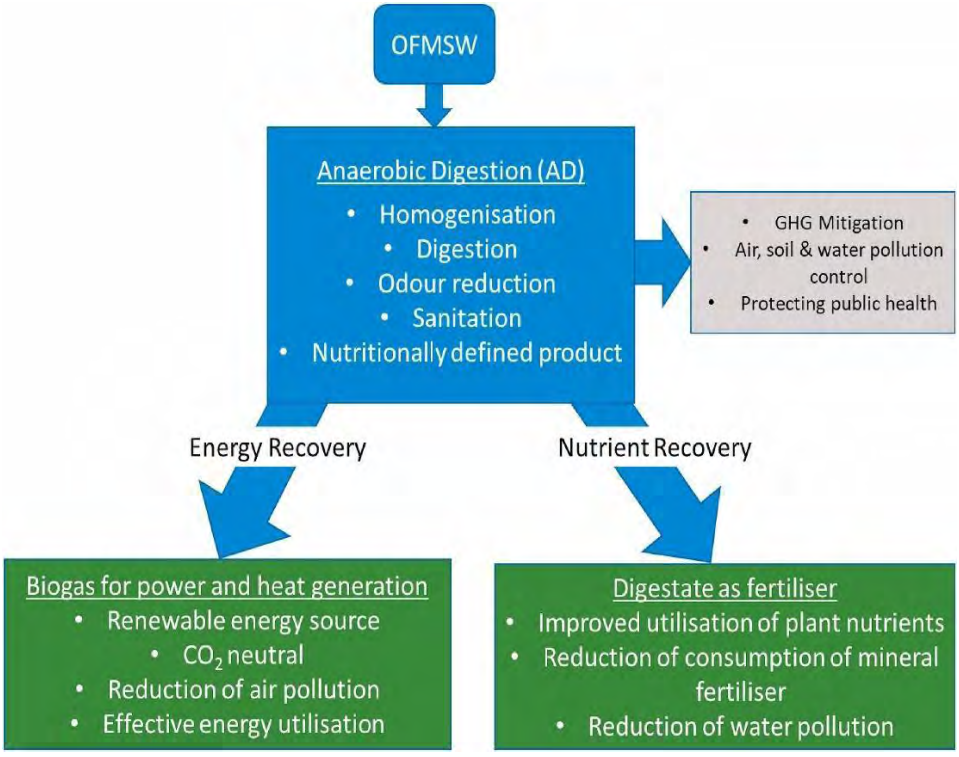

Fig 2.3. Applications of Anaerobic Digestion (Wilkie, 2005)

2.7.2. Steps in Anaerobic digestion:

It is a series of various metabolic steps involving hydrolysis, acidogenesis, acetogenesis and methanogenesis and each stage requires specific microbes such as hydrolytic, acid forming, acetate forming and methane forming bacterial species. Product formed at one step acts as a substrate for the next step (Wilkie, 2005).

2.7.2.1. Hydrolysis

Polymers are broken down into dimers or monomers through lysis. Most insoluble proteins, lipids, and carbohydrates are converted into soluble monosaccharides, amino acids, and fatty acids by the action of hydrolytic enzymes. Hydrolytic strains release extracellular enzymes referred to as hydrolyses, including as amylases, proteases, and lipases. If a substrate that is much more difficult to decompose ad degrade is added in the reactor like the waste without grinding it will limit the rate of reaction and reaction might get stuck in this level because of less or no degradation. It does not get in soluble form and uptake of substrate by bacteria decreases resulting in the failure of experiment. The efficiency of hydrolysis mainly depends upon different factors like size of particles, pH, enzyme, adsorption of enzyme diffusion (Amjid et al., 2011).

Table 2.1: Shows different substrates and enzymes produced by different bacteria to convert them to monomers (Menzel et al., 2020)**.**

Substrates to be degraded	**Enzymes**	**Example of bacteria**	**Products**
Polysaccharides	saccharolytic cellulase	Cellulomonas	Simple sugars
proteins	Proteolytic protease	bacillus	Amino acids
lipids	Lipolytic lipase	mycobacterium	Fatty acids +glycerol

2.7.2.2. Acidogenesis:

The hydrolysis products, which are the easily soluble chemical components, are produced in the second step. These products include aldehydes, hydrogen, and carbon dioxide in addition to organic acids like acetic, formic, propionic, butyric, and pentanoic acids, as well as alcohols like ethanol in order and methanol. The microbes engaged in the AD process use these degraded products as a source of energy. Because there are numerous populations of microorganisms involved, acidogenesis occurs via a couple of processes. Dehydrogenation and hydrogenation are the two categories into which this process falls (Kim et al., 2008). Hydrogen, CO_2, and acetate are the basic byproducts. Methanogens that may directly utilize the energy sources acetate, carbon dioxide, and hydrogen to produce methane. Electrons are accumulated in the solution as a result of the formation of lactate, ethanol, which is butyrate, and propionate, among other strongly volatile fatty acids that are produced (Xiao et al., 2019).

Simple sugars + fatty acids + amino acids → organic acids, including acetate + alcohols

$C6H12O6 \rightarrow 2CH3CH2OH + 2CO2\ C6H12O6 + 2H2O$

$2CH3CH2COOH + 2H2O\ C6H12O6 \rightarrow 3CH3COOH$

2.7.2.3. Acetogenesis:

In this stage, the acidogenesis products are converted to acetate and hydrogen by bacteria that produce acetate, such as those from the genera *Syntrophomonas* and *Syntrophobacter*. *Methanobacterium suboxydans* and *Methanobacterium propionicum* are the bacteria responsible for the conversion of the pentanoic acid to the compound's propionic acid and acetic acid, respectively (Bajpai, 2017). Hydrogen is released as a result of acetogenesis, which is harmful to this process. For this purpose, a symbiotic relation is needed between acetogens and autotrophic methane bacteria for utilizing hydrogen. That relationship is referred to as syntrophy. Biogas production efficiency is estimated by this step of anaerobic digestion because almost 70% of methane is produced from acetate utilization by methanogens. Acetate acts as a key ingredient in the process of biogas production (Amani et al., 2010).

Organic acids + alcohols → acetate

$CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + 2H_2 + CH_3CH_2COO^- + 3H_2O$

$CH_3COO^- + H^+ + HCO_3^- + 3H_2C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$

2.7.2.4. Methanogenesis:

Last step is methane production by utilization of substrate produced as a product of last step that was acetate, carbon dioxide, hydrogen, methanol, methylamine, or dimethyl sulfide. Methanogenic bacteria are substrate specific microbes acting on a few selected substrates such as acetate and mixture of carbon dioxide and hydrogen. Autotrophic bacteria reduce CO_2 by over 30%, which produces methane. H_2 from the solution mixture that was created throughout each the procedures of the acetogenesis and acidogenesis is utilized in this CO_2 reduction procedure. When low production of Hydrogen occurs during acetogenesis this will increase the amount of CO_2 in the end product.

Acetate → $CH_4 + CO_2$

$CH_3COOH \rightarrow CH_4 + CO_2$

$CO_2 + 4H_2 = CH_4 + 2H_2O$

2.7.3. Substrates Used by Methanogenic Bacteria:

Methanogenic archaea are substrate specific utilizing a few organic compounds to produce methane. On this basis methanogenic archaea are divided into two major groups:

• **Hydrogenotrophic Methanogens:** They use carbon dioxide and hydrogen as their substrate. One of the key factors that maintain stability of anaerobic reactors is the partial pressure of hydrogen. Thus, the activity of these methanogens is necessary to maintain a stable state for anaerobic digesters. Effectiveness of these archaea is vital for easily degradable substrate like ethanol and acetate as well as hardly degradable substrates like oil etc (Y. Zhao et al., 2019).

• **Acetotrophic Methanogens:** Acetotrophic methanogens are those which undergo the process of methyl group reduction. Species of Methanosarcinales genus use acetate as their

substrate. Almost 70% of methane is the product of Acetotrophic archaea and they also yield carbon dioxide (Akcakaya et al., 2022).

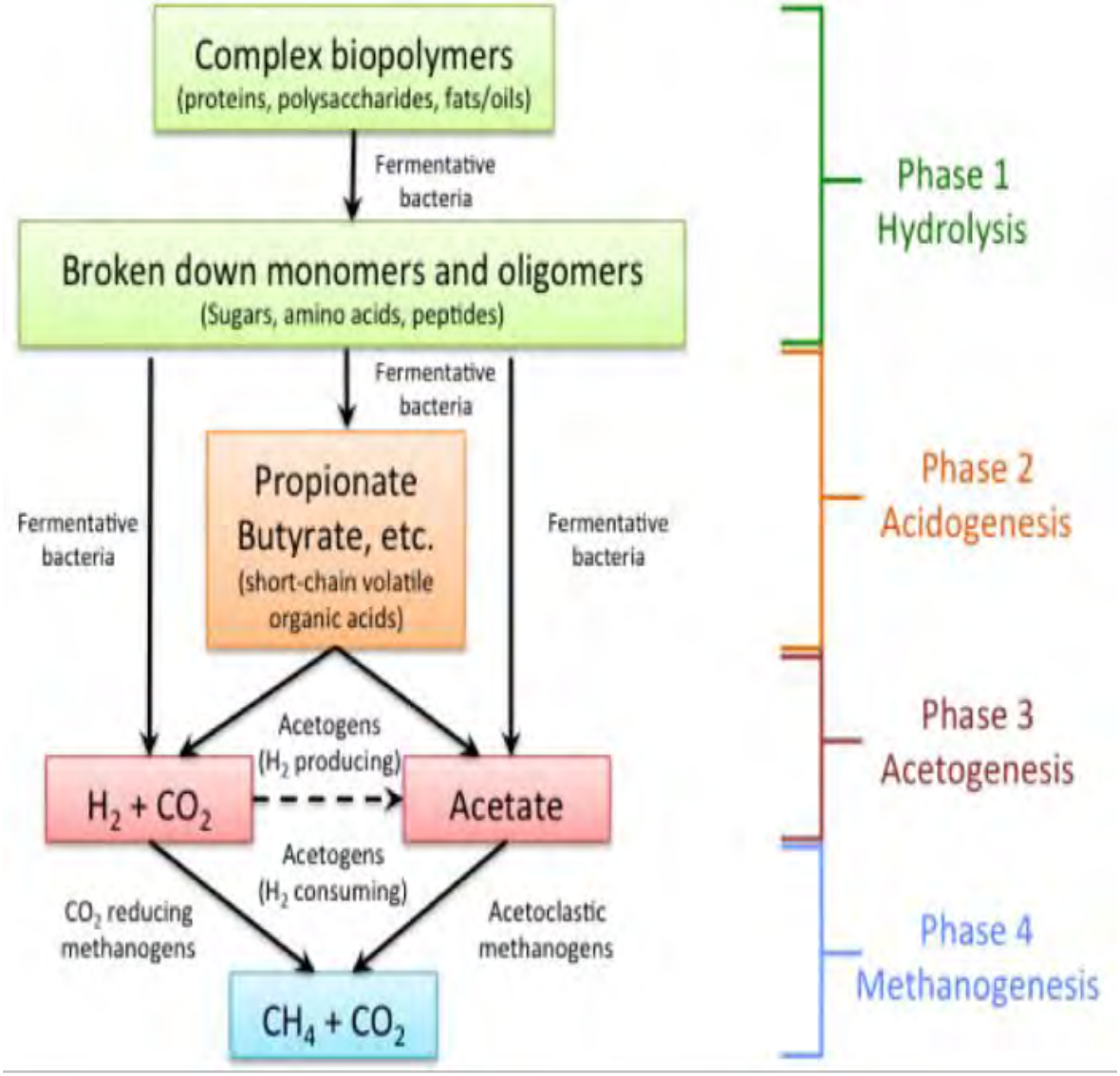

Fig 2.4. Diagrammatic sketch of anaerobic digestion process (Mao et al., 2015)

Table 2.2: The biogas composition (Bharathiraja et al., 2018)

Compound	Chemical formula	Yield in %
Methane	CH_4	50–75
Carbon dioxide	CO_2	25–45
Water vapor	H_2O	2–7
Nitrogen	N_2	Less than 2
Oxygen	O_2	Less than 2
Hydrogen sulfide	H_2S	Less than1
Hydrogen	H_2	Less than1
Ammonia	NH_3	Less than 1

2.7.4. Types of Anaerobic Digestion:

2.7.4.1. On the basis of Digester feed:

2.7.4.1.1. Batch anaerobic digestion:

In batch anaerobic digestion system, the calculated fixed amount of substrate is fed into the reactor along with the inoculum mostly taken from a running reactor and then the reactor is closed and operated for 30-170 days for biogas production. This approach involves loading a fresh batch of substrates into the batch reactor, running it, discharging the reactor, and then loading it once more (Kundu et al., 2023). Due to their relative technological simplicity, robustness, low capital cost, low maintenance requirements, and low parasitic energy loss, batch reactors provide a number of advantages. However, they also have disadvantages like less amount of wastes is treated with batch reactors as they are run with low OLR, the substrate materials may get deposit in the bottom which may results in less biogas yield and may cause explosion during unloading of the reactor (Rocamora et al., 2020).

2.7.4.1.2. Continuous anaerobic reactor:

In continuous anaerobic digestion process, a fresh substrate is feed continuously into the reactor while removing an equal amount of digestate from the same reactor on daily basis. With a constant input of substrate, all the reactions occur at a stable rate with approximately stable and constant biogas yield. Continuous anaerobic digestion is advantageous as high amount of waste is treated via high OLR (Bharati Barua & Kalamdhad, 2019). However, the effluent comes from the continuous anaerobic digestion process containing a mixture of fully digested substrate materials which results in incomplete digestion that effect reactor performance. Some designs specify the passage of the digested matter inside the chamber, for instance by using interior walls, in order to reduce the elimination of partially digested material (Srisowmeya et al., 2020).

2.8. Factors affecting AD.

2.8.1. Temperature:

A key component of the AD process is temperature stability. The operational temperature is selected upon the criterion of which substrate is used and many other conditions. Temperature has an indirect relation with retention time such that the higher the temperature lesser retention time will be requiring (Hosseini Beinabaj et al., 2023). The temperature at which digester is working effect the toxicity of ammonia. It has a direct relation that when temperature is high ammonia toxicity is high and it can be decreased by reducing temperature. Ammonia, Hydrogen, methane content and VFA solubility mainly depend on temperature of digester. The viscosity of substrate used in anaerobic digester is inversely proportional to temperature. As the friction between the layers decreases when temperature is higher, it facilitates the diffusion process of molecules in substrate (Beugre & 4462349, n.d.).

2.8.2. pH

pH is basically the measure of hydrogen ions in the solutions. It determines the acidity/alkalinity of the substrate mixture in anaerobic digestion and is represented in parts per million (ppm). As methanogenic archaea are sensitive to slight change in pH thus it must be maintained in a specific region. pH measures the degradation and utilization of VFAs in the reactor (Zhou et al., 2021). Many studies show that methane gas production occurs between the range of 5.5 to 8.5, and the optimum range is 7-8 for methane forming microbes. Acid forming bacteria has a lower range of pH such that the optimum range for mesophilic digesters is 6.5 to 8 and process is negatively affected when pH fluctuates. The value of ph. increases when ammonia is present in the mixture as a result of protein degradation or from ammonia in the feed supply. While volatile fatty acids are produced by acid-forming bacteria, their buildup lowers the pH level (Eryildiz et al., 2020). The majority of the time, a bicarbonate buffer system is used to maintain the pH of an anaerobic digester. Consequently, the pH is largely dependent on two key factors. The amount of either alkaline or acidic components found in the solution mixture, and secondly the partial's pressure of carbon dioxide (CO_2) in the solution. When bases or acids levels surpass the ideal range, the solution's buffer capacity kicks in. Anaerobic digestion is unsuccessful when the buffer ceases operating because it causes a significant pH change (Lu et al., 2020).

2.8.3. Fatty Acids that are volatile:

The stability of the anaerobic digestion process depends heavily on volatile fatty acids, which are the intermediate outcome of the process. The greatest number of carbons in volatile fatty acids, which can have up to six carbon atoms, are acetate, propionate, butyrate, and lactate. Sometimes in anaerobic digesters volatile fatty acid accumulation occurs which leads to a drop of pH in the digester (Al-Sulaimi et al., n.d.). Sometimes buffering capacity of digester does not let the pH fall. The pH remains almost at the neutral range because of buffering capacity of substrate. Substrate like animal manure has a high alkalinity value if volatile fatty acid accumulation occurs the alkalinity of substrate normalizes the pH and does not allow to detect VFA accumulation inside the reactor. So volatile fatty acid amount will be so high inside the reactor and that would lead to inhibition of anaerobic digester (Wang et al., 2023). Much research shows that the same concentration of VFA in two different reactors act differently,

why is that so in one reactor it will act normally but in second one it will inhibit the whole process. One of the sane explanations is presence of different microbiota inside the reactor as the micro-organism differ from one another so does their effect on volatile fatty acid assimilation as scientists call this as a black box (Yin et al., 2021;.Roopnarain et al., 2021)

2.8.4. Ammonia

NH_3 is a necessary factor for anaerobic digestion process. In anaerobic digestion protein acts as a source of ammonia as degradation of amino acid yield ammonia. Ammonia has its characteristic pungent smell. High ammonia concentration in the anaerobic digester will lead to ammonia toxicity on the process, free ammonia in the reaction mixture will lead to inhibit the process of anaerobic digestion (Jiang et al., 2019). When animal slurries are used in process as substrate that leads to higher ammonia concentration, due to presence of urine. The ratio needs to be restricted to kept below 80 mg/L since toxicity occurs at higher concentrations. High levels of ammonia can be toxic to methanogenic archaea (Puig-Castellví et al., 2020). Due to the direct relationship between ammonia toxicity and temperature, digesters operating at thermophilic temperatures are more likely to be inhibited by the greater ammonia concentrations (Yan et al., 2020).

2.8.5. Toxins, harmful substances, and macro- and micronutrients

As crucial for the growth and advancement of microorganisms as macro nutrients including carbon, sulphur, nitrogen, and phosphorus are, trace metals like cobalt, nickel, iron, the element selenium molybdenum, or tungsten are. The ideal ratio of the macronutrients C:N:P:S (carbon, nitrogen, phosphorus, and sulphur) is 600:15:5:1. The process might become unstable and even come to a halt due to inadequate supply of minerals and micronutrients as well as high substrate digestibility (Fermoso et al., 2023).

The presence of hazardous chemicals is a significant element that influences the anaerobic digestion process. Toxic substances can enter the reactor in one of two ways: either with the feedstock or as a result of the process itself (Adedeji et al., 2023).

2.8.6. Organic Loading Rate

Designing and processing of a biogas plant depends on both technical and economic feasibility. To attain the highest amount of biogas yield through the degradation of whole substrate would need longer retention time and a large size digester.Based on the maximum feasible biogas yield and a reasonable plant economy, retention time is required. In a nutshell, OLR is a crucial component that establishes the volume and timing of the amount of organic matter to be fed into the reactor (Nkuna et al., 2022). The following equation provides the definition of OLR:

OLR = Concentration × (Flow rate / volume of reactor)

2.8.7. Hydraulic Retention Time

The anaerobic digester's time of hydraulic retention is a crucial component of its operation. The HRT measures how long the substrate usually undergoes treatment inside the reactor. The amount of substrate and reactor volume injected into the reactor each unit of time affects HRT (Bella & Rao, n.d.). based on the equation below

$$HRT = VR / V$$

HRT= hydraulic retention time (days)

VR = digester volume (m^3)

V= Volume of substrate fed per unit time (m^3 /d)

From this equation we can conclude that when organic load increases it decreases the HRT. While deciding the retention time one thing must be kept in mind that it must be more than or equal to doubling time of micro-organism involved in the process. Because removal of microorganisms occurs with the effluent so they must get doubled to perform their work properly. For anaerobic microorganisms, the double-growth period typically lasts 10 days. The substrate flow may be good with a short HRT, but the gas generation may be less. Therefore, the HRT must be modified to account for the substrate's rate of breakdown. The approximate

HRT, the daily feed OLR, and decomposition can be used to determine the reactor volume (Tshemese et al., 2023).

2.8.9. Seeding of biogas plant:

Anaerobic Digester hosts so many diverse classes of microorganism that involve hydrolytic, acidogenic, acetogenic and most importantly methanogenic. In the case of methanogenic microorganism these reactors are quite rich they contain two types of methanogens, first one is Acetoclastic and secondly hydrogenotrophic. Both of the groups maintain the stability and function of anaerobic digestion (Liang et al., 2020). *Methanosarcina sp*. are able to convert the product of acetogenesis i.e., acetate and carbon dioxide and hydrogen to methane gas. Others compounds like other VFA's Methanosarcina sp. can also convert it to methane. Mainly *Methanosarcina* can convert these all-intermediate compounds to methane thus all other groups of anaerobic bacteria become jobless because of these species (Mutungwazi et al., 2020).

2.8.10. Different micro-organism involved with different substrate:

It was found that the following two kinds were discovered to predominate in a reaction mix when animal dung was employed as the substrate for an anaerobic digester: *Methanoculleus thermophilicus* (hydrogenotrophic) and Methsnosarcina thermophila (acetotrophic) (Mutungwazi et al.,2020). And when fruit and vegetable waste were treated the main and dominating species were of hydrogenotrophic micro-organism and they were: *Methanosphaera stadtmanae* and *Methhanobrevibacterwolinii.* And the acetotrophic micro-organism that were found in the reaction mixture are from genus *Methanosarcina*. Different studies showed that when municipal waste and sewage sludge degradation occur in anaerobic digestion following species of acetotrophic micro-organism *Methanosaeta conciliiwas*. Were found (Gerardi, 2003).

2.9. Ricinus communis: a potential feedstock for biogas production

The castor plant is a large, palmate, and lobed plant with deep lobes and brilliant green leaves. Its thick, hollow stems give it a treelike appearance, with violet or reddish hues and tiny hairs. The plant produces small, unisexual flowers, with female flowers at the bottom and male flowers at the top. After pollination, female flowers transform into seed pods or capsules,

which release seeds as they ripen (Severino & Auld, 2013). The castor plant's deep root system allows it to thrive in various soil types and endure drought conditions. Seed production starts from six month and it continues 6-7 months yielding throughout the year. It shows slow growth in winter and sometimes plants die while in spring plants show rapid growth (Kumar et al., 2015). Its output volume starts to decline after the third generation, at which point planting must be recommenced. It acts as an annual crop in locations that get frost. It can thrive on marginal, underdeveloped land. It can withstand a variety of climatic conditions, has cheap cultivation costs, and yields more oil (between 45 & 50%). Castor seed is used to extract oil, which is mostly made up of non-drying, inedible ricinoleic acid triglycerides and accounts for over 95% of castor seed use (Patel et al., 2017).

Although the seeds of castor and seed cake are highly poisonous, their usage is restricted for a variety of purposes, most notably as food for humans and animals. Castor plants are utilized as urban decorative plants (Worbs et al., 2011). As a result, castor wastes such as the stems, seed cake, and leaflets could be used as feedstock for the manufacture of ethanol and biogas, while the oil from castor beans is helpful for the creation of biodiesel.

Commercial castor cultivation for the production of 220,000 tons of oil per year is done in 30 different nations. A lot of castor oil is produced in the nation of Brazil, Russia, China, Thailand, and India. The majority of the exports, 70%, come from India. 1.8 million tons more of castor oil were produced worldwide each year in 2018 (Panhwar et al., 2016).

2.9.1. Castor Seeds:

Castor plant seeds, also referred to as castor beans, are oval-shaped, substantial, lustrous, and have a distinct mottled pattern. They typically have a length of 1 to 1.5 centimeters and 5 to12 mm width. Seed production starts from the first year. Castor seeds have a thick, hard bright brown- red in color shell called hull that protects the white, oily endosperm inside, which is where castor oil is made. Hull is easily separated from kernel. The hull contributes 20 to 30% of seed weight. Whole seed has 47% to 51% oil. 40% and 60% of seed has triglyceride rich oil that is mainly ricinolein (Carrino et al., 2020).

2.9.2. Castor seed cake:

Castor bean farming and processing have increased over the past several years as a result of

the increase in vegetable oil output for biodiesel. The castor bean cake, which has great chemical qualities for use in farming and contains a high amount of nitrogen along with other crucial elements, is a significant co-product of the extraction of castor oil. 530 kg of cake are produced for every ton of castor seeds that are processed (Mondal et al., 2019). Due to its high nutrient content, the beans of castor cake has been used as an organic fertilizer all over the world. Because it is a great source of nitrogen and possesses nematicide and pesticide qualities, castor cake is frequently employed as an organic fertilizer. It contains about 43% protein. The industrial technology for detoxication determines how it is used in animal feed. A recipe's growth in its supply chains and subsequent profitability are made possible by treating this material properly (de Oliveira Sousa et al., 2022).

2.9.3. Biogas Production from castor seed cake:

As castor seed cake has many nutritional values, but due to the presence of ricin it is unfit for human consumption. The anaerobic digestion process is commonly employed for biogas production from organic waste materials. Castor seed cake can be used as a substrate in anaerobic digesters, where microorganisms break down the organic matter to produce biogas. The high protein content of castor seed cake provides a favorable substrate for microbial activity, leading to efficient biogas production (Saha, 2020).

A number of studies have been conducted on biogas potential of castor seed cake. Mono-digestion of seed cake have resulted low amount of biogas due to potentially lower values of carbon to nitrogen in the feedstock. Combination of castor seed cake with other feedstocks such as cattle manure eventually increased the overall biogas yield which was due to the adjustment of C/N ratio in the digestors (Quezada-Morales et al., 2023).

The current study's objective was to assess the potential for castor seed cake-derived biogas as a whole. For phytochemical analysis, solvent extraction method was employed. Leftover residues, potential waste material, was also used as feedstock for biogas production. In this study batch and two stage continuous reactors were set up to calculate the biogas yield. Five type of solvents were used for phytochemical extraction; i) Methanol ii) n-hexane iii) Chloroform iv) Methyl acetate v) Ethyl acetate. Residues after the extraction process, whole seed powder, seed cake and oil were used as a feedstock for biogas production.

Chapter 3

MATERIALS AND METHODS

Material and Methods:

3.1. Substrate for Anaerobic Digestion

The current study was designed for the biogas production from residues left after the solvent extraction, seed cake, castor oil and whole seed powder. All these substrates were obtained from Sustainable Bioenergy and Biorefinery Lab, Quaid-i-Azam University Islamabad.

3.2. Substrate Characterization (Total and Volatile solids determination):

For the determination of different analysis like Total solids (We employed various equipment in accordance with the regulations to measure things including Total solids (TS), Volatile solids (VS), comprising all the feedstocks, and animal dung TS), Volatile solids (VS), of all the feedstocks and cattle manure we used different apparatus according to the standards. We determined the VS and TS according to the standard method of National Renewable Energy Laboratory's Analytical procedure by Archer et al., 2014.

3.2.1. Total solids (TS) of substrate

To determine the TS of the sample first the crucibles were burned for two hours at 550 °C in a muffle furnace with all of the organic material attached. Then the crucibles were cool down at room temperature.

After cooling these crucibles were weighted at the electrical balance.

Then the sample was added into the crucibles and weighted again along the sample.

The weight of the crucibles was subtracted from the sample's weight to determine the sample's weight. Three copies of each sample were taken.

The sample was then dried entirely dehydrated for 24 hours within an oven at 105 °C. The crucible and the dry sample were then measured and the dry sample's weight was calculated by subtracting the weight of each of the sample's filled crucibles from the entire sample's weight.

Using the equation listed below, the sample's TS was calculated.

$$\text{TS \% of Sample} = \frac{\text{(Weigh of Dried Sample)}}{\text{(Weigh of Initial Sample)}} \times 100$$

- TS was calculated in triplicates and mean value was considered as TS of sample.

3.2.2. Volatile solids (VS) of substrate

- The dried sample was burned at 550 °C for two hours in the muffle furnace to estimate the sample's VS.
- At this temperature the organic materials are volatilized and the remaining ash was consist of inorganic material.
- After the incineration the crucibles were kept at room temperature to cool down and then they were weighted.
- The weight of ash was determined by subtracting the weight of empty crucible from the weight of the ash containing crucible.
- The VS of the sample was calculated by the following equation.

$$\text{VS\% of the TS} = \frac{\text{(Weigh of Dried Sample)} - \text{(Weigh of ash)}}{\text{(Weigh of Dried Sample)}} \times 100$$

3.3. Inoculum development:

Inoculum generation was performed in order to initiate an anerobic digesting process. Highly active anaerobic bacteria that support breakdown make up the inoculum. Cattle may serve as the vaccine. dung diluted with water and utilized as a slurry, Alternatively, it might be taken from an active anaerobic digester.

Fresh cow manure was obtained from a local dairy farm for inoculum formation. Water was added in 7:3 to make a semi thin homogenized paste of the manure by vigorous shaking and

was screened for solid inert material. The mixture was added to reactors plugged with rubber corks. Two holes were made into the cork and pipes were fitted into them. One pipe supplied the reactor, while the other was used for gas collection. A gas collecting pipe is equipped with a gas bag for the collection of biogas. As soon as the microorganisms became active and the anaerobic reaction reached a stage of biogas generation, reactors containing the combination were acclimatized at 37 °C for a few weeks. This process involved flushing the reactors with nitrogen gas to remove any oxygen that may have been present.

3.4. Reactor Setup:

The present study was designed for the evaluation of biogas production potential of castor seeds. Both batch and continuous reactors (two stage) were designed for experiments.

3.4.1. Anaerobic digester setup for batch process:

For designing batch reactors, glass bottles of working volume of 200ml was used. Opening of the reactor was covered with a rubber cork. One opening in the cork with a copper pipe was serving as a gas outlet plugged in glass bottles. Plastic gas tight bags with capacity of 1000 ml were attached to the reactor through a transparent plastic pipe having one side attached with the copper pipe in the rubber cork while the other side was attached with the bag.

Start-up of the batch experiment:

To evaluate the biogas potential of the castor seeds, eight type of feedstocks were used. Each setup was run in triplicate. Inoculum was used a negative control while cellulose was used as a positive control. Initially all the reactors were fed with calculated amount of inoculum and substrate. Nitrogen flushing was done for 45 seconds to remove excess of oxygen and to avoid reactor failure. With the help of diluted NaOH and H_2SO_4 solutions, the pH was raised to 7. With the use of an incubator, the temperature was maintained at 37°C. Each day, the reactors were given a thorough shakedown before the biogas was measured with a 60ml syringe. For 72 days, the experiment was carried out. The generation of biogas and biomethane was monitored daily for the first 21 days, but every third day after that. Biogas was fed through a reactor containing a scrubbing solution (3M NaOH), which efficiently collected CO_2 and released methane, in order to calculate the amount of biomethane produced. The measured

amounts of biogas and biomethane were converted to normalized liters (at standard pressure and temperature) and reported.

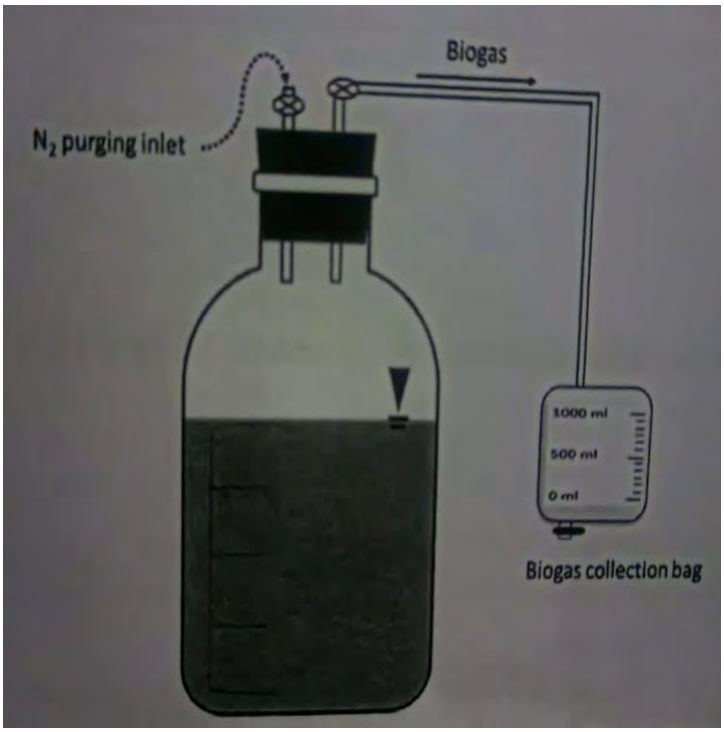

Fig 3.1. Schematic diagram of batch anaerobic digester setup

Calculations for the substrate:

Amount of Substrate=Working Volume*VS of Inoculum/VS of Inoculum*4*VS of Substrate

Amount of Inoculum=Working Volume - Amount of Substrate

Table 3.1: Addition of calculated amount of substrates and inoculum in reactors

Reactor	Inoculum added (ml)	Substrate added |(g)
Ethyl acetate residues	170.59	29.41
Methyl acetate residues	172.95	27.05
Methanol residues	179.6	20.4
n-Hexane residues	184.28	15.72
Chloroform residues	182.58	17.42
Seed cake	182.16	17.84
Whole seed	191.01	8.99
Positive control	198.4	1.6
Negative control	200ml	0

3.4.2. Anaerobic digester setup for continuous process:

In the continuous process, two stage reactors were performed. The 500 ml glass reagent bottles that made up the reactors' working volume were used as the reactors' total volume. Empty space was remained for biogas accumulation in the reactor as well as shaking the mixture in the reactor. To seal the bottles, rubber stoppers with two hosesat the top of hydrolytic reactor 1 (R-1) and three hoses at the top of reactor 2 (R-2) were utilized (methanogenic reactor). The gas generated in R-1 is transported to R-2 through a pipe. Each reactor includes hose gas line for feeding the substrate and collecting effluent.

3.5. Operational Parameters

Following operational parameters were studied. Operational parameters include the FR, OLR, SRT, and concentration of the effluent.

- Hydraulic retention time (HRT)
- Organic loading rate (OLR)
- Flow rate
- Concentration of effluent
- Weight of the substrate

3.5.1. Hydraulic Retention Time (HRT)

Reactors had a 10-day hydraulic retention period. This time is more than the doubling time of methanogens and most of organic matter can be degrade in this much time. Set up for each OLR was run for 25-30 days with 2 to 3 retention times.

3.5.2. Organic Loading Rate (OLR)

It's the measure of organic content per component volume of reactor in a specific period of time. Over the course of around three retention periods, the reactors were run at OLR of 1g VS/L/day.

3.5.3. Flow rate of effluent

Flow rate of effluent can be calculated by using formula:

Flow rate = working volume of the reactor/ HRT

Reactor working volume=

250ml

0.25 L (250 ml)

Retention time = 10 days

Flow rate = 0.25 L / 10 days

Flow rate = 25 ml/day (0.025 L/day)

3.5.4. pH

pH for continuous reactor will be checked on daily basis and will be recorded i.e., 6.9 on average.

3.5.5. Temperature

The reactor temperature will be maintained at 39°C. In this study, a water bath at 39°C was used for temperature maintenance. Since we know that methanogens and other microorganisms in general, are quite sensitive, with a one-degree difference causing variances in the results.

3.6. Measurement of Biogas

The biogas generated by the reactor will be collected using a water displacement system or gas bags. Biogas is often measured using syringes in water displacement methods, and results are recorded. Plastic bags were used to collect biogas while in the gas bags. The benefit of employing awater displacement method over gas bags is that we can add a little quantity of acid to the water. Consequently, CO_2 is not absorbed by the water, and biogas can be collected and measured.

3.7. Measurement of Methane Content

Scrubbing process is employed to determine the methane content. Biogas collected in the gas bags linked to R-2 was measured, and each day, the gas was removed from the bags using a 70 ml syringe. A glass container containing 3M NaOH scrubbing solution was attached to each reactor when the experiment reached steady state (the point where the rate of gas production almost became constant). The biogas passes through this solution first, absorbing CO_2 and some concentrations of H_2S from it, and the remaining CH_4 then accumulates in the gas bags, which is measured using a syringe. As a result, only CH_4 will be left in the syringes while CO_2 is absorbedinto the NaOH solution. So, using that information, we can determine how much methane the reactor has produced overall.

3.8. VFA And Alkalinity Estimation:

The VFA and alkalinity of the effluent was determined according to APHA Standard Methods, following procedure was used.

10 to 20 ml of sample was taken in small flask or beaker and the initial pH was measured.

After adding 0.1 mol $H2SO_4$, the sample's pH was reduced to 4.3, whereas the total quantity of acid used to do so was recorded for the purpose of calculating the alkalinity. Then pH of sample was brought to 3.5 adding more acid.

Boiled the sample for 2-3 min on an electric hot plate.

Following the addition of 0.1 mol of NaOH to get the sample's pH to 7, the system's VFA was determined using the NaOH concentration.

3.8.1. Calculations for VFAs And Alkalinity:

Alkalinity (mg/L) = V mL of acid consumed X Normality of the acid used X 50000/ Volume mL of sample

VFA (mg/L) = V mL of alkali consumed X Normality of the alkali used X 50000/ Volume mL of sample

Chapter 4

RESULTS

Results:

Castor seeds and residues left after solvent extraction were used as a feedstock for the biogas production. The total solids and volatile solids of the substrate and inoculum was determined and shown in the Table 4.1.

Table 4.1. Characterization of feedstock

Characterization	Inoculum	Whole Seed	Seed Cake	Methanol residues	Methyl acetate	Ethyl acetate	n-Hexane	Chloroform
Total Solids (%)	4.60	98.92	93.2	85.99	78.57	90.47	90.71	94.41
Volatile Solids (%)	3.41	1.08	6.75	14.01	21.43	9.53	9.29	5.59
Moisture Content (%)	95.40	95.67	93.3	83.90	75.47	99	78.26	92.57
Average VS of TS (%)	74.27	94.63	87.0	72.14	59.30	89.56	70.99	87.39

4.1. Batch Anaerobic digestion:

Batch experiment was designed to determine the biogas potential of castor seeds, seed cake and multiple residues left after the solvent extraction process. Triplicate reactors were run for all the setups by keeping the temperature constant for 30 days until no or less biogas production was observed. Inoculum was used a negative control with no substrate feeding was done while cellulose was used as a positive control. The reported results present the average biogas yield from substrate after excluding the biogas produced from the negative control.

During the batch anaerobic digestion maximum biogas yield was exhibited by the oil which was 619 Nml/g of VS (0.619 NL/gVS). After 30 days maximum reactors reduced biogas production due to the complete degradation of the added feedstock. Methanol and Ethyl acetate residues also produced good amount of biogas, 499 Nml/g of VS and 564 Nml/g of VS respectively. Amount of biogas produced from different feedstocks during the whole experiment is showed in the Figure 4.1.

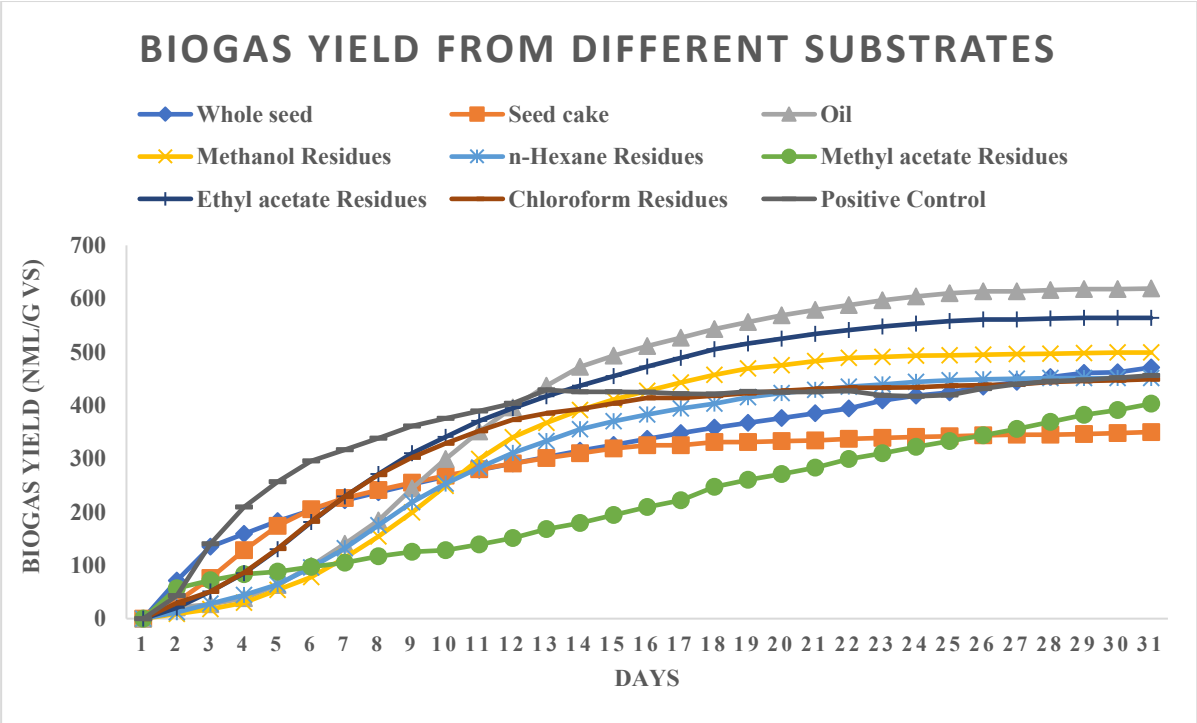

Fig 4.1. Overall trend of biogas production rate from castor seed, seed cake, oil and residues obtained after the solvent extraction process in batch anaerobic digestion

Fig 4.2 shows the overall biogas production form all substrates in the batch experimentation. Oil produced highest biogas 619 Nml/g of VS (0.619 NL/g VS) followed by Ethyl acetate and Methanol residues which produced 564 Nml/g of VS (0.564 NL/g VS) and 499 Nml/g of VS (0.499 NL/g VS) respectively. Seed cake showed minimum biogas production 350 Nml/g of VS (0.350 NL/g VS).

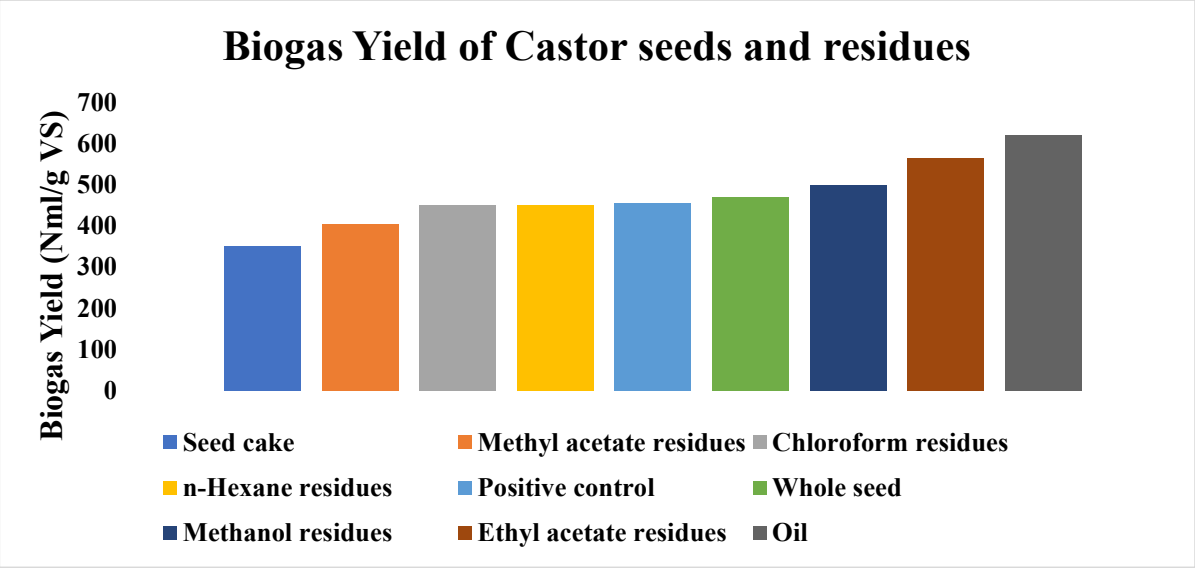

Fig 4.2. The volumetric biogas production rate in batch process from castor seeds and residues

4.2. CH_4 Content:

Amount of methane gas present in the biogas was also calculated by using the scrubbing solution. 3M NaOH solution was prepared and biogas collected from the gas bags was passed through the solution. NaOH absorbed the carbon dioxide and the remaining gas was measured as methane content in the biogas. Figure 4.3 depicts the percent methane content present in the biogas obtained from whole seeds, seed cake, oil and residues. Maximum methane content was shown by seed cake and chloroform residues, 81% and 77% of the biogas respectively.

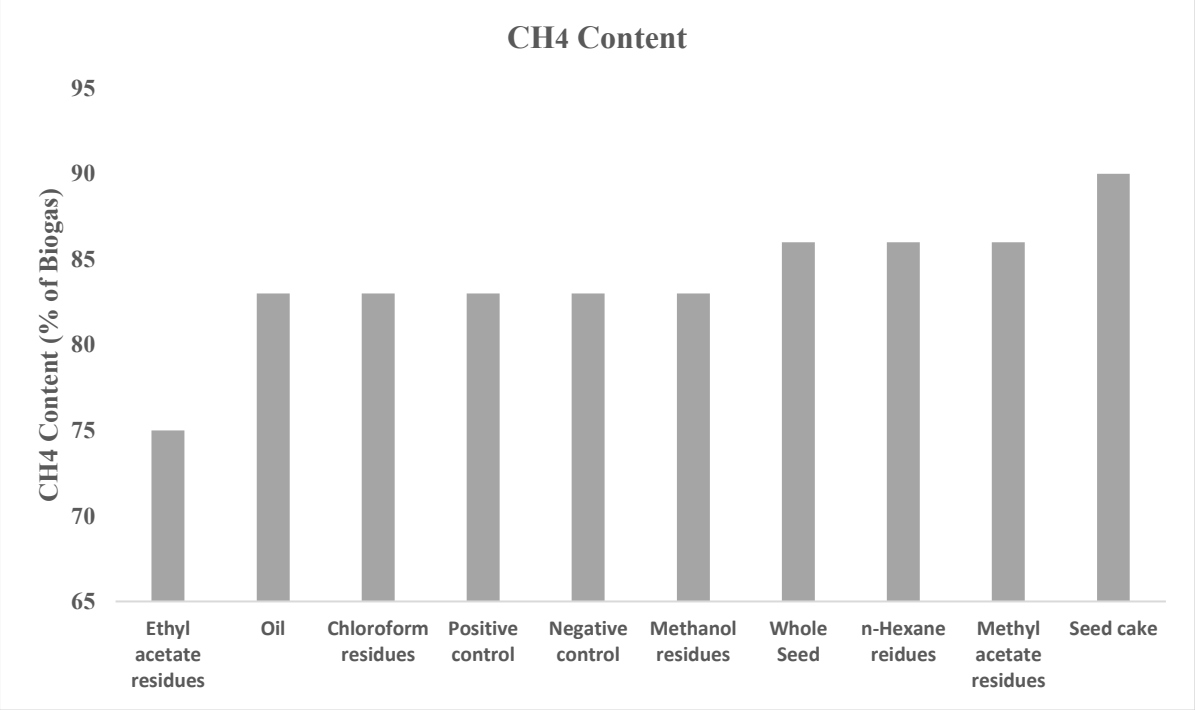

Fig 4.3. Effect of the feedstock on the methane content present in the biogas

4.3. Volatile Fatty acids and alkalinity in the digestate:

After the completion of the batch experiment, volatile fatty acids and alkalinity of the digestate of the reactors were determined. Volatile fatty acids and alkalinity are represented in mg/L in the figure 4.4.

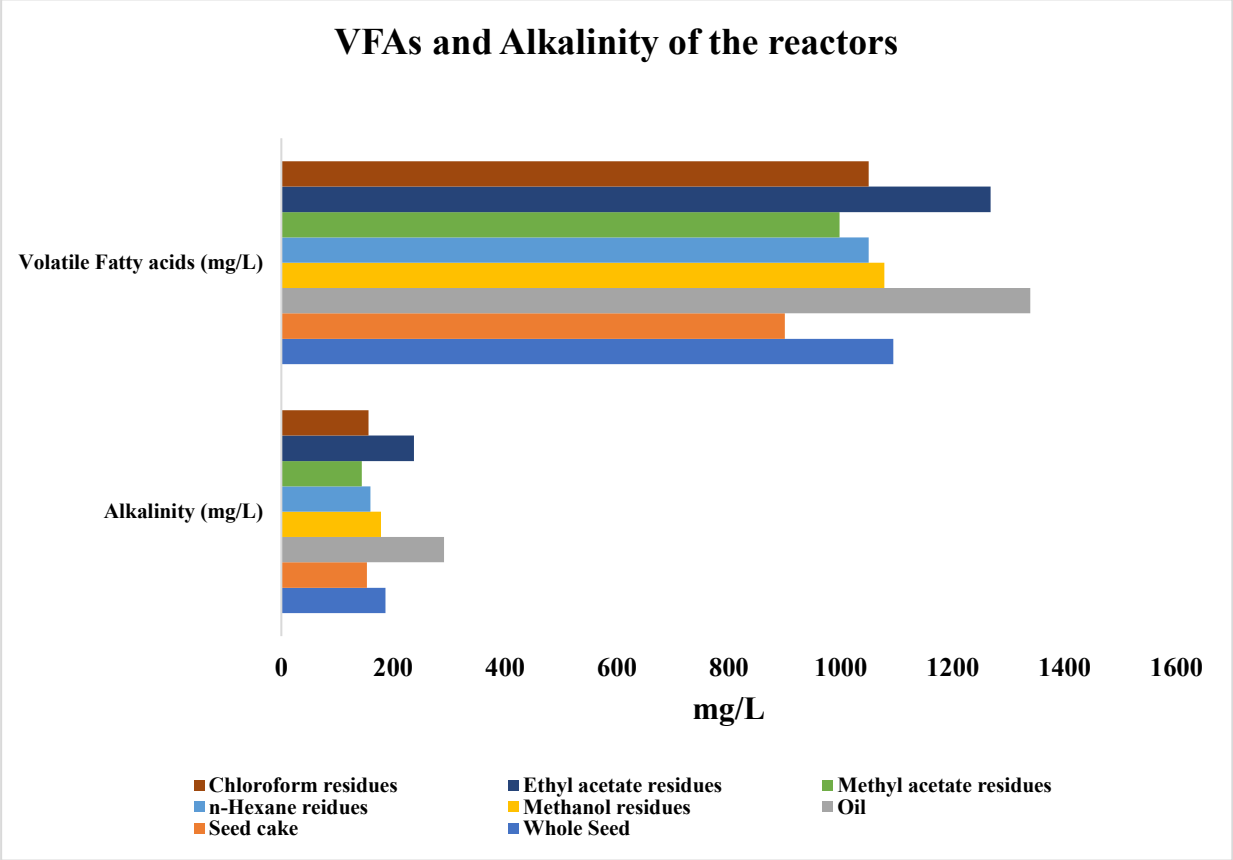

Fig 4.4. Changes in VFAs and Alkalinity during the batch anaerobic digestion process

4.4. Continuous anaerobic digestion:

In order to calculate the daily biogas production from castor seeds, seed cake, oil and residues, continuous two stage anaerobic reactors were set up. All these reactors were run at certain operational parameters. HRT and OLR was kept constant at 10 days and 1g of VS/L/day. Reactor setup was continued for almost one HRT after achieving the steady state (daily same amount of biogas production) in the reactors. VFAs, Alkalinity and pH of the reactors were

calculated on regular basis and after the completion of the reaction % VS reduction of the substrates through anaerobic digestion process was calculated.

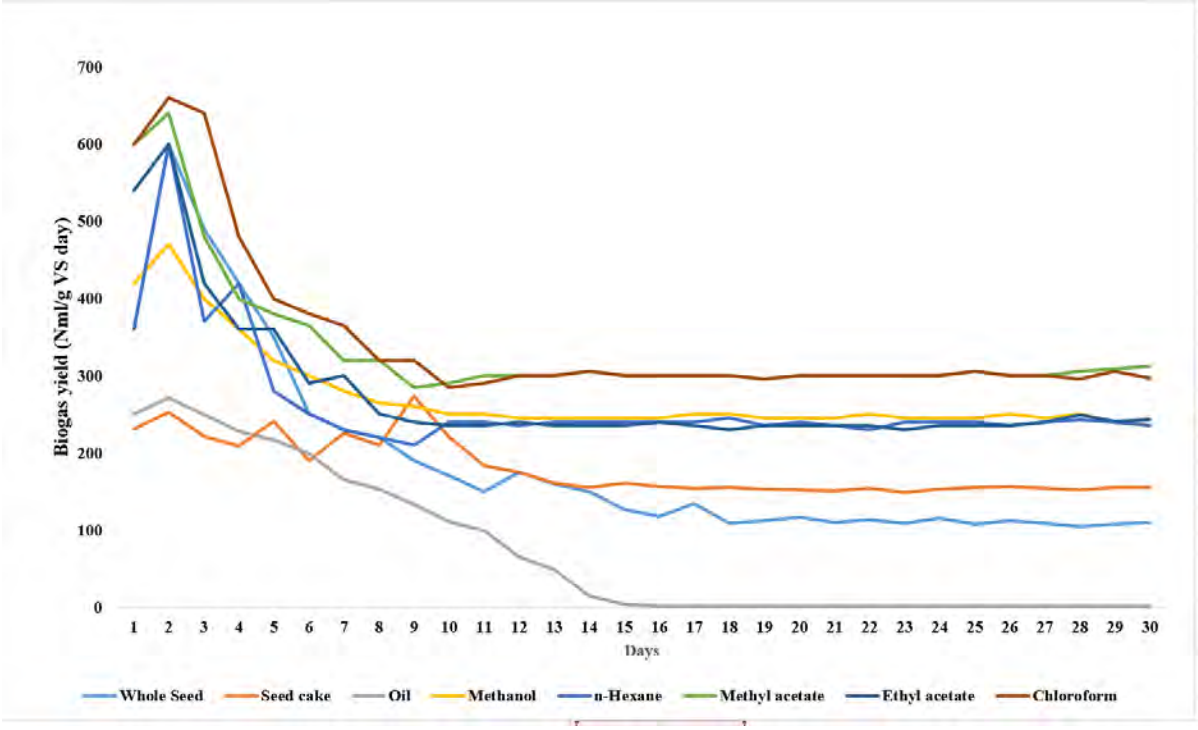

Figure 4.5. Overall trend of biogas production rate from castor seeds, oil and residues in a two-stage anaerobic digestion process

In the continuous reactors' methyl acetate showed the highest amount of biogas production, while whole seed produced very low amount of biogas production per day. Amount of biogas produced was calculated after achieving the steady state of the reactor

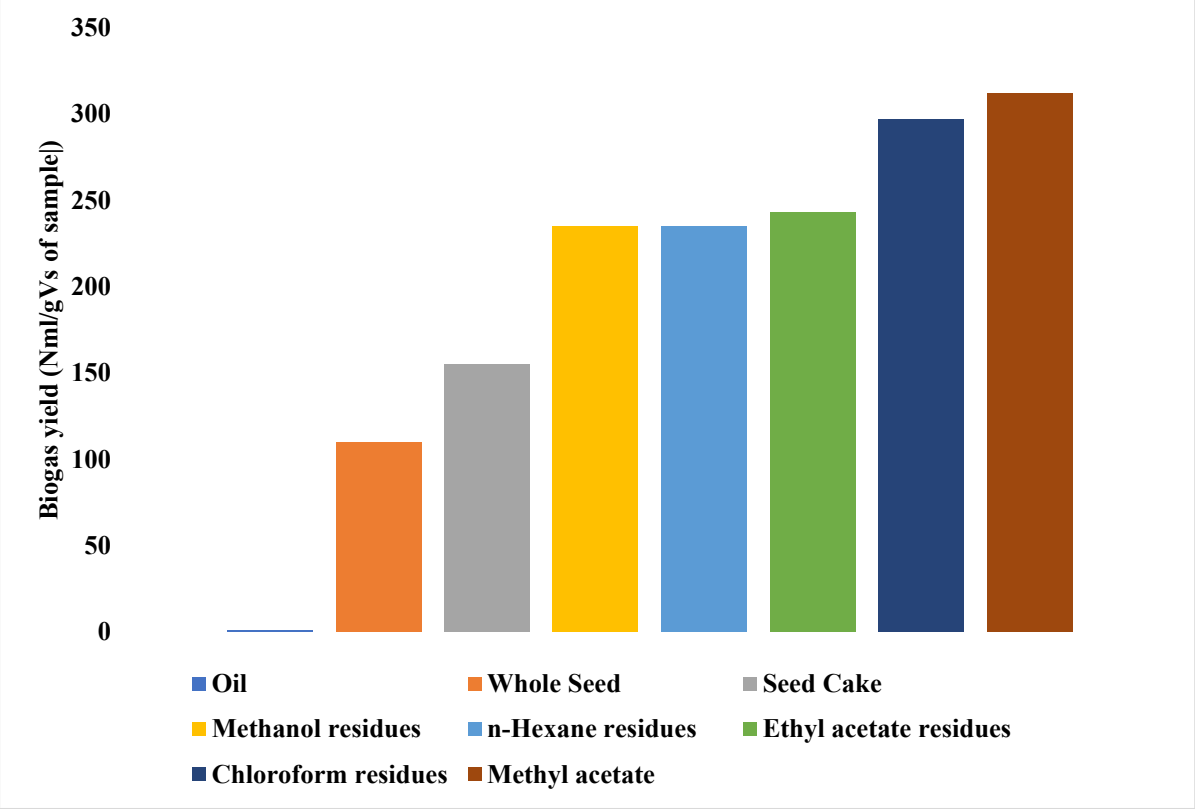

Fig 4.6. The volumetric biogas production rate in continuous reactors from castor seeds and residues

4.5. VFA and Alkalinity of the reactors:

VFAs production in acidogenic reactors was in the range of 1750-2000 mg/L while VFA accumulation in the methanogenic reactors was in the range of 550-720 mg/L.

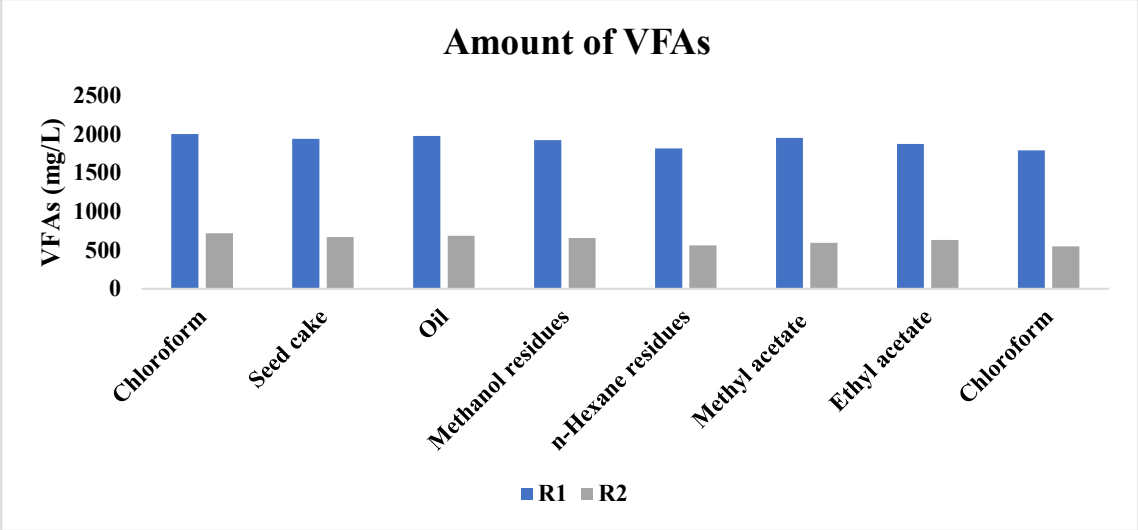

Fig 4.7. Effect of different substrates on the production and accumulation of VFAs in the acidogenic (R1) and methanogenic (R2) reactors

The alkalinity of the acidogenic reactors was in the range of 850-1050 mg/L while that of the methanogenic reactors was in the range of 550-720 mg/L

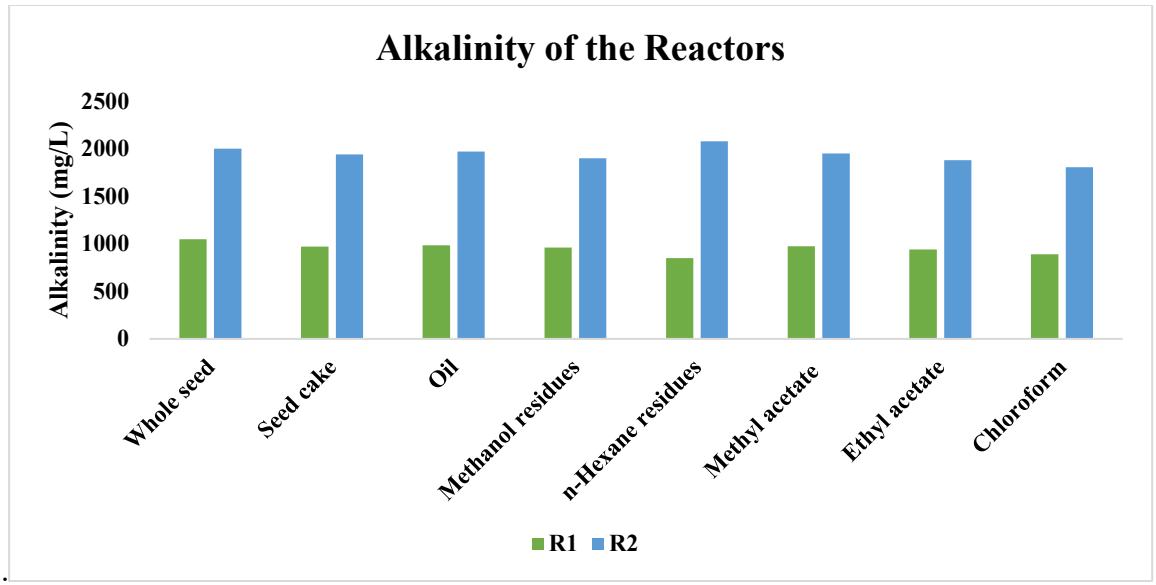

Fig 4.8. Alkalinity range in acidogenic (R1) and methanogenic (R2) reactors in different substrates

VFAs/alkalinity ratio of all the reactor setups is shown in the figure 4.8.

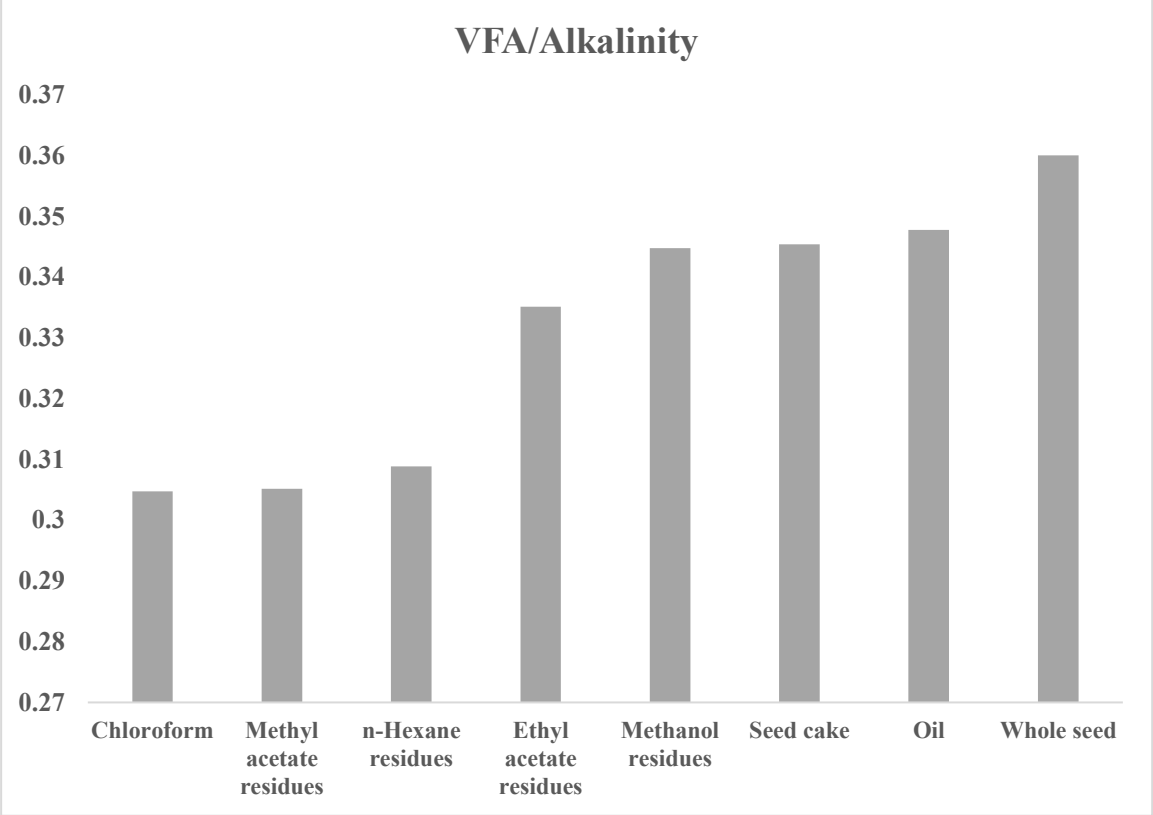

Fig 4.9. The VFA/Alkalinity ratio in all the reactors in continuous reactors

VS reduction in all the reactors was calculated after the reaction completion of the reaction. Maximum amount of VS reduction was achieved in methyl acetate residues reactor followed by the n-Hexane and Ethyl acetate residues reactors.

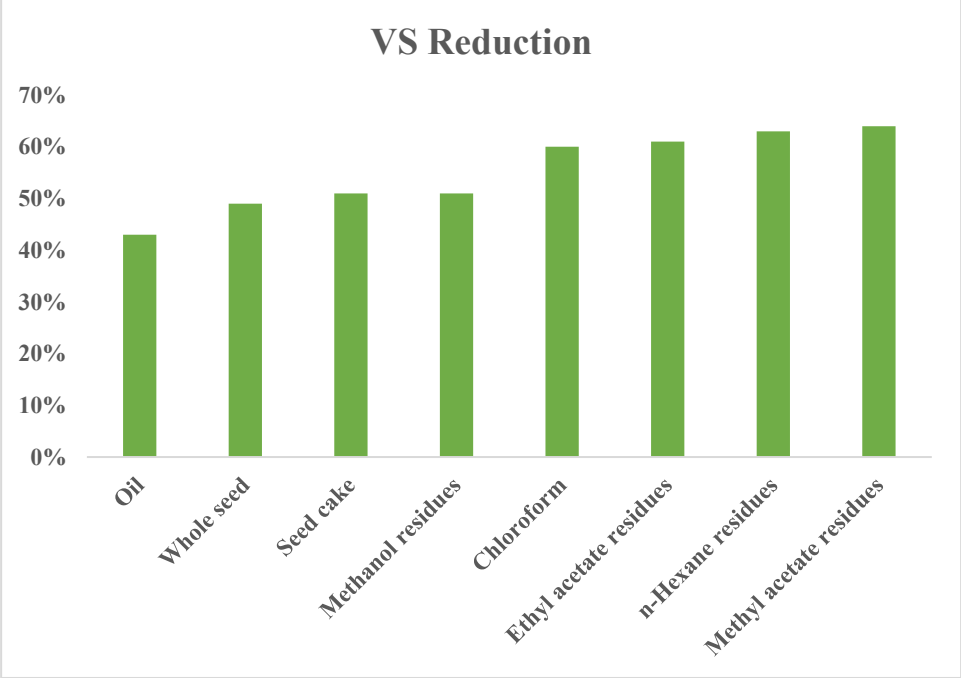

Fig 4.10. The % VS reduction in all reactors during anaerobic digestion

4.6. pH of the reactors:

The pH of the acidogenic reactor was in the range of 4.9-5.5 which is optimum for the process of hydrolysis and acidogenesis while the pH of the methanogenic reactor was in the range of 6.8-7.2 which is ideal for growth of methanogenic.

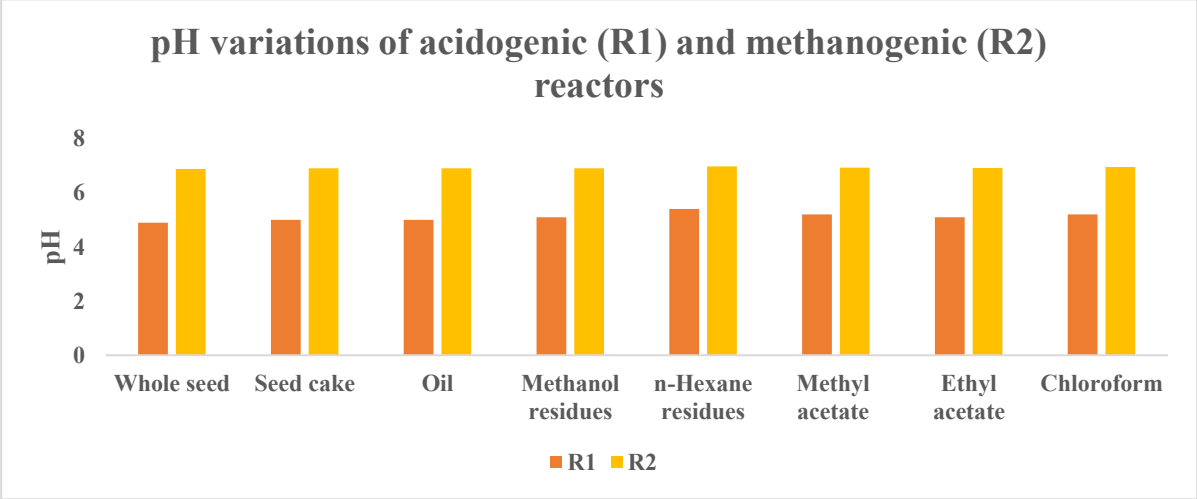

Fig 4.11. pH variations of acidogenic (R1) and methanogenic (R2) reactors

Chapter 5

DISCUSSION

Discussion

Current study was conducted to evaluate the biogas potential of castor seeds, seed cake, oil and different types of residues left after the solvent extraction in the both batch and continuous processes. For the proper feedstock addition, it was first subjected to the analysis of different parameters e.g., total solids, volatile solids and moisture content. Characterization of all the feedstocks is listed in the table 4.1.

Batch experiment was designed to determine the biogas potential of castor seeds, seed cake and multiple residues left after the solvent extraction process. Triplicate reactors were run for all the setups by keeping the temperature constant for 30 days until no or less biogas production was observed. During this time period maximum reactors reduced biogas production due to the complete degradation of the added feedstock. During the batch anaerobic digestion maximum biogas yield was exhibited by the oil which was 619 Nml/g of VS (0.619 NL/g VS).

After 30 days maximum reactors reduced biogas production due to the complete degradation of the added feedstock. Methanol and Ethyl acetate residues also produced good amount of biogas, 499 Nml/g of VS and 564 Nml/g of VS respectively. Amount of biogas produced from different feedstocks during the whole experiment is shown in the Figure 4.1. Very few studies have been reported till now on the production of biogas from castor seeds and residues. Seed cake produced 350 Nml/ g of VS of substrate biogas. Hamed Bateni et al., conducted similar type of work from *Eruca sativa* seeds and reported that after the oil extraction remaining residues called seed extracts have great potential for biogas production.During batch anaerobic reaction oil produced maximum amount of biogas 619 Nml/g of VS. Compared to proteins and carbohydrates, oils have a larger energy content. Accordingly, a higher yield of biogas is produced when oils are broken down since they release more energy. Carbon-to-nitrogen ratio of oils is typically lower. Because it encourages the growth of methane producing bacteria, a lower carbon-to-nitrogen ratio is advantageous for the generation of biogas. In this study although oil gave maximum yield but its degradation was comparatively slower than other substrates (Pastor et al., 2013).

Continuous reactors were also set up to calculate the biogas yield from *Ricinus communis* seeds. For this purpose, all the reactors were run at constant OLR 1g/VS of the substrate and HRT was chosen 10 days. This time is mostly enough for the degradation of the organic matter being fed in the anaerobic digester and microorganisms increase their number significantly (Wainaina et al., 2019). In continuous reactor setup oil produced lowest amount of biogas and this reactor was failed at the end as it stopped producing biogas. It's because lipids found in oils can build up and create the scum-like layer that is seen on the reactor's surface. The operation of the reactor may be hampered and the system may become clogged as a result of this scum layer's ability to obstruct the transfer of gases and nutrients. Some oils, particularly those originating from specific sources or containing significant concentrations of pollutants, might be hazardous to the bacteria that produce biogas. If the toxicity levels are high, this toxicity may even cause system failure. It might limit the microbial activity, resulting in decreased biogas generation. Another major reason is that due to the high amount of carbon present in oils it can cause the accumulation of VFAs in the reactor which may lead to the reactor failure (Alhraishawi et al., 2020). Pastor et al (2013) also studied the biogas production from used oils. He also reported that during batch process despite of the fact that biogas production was delayed, but it produced maximum amount of biogas as compared to all other substrates. But this trend was reversed in the continuous process. Oil alone did not produce significant amount of biogas and eventually reactor was failed. He also studied the co-digestion of oil with sludge in different concentrations and found that less than 1% oil concentration in the reactor enhanced the biogas yield but higher concentrations had adverse effects on the biogas production (Pastor et al., 2013).

The results of the current study demonstrate that the *Ricinus communis L* seeds are good option to be used as a substrate for biogas production. Seed cake and residues obtained after the solvent extraction process, which is a waste material, can be utilized to produce biogas to resolve country's energy crisis and environmental problem as well.

CONCLUSION

Conclusion:

Increased population and globalization have created number of problems that include energy shortage and climate change. Need of the hour is to develop sustainable development goals to combat the energy shortage and constantly increasing global warming. To deal with energy requirements for daily use there is a need for new sustainable, renewable, and cheap sources. For this purpose, many countries are making biofuels from biomass. For countries like Pakistan, it's better to obtain energy from cheap sources. *Ricinus communis* seed is a renewable feedstock for biogas production. This study was carried out to study the biogas potential of *Ricinus communis L* seeds, seed cake, oil and residues obtained after the solvent extraction process. Both batch and continuous reactors were set up at controlled temperature 37°C. during batch anaerobic digestion process *Ricinus communis* seeds and residues showed remarkable potential for biogas production. Ethyl acetate residues and castor oil produced maximum amount of biogas. During the continuous reactors, the organic loading rate of this setup was maintained at 1g of VS of substrate and hydraulic retention time was kept constant at 10 days and flow rate was 25 ml daily. Maximum biogas yield was achieved by methyl acetate residues which was 312 Nml/ g of VS of substrate added per day, followed by chloroform 297 Nml/ g of VS of substrate per day. Minimum yield was observed in the whole seed reactor 110 Nml/ g of VS of substrate per day. VFAs and alkalinity of the reactors were also measured regularly. Maximum VS reduction was achieved in the methyl acetate residues around 64% followed n-Hexane and ethyl acetate residues in which VS reduction was observed around 63 and 61% respectively. This study indicates that *Ricinus communis* seed is potent feedstock for biogas production.

FUTURE PROSPECTS

Future Prospects:

- Effect of co-digestion of castor seed residues with fruit and vegetable waste, cattle manure and other carbon rich substrates can be evaluated in both batch and continuous reactors.
- Investigation of the effects of the solvents on the microbial communities during anaerobic digestion steps
- Effect of different HRT on biogas production
- Biogas production potential of these substrates can also be studied at thermophilic temperatures.
- Effects of acidic and alkaline pretreatments of castor seed residues on biogas and biomethane production can also be studied.
- Effect of different leftover residues on the stages of anaerobic digestion and microbial communities involved can also be studied.

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