Development of Fermentation Process for Production of Bio-based Energy Carriers from *Spirodela polyrhiza*

By

Qurrat Ul Ain Rana

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
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A thesis submitted in partial fulfillment of the requirements for the Degree of

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Qurrat Ul Ain Rana

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Islamabad
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Dedication

This thesis is dedicated to all the torch bearers and seekers of knowledge who are consistently and continuously trying to breach the barriers of unknown for the betterment of this world.

Author's Declaration

I **Ms. Qurrat Ul Ain Rana** hereby state that my Ph.D. thesis titled **"Development of Fermentation Process for Production of Bio-Based Energy Carriers from *Spirodela polyrhiza*"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

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Student Name: **Ms. Qurrat Ul Ain Rana** Signature: Qurrat ul ain Rana

Examination Committee:

a) External Examiner 1: Signature:

Dr. Syed Muhammad Jamal
Associate Professor
Department of Biotechnology
University of Malakand, Chakdara
Dir (L), Khyber Pukhtoonkhwa

b) External Examiner 2: Signature:

Dr. Hazrat Ali
Senior Scientist
National Institute of Biotechnology and Genetic Engineering
P.O. Box 577, Jhang Road, Faisalabad

Supervisor Name: **Dr. Malik Badshah** Signature:

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature:

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List of Acronyms

$(NH_4)_2SO_4$	Ammonium Sulphate
° C	Degree Celsius
µl	microliter
µmol	micromoles
AD	Anaerobic Digestion
AIR	Alcohol Insoluble Residues
Apo	Apiose
Ara	Arabinose
CCD	Central Composite Design
CFU	Colony Forming Units
CTAB	Cetyltrimethylammonium Bromide
DM	Dry Mass
DNS	Dinitrosalicylic Acid
E	Energy
Fuc	Fucose
FW	Fresh Weight
g	grams
Gal	Galactose
GalA	Galacturonic Acid
GHG	Green House Gasses
Glc	Glucose
GlcA	Glucuronic Acid
H_2SO_4	Sulphuric Acid
HCL	Hydrochloric Acid
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
IL	Ionic Liquid
ISR	Inoculum to Substrate Ratio
KH_2PO_4	Potassium Dihydrogen Phosphate
LED	Light Emitting Diode
Man	Mannose
mg	milligram
$MgSO_4$	Magnesium Sulphate
ml	milliliter
nm	nanometer
NPK	Nitrogen Phosphate Potassium
OD	Optical Density
PAD	Pulse Amperometric Detection
Rha	Rhamnose
RPM	Revolution Per Minute
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulfate
TFA	Trifloroacetic Acid
TS	Total Solids
UV	Ultra Violet

VS	Volatile Solids
WLN	Wallerstein Differential Nutrient Agar
Xyl	Xylose
YFM	Yeast Fermentation Media

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Qurrat Ul Ain Rana

Abstract

Energy is the driving force for socioeconomic development. Currently, world's energy needs are fulfilled by non-renewable energy sources like fossil fuels which are depleting readily and come with the price of environmental pollution. This has led to the quest for alternative energy sources like renewable biofuels. Bioethanol and biogas are two of the promising biofuels that can help in minimizing dependence on crude oil and natural gas, respectively. However, currently bioethanol is produced from food crops that is raising food versus fuel feud. *Spirodela polyrhiza* is one of the novel feedstock that can be used to produce bioethanol at large scale without causing any food and fuel competition. *Spirodela polyrhiza* is a hydrophyte with high growth rate, ability to accumulate high starch content in its biomass and has high biomass yield which makes it an excellent alternative feedstock for the production of bioethanol and biogas. In addition to high starch accumulation in its biomass, *Spirodela polyrhiza* has also the ability to to phytoremediate wastewater like agricultural runoff. The excessive use of agrochemicals to fulfill the high demand of agricultural products has been imposing numerous negative impacts on the environment for decades worldwide. Untreated wastewater with high chemical load and unutilized nutrients of fertilizers and pesticides released from agricultural farms results in contamination and intoxication of clean water bodies. Utilization of *Spirodela polyrhiza* for the treatment of agriculture wastewaters is an efficient and cost-effective method due to its excellent nutrient uptake abilities. Production of valuable *Spirodela polyrhiza* biomass during phytoremediation of agricultural runoff also serves as an alternative feedstock for the production of biofuel (bioethanol) which is another precedence of using this method. This research work comprised of four phases.

In phase 1 *Spirodela polyrhiza* from a local pond was collected and its growth was established in Hoagland growth media. The plant was then given nutrient starvation stress to enhance its starch content by 78%. The high starch containing plant biomass was acid pretreated and 99.3% starch to glucose conversion was achieved. In order to ferment plant sugars, yeast strain *Saccharomyces cerevisiae* QG1 MK788210 was indigenously isolated. In order to achieve high ethanol yield the isolated yeast strain was statistically optimized using Plackett-Burman and Central Composite Design. The fermentation of plant sugar by *Saccharomyces cerevisiae* QG1 MK788210 resulted in 100% theoretical ethanol yield thus successfully achieving complete conversion of *Spirodela polyrhiza* starch to bioethanol. The study conducted demonstrated effective optimization of indigenously isolated yeast

strain *Saccharomyces cerevisiae* QG1 MK788210 to deliver high ethanol yield from *Spirodela polyrhiza*. Application of nutrient stress also indicated *Spirodela*'s excellent absorptive capacity of various nutrients from water which indicates that it can also be used as biophytoremediator for nutrient polluted wastewaters like agricultural runoff.

In phase 2 of this research work, the potential of *Spirodela polyrhiza* for taking nitrogen and phosphates from the agricultural runoff was determined by growing it in the agricultural runoff under lab conditions**.** Up to 70% and 78% removal of nitrogen and phosphates respectively was achieved during the treatment of agricultural runoff by *Spirodela polyrhiza*. The starch content of *Spirodela polyrhiza* was elevated by 58% during 10-12 days of nutrient starvation at the end of the agricultural runoff treatment process. The biomass obtained was pretreated with dilute acid that liberated 96.4% glucose from the starch-rich biomass. In the last step, glucose was used as a substrate for the fermentation process carried out by *Saccharomyces cerevisiae* QG1 MK788210 yeast strain which yielded 99% volumetric conversion of glucose into bioethanol.

In the phase 3 of this research work, we evaluated the potential of *Spirodela polyrhiza* for production of both bioethanol and biogas in a sequential process and analyzed the ability to generate energy using this process. *Spirodela* was given nutrient stress to enhance its starch content by 80%. The plant biomass with high starch was dilute acid pretreated and starch to glucose conversion rate of 99.6% was achieved. The plant derived glucose was fermented with indigenously isolated and optimized yeast strain and 99.8% theoretical ethanol yield was obtained. The fermentation vinasse was anaerobically digested and 0.88 NL/g VS biogas yield was obtained. Further output energy analysis of processes producing bioethanol, biogas and both bioethanol and biogas sequentially from *Spirodela* feedstock was carried out which showed that highest amount of energy i.e., 18.5 MJ/g VS is produced in the process where high starch containing *Spirodela* plant biomass was used for the production of biomethane only. The results obtained showed that high starch *Spirodela* biomass is best used in terms of energy when it is anaerobically digested for production of biogas only. However, the type of fuel produced depends upon the need for which fuel production is desired.

By now, we have established that high starch *Spirodela* biomass can effectively be used for bioethanol production. In phase 4 of this research work the cell walls of duckweeds were studied and the results depicted that the duckweed cell walls have high proportion of three fermentable sugars that are galactose, glucose and xylose. The starch content of two duckweed specie, *Spirodela polyrhiza* and *Lemna minor* was enhanced by 80% and 72%

by applying nutrient stress, respectively. The cell wall analysis of high starch duckweeds was carried out and three fermentable sugars i.e., galactose, xylose and glucose were identified. The cell walls of these high starch duckweed biomass were treated with sequential hydrothermal and enzymatic saccharification and ionic liquid cholinium phosphate. The sequential hydrothermal pretreatment and enzymatic saccharification in case of *Lemna* and IL pretreatment in case of *Spirodela* were effective in releasing high amount of xylose and glucose from cell wall which, when fermented can effectively enhance the overall bioethanol yield from high starch duckweed biomass rather than utilizing duckweed starch alone.

Introduction

Energy is one of the prime factors that drives the development of a country. Energy stimulates the socioeconomic growth of developing countries. Thus, having access to the energy supplies is the basic need of modern society (Moreau & Vuille, 2018). Since 1900 expansion of human population and industrialization have raised energy utilization worldwide. Currently, fossil fuels like natural gas and crude oil are in use for energy production but due to their non-renewable nature the amount of these resources will be declined after year 2025. It has also been anticipated that oil production will also decrease worldwide from 25 billion barrels to 5 billion barrels around the year 2050 (Campbell, 2013). This unpreventable shrinking of fossil fuels and continuous increase in their demand led the world to explore for alternative energy sources.

To overcome the shortcomings of fossil fuels, renewable bioenergy is considered as the best alternative which can fulfil the global energy demand. Bioethanol, the alternative source to petroleum, is produced through sugar fermentation. It is a renewable source of energy in the transportation sector and eco-friendly in nature (Budzianowski, 2017; Wyman, 1999). Bioethanol production from the renewable biomass and its utilization in the replacement of fossil fuels, lowers the greenhouse gases emission. Its production can also provide job opportunities to the locals which can lead to social sustainability as well. During the last decade bioethanol's production has increased up to 85.2 billion litres per year and it is consumed as a transport fuel in the form of gasoline additive (Nahar & Sunny, 2020). Producing around 47% of total bioethanol, US stands at the highest point followed by Brazil in bioethanol production (Balat et al, 2008). 'Flex fuel' cars are widely used in Brazil which can use blend of bioethanol and gasoline (Amorim et al., 2011; Wang et al., 1999).

Different types of feedstock can be used for the production of bioethanol including starch, sugar and cellulosic biomass (Lennartsson et al., 2014). Currently, corn grains are widely used for bioethanol production. But to produce large amount of corn a large cropland is required. It is also considered as an important feed source (Endo et al., 2008). Due to extensive agricultural practices required and potential damage to soil, many environmental concerns have been raised for corn production as an energy crop (Pimentel, 2003).

Sugarcane juice and cane molasses are also used for the production of bioethanol (Wilkie et al, 2000; Ghosh & Ghose, 2003). However, tropical area is required for the production of sugarcane and hence very less land is available for its cultivation. Some other feedstocks which can be extensively used for the bioethanol production are cassava and sweet potato, but these all are also used for food and causes food to fuel competition (Papong & Malakul, 2010; Zhang et al., 2010). Another cheap and abundantly available feedstock for bioethanol production is lignocellulosic biomass but its conversion to bioethanol is limited due to its recalcitrant nature (Sarkar et al., 2012). So, starch-based bioethanol production is extensively used worldwide due to feedstock availability and advanced technology in this area (Demirbas, 2007). But starch enriched crops like wheat, corn, sweet potatoes and rice can lead to agricultural land utilization and food shortage (Wyman, 1994). Hence, there must be a source of starch for the production of bioethanol that can reduce the need for cropland and lead to a more sustainable process.

In addition to bioethanol, another versatile renewable bioenergy fuel which can effectively be used as an alternative to fossil fuels in power and heat production is biogas. Bimethane i.e., methane rich biogas can also replace natural gas. Biogas can also be utilized as gaseous vehicle fuel. Organic waste materials like municipal solid waste (sludge/wastewater), industrial waste and agricultural waste are widely used for the biogas production through anaerobic digestion (Weiland, 2010). Organic molecules of large molecular waste e.g., cellulose, proteins, carbohydrates and lipids. Hydrolysing bacteria converts these molecules into smaller subunits like peptides, amino acids, fatty acids and sugars and some small amount of hydrogen, carbon dioxide and acetic acid. Anaerobic bacteria during the process of acidogenesis, further converts these smaller molecules into volatile fatty acids such as butyric and propionic acids. During acetogenesis these VFAs are degraded into hydrogen, carbon dioxide and acetic acid. And at the last stage (methanogenesis) of this anaerobic digestion process methane gas is produced through the digestion of acetic acid and hydrogen (Weiland, 2010; Bagi et al., 2007).

Keeping in view the problems like utilization of cropland and food crops for bioethanol production and recalcitrant nature of cheaper feedstocks that can be utilized for both bioethanol and biogas production, it has become necessary to explore novel feedstocks for development of biogas and bioethanol industry that are environmental friendly and more sustainable. Duckweed is a tiny plant that floats on the water surface, multiply through

the vegetative budding of fronds (leaf like thallus). Duckweeds are monocotyledonous plants distributed in five different genera (Lemna, Wolffia, Landoltia, Wolfiella and Spirodela) and 37 diverse species. On dry basis 3-75% increase in starch content can be obtained from different species on applying different growing conditions (Reid & Bieleski, 1970; Hasan et al., 2018). Variation in plant growing conditions for example nutrient concentration in the growth medium, phosphate concentration and pH can also increase the total starch accumulation in the plant (Jong & Veldstra, 1971; Mclaren & Smith, 1976). Due to these capabilities, duckweed is a promising feedstock for the biofuels production.

By doubling its mass every 16 to 24 hours under suitable environmental conditions, its growth rate is higher than many other plants (Peng et al., 2007). According to Cheng et al. (2002), cultivation of duckweed plants in swine lagoon could reach to the maximum level of 29 g $m^{-2}.day^{-1}.ha^{-1}$ (on dry basis) and on annual basis if its growth continues for the whole year (365 days) is 106 t $ha^{-1}.year^{-1}$ (dry basis) (Cheng et al., 2002). On comparing this annual yield with other crops like wheat 3.15 t $ha^{-1}.y^{-1}$, corn 7.48 t $ha^{-1}.y^{-1}$ and barley 3.70 t $ha^{-1}.y^{-1}$, duckweed is a non-feed potential starch crop for the bioethanol production (Nass, 2011).

Duckweed can also be used for wastewater treatment due to its tolerance to high nutrient concentration and its ability to uptake nutrients. It can be used for tertiary treatment of wastewater and nutrient recovery. These processes make duckweed an environment friendly and promising feedstock for biofuels production (Alaerts et al., 1996; Shen et al., 2006).

This research work comprised of four phases. In the first phase *Spirodela polyrhiza* was grown under nutrient starvation condition to enhance its starch content. High starch containing plant biomass was then acid pre-treated for conversion of starch to glucose. The plant sugar was then fermented via indigenously isolated and optimized *Saccharomyces cerevisiae* QG1 MK788210 for bioethanol production.

In the second phase *Spirodela polyrhiza* was grown in agricultural runoff that was synthesized at the lab to test the capability of the plant to uptake nitrogen and phosphorus from the agricultural runoff. The biomass generated by the plants during their growth in agricultural runoff was used further for the production of bioethanol. The biomass was given acidic pretreatment and bioethanol was produced via fermentation process carried out by yeast strain *Saccharomyces cerevisiae* QG1 MK788210.

In the third phase *Spirodela* was grown under nutrient stress condition to enhance its starch content. The plant starch was then fermented to ethanol whereas, the fermentation vinasse was anaerobically digested for production of biogas. Further, a comparative analysis of the energy output in four different biofuel production processes i.e., utilization of high starch *Spirodela* biomass for ethanol production only, utilization of high starch *Spirodela* biomass for ethanol production and then utilization of fermentation vinasse for biomethane production, utilization of high starch *Spirodela* biomass for biomethane production only and utilization of fresh *Spirodela* biomass for biomethane production only, was also carried out to identify the process and the biofuel which gives maximum energy output by using *Spirodela* as feedstock.

In the fourth and last phase two duckweed specie, *Spirodela polyrhiza* and *Lemna minor* were grown in nutrient starved environment to increase their starch content. The cell wall material of high starch duckweed biomass was prepared and subjected to TFA hydrolysis for cell wall analysis. The cell wall material was further processed using two intensive pre-treatments that were hydrothermal treatment followed by enzymatic saccharification and ionic liquid pretreatment to enhance the release of two fermentable sugars i.e., glucose and xylose from cell wall which can easily be fermented to ethanol thereby enhancing the overall ethanol yield of high starch duckweed biomass.

1.2 Aim of the Study

To develop fermentation process for production of bioethanol and biomethane from *Spirodela polyrhiza* feedstock.

1.3 Objectives

- To isolate fermentative yeast and optimize its physicochemical parameters for high ethanol yield in batch reactor
- To enhance *Spirodela polyrhiza* starch content, convert starch to glucose by pretreatment and ferment plant derived glucose to bioethanol.
- To treat agricultural runoff by *Spirodela polyrhiza* and utilize the plant biomass grown during phytoremediation for bioethanol production.
- To evaluate biogas potential of high starch containing *Spirodela* biomass, fresh *Spirodela* biomass and vinasse and comparative analysis of the total energy content produced in these three conditions.
- To analyze the cell walls of *Spirodela polyrhiza* and identify sugars that can be utilized for production of biofuels and bioproducts

References

1. Ahmed, F. E. (2001, September 15). Toxicology and human health effects following exposure to oxygenated or reformulated gasoline. *Toxicology Letters*, Vol. 123, pp. 89–113. https://doi.org/10.1016/S0378-4274(01)00375-7
2. Alaerts, G. J., Mahbubar, R., & Kelderman, P. (1996). Performance analysis of a full-scale duckweed-covered sewage lagoon. *Water Research*, *30*(4), 843-852.
3. Amorim, H. V., Lopes, M. L., De Castro Oliveira, J. V., Buckeridge, M. S., & Goldman, G. H. (2011, September 7). Scientific challenges of bioethanol production in Brazil. *Applied Microbiology and Biotechnology*, Vol. 91, pp. 1267–1275. https://doi.org/10.1007/s00253-011-3437-6
4. Bagi, Z., Ács, N., Bálint, B., Horváth, L., Dobó, K., Perei, K. R., … Kovács, K. L. (2007). Biotechnological intensification of biogas production. *Applied Microbiology and Biotechnology*, *76*(2), 473–482. https://doi.org/10.1007/s00253-007-1009-6
5. Balat, M., Balat, H., & Öz, C. (2008). Progress in bioethanol processing. *Progress in Energy and Combustion Science*, *34*(5), 551–573. https://doi.org/10.1016/J.PECS.2007.11.001
6. Budzianowski, W. M. (2017). High-value low-volume bioproducts coupled to bioenergies with potential to enhance business development of sustainable biorefineries. *Renewable and Sustainable Energy Reviews*, *70*, 793–804. https://doi.org/10.1016/J.RSER.2016.11.260
7. Campbell, C.J. (2013). The Oil Age in Perspective. *Energy Exploration & Exploitation*, *31*(2), 149–165. https://doi.org/10.1260/0144-5987.31.2.149
8. Campbell, Colin J., & Laherrère, J. H. (1998). The End of Cheap Oil. *Scientific American*, Vol. 278, pp. 78–83. https://doi.org/10.2307/26057708
9. Cheng, J., Landesman, L., Bergmann, B. A., Classen, J. J., Howard, J. W., & Yamamoto, Y. T. (2002). Nutrient removal from swine lagoon liquid by Lemna minor 8627. *Transactions of the ASAE*, *45*(4), 1003.
10. Demirbas, A. (2007). Progress and recent trends in biofuels. *Progress in Energy and Combustion Science*, *33*(1), 1–18. https://doi.org/10.1016/j.pecs.2006.06.001

11. Drosg, B., Braun, R., Bochmann, G., & Al Saedi, T. (2013). Analysis and characterisation of biogas feedstocks. In *The Biogas Handbook: Science, Production and Applications* (pp. 52–84). https://doi.org/10.1533/9780857097415.1.52
12. Endo, A., Nakamura, T., Ando, A., Tokuyasu, K., & Shima, J. (2008). Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in Saccharomyces cerevisiae. *Biotechnology for Biofuels*, *1*(1), 1–6. https://doi.org/10.1186/1754-6834-1-3
13. ETH, E. L.-, Rübel, S., Zürich, undefined, Switzerland, undefined, & 1986, undefined. (n.d.). *Biosystematic Investigations on the Family of Duckweeds: The Family of Lemnaceae*.
14. Ghosh, P., & Ghose, T. K. (2003). Bioethanol in India: recent past and emerging future. *Advances in Biochemical Engineering/Biotechnology*, Vol. 85, pp. 1–27. https://doi.org/10.1007/3-540-36466-8_1
15. Hasan, M. M., Hasan, M., & Saeed, T. Evaluation of duckweed based waste stabilization pond system for domestic wastewater treatment.
16. JONG, J. G. T.-D., & VELDSTRA, H. (1971). Investigations on Cytokinins. II. Interaction of Light and Cytokinins as Studied in Lemna minor. *Physiologia Plantarum*, *24*(2), 239–241. https://doi.org/10.1111/j.1399-3054.1971.tb03486.x
17. Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. *Bioresource Technology*, *165*(C), 3–8. https://doi.org/10.1016/j.biortech.2014.01.127
18. McCarthy, J. E., & Tiemann, M. (2006). MTBE in gasoline: clean air and drinking water issues.
19. Mclaren, J. S., & Smith, H. (1976). The effect of abscisic acid on growth, photosynthetic rate and carbohydrate metabolism in *Lemna minor* l. *New Phytologist*, *76*(1), 11–20. https://doi.org/10.1111/j.1469-8137.1976.tb01433.x
20. Moreau, V., & Vuille, F. (2018). Decoupling energy use and economic growth: Counter evidence from structural effects and embodied energy in trade. *Applied energy*, *215*, 54-62.

21. Nahar, K., & Sunny, S. A. (2020, March 1). Duckweed-based clean energy production dynamics (ethanol and biogas) and phyto-remediation potential in Bangladesh. *Modeling Earth Systems and Environment*, Vol. 6, pp. 1–11. https://doi.org/10.1007/s40808-019-00659-y
22. NASS, U. (2011). *Crop Production 2010 Summary*.
23. Papong, S., & Malakul, P. (2010). Life-cycle energy and environmental analysis of bioethanol production from cassava in Thailand. *Bioresource Technology*, *101*(1 SUPPL.), S112–S118. https://doi.org/10.1016/j.biortech.2009.09.006
24. Peng, J. F., Wang, B. Z., Song, Y. H., & Yuan, P. (2007). Modeling N transformation and removal in a duckweed pond: model development and calibration. *Ecological modelling*, *206*(1-2), 147-152.
25. Pimentel, D. (2003). Ethanol fuels: Energy balance, economics, and environmental impacts are negative. *Natural Resources Research*, *12*(2), 127–134. https://doi.org/10.1023/A:1024214812527
26. Reid, M. S., & Bieleski, R. L. (1970). Response of Spirodela oligorrhiza to phosphorus deficiency. *Plant physiology*, *46*(4), 609-613.
27. Sarkar, N., Ghosh, S. K., Bannerjee, S., & Aikat, K. (2012). Bioethanol production from agricultural wastes: An overview. *Renewable Energy*, *37*(1), 19–27. https://doi.org/10.1016/j.renene.2011.06.045
28. Shen, G., Xu, J., Hu, S., Zhao, Q., Environment, Y. L.-J. of E. and R., & 2006, undefined. (n.d.). *Nitrogen removal pathways in shallow-water duckweed-based wastewater treatment systems*.
29. Wang, M., Saricks, C., & Santini, D. (1999). Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions.
30. Weiland, P. (2010, September 24). Biogas production: Current state and perspectives. *Applied Microbiology and Biotechnology*, Vol. 85, pp. 849–860. https://doi.org/10.1007/s00253-009-2246-7
31. Wilkie, A. C., Riedesel, K. J., & Owens, J. M. (2000, August 1). Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass and Bioenergy*, Vol. 19, pp. 63–102. https://doi.org/10.1016/S0961-9534(00)00017-9
32. Wyman, C. E. (1994). Ethanol from lignocellulosic biomass: Technology,

economics, and opportunities. *Bioresource Technology*, *50*(1), 3–15.

33. Wyman, C. E. (1999). B IOMASS E THANOL : Technical Progress, Opportunities, and Commercial Challenges. *Annual Review of Energy and the Environment*, *24*(1), 189–226. https://doi.org/10.1146/annurev.energy.24.1.189
34. Zhang, L., Chen, Q., Jin, Y., Xue, H., Guan, J., Wang, Z., & Zhao, H. (2010). Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG). *Fuel Processing Technology*, *91*(12), 1845–1850. https://doi.org/10.1016/j.fuproc.2010.08.009

Literature Review

2.1. Energy Crisis

For continued human development more energy sources and effective utilization of energy is necessary. During the past decades, rapid increase in industrialization, evolution of civilization increasing human population, modernization, mechanization, and urbanization has increased the global demand of energy. In past humans were promptly using fossil fuels to fulfil their energy requirements. Commonly used fossil fuels are coal, gas and oil. About 80% of global energy demand is fulfilled by the fossil fuels.

About 85 million barrels' oil is consumed on daily basis. It is being estimated that by 2030 this figure will increase up to 113 million barrels. Non-renewable energy resources are being used more vigorously and their depletion is occurring at a speedy rate so that it is estimated that in near future fossil fuels would not be available to meet our energy demands. According to Campbell and Laherrere (1998) the remaining crude oil reserves are quite less and the annual global oil production will decrease from recent 25 billion barrels to near about 5 billion barrels by 2050. In the whole world, the economy of any country mainly depends on oil. The decline in availability of oil will adversely affect the economy of whole world. So, in a great interest there is a strong need to discover renewable sources as alternative energy sources (Woli & Paz, 2013).

Huge amount of energy consumption throughout the world has adversely affected the earth's ecosystem. Production of nuclear energy and fossil fuels consumption has caused environmental degradation (Asif, 2009). If the global energy demands are fulfilled without any check and balance, it would cause adverse effects on ecosystem and would endanger the lives of future generation.

Recently the focus is diverted from non-renewable energy resources to renewable energy resources like solar energy, wind energy, nuclear energy and water energy etc. About 6.5% and 13.1% of total energy demand is provided by nuclear power and renewable energy respectively (Huacuz, 2005). The advantage of utilizing renewable energy resources is that they are environmental friendly, do not cause air pollution, and have reduced greenhouse gas emissions. (Wyman, 1999).

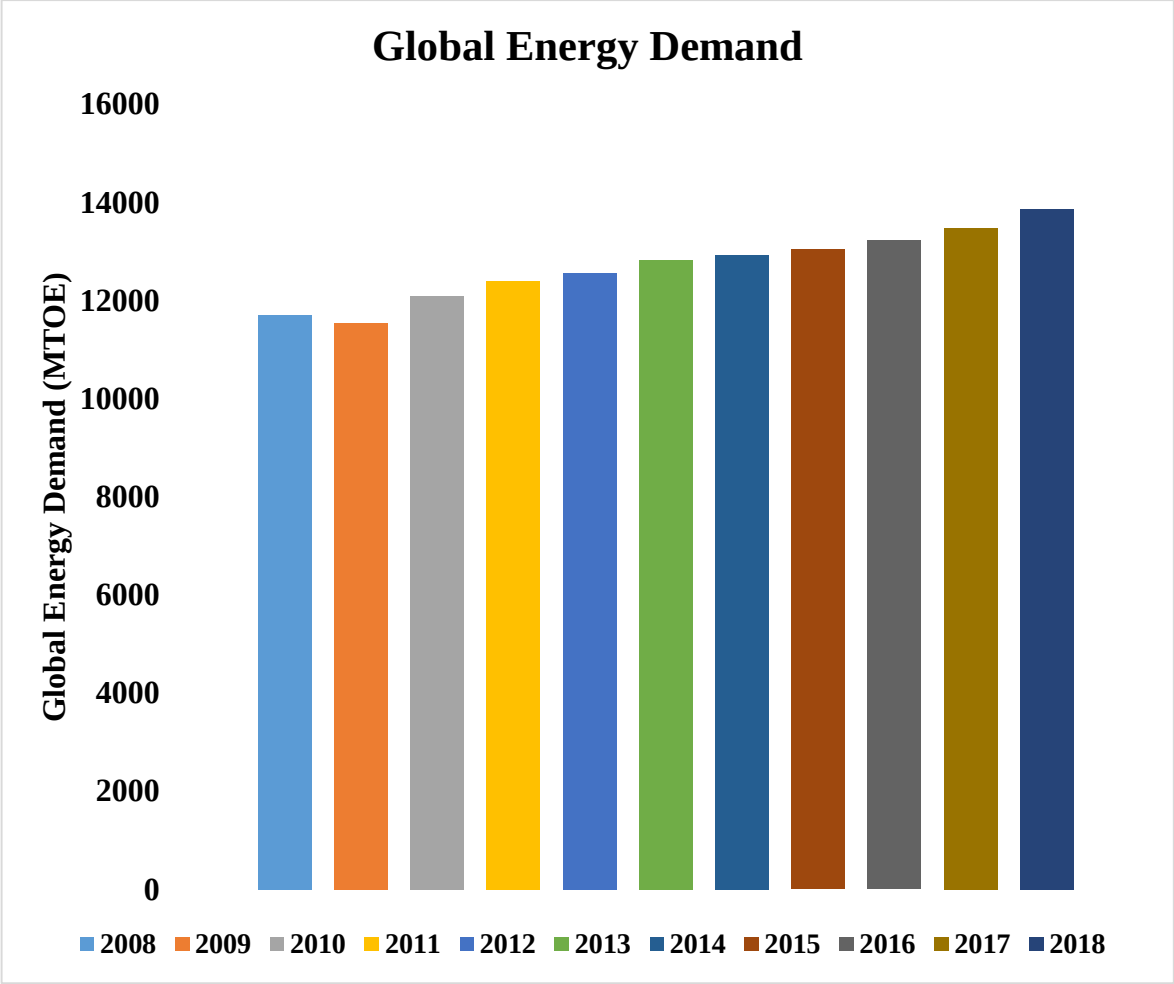

Fig 2.1. Increase in global energy demand during 2008-18
(Source: BP Statistical review of World Energy, 2019)

2.2. Fossil fuels and other methods of energy production:

The index of energy consumption has increased constantly due to increase population and industrialization. World is dependent on the fossil energy sources like oil, natural gas and coal which are used for the production of electricity, other chemical compounds and as fuel. The reservoirs of fossil fuels are limited and world could face shortage of oil and fuel in near future (Uihlein & Schebek, 2009).

Oil is primary energy source for transportation and per day demand of oil around the world is 84 barrels that will increase up to 116 by 2030 as estimated. Dependence on oil has increased even more as the chemical production is increased. Moreover, about 4% of oil extracted is used for the synthesis of chemicals and plastic polymerization (Amenaghawon, et al., 2014). In the last few decades due to constant and excessive use of

oils, the level of pollution and greenhouse gas in the air has been increased many folds ("World Economic Outlook, April 2007: Spillovers and Cycles in the Global Economy - International Monetary Fund. Research Dept - Google Books," 2007).

Alternative sources for the production of energy are mandatory to avoid the dependence on oil and to protect environment. Immediate and well-defined actions are required to overcome this issue including behavioral change, technological upgradation of motor vehicles and awareness in public to limit the use of oil by using public transportation. Recent and innovative technologies are also required for the production of different types of biofuels that can be utilized instead of conventional fossil fuels (Oliveira et al., 2013).

2.3. Sustainable Method and Green Technology

Three main technical changes are included in sustainable energy development process: less demand and more saving of energy, utilizing efficient ways for the production of energy and introduction of renewable resources as alternate of fossil fuels (Lior, 2002). Plans must be designed on large scale for the coherent energy systems to integrate sustainable energy methods and efficiency of the system and energy saving must be considered. Expansion of renewable energy index in the supply system is the major problem (Qureshi, 2005). Around the world, renewable bioenergy is considered as the best option for energy production but only 15% of energy supply on the globe is renewable energy and wood fuel and hydropower systems are major energy producing sources in most of the developing countries. All this energy is generated via solar, wind and tidal sources that share a small contribution out of the total energy demand (Woli & Paz, 2013).

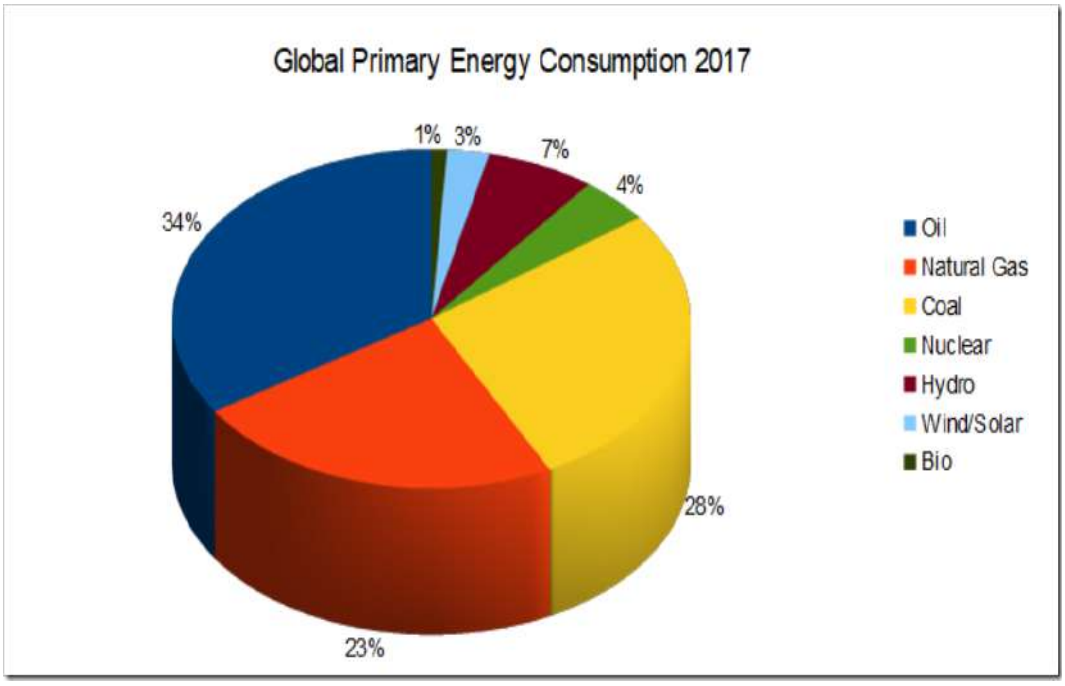

Fig 2.2. Global Energy Consumption (Paul Homewood, 2018)

2.4. Biofuels

Energy for industrial use can be generated by renewable resources like air, geothermal reservoirs, tides, solar heat and biomass but for fuel and energy production for chemical industries world has to depend on biomass as an alternate in future (Lynd, Weimer, Zyl, & Pretorius, 2002). Biofuels like bioethanol, biodiesel and bio-hydrogen could replace the petroleum-based fuels and these biofuels can be obtained from algal biomass or from sugarcane, switchgrass or corn. Wind mills and solar farm can be used for the production of electricity. Individual's energy consumption includes its energy expenditure on electricity usage, cooking of food, other goods and energy utilized for the transportation purposes. To minimize the import of fuels and to be self-sufficient for energy supply, development of biomass based biofuels is the best option. These biofuels lead to the reduction in greenhouse gas emissions by 80%. Plants utilize CO_2 from atmosphere during the photosynthesis process and resulted biomass is used for fuels production. Released CO_2 during the burning of these biofuels is recycled back in the process hence no extra CO_2 is released in the atmosphere (Pereira & Ortega, 2010).

2.4.1. Biogas

On organic farms, biogas has been introduced as motor fuel in short term usage. Anaerobic break down of organic material liberates biogas from which CO_2 is removed for elevation of energy content when used as fuel and this fuel could be preserved at high

pressure. Instead of propane or natural gas, biogas is a good option for the production of electricity. Wide variety of substrates can be utilized for the anaerobic digestion process which is carried out by the anaerobic microorganisms. Vegetable waste, garden waste, cattle manure and algal biomass can be used for this purpose (Dhussa, 2004).

2.4.2. Biodiesel

As a substitute of diesel, biodiesel is considered as best alternative because it could be used in ignition engines without any technical alterations in conventional engines. A combination of methyl esters and long chain fatty acids, derived from harmless and bio-based source like vegetable oils, fats of animal or cooking oils (UFO), is termed as biodiesel. In many regions in the world, edible oils are used as feedstock for the production of about 95% of biodiesel and its properties make it more suitable to be used as substitute of conventional diesel (Santos, Malveira, Cruz, & Fernandes, 2010). But the problem with using edible oils for biodiesel production is the competition in market for edible oils that will affect the price of both biodiesel and edible oils. This condition will also lead to deforestation due to requirement of more land for cultivation purposes (Kumar et al., 2007).

2.4.3. Green Diesel

Hydrocracking is basic step in formation of green diesel which may also be termed as hydrogen derived renewable diesel. It requires higher pressure and temperature and specific catalysts that are involved in the breakdown of complex polymer of edible oils into simpler monomers and oligomers of the compounds that can be easily used in the engine oils. It has the same chemical characteristics as that of petroleum derived diesel as compared to biodiesel (Vonortas & Papayannakos, 2014). Another advantage is that there is no need of separate infrastructure, pipelines or new engines for its use but its high cost is major hindrance in its used on a large scale (Pragya & Pandey, 2016).

2.4.4. Bio ethers

Also termed as oxygenated fuels, these are cost effective compounds and can also be used as octane rating enhancers. Production of bio ethers can be done by the reaction of bioethanol with iso-olefins like butylene. These reduces the greenhouse gas emissions (GHG) and improves the engine efficiency. Bio ethers improves the air quality by decreasing the emission of ozone at ground level (Prieto, Sánchez, & Pereda, 2019).

2.4.5. Bioethanol

Bioethanol could be derived from either sugar containing feedstock like sugarcane and molasses or from starch like cassava, corn or rice. As these raw materials are edible crops and cannot be used excessively in future, in many countries alternate sources for raw material are already used. Potential sources of sugar raw material which are gaining the attention of researchers are agricultural lignocellulose, hardwood or softwood. Lignocellulose is counted as best option for bioethanol because of its less cost and no competition with food supply but its conversion to bioethanol is difficult because of its recalcitrant nature (Quintero et al., 2008).

Bioethanol, unlike fossil fuels is extracted from sugar via fermentation. It is used as gasoline alternate and is used in oxygenated fuels like gasohol since 1980s in which 10% by volume of gasoline is ethanol. The transportation sector is utilizing 4540 million liters of bioethanol per annum that is just 1% of gasoline consumption (Wang et al., 1999). Production of flexible fueled vehicles can enhance the usage of fuel blends (E85 which contains only 15% gasoline while rest of 85% is ethanol) with ethanol which can control the petroleum consumption and reduce emission of greenhouse gas significantly. Methyl Tertiary butyl ether (MTBE) is used to provide safer combustion and is commonly added into the gasoline (Browner, 2000). However, it is a chemical compound that has toxic effects on the quality of groundwater. This chemical is banned by the Environmental Protection Agency of US (McCarthy & Tiemann, 2006). As the fossil fuels are depleting at a rapid rate renewable sources of energy like biofuels production is gradually increased across the world.

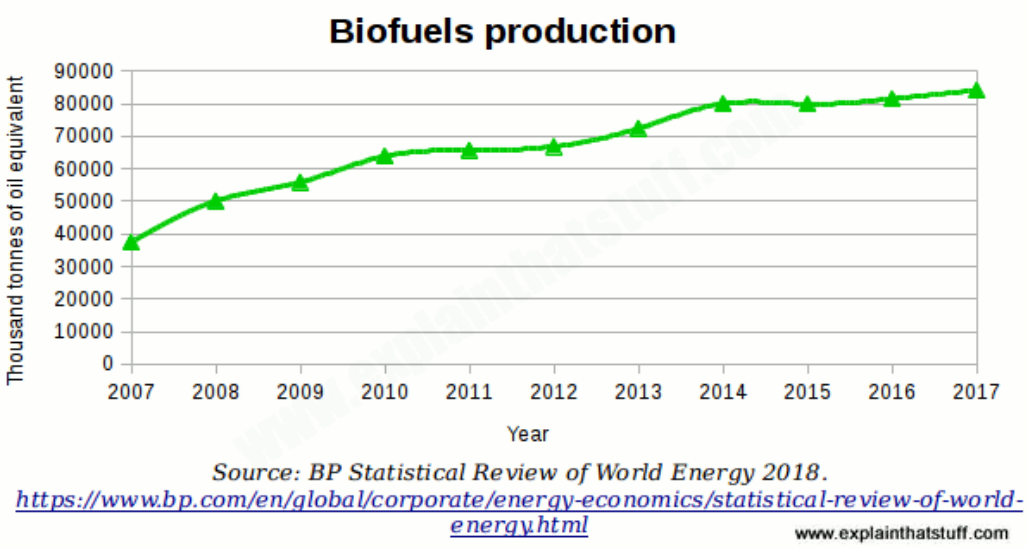

Fig 2.3. Worldwide biofuel production

2.5. Types of Biofuels

2.5.1. First Generation Biofuels Production

Generally, agricultural by-products like palm oil, sugarcane juice and soybean are used as source for biodiesel and biofuel production. Overall, first generation biofuels are the biofuels that are derived from food crops. As plants are used for production of biofuel and they can uptake carbon dioxide from air as they grow, they could change the climatic conditions caused by fossil fuel combustion by lowering the emission of greenhouse gases (Mabee, 2006). So, switching from fossil fuels to biofuels will impart positive impacts on the environment and will control the global warming.

Apart from a lot of benefits, there are some issues related with the first generation biofuels as well. First, the agricultural products that are used are sources of fats and oils like rapeseed oils and palm oil. In US and South Africa, sugarcane and corn are used as feedstock for biofuels which also are a major part of human food chain so with the increasing demand of biofuels the costs of these food crops will also increase drastically. In the end, consumers will suffer by the hike in price of the food commodities (Demirbas, 2007).

Another concern is loss of plantation, as if biofuel is extracted through plant material than a lot of plantation is needed that will demand a large are of land. This will result in deforestation dues to which flora and fauna of the Earth will be disturbed and ecological sustainability will be affected greatly along with increase in pollution in environment (Goldemberg et al.,2008).

2.5.2. Second Generation Biofuels Production

Due to the afore mentioned issues, first generation biofuels do not seem as ultimate source for the bioenergy production. To solve these problems, a new technique emerged as an alternate option termed as second generation biofuel production (Zabed et al., 2016). For the extraction of second generation biofuel, low cost and non-edible cellulosic biomass was required, that is available easily in large quantity (Harvey, 2007). For the production of second generation biofuel no valuable food crop wass needed and the food-fuel contradiction does not arise. Moreover, the price of second generation biofuels was also be equal to that of gasoline as inexpensive raw material was used.

2.5.3. Third Generation Biofuels Production

Plants are the major source of feedstock for the second generation biofuels production but these require continuous supply of nutrients and irrigation. Due to the shortage of sources and low conversion rate, these feedstocks are not suitable practically and economically for the continuous energy supply (Lam & Lee, 2012). To overcome this issue, different oleaginous microorganisms (yeast, bacteria, fungi and microalgae) are used for production of third generation biofuels. Due to high biomass production, better growth tendencies and ability to store maximum lipid content in biomass (20-77%), microalgae is widely used for biofuels production (Leong et al., 2018). Duckweed, member of family Lemnaceae, is group of aquatic plants. Due to the ability of duckweed to grow rapidly, having high tolerance against elevated level of nutrients and accumulation of starch content in the biomass, it is considered as a suitable feedstock for the third generation biofuels. Duckweed could be found in all geographical regions around the globe and this family consists of four genera and about 40 species (Mclaren & Smith, 1976).

2.5.4. First Generation Bioethanol Production

Production of Bioethanol from food sources like cassava, corn, sugar beet and sugars is termed as first generation bioethanol production. These conventional raw materials for bioethanol production imposes a great danger of food to fuel competition which subsequently increases the price of food items and it also increase the costs of bioethanol production which is a great challenge for its use on large scale and at industrial level (Lennartsson et al., 2014).

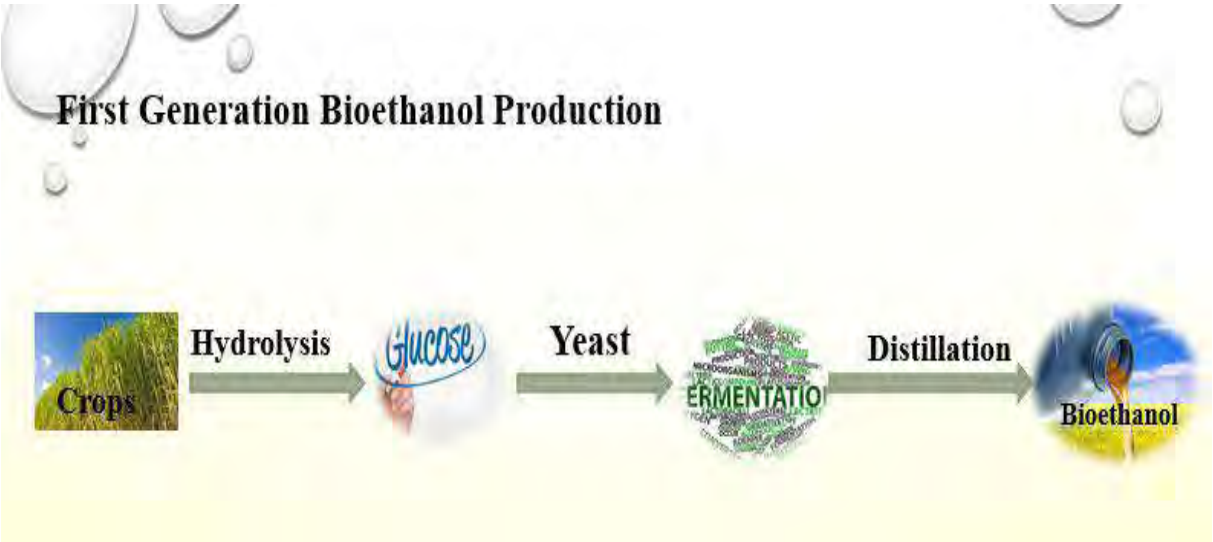

Fig 2.4. First Generation bioethanol production

2.5.6. Second Generation Bioethanol production

The bioethanol derived from non-edible cellulosic, lignin and hemicellulosic sources are termed as second generation bioethanol. Both cellulose and hemicellulose apart from lignin, are composed of long chain polysaccharides which give pentose mixture as by product after hydrolysis (Zabed et al., 2014). Hexose, a type of glucose is utilized as substrate by the enzymes during the fermentation process which converts sugar into ethanol but in the case of second generation, an extra step for hydrolysis of lignocellulosic biomass is required (Kim & Lee, 2002).

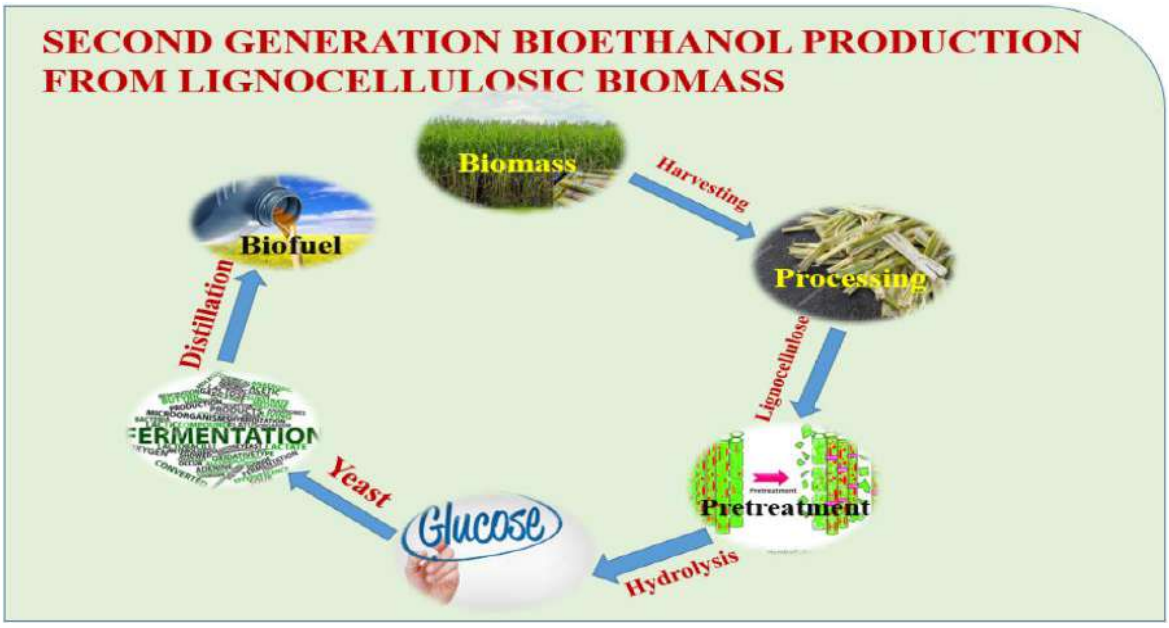

Fig 2.5. Second generation bioethanol production from lignocellulosic biomass

2.5.7. Third Generation Bioethanol Production

Feedstock which is commonly used for the production of third generation bioethanol can reduce the competition of feedstock from different agricultural plants. Algae (microalgae - phytoplankton), macroalgae (sea weeds) and duckweeds are the promising feedstock for the higher level of bioethanol production. Both are aquatic plants and reduce the use of agricultural land for cultivation purposes. Due to high carbohydrate and lipid content, easy cultivation and proton conversion, algal biomass is mainly used for the bioethanol and biodiesel production. Recalcitrance of algal biomass is much less due to low amount of hemicellulose and lignin which makes it a better feedstock (Jambo et al., 2016). Duckweed is a small aquatic plant with least cultivation cost, efficient ability of up taking nutrients and produce higher biomass yield which can be used for the bioethanol production. Duckweed has the ability to produce and accumulate starch in the biomass. The accumulation of starch in the duckweed is affected by two processes, starch production by photosynthesis and metabolism related consumption of starch (Cheng et al., 2002,). About 3-75% starch accumulation has been reported in different duckweed species. Starch concentration in duckweed biomass can be increased by manipulating nutrient concentration and growth conditions such as pH, light and temperature (Cui et al., 2011). Doubling time of duckweed biomass is about 16-24 hours at favorable environmental conditions. Due to its ability to produce high biomass duckweed can produce more

bioethanol as compared to first and second generation bioethanol feedstocks (Peng et al., 2007).

2.6. Biogas production

In the absence of oxygen, different bacteria (both anaerobes and facultative anaerobes) produce a mixture of different gases generally termed as biogas. Composition of biogas is CH_4 (40-75%) and CO_2 (15-60%) along with some other gases such as H_2S (0.005-2%), N_2 (0-2%), CO (<0.6%) and water vapours (5-10%) (Ryckebosch et al., 2011). Technological advancements have been made for the biogas production but commonly used method is anaerobic digestion.

2.7. Anaerobic digestion

Both obligate and facultative anaerobes are responsible for the anaerobic digestion. Biogas production is a complex process and involves a number of steps such as hydrolysis, acidogenesis followed by acetogenesis and methanogenesis and different microbial communities in available consortia depend on one another to carry out this sequential process. Hydrolysis is an important step during the process because it is involved in the breakdown of complex compounds which results in the production of volatile fatty acids and different intermediates. If the concentration of these intermediate products exceeds the optimum range, they inhibit the microbial action during the process. Methanogenesis can also be the rate limiting step during the anaerobic digestion if easily degradable feedstock is used (Lu et al., 2008). On broader term the whole process can be divided into two phases; fermenting phase and the methanogenic phase. Physiology, nutritional requirements, sensitivity to environment and growth kinetics are different for the microbial species responsible for these two phases. For higher biogas yield it is essential to maintain stability between microbial communities of both phases because any minute disturbance in conditions may alter their behaviour that eventually leads to the decreased biogas production. Kinetics control, pH or membranes can be used to keep these microbial communities separate (Adekunle, 2015).

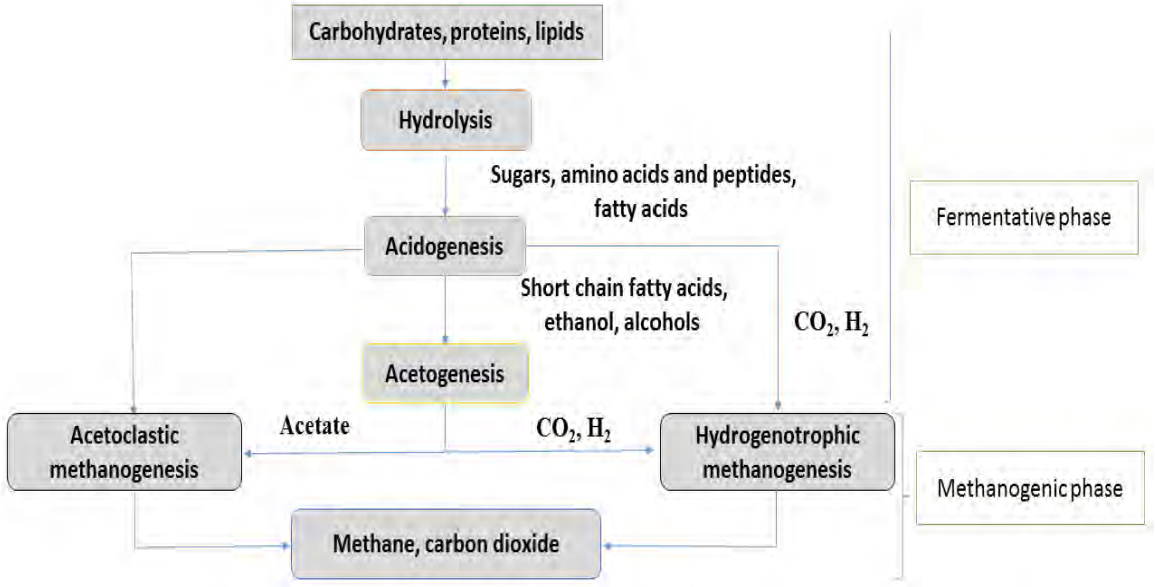

Fig 2.6. Different phases of anaerobic digestion process

2.7.1. Hydrolysis

At the very first step different enzymes are involved in the hydrolysis of complex insoluble organic compounds which mainly constitute of fats, cellulose, hemicellulose and proteins and convert them into their monomeric soluble compounds such as fatty acids, monosaccharides and amino acids. These soluble compounds are effectively consumed by microbial consortia as cell carbon or energy source. Mixed species of obligate and facultative anaerobes including *Clostridia, Butyrivibrio, Streptococci, Fusobacteria, Micrococci* and *Bacteroides* secretes the variety of enzymes such as amylases, proteases, xylanases and cellulases which are involved in the breakdown of their respective substrates such as fats, proteins, xylose and carbohydrates respectively (Cirne, et al., 2007); Riviere et al., 2009); Schnurer, 2010). Efficient hydrolysis in anaerobic digestion is required because in this process polymeric complex molecules are degraded into monomeric particles to enhance bioavailability of these compounds to microbial consortia. Different enzymes act on the specific sites of complex molecules and are involved in the depolymerization process. Some microorganisms secrete only single type of enzymes which act on specific substrates such as proteins and sugars. While some microbial communities produce variety of enzymes that are involved in the degradation of number of compounds which can be further used as source of energy. Protein degrading microbes are called proteolytic while sugar degrading microorganisms are termed as saccharolytic bacteria. Substrate structure is mainly responsible for the rate of hydrolysis. Proteins are

easily degradable polymeric compounds as compared to cellulose and hemicellulose which takes much longer time for degradation (Schnurer et al., 2013).

2.7.2. Acidogenesis

In anaerobic digestion, hydrolysis and acidogenesis are 10 times faster than the acetogenesis and methanogenesis while acidogenesis being the fastest step in the process. During the acidogenesis step, the monomeric substances produced during the hydrolysis such as amino acids, long chain fatty acids and sugars further transform into intermediate compounds such as alcohols, CO_2, H_2 and some short chain fatty acids such as valeric acid, acetic acid, butyric acid, propionic acid and some other short chain fatty acids. Microbial communities involved in the acidogenesis are strict and facultative anaerobic bacteria including, *Lactobacillus, Salmonella, Bacillus, Streptococcus* and *Escherichia coli*. These short chain fatty acids drops the reactor pH in the range of 4-5.5 which favours the activation of hydrolytic and acidogenic bacteria (Demirel & Yenigün, 2002). Production of hydrogen at this stage triggers the formation of further products. Partial pressure of hydrogen has inverse relation with the production of reduced compounds (Gerardi, 2003).

2.7.3. Acetogenesis

Different intermediate products of acidogenic phase are further converted into some other products such as hydrogen, carbon dioxide, acetate and different alcohols. Acetogenic bacteria follow the acetyl Co-A pathway to produce carbon dioxide, hydrogen and acetate at their optimum pH 6. Microbial consortia involved in the acetogenesis is, *Syntrophobacter wolinii* and *Syntrophomonas wolfeii*. Variety of enzymes are responsible for this process but these are highly sensitive to presence of oxygen, environmental and operational parameters (Palatsi et al., 2009).

In this phase anaerobic oxidation reactions are carried out by microbial communities. Acetogenic reactions produce intermediate products which are then used as a substrate for the next step i.e. methanogenesis. During these anaerobic oxidation reactions, protons (H+) are the final electron acceptor molecules and involved in the production of H_2 molecules but these processes highly depend on the partial pressure of hydrogen. Methanogenic bacteria utilize this hydrogen to produce methane. Acetogenic bacteria are responsible for the hydrogen production but these microbes can efficiently perform their function at low partial pressure of hydrogen. So, these microbes establish

some symbiotic relationships with other microorganisms that consume hydrogen and transfer of hydrogen occurs between these microbial consortia (Gerardi, 2003).

2.7.4. Methanogenesis

It is the last phase of anaerobic digestion, where the intermediate compounds formed during the previous phases are transformed into CO_2 and CH_4 by the archaea or methanogenic bacteria. Methanogenic bacteria are generally classified into two groups; hydrogenotrophic methanogens and acetoclastic methanogens. Acetoclastic bacteria utilizes acetate as a substrate while hydrogenotrophic bacteria consumes hydrogen and carbon dioxide to produce final product methane. *Methanosarcina thermophila* and *Methanosaeta* are the important species of acetoclastic methanogens while *Methanoculles receptaculi* and *Methannospirillum hungatei* are the main species of hydrogenotrophic methanogens. Hydrogenotrophic bacteria requires 6 hours while the acetoclastic methanogens require almost 2.6 days to increase their microbial biomass (Christy, & Gopinath, 2014). Acetoclastic bacteria are mainly responsible for the production of methane. Almost 70% of total methane is produced by these bacteria while the rest of methane is generated through the redox reactions of carbon dioxide and hydrogen carried out by hydrogenotrophic bacteria. Methanogenesis is the rate limiting step in the anaerobic digestion because acetoclastic bacteria are sensitive to any change in the concentration of VFAs generated in acidogenic and acetogenic steps. Generation time for methanogens ranges from hours to days (Palatsi et al., 2009). Increase in the concentration of volatile fatty acids in earlier stages inhibits the methanogens and alters the reactor stability and eventually reduction in biogas yield. Biogas yield can be increased by the coordination in speed of all phases of anaerobic digestion and equilibrium state is required for the microbial communities involved in the whole process. pH is an important factor that is responsible for the proper functioning of methanogenic bacteria. Optimum pH range is 6.5-8 which is required for better performance of methanogens (Schmidt et al., 2009). If rate of organic acids production is higher than the utilization of acids by methanogenic bacteria, then the pH of the reactor drops and reduces the efficiency of methanogenic bacteria and eventually leads to reduction in biogas yield.

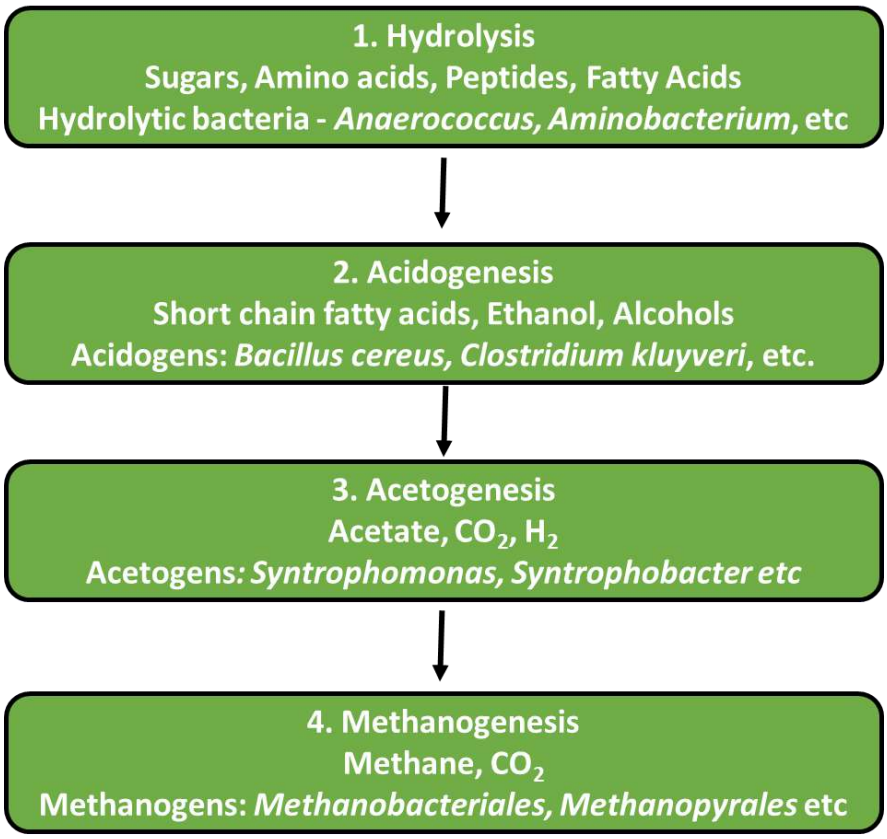

Fig 2.7. Microbiology of Anaerobic Digestion

2.8. Duckweed for Biofuel Production

Duckweed is a tiny plant that floats on the water surface and multiply through the vegetative budding of fronds (leaf like thallus). Duckweeds are monocotyledonous plants distributed in five different genera (*Lemna, Wolffia, Landoltia, Wolfiella* and *Spirodela*) and 37 diverse species. On dry basis 3-75% increase in starch content can be obtained from different species on applying different growing conditions (Reid & Bieleski, 1970; Hasan et al., 2018). Variation in plant growing conditions for example nutrient concentration in the growth medium, phosphate concentration and pH can also increase the total starch accumulation in the plant (Jong & Veldstra, 1971; Mclaren & Smith, 1976). Due to these capabilities, duckweed is a promising feedstock for the biofuels production.

By doubling its mass every 16 to 24 hours under suitable environmental conditions, its growth rate is higher than many other plants (Peng et al., 2007). According to Cheng et al. (2002), cultivation of duckweed plants in swine lagoon could reach to the maximum level of 29 g $m^{-2}.day^{-1}.ha^{-1}$ (on dry basis) and on annual basis if its growth continues for the whole year (365 days) is 106 t $ha^{-1}.year^{-1}$ (dry basis) (Cheng et al., 2002). On comparing

this annual yield with other crops like wheat 3.15 t $ha^{-1}.y^{-1}$, corn 7.48 t $ha^{-1}.y^{-1}$ and barley 3.70 t $ha^{-1}.y^{-1}$, duckweed is a potential starch crop for the bioethanol production (Nass, 2011).

Duckweed can also be used for wastewater treatment due to its tolerance to high nutrient concentration and nutrient uptake. It can be used for tertiary treatment of wastewater and nutrient recovery. These processes make duckweed an environment friendly and promising feedstock for biofuels production (Alaerts et al., 1996; Shen et al., 2006).

2.8.1. Accumulation of Starch in Duckweed

Many governments and researchers have drawn their attention towards the duckweed biomass due to its increased rate of biomass production and potential use as feedstock for bioenergy generation (Zhao et al., 2012). During daylight plants through photosynthesis generate starch, some of which is utilized during the cellular respiration while the extra amount is stored as starch granules in the chloroplasts (Fig. 1.). At night in the absence of photosynthesis process plants utilize this stored starch for metabolism process by degrading and exporting of stored starch outside the chloroplast.

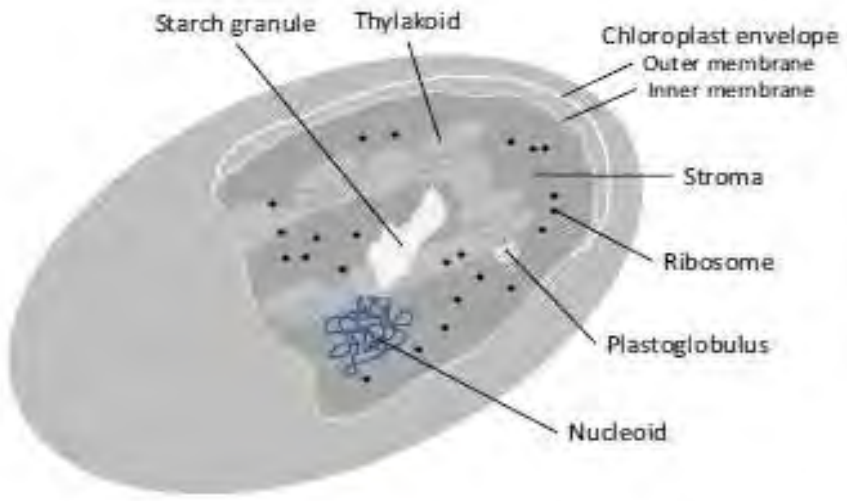

Fig 2.8. Structure and components of the chloroplast (Cui & Cheng, 2015)

This degradation of starch is done by different enzymes which allow the hydrolytic or phosphorolytic degradation of starch (Smith et al., 2004) (Lloyd & Kossmann, 2005) (Y. L. Ae & Sharkey, 2004). Pathways showing the degradation of transitory starch

(temporarily stored starch easily used for the metabolism) are shown in figure below. For loosening of starch structure and phosphorylation of semicrystalline granules, storage form of starch in chloroplasts, plants use different enzymes for example glucan water dikinase (GWD) (Ritte et al., 2012) and phosphoglucan water dikinase (PWD) (Kö et al., 2005).

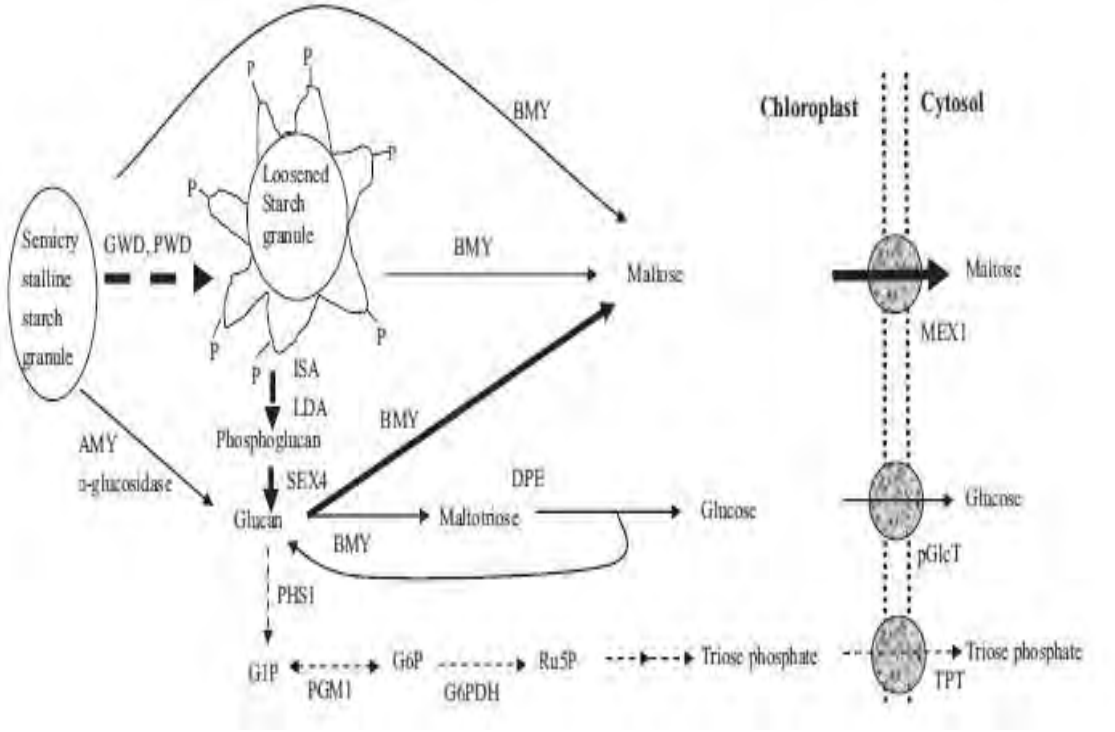

Fig 2.9. Pathways of starch degradation in the chloroplast. The size of the arrows indicates estimated flux while the Dotted lines indicate phosphorolytic starch degradation. Solid lines indicate hydrolytic starch degradation. AMY: a-amylase; BMY: b-amylase; DPE: disproportionating enzyme; G1P: glucose-1- phosphate; G6P: glucose-6-phosphate; G6PDH: glucose-6-phosphate dehydrogenase; GWD: glucan-water dikinase; ISA: isoamylase; LDA: limit dextrinase; MEX1: maltose transporter; pGlcT: plastid glucose transporter; PGM1: plastidial phosphoglucomutase; PHS1: a-glucan phosphorylase; PWD: phosphoglucanwater dikinase; Ru5P: ribulose 5-phosphate; TPT: triose phosphate translocator; SEX4: phosphoglucan phosphatase (Cui & Cheng, 2015)

Limit dextrinase (LDA) and isoamylase (ISA) are the debranching enzymes used by the plants (Ishizaki et al, 1983) (Doehlert, 1991) and present in the chloroplasts (Okita et al., 1979) (Ludwig et al., 1984) (Kakefuda et al., 1986) (Li et al, 1992) (Zeeman et al., 1998). Both of these enzymes act on the starch granules, loosen their structure and release phosphoglucans (Dinges, Colleoni, James, & Myers, 2003) (Hussain et al., 2003). Then

phosphoglucan phosphatase (SEX4) act on the phosphoglucans and cut off the phosphate groups resulting in the formation of linear structured glucans (Kö et al., 2005) (Hejazi, Fettke, Kö, Zeeman, & Steup, 2010), which can be further hydrolysed by the α-amylase (AMY) and α-glucosidase (Sun', Duke, & Henson, 1995) (Frandsen & Svensson, 1998). ß-amylase is used to produce maltose by the degradation of linear and branched glucans (Scheidig, Fröhlich, Schulze, Lloyd, & Kossmann, 2002) (Chia et al., 2004) (Y. L. Ae & Sharkey, 2004) (Sharkey, Laporte, Lu, Weise, & Weber, 2004) (S. E. W. Ae, Weber, & Sharkey, 2004). Disproportionating enzyme (DPE) is involved in the different types of catalytic reactions in which it transfers a part of molecule from donor to acceptor part.

Donor molecules can be larger molecules (maltosyl residues) or smaller molecules (maltotriose), while the acceptor part may consist of malto-oligosaccharide, polyglucan or even larger molecules like glucose (Kakefuda et al., 1986) (Lin, Spilatro, & Preiss, 1988) (Takaha$, Yanasej, Okadaq, & Smith$, 1993). Glc transporter (Trethewey & ap Rees, 1994) and maltose transporter (J. Chen, 2004) are involved for the transportation of glucose and maltose respectively from chloroplast to cytosol. The main enzyme involved in the breakdown of starch in the leaves is α-glucan phosphorylase that acts on the non-reducing ends of chains and releases glucose-1-phosphate (Stitt & Heldt, 1981) (Kruger, 1983).

Enzyme phosphorylase does not have a major contribution towards the starch degradation. Addition of phosphate in the nutrient medium stimulates the α-glucan phosphorylase which triggers the production of phosphorylated compounds (3 phosphoglycerate, triosephosphates and hexose-phosphates). Most of these compounds are not transported as hexose phosphate but in the form of 3-PGA or triose-phosphates through the phosphate or triose phosphate translocator (TPT).

Maltose and glucose, released compounds during the starch hydrolysis, are transported from plastid and act as a substrate for the synthesis of sucrose. So it seems TPT has a minor role in the export of carbohydrates to cytosol (Zeeman et al., 2010). But TPT is involved in the export of triose phosphate (form of carbon) from chloroplast during daytime.

Schleucher et al. suggested that at night time carbon in the form of hexoses (not as trioses) is transported from chloroplasts (Rgen et al., 1998) but Weise et al. indicated that during night most of the carbon leaves the chloroplasts in the form of maltose.

To inhibit the starch degradation in duckweed nutrient deficiency, addition of inhibitory chemicals and low temperature can play an important role. Decrease in extent of starch degradation and elevated rate of photosynthesis can result in increase in starch generation and accumulation. Increased intensity of light and carbon dioxide concentration are two factors that can increase the rate of photosynthesis, but these are difficult to achieve and are not cost effective. While change in the growth conditions (i.e. nutrients, inhibitory chemicals and temperature) is somewhat easy and cheap method.

2.8.2. Nutrient Starvation

Growth medium deficit in the nutrients like nitrogen (Eyster, 1978), potassium (Botany & 1939) and phosphorus (Reid & Bieleski, 1970) can enhance the starch accumulation and reduced amount of starch use by the cells in the duckweed plants. *Lemna gibba* responded to the deficiency of phosphate by accumulating starch in leaves (Thorsteinsson & Tillberg, 1987) (Ciereszko, 2000). Water contains only small amount of nutrients and is a suitable medium for nutrient stress and starch accumulation. Cheng and Stomp (2009) transferred *Spirodela polyrhiza* from a high nutrient medium to a simple tap water and observed the elevated level of starch accumulation from 25-45.8% on dry basis (Cheng & Stomp, 2009). Tao et al (2013) studied the effect of nutrient stress by shifting *Landoltia punctate* from enriched nutrient medium to distilled water and checked its starch concentration at different time intervals. Under the nutrient starvation conditions, activity of the enzyme involved in the starch generation, ADP glucose pyrophosphorylase, and the starch accumulation has increased. The profile of *L. punctata* under nutrient starvation, revealed by using RNA sequencing during comparative gene expression analysis, shows that starch accumulation is resulted by down-regulated global metabolic status and redirected metabolic flux of fixed CO_2 into the starch synthesis branch (Tao et al., 2013).

Cui et al., studied the effect of temperature and light on the starch accumulation on *Spirodela polyrhiza*. Plant grown at 5°C had increased starch content up to 114% higher as compared to the plants grown at 25°C. Elevated starch accumulation has been observed at all temperatures (5°C, 15°C and 25°C) with increase in daily light provided to the plants (Cui, 2010). Higher light exposure and lower temperature increase the starch concentration. As light plays an integral role in the starch generation so the amount of starch in the plant increases during the daytime while its concentration decreases during the night time. Another factor, temperature, also has an important effect on starch accumulation along with

the day and night time (Cui et al., 2010). If temperature remains same during the day time and drops at night time it eventually increases the starch accumulation but if during day temperature fluctuates and remains same at night time it did not affected the overall accumulation. At night time relatively decreased temperature enhances starch accumulation.

Xu et al (2011) studied outdoor cultivation of duckweed in swine farm in Zebulon, North Carolina, USA, and studied the effect of natural climate conditions on the cultivation of duckweed and starch accumulation. This was the first detailed report on the production of starch enriched duckweed and the resulted biomass was commercially converted into bioethanol (Xu, 2011). Farrell (2012) also studied the effect of nutrient deficiency on starch accumulation. He cultivated duckweed species in a 23 hector lagoon and found that starch concentration in the duckweed has been increased from 10% to 19% under nutrient stress (Bay Farrell, 2012). In order to investigate the different species of duckweed, factors responsible for the growth and starch generation were investigated: nutrient supply to the field, duckweed density per unit area and frequency at which plants were harvested. After the selection procedure and the results obtained, the species involved in the highest starch accumulation in leaves i.e. 52.9% (highest starch accumulation at pilot scale) was selected for the further starch production (Xiao, Fang, Jin, Zhang, & Zhao, 2013).

2.8.3. Cation Exposure

Availability and concentration of sodium ions in the growth medium directly effects the duckweed growth and starch accumulation in the plants. Xu et al (2011) studied the effect of sodium on starch generation and accumulation in *Spirodela polyrhiza*. *S.polyrhiza* was cultivated in growth medium with different NaCl concentration i.e. 10, 20 and 30 mM and observed starch accumulation was, 13.4%, 15.7% and 18.7% respectively as compared to the control with no sodium mineral in the growth medium (Xu, 2011). Heavy metals accumulated in the environment are of great concerns (Wang et al., 1999). Duckweeds are widely used to check the quality of water and are also involved in the heavy metals uptake during the wastewater treatment (Boniardi et al., 1994.) (Cheng, 2002) (Tunçsiper, 2019). These heavy metals also cause nutrient stress and stimulate the starch generation and accumulation in the duckweed. Appenroth et al (2003) investigated the effect of chromate ions on the duckweed growth. He subjected the plants with chromate ions at lower concentrations with different intervals of time i.e. 100 µM for two days and 500 µM for

one day. Chromate concentration > 50µM had negative effect on the plant growth but it also lead to the accumulation of starch granules when the plants were studied under transmission electron microscope (Appenroth, wet al., 2003). Appenroth et al (2010) also studied the extent of growth and starch accumulation in the presence of Ni^{+2} ions in the two duckweed species, *Lemna minor* and *Spirodela polyrhiza*, revealing that it had positive effect on the overall growth of plants and accumulation of starch granules. But these heavy metals in high concentration also create stress conditions for the plant growth and starch accumulation (Appenroth, et al., 2010).

2.8.4. Abscisic Acid (ABA) Inhibition

Under stress conditions (cation exposure and nutrient starvation), plants usually contain higher concentration of Abscisic acid (ABA) which decreases when the stress conditions are over (Bauer, et al., 1976.) (Mclaren & Smith, 1976). Huber and Sankhla (1979) found that increase in the ABA by 150% occurd when 85mM of NaCl was added in the nutrient medium. ABA affects different physiological processes like senescence, germination of seeds, growth and dormancy (Addicott & Lyon, 1969). Dekock et al (1978) studied the effects of ABA on the growth of *Lemna* and *Spirodela* species and found that ABA even found in very low quantity like 1 ppm reduced the growth of plants as compared to the control without any ABA (Dekock et al., 1978).

The growth of *L. gibba*, measured as fresh weight elevation, was strongly inhibited by ABA whereas, the organic material production was somewhat affected when determined as dry weight increase (Tillberg et al., 1981). Starch accumulation in duckweed was triggered by the inhibitory effect of ABA and nutrient starvation. When grown in the presence of 10^{-6} M ABA for 6 days, the fresh weight of *L.minor* declined about 60% however, 220% elevation in dry mass and nearly 500% increase in starch content of duckweed was reported by Mclaren & Smith, (1976). In spite of inhibiting the growth of duckweed, ABA activates colossal starch accumulation as dry biomass.

When treated with ABA, instant reaction of declined gaseous exchange due to stomatal closure is shown by most plants. However, ABA treatment shows no impact on stomatal functioning of *L. minor* and *L. gibba* (Bauer et al., 1976) (Tillberg et al., 1981). Bauer et al., (1976) reported that the *Lemna* species shows non-stomatal inhibition on photosynthesis. Starch accumulation takes place in duckweed by addition of a certain concentration of ABA in growth media. As per Dekock et al., (1978) significant decrease

in growth and starch accumulation in *L. gibba* is observed in the presence of 3 9 10^{-6}M ABA however, starch grains disappeared at 10^{-7} M ABA and duckweed cells grew rapidly. It is noticeable that ABA stimulates starch accumulation and hinders the growth of duckweed.

2.8.5. Other Chemical Growth Inhibitors

Starch accumulation in duckweeds is also affected by some chemicals inhibitory to microbial metabolism or plants. A strong inhibitor of L-phenylalanine ammonia-lyase (PAL) i.e 2-aminoindan-2-phosphonic acid (AIP) reported by (Janas, Osiecka, & Zoń, (1998) gave rise to starch enrichment in duckweed. When treated with 100 µM AIP during the first 5 days and following 3 days the starch content in dry mass of duckweed elevated by 130% and 370% respectively. Less accumulation of starch in duckweed was observed on treatment with other PAL inhibitors such as 1-amino-2- phenylethylphosphonic acid (PheP) and 1-amino-3-phenylpropylphosphonic acid (PhPP) (Janas, 1995.). Starch accumulation started in the chloroplasts of fronds and root plastids of *L. pausicostata* when treated with Apicidin, a cyclic tetrapeptide fungal toxin, and till the later stages of a 72-h treatment, starch inclusions continued to increase (Abbas, Gronwald, Plaisance, Paul, & Lee, 2001).

2.8.6. Turion Formation in Duckweed

For surviving under unfavourable circumstances like nutrient starvation or temperature fluctuation, most species of duckweed produce special ‘resting fronds’ (Xiao et al., 2013). Resting fronds are of three different types including fronds that do not sink to the bottom of the water, that sinks to the bottom and the turions that are morphologically different from normal fronds and sink to the bottom of the water but do not grow any further.

Including *S. polyrhiza*, being firstly reported, about 10 duckweed species produce turions. The special characteristics of turions in *S. polyrhiza* grabbed the attention of researchers as these turions are 0.5–3.5-mm wide, olive-green to dark brown in colour, orbicular to teniform in shape and have nonvisible rudimentary roots (Rubel, 1986). The turions cells can gather tannins, anthocyanins, and mainly starch grains that constitute up to 70% of dry mass, because the cells of turions could attain size of vegetative frond cells. Phosphate, nitrate or sulphate limitation or ABA application can induce the turion

formation in duckweed and play role in high starch accumulation that is main point of interest for researcher.

2.9. Biotransformation of Duckweed for Biofuel Production

Currently corn starch is considered as major feedstock for biofuel production via fermentation but duckweed could serve as an excellent alternative feedstock and can also be used for biogas synthesis via anaerobic digestion.

2.9.1. Bioethanol Production

Ethanol could be synthesized from starch rich duckweed by following two steps: saccharification and fermentation. The first step involves saccharification of starch and other carbohydrates to produce fermentable sugars by enzyme catalysis whereas, in second step yeasts or bacteria carry out fermentation of the sugars to ethanol. To maximize the sugar release from duckweed, multiple enzymatic hydrolysis have been tested including the use of α-amylase, pullulanase and amyloglucosidase for hydrolysis through which theoretically 96.8% of sugar recovery was attained (Cui et al., 2000).

In order to recover sugar from duckweed cell wall Gray, et al., (2012) tested few mixtures of cellulase enzymes for degrading cell wall. By utilizing commercial cellulase at 4.35 FPU g^{-1} substrate in conjunction with beta-glucosidase at 100 Ug^{-1} substrate, saccharification could be achieved within about 8 h but those enzymes are quite costly. Saccharification efficiency was enhanced by pectinase hydrolysis which release glucose from *L. punctata* mash and glucose yield of 218.6 -3.1 mgg^{-1} dry matter was achieved, which is a 142% more as compared to untreated mash (Chen et al., 2009.). For saccharification of the S. *polyrhiza* starch to fermentable sugars Xu et al. (2011) conducted a study according to which duckweed starch content was increased up to 64.9% when transferred from nutrient-rich wastewater into well water for 10 days.

Through enzymatic hydrolysis and yeast fermentation, starch was converted to ethanol achieving 94.7% of the theoretical starch conversion to ethanol. According to a study duckweed cultivation could provide starch yield of 9420 $kg.ha^{-1}.year^{-1}$, leading to an ethanol yield of 6420 $ha^{-1}year^{-1}$, about 50% higher than that obtained using corn which makes duckweed-to-ethanol conversion a promising technology to supplement corn-based ethanol production (Cui et al., 2009).

2.9.2. Biogas Production

Utilization of organic waste materials like animal manure for biogas production through anaerobic digestion has been practiced since decades. The biomass of duckweed, a common aquatic plant growing on agricultural runoff containing water bodies, could be used in farm-scale anaerobic digesters in order to produce biogas. In laboratory-scale anaerobic digesters, mixed poultry manure and iron-enriched fresh duckweed was used by (Clark & Hillman, (1996) in batch and semi-continuous operations leading to conclusion that 150 ml biogas g^{-1} dry biomass could be produced from powdered duckweed. Biogas production could be elevated up to 44% by adding duckweed to digester. By adding five different concentrations of dry duckweed along with dairy manure in anaerobic digestion (Triscari et al., 2009) find out that the addition of 0.5–2.0% duckweed enhanced methane and total biogas production significantly. Similarly, according to Weidong et al., (2013), at a ratio of swine manure to duckweed of 1:1 in a 4 m^3 pilot-scale plug-flow baffled digester, COD conversion rate and volumetric biogas production were 0.31 Lg^{-1} COD, 63.2% and 1.00 $m^{-3}.day^{-1}$, respectively. Whereas, in the control anaerobic digester containing swine manure as sole substrate, biogas yield, COD conversion rate and volumetric biogas production were 0.28 Lg^{-1} COD, 57.1% and 0.71 $m^{-3}day^{-1}$, respectively was observed which indicates that biogas production in the anaerobic digester improved significantly by addition of duckweed.

References

1. Adekunle, K. F., & Okolie, J. A. (2015). A review of biochemical process of anaerobic digestion. *Advances in Bioscience and Biotechnology*, *6*(03), 205. https://www.scirp.org/html/7-7301031_55061.htm
2. Christy, P., Gopinath, L., Energy, D. D.-R. and S., & 2014, undefined. (n.d.). A review on anaerobic decomposition and enhancement of biogas production through

enzymes and microorganisms. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S1364032114001671

3. Cirne, D. G., Lehtomäki, A., Björnsson, L., & Blackall, L. L. (2007). Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops. *Journal of Applied Microbiology*, *103*(3), 516–527. https://doi.org/10.1111/j.1365-2672.2006.03270.x
4. Demirel, B., & Yenigün, O. (2002). Two-phase anaerobic digestion processes: A review. *Journal of Chemical Technology and Biotechnology*, Vol. 77, pp. 743–755. https://doi.org/10.1002/jctb.630
5. Gerardi, M. H. (2003). *The microbiology of anaerobic digesters*. John Wiley & Sons.
6. Jain, S., Jain, S., Wolf, I., Lee, J., Sustainable, Y. T.-R. and, & 2015, undefined. (n.d.). A comprehensive review on operating parameters and different pretreatment methodologies for anaerobic digestion of municipal solid waste. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S1364032115007388
7. Lu, J., Gavala, H., Skiadas, I., … Z. M.-J. of, & 2008, undefined. (n.d.). Improving anaerobic sewage sludge digestion by implementation of a hyper-thermophilic prehydrolysis step. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S030147970700182X
8. Methanogenesis and methanogenic partnerships. (1982). *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *297*(1088), 599–616. https://doi.org/10.1098/rstb.1982.0064
9. Palatsi, J., Laureni, M., Andrés, M., … X. F.-B., & 2009, undefined. (n.d.). Strategies for recovering inhibition caused by long chain fatty acids on anaerobic thermophilic biogas reactors. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0960852409004490
10. Raheman, H., bioenergy, S. M.-B. and, & 2012, undefined. (n.d.). Biogas production potential of jatropha seed cake. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0961953411006611
11. Riviere, D., Desvignes, V., … E. P.-T. I., & 2009, undefined. (n.d.). Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *Nature.Com*. Retrieved from https://www.nature.com/articles/ismej20092/

12. Ryckebosch, E., Drouillon, M., & Vervaeren, H. (2011, May 1). Techniques for transformation of biogas to biomethane. *Biomass and Bioenergy*, Vol. 35, pp. 1633–1645. https://doi.org/10.1016/j.biombioe.2011.02.033
13. Schmidt, S., Biegel, E., (BBA), V. M.-B. et B. A., & 2009, undefined. (n.d.). The ins and outs of Na+ bioenergetics in Acetobacterium woodii. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0005272809000048
14. Schnurer, A., U, A. J.-S. W. M., & 2010, undefined. (n.d.). Microbiological handbook for biogas plants. *Emrg.It*. Retrieved from http://www.emrg.it/Lezioni_Energia_Rinnovabile/Microbiological_handbook_for_biogas.pdf
15. Singh, R., Vyas, D., Srivastava, N., energy, M. N.-R., & 2008, undefined. (n.d.). SPRERI experience on holistic approach to utilize all parts of Jatropha curcas fruit for energy. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0960148107003175
16. Huacuz, J. M. (2005). The road to green power in Mexico—reflections on the prospects for the large-scale and sustainable implementation of renewable energy. *Energy Policy*, *33*(16), 2087–2099. https://doi.org/10.1016/J.ENPOL.2004.04.004
17. Woli, P., & Paz, J. (2013). International Journal of Environment and Bioenergy, 2013, 7(1): 28-42 International Journal of Environment and Bioenergy Biomass Yield and Utilization Rate Effects on the Sustainability and Environment-friendliness of Maize Stover-and Switchgrass-based Ethanol Production. *Int. J. Environ. Bioener*, *7*(1), 28–42. Retrieved from www.ModernScientificPress.com/Journals/IJEE.aspx
18. Asif, M. (2009). Sustainable energy options for Pakistan. *Renewable and Sustainable Energy Reviews*, *13*(4), 903–909. https://doi.org/10.1016/J.RSER.2008.04.001
19. Wyman, C. E. (1999). B IOMASS E THANOL : Technical Progress, Opportunities, and Commercial Challenges. *Annual Review of Energy and the Environment*, *24*(1), 189–226. https://doi.org/10.1146/annurev.energy.24.1.189
20. Uihlein, A., & Schebek, L. (2009). Environmental impacts of a lignocellulose feedstock biorefinery system: An assessment. *Biomass and Bioenergy*, *33*(5), 793–802. https://doi.org/10.1016/J.BIOMBIOE.2008.12.001

21. Amenaghawon, N. A., Ogbeide, S. E., & Okieimen, C. O. (2014). Application of statistical experimental design for the optimisation of dilute sulphuric acid hydrolysis of cassava bagasse. *Acta Polytechnica Hungarica*, *11*(9), 239-250. 3rhttps://www.uni-obuda.hu/journal/Amenaghawon_Ogbeide_Okieimen_55.pdf
22. International Monetary Fund. Research Dept. (2007). *World Economic Outlook, April 2007: Spillovers and Cycles in the Global Economy*. International Monetary Fund.
23. Oliveira, F. M. V., Pinheiro, I. O., Souto-Maior, A. M., Martin, C., Gonçalves, A. R., & Rocha, G. J. M. (2013). Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products. *Bioresource Technology*, *130*, 168–173. https://doi.org/10.1016/j.biortech.2012.12.030
24. Lior, N. (2002). Thoughts about future power generation systems and the role of exergy analysis in their development. *Energy Conversion and Management*, *43*(9–12), 1187–1198. https://doi.org/10.1016/S0196-8904(02)00007-9
25. Qureshi, M. A., & Afghan, S. (2005). Sugarcane cultivation in Pakistan. *Sugar Book Pub. Pakistan Society of Sugar Technologist*.
26. Woli, P., & Paz, J. (2013). International Journal of Environment and Bioenergy, 2013, 7(1): 28-42 International Journal of Environment and Bioenergy Biomass Yield and Utilization Rate Effects on the Sustainability and Environment-friendliness of Maize Stover-and Switchgrass-based Ethanol Production. *Int. J. Environ. Bioener*, *7*(1), 28–42. Retrieved from www.ModernScientificPress.com/Journals/IJEE.aspx
27. Lynd, L. R., Weimer, P. J., Zyl, W. H. Van, & Pretorius, I. S. (2002). Microbial celullosa utilizaton: Fundamentals and Biotechnology. *Bioresource Technology*, *66*(3), 506–577. https://doi.org/10.1128/MMBR.66.3.506
28. Pereira, C. L. F., & Ortega, E. (2010). Sustainability assessment of large-scale ethanol production from sugarcane. *Journal of Cleaner Production*, *18*(1), 77–82. https://doi.org/10.1016/j.jclepro.2009.09.007
29. Dhussa, A. (2004). Biogas in India. *Globalmethane.Org*. Retrieved from http://www.globalmethane.org/documents/partners_india_ag_update.pdf
30. Santos, F. F. P., Malveira, J. Q., Cruz, M. G. A., & Fernandes, F. A. N. (2010).

Production of biodiesel by ultrasound assisted esterification of Oreochromis niloticus oil. *Fuel*, *89*(2), 275–279. https://doi.org/10.1016/J.FUEL.2009.05.030

31. Kumar Tiwari, A., Kumar, A., & Raheman, H. (2007). Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: An optimized process. *Biomass and Bioenergy*, *31*(8), 569–575. https://doi.org/10.1016/J.BIOMBIOE.2007.03.003
32. Qureshi, M. A., & Afghan, S. (2005). Sugarcane cultivation in Pakistan. *Sugar Book Pub. Pakistan Society of Sugar Technologist*. Comparative analysis of biodiesel versus green diesel. *Wiley Interdisciplinary Reviews: Energy and Environment*. https://doi.org/10.1002/wene.78
33. Pragya, N., & Pandey, K. K. (2016). Life cycle assessment of green diesel production from microalgae. *Renewable Energy*. https://doi.org/10.1016/j.renene.2015.08.064
34. Prieto, M. G., Sánchez, F. A., & Pereda, S. (2019). A Group Contribution Equation of State for Biorefineries. GCA-EOS Extension to Bioether Fuels and Their Mixtures with n-Alkanes. *Journal of Chemical and Engineering Data*. https://doi.org/10.1021/acs.jced.8b01153
35. Quintero, J. A., Montoya, M. I., Sánchez, O. J., Giraldo, O. H., & Cardona, C. A. (2008). Fuel ethanol production from sugarcane and corn: Comparative analysis for a Colombian case. *Energy*, *33*(3), 385–399. https://doi.org/10.1016/j.energy.2007.10.001
36. Wang, M., Saricks, C., & Santini, D. (1999). Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions. Retrieved from https://trid.trb.org/view/497419
37. Browner, C. (2000). *Remarks as prepared for delivery to press conference on March 20, 2000*. Retrieved from https://scholar.google.com.pk/scholar?
38. McCarthy, J., & Tiemann, M. (2006). MTBE in Gasoline: Clean Air and Drinking Water Issues. *Congressional Research Service Reports*. Retrieved from https://digitalcommons.unl.edu/crsdocs/26
39. Mabee, W. (2006). *Economic, Environmental and Social Benefits of 2 nd-Generation Biofuels in Canada*. Retrieved from www.biocap.ca
40. Demirbas, A. (2007). Progress and recent trends in biofuels. *Progress in Energy*

and Combustion Science, *33*(1), 1–18. https://doi.org/10.1016/j.pecs.2006.06.001

41. Goldemberg, J., Coelho, S. T., & Guardabassi, P. (2008). The sustainability of ethanol production from sugarcane. *Energy Policy*, *36*(6), 2086–2097. https://doi.org/10.1016/j.enpol.2008.02.028
42. Zabed, H, Sahu, J. N., Boyce, A. N., & Faruq, G. (2016). Fuel ethanol production from lignocellulosic biomass : An overview on feedstocks and technological approaches. *Renewable and Sustainable Energy Reviews*, *66*, 751–774. https://doi.org/10.1016/j.rser.2016.08.038
43. Harvey, J. (2007). *A versatile solution? Growing Miscanthus for bioenergy*. Retrieved from http://www.bioagrolife.com
44. Maeda, R. N., Barcelos, C. A., Santa Anna, L. M. M., & Pereira Jr, N. (2013). Cellulase production by Penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. *Journal of Biotechnology*, *163*(1), 38-44.
45. Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. *Bioresource Technology*, *165*(C), 3–8. https://doi.org/10.1016/j.biortech.2014.01.127
46. Zabed, Hossain, Faruq, G., Sahu, J. N., Azirun, M. S., Hashim, R., & Nasrulhaq Boyce, A. (2014). Bioethanol Production from Fermentable Sugar Juice. *The Scientific World Journal*, *2014*, 1–11. https://doi.org/10.1155/2014/957102
47. Kim, S. B., & Lee, Y. Y. (2002). Diffusion of sulfuric acid within lignocellulosic biomass particles and its impact on dilute-acid pretreatment. *Bioresource Technology*, *83*(2), 165–171. https://doi.org/10.1016/S0960-8524(01)00197-3
48. Leong, W. H., Lim, J. W., Lam, M. K., Uemura, Y., & Ho, Y. C. (2018, August 1). Third generation biofuels: A nutritional perspective in enhancing microbial lipid production. *Renewable and Sustainable Energy Reviews*, Vol. 91, pp. 950–961. https://doi.org/10.1016/j.rser.2018.04.066
49. Lam, M. K., & Lee, K. T. (2012, May 1). Microalgae biofuels: A critical review of issues, problems and the way forward. *Biotechnology Advances*, Vol. 30, pp. 673–690. https://doi.org/10.1016/j.biotechadv.2011.11.008
50. McLAREN, J. S., & Smith, H. (1976). The effect of abscisic acid on growth,

photosynthetic rate and carbohydrate metabolism in Lemna minor L. *New Phytologist*, *76*(1), 11-20. https://doi.org/10.1111/j.1469-8137.1976.tb01433.x

51. Jambo, S. A., Abdulla, R., Mohd Azhar, S. H., Marbawi, H., Gansau, J. A., & Ravindra, P. (2016, November 1). A review on third generation bioethanol feedstock. *Renewable and Sustainable Energy Reviews*, Vol. 65, pp. 756–769. https://doi.org/10.1016/j.rser.2016.07.064
52. Cheng, J., Landesman, L., … B. B.-T. of the, & 2002, undefined. (n.d.). Nutrient removal from swine lagoon liquid by Lemna minor 8627. *Elibrary.Asabe.Org*. Retrieved from https://elibrary.asabe.org/abstract.asp?aid=9953
53. Cui, W., Xu, J., Cheng, J. J., & Stomp, A. M. (2011). Starch accumulation in duckweed for bioethanol production. *Biological Engineering*, *3*(4), 187–197. https://doi.org/10.13031/2013.37123
54. Peng, J., Wang, B., Song, Y., modelling, P. Y., & 2007, undefined. (n.d.). Modeling N transformation and removal in a duckweed pond: Model application. *Elsevier*. Retrieved from https://www.sciencedirect.com
55. Abbas, H. K., Gronwald, J. W., Plaisance, K. L., Paul, R. N., & Lee, Y. W. (2001). Histone deacetylase activity and phytotoxic effects following exposure of duckweed (Lemna pausicostata L.) to apicidin and HC-toxin. *Phytopathology*, *91*(12), 1141–1148. https://doi.org/10.1094/PHYTO.2001.91.12.1141
56. Addicott, F. T., & Lyon, J. L. (1969). Physiology of Abscisic Acid and Related Substances. *Annual Review of Plant Physiology*, *20*(1), 139–164. https://doi.org/10.1146/annurev.pp.20.060169.001035
57. Ae, S. E. W., Weber, A. P. M., & Sharkey, T. D. (2004). Maltose is the major form of carbon exported from the chloroplast at night. *Springer*, *218*(3), 474–482. https://doi.org/10.1007/s00425-003-1128-y
58. Ae, Y. L., & Sharkey, T. D. (2004). The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. *Springer*, *218*(3), 466–473. https://doi.org/10.1007/s00425-003-1127-z
59. Ahmed, F. E. (2001, September 15). Toxicology and human health effects following exposure to oxygenated or reformulated gasoline. *Toxicology Letters*, Vol. 123, pp. 89–113. https://doi.org/10.1016/S0378-4274(01)00375-7
60. Alaerts, G. J., Mahbubar, R., & Kelderman, P. (1996). Performance analysis of a

full-scale duckweed-covered sewage lagoon. *Water Research*, *30*(4), 843-852. Retrieved from https://www.sciencedirect.com/science/article/pii/0043135495002340

61. Amorim, H. V., Lopes, M. L., De Castro Oliveira, J. V., Buckeridge, M. S., & Goldman, G. H. (2011, September 7). Scientific challenges of bioethanol production in Brazil. *Applied Microbiology and Biotechnology*, Vol. 91, pp. 1267–1275. https://doi.org/10.1007/s00253-011-3437-6
62. Appenroth, K. J., Keresztes, Á., Sárvári, É., Jaglarz, A., & Fischer, W. (2003). Multiple effects of chromate on Spirodela polyrhiza: Electron microscopy and biochemical investigations. *Plant Biology*, *5*(3), 315–323. https://doi.org/10.1055/s-2003-40801
63. Appenroth, K. J., Krech, K., Keresztes, A., Fischer, W., & Koloczek, H. (2010). Effects of nickel on the chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in biomonitoring and phytoremediation. *Chemosphere*, *78*(3), 216-223. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0045653509013307
64. Asatsuma, S., Sawada, C., Itoh, K., … M. O.-P. and C., & 2005, undefined. (n.d.). Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. *Academic.Oup.Com*. Retrieved from https://academic.oup.com/pcp/article-abstract/46/6/858/1823295
65. Bagi, Z., Ács, N., Bálint, B., Horváth, L., Dobó, K., Perei, K. R., … Kovács, K. L. (2007). Biotechnological intensification of biogas production. *Applied Microbiology and Biotechnology*, *76*(2), 473–482. https://doi.org/10.1007/s00253-007-1009-6
66. Balat, M., Balat, H., & Öz, C. (2008). Progress in bioethanol processing. *Progress in Energy and Combustion Science*, *34*(5), 551–573. https://doi.org/10.1016/J.PECS.2007.11.001
67. Bauer, R., Huber, W., & Sankhla, N. (1976). Effect of abscisic acid on photosynthesis in Lemna minor L. *Zeitschrift für Pflanzenphysiologie*, *77*(3), 237-246.Retrieved from https://www.sciencedirect.com/science/article/pii/S0044328X76802000
68. Boniardi, N., Vatta, G., Rota, R. E. N. A. T. O., Nano, G. I. U. S. E. P. P. E., &

Carra, S. (1994). Removal of water pollutants by Lemna gibba. *The Chemical Engineering Journal and the Biochemical Engineering Journal*, *54*(2), B41-B48. Retrieved from https://www.sciencedirect.com/science/article/pii/0923046794060517

69. Botany, H. W.-A. of, & 1939, undefined. (n.d.). The Interaction of Factors in the Growth of Lemna: XIV. The Interaction of Potassium and Light Intensity in Relation to Growth and Assimilation. *JSTOR*. Retrieved from https://www.jstor.org/stable/42906734
70. Budzianowski, W. M. (2017). High-value low-volume bioproducts coupled to bioenergies with potential to enhance business development of sustainable biorefineries. *Renewable and Sustainable Energy Reviews*, *70*, 793–804. https://doi.org/10.1016/J.RSER.2016.11.260
71. Campbell, C.J. (2013). The Oil Age in Perspective. *Energy Exploration & Exploitation*, *31*(2), 149–165. https://doi.org/10.1260/0144-5987.31.2.149
72. Campbell, Colin J., & Laherrère, J. H. (1998). The End of Cheap Oil. *Scientific American*, Vol. 278, pp. 78–83. https://doi.org/10.2307/26057708
73. Chen, J. (2004). A Previously Unknown Maltose Transporter Essential for Starch Degradation in Leaves. *Science.Sciencemag.Org*. https://doi.org/10.1126/science.1091811
74. Chen, Q., Jin, Y., Zhang, G., Fang, Y., Xiao, Y., Energies, H. Z.-, & 2012, undefined. (n.d.). Improving production of bioethanol from duckweed (Landoltia punctata) by pectinase pretreatment. *Mdpi.Com*. Retrieved from https://www.mdpi.com/1996-1073/5/8/3019
75. Cheng, J. J., & Stomp, A. M. (2009). Growing Duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *Clean - Soil, Air, Water*, Vol. 37, pp. 17–26. https://doi.org/10.1002/clen.200800210
76. Cheng, J., Landesman, L., Bergmann, B. A., Classen, J. J., Howard, J. W., & Yamamoto, Y. T. (2002). Nutrient removal from swine lagoon liquid by Lemna minor 8627. *Transactions of the ASAE*, *45*(4), 1003. Retrieved from https://elibrary.asabe.org/abstract.asp?aid=9953
77. Cheng, J., Landesman, L., Bergmann, B. A., Classen, J. J., Howard, J. W., & Yamamoto, Y. T. (2002). Nutrient removal from swine lagoon liquid by Lemna

minor 8627. *Transactions of the ASAE*, *45*(4), 1003. Retrieved from https://elibrary.asabe.org/abstract.asp?aid=9953

78. Chia, T., Thorneycroft, D., Chapple, A., Messerli, G., Chen, J., Zeeman, S. C., … Smith, A. M. (2004). A cytosolic glucosyltransferase is required for conversion of starch to sucrose in Arabidopsis leaves at night. *Plant Journal*, *37*(6), 853–863. https://doi.org/10.1111/j.1365-313X.2003.02012.x
79. Ciereszko, I., Physiology, A. B.-J. of P., & 2000, undefined. (n.d.). Sucrose metabolism in leaves and roots of bean (Phaseolus vulgaris L.) during phosphate deficiency. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0176161700802254
80. Clark, P. B., & Hillman, P. F. (1996). Enhancement of anaerobic digestion using duckweed (lemna minor) enriched with iron. *Water and Environment Journal*, *10*(2), 92–95. https://doi.org/10.1111/j.1747-6593.1996.tb00015.x
81. Cui, W. (2010). Growing Duckweed for Bioethanol Production. *Elibrary.Asabe.Org*. https://doi.org/10.13031/2013.29907
82. Cui, W., & Cheng, J. J. (2015). Growing duckweed for biofuel production: A review. *Plant Biology*, *17*(s1), 16–23. https://doi.org/10.1111/plb.12216
83. Cui, W., Xu, J., Louisville, J. C.-, Kentucky, undefined, 7-10, A., 2011, undefined, & 2011, undefined. (n.d.). Duckweed starch accumulation for bioethanol production. *Elibrary.Asabe.Org*. Retrieved from https://elibrary.asabe.org/abstract.asp?aid=37783
84. DeKock, P. C., Vaughan, D., & Hall, A. (1978). Effect of abscisic acid and benzyl adenine on the inorganic and organic composition of the duckweed, Lemna gibba L. *New Phytologist*, *81*(3), 505-511. https://doi.org/10.1111/j.1469-8137.1978.tb01623.x
85. Demirbas, A. (2007). Progress and recent trends in biofuels. *Progress in Energy and Combustion Science*, *33*(1), 1–18. https://doi.org/10.1016/j.pecs.2006.06.001
86. Farrell, J. B. (2012). Duckweed uptake of phosphorus and five pharmaceuticals: microcosm and wastewater lagoon studies. Retrieved from https://digitalcommons.usu.edu/etd/1212
87. Dinges, J. R., Colleoni, C., James, M. G., & Myers, A. M. (2003). Mutational Analysis of the Pullulanase-Type Debranching Enzyme of Maize Indicates Multiple

Functions in Starch Metabolism. *The Plant Cell*, *15*, 666–680. https://doi.org/10.1105/tpc.007575

88. Doehlert, D., Physiology, C. K.-J. of P., & 1991, undefined. (n.d.). Two classes of starch debranching enzymes from developing maize kernels. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0176161711802427
89. Drosg, B., Braun, R., Bochmann, G., & Al Saedi, T. (2013). Analysis and characterisation of biogas feedstocks. In *The Biogas Handbook: Science, Production and Applications* (pp. 52–84). https://doi.org/10.1533/9780857097415.1.52
90. Effects, M. L. A. T., Fate, E., & 1995, undefined. (n.d.). Algae and vascular plant tests. *Books.Google.Com*.
91. Endo, A., Nakamura, T., Ando, A., Tokuyasu, K., & Shima, J. (2008). Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in Saccharomyces cerevisiae. *Biotechnology for Biofuels*, *1*(1), 1–6. https://doi.org/10.1186/1754-6834-1-3
92. Ziegler, P., Sree, K. S., & Appenroth, K. J. (2016). Duckweeds for water remediation and toxicity testing. *Toxicological & Environmental Chemistry*, *98*(10), 1127-1154.
93. Eyster, H. C. (1978). Brief Note Nutrient Concentration Requirements for Chlorella Sorokiniana. Retrieved from https://kb.osu.edu/bitstream/handle/1811/22516/1/V078N2_079.pdf
94. Frandsen, T. P., & Svensson, B. (1998). Plant α-glucosidases of the glycoside hydrolase family 31. Molecular properties, substrate specificity, reaction mechanism, and comparison with family members of different origin. *Plant Molecular Biology*, Vol. 37, pp. 1–13. https://doi.org/10.1023/A:1005925819741
95. Ghosh, P., & Ghose, T. K. (2003). Bioethanol in India: recent past and emerging future. *Advances in Biochemical Engineering/Biotechnology*, Vol. 85, pp. 1–27. https://doi.org/10.1007/3-540-36466-8_1
96. Gray, K. A., Zhao, L., & Emptage, M. (2012). *Bioethanol*. https://doi.org/10.1016/j.cbpa.2006.02.035
97. Hasan, M. M., Hasan, M., & Saeed, T. (2018). *EVALUATION OF DUCKWEED BASED WASTE STABILIZATION POND SYSTEM FOR DOMESTIC*

WASTEWATER TREATMENT. Retrieved from https://www.researchgate.net/publication/323257940

98. Hejazi, M., Fettke, J., Kö, O., Zeeman, S. C., & Steup, M. (2010). The Laforin-Like Dual-Specificity Phosphatase SEX4 from Arabidopsis Hydrolyzes Both C6-and C3-Phosphate Esters Introduced by Starch-Related Dikinases and Thereby Affects Phase Transition of a-Glucans 1[W]. *Plant Physiology Ò*, *152*, 711–722. https://doi.org/10.1104/pp.109.149914
99. Hussain, H., Mant, A., Seale, R., Zeeman, S., Hinchliffe, E., Edwards, A., … Bustos, R. (2003). Three Isoforms of Isoamylase Contribute Different Catalytic Properties for the Debranching of Potato Glucans. *The Plant Cell*, *15*, 133–149. https://doi.org/10.1105/tpc.006635
100. ISHIZAKI, Y., TANIGUCHI, H., MARUYAMA, Y., & NAKAMURA, M. (1983). Debranching Enzymes of Potato Tubers (Solanum tuberosum L.). *Journal of the Japanese Society of Starch Science*, *30*(1), 19-29. Retrieved from https://www.jstage.jst.go.jp/article/jag1972/30/1/30_1_19/_article/-char/ja/
101. Janas, K. M., Osiecka, R., & Zoń, J. (1998). Growth-retarding effect of 2-aminoindan-2-phosphonic acid on Spirodela punctata. *Journal of Plant Growth Regulation*, *17*(3), 169–172. https://doi.org/10.1007/PL00007031
102. Janas, K., Supplement, R. O.-B. B. of P., & 1995, undefined. (n.d.). Effect of aminophosphonates on starch accumulation in Spirodela punctata. *Agro.Icm.Edu.Pl*. Retrieved from http://agro.icm.edu.pl/agro/element/bwmeta1.element.agro-bf2158b9-8b27-4c55-9835-45feaf99e6d1
103. JONG, J. G. T.-D., & VELDSTRA, H. (1971). Investigations on Cytokinins. II. Interaction of Light and Cytokinins as Studied in Lemna minor. *Physiologia Plantarum*, *24*(2), 239–241. https://doi.org/10.1111/j.1399-3054.1971.tb03486.x
104. Kakefuda, G., Duke, S. H., & Hostak, M. S. (1986). Chloroplast and extrachloroplastic starch-degrading enzymes in Pisum sativum L. *Planta*, *168*(2), 175–182. https://doi.org/10.1007/BF00402961
105. Kö, O., Pusch, K., Tiessen, A., Geigenberger, P., Steup, M., & Ritte, G. (2005). Identification of a Novel Enzyme Required for Starch Metabolism in Arabidopsis Leaves. The Phosphoglucan, Water Dikinase 1[w]. *Am Soc Plant Biol*.

https://doi.org/10.1104/pp.104.055954

106. Kruger, N., Planta, T. A. R.-, & 1983, undefined. (n.d.). Maltose metabolism by pea chloroplasts. *Springer*. Retrieved from https://link.springer.com/content/pdf/10.1007/BF00397712.pdf

107. Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. *Bioresource Technology*, *165*(C), 3–8. https://doi.org/10.1016/j.biortech.2014.01.127

108. Li, B., Servaites, J. C., & Geiger, D. R. (1992). Characterization and Subcellular Localization of Debranching Enzyme and Endoamylase from Leaves of Sugar Beet1. In *Plant Physiol* (Vol. 98). Retrieved from www.plantphysiol.org

109. Lin, T.-P., Spilatro, S. R., & Preiss, J. (1988). Subcellular Localization and Characterization of Amylases in Arabidopsis Leaf . *Plant Physiology*, *86*(1), 251–259. https://doi.org/10.1104/pp.86.1.251

110. Lloyd, J., Kossmann, J., science, G. R.-T. in plant, & 2005, undefined. (n.d.). Leaf starch degradation comes out of the shadows. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S1360138505000166

111. Ludwig, I., Ziegler, P., & Beck, E. (1984). Purification and Properties of Spinach Leaf Debranching Enzyme. *Plant Physiology*, *74*(4), 856–861. https://doi.org/10.1104/pp.74.4.856

112. McCarthy, J., & Tiemann, M. (2006). MTBE in Gasoline: Clean Air and Drinking Water Issues. *Congressional Research Service Reports*. Retrieved from https://digitalcommons.unl.edu/crsdocs/26

113. Mclaren, J. S., & Smith, H. (1976). The effect of abscisic acid on growth, photosynthetic rate and carbohydrate metabolism in *Lemna minor* l. *New Phytologist*, *76*(1), 11–20. https://doi.org/10.1111/j.1469-8137.1976.tb01433.x

114. Nahar, K., & Sunny, S. A. (2020, March 1). Duckweed-based clean energy production dynamics (ethanol and biogas) and phyto-remediation potential in Bangladesh. *Modeling Earth Systems and Environment*, Vol. 6, pp. 1–11. https://doi.org/10.1007/s40808-019-00659-y

115. Nass, U. (2011). *Crop Production 2010 Summary*.

116. Okita, T. W., Greenberg, E., & Kuhn2, D. N. (1979). Subcellular

Localization of the Starch Degradative and Biosynthetic Enzymes of Spinach Leaves1. In *Plant Physiol* (Vol. 64). Retrieved from http://www.plantphysiol.org/content/64/2/187.short

117. Papong, S., & Malakul, P. (2010). Life-cycle energy and environmental analysis of bioethanol production from cassava in Thailand. *Bioresource Technology*, *101*(1 SUPPL.), S112–S118. https://doi.org/10.1016/j.biortech.2009.09.006

118. Peng, J., Wang, B., Song, Y., modelling, P. Y., & 2007, undefined. (n.d.). Modeling N transformation and removal in a duckweed pond: Model application. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0304380007001858

119. Pimentel, D. (2003). Ethanol fuels: Energy balance, economics, and environmental impacts are negative. *Natural Resources Research*, *12*(2), 127–134. https://doi.org/10.1023/A:1024214812527

120. Reid, M. S., & Bieleski, R. L. (1970). Response of Spirodela oligorrhiza to Phosphorus Deficiency. In *Plant Physiol* (Vol. 46). Retrieved from www.plantphysiol.org

121. research, W. W.-E., & 1990, undefined. (n.d.). Literature review on duckweed toxicity testing. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0013935105801471

122. Rgen Schleucher, J., Vanderveer, P. J., & Sharkey, T. D. (1998). Export of Carbon from Chloroplasts at Night 1. In *Am Soc Plant Biol*. Retrieved from www.plantphysiol.org

123. Ritte, G., Lloyd, J. R., Eckermann, N., Rottmann, A., Kossmann, J., & Steup, M. (n.d.). The starch-related R1 protein is an-glucan, water dikinase. In *National Acad Sciences*. Retrieved from www.pnas.orgcgidoi10.1073pnas.062053099

124. Sarkar, N., Ghosh, S. K., Bannerjee, S., & Aikat, K. (2012). Bioethanol production from agricultural wastes: An overview. *Renewable Energy*, *37*(1), 19–27. https://doi.org/10.1016/j.renene.2011.06.045

125. Scheidig, A., Fröhlich, A., Schulze, S., Lloyd, J. R., & Kossmann, J. (2002). Downregulation of a chloroplast-targeted β-amylase leads to a starch-excess

phenotype in leaves. *Plant Journal*, *30*(5), 581–591. https://doi.org/10.1046/j.1365-313X.2002.01317.x

126. Sharkey, T. D., Laporte, M., Lu, Y., Weise, S., & Weber, A. P. M. (2004, May). Engineering plants for elevated CO2: A relationship between starch degradation and sugar sensing. *Plant Biology*, Vol. 6, pp. 280–288. https://doi.org/10.1055/s-2004-817911

127. Shen, G., Xu, J., Hu, S., Zhao, Q., & Liu, Y. (2006). Nitrogen removal pathways in shallow-water duckweed-based wastewater treatment systems. *Journal of Ecology and Rural Environment*, *22*(1), 42-47.

128. Smith, S. M., Fulton, D. C., Chia, T., Thorneycroft, D., Chapple, A., Dunstan, H., … Smith, A. M. (2004). Diurnal Changes in the Transcriptome Encoding Enzymes of Starch Metabolism Provide Evidence for Both Transcriptional and Posttranscriptional Regulation of Starch Metabolism in Arabidopsis Leaves 1. *Am Soc Plant Biol*. https://doi.org/10.1104/pp.104.044347

129. Stitt, M., & Heldt, H. W. (1981). Physiological rates of starch breakdown in isolated intact spinach chloroplasts. In *Plant Physiol* (Vol. 68). Retrieved from www.plantphysiol.org

130. Sun', Z., Duke, S. H., & Henson, C. A. (1995). The Role of Pea Chloroplast a-Glucosidase in Transitory Starch Degradation'. In *Plant Phvsiol* (Vol. 108). Retrieved from www.plantphysiol.org

131. Takaha, T., Yanase, M., Okada, S., & Smith, S. M. (1993). Disproportionating enzyme (4-alpha-glucanotransferase; EC 2.4. 1.25) of potato. Purification, molecular cloning, and potential role in starch metabolism. *Journal of Biological Chemistry*, *268*(2), 1391-1396.Retrieved from https://www.jbc.org/content/268/2/1391.short

132. Tao, X., Fang, Y., Xiao, Y., Jin, Y. L., Ma, X. R., Zhao, Y., … Wang, H. Y. (2013). Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (Landoltia punctata) under nutrient starvation. *Biotechnology for Biofuels*, *6*(1). https://doi.org/10.1186/1754-6834-6-72

133. Thorsteinsson, B., & Tillberg, J. -E. (1987). Carbohydrate partitioning, photosynthesis and growth in Lemna gibba G3. II. Effects of phosphorus limitation. *Physiologia Plantarum*, *71*(3), 271–276. https://doi.org/10.1111/j.1399-

3054.1987.tb04341.x

134. Tillberg, E., Dons, C., … M. H.-P., & 1981, undefined. (n.d.). Effect of abscisic acid on CO2 exchange in Lemna gibba. *Wiley Online Library*. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1399-3054.1981.tb02707.x

135. Trethewey, R. N., & ap Rees, T. (1994). The role of the hexose transporter in the chloroplasts of Arabidopsis thaliana L. *Planta*, *195*(2), 168–174. https://doi.org/10.1007/BF00199675

136. Triscari, P., Reno, S. H.-2009, Nevada, undefined, & 2009, undefined. (n.d.). Anaerobic digestion of dairy manure combined with duckweed (Lemnaceae). *Elibrary.Asabe.Org*. Retrieved from https://elibrary.asabe.org/abstract.asp?aid=27445

137. Tunçsiper, B. (2019). Combined natural wastewater treatment systems for removal of organic matter and phosphorus from polluted streams. *Journal of Cleaner Production*, *228*, 1368–1376. https://doi.org/10.1016/j.jclepro.2019.04.211

138. Wang, M., Saricks, C., & Santini, D. (1999). Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions. Retrieved from https://trid.trb.org/view/497419

139. Wang, S., Xu, X., Sun, Y., Liu, J., bulletin, H. L.-M. pollution, & 2013, undefined. (n.d.). Heavy metal pollution in coastal areas of South China: a review. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0025326X13005183

140. Weidong, H., Dongxu, Z., Eng, X. W.-C. J. E., & 2013, undefined. (n.d.). Anaerobic fermentation of duckweed and swine manure in a plug-flow anaerobic digestion system. *En.Cnki.Com.Cn*. Retrieved from http://en.cnki.com.cn/Article_en/CJFDTotal-HJJZ201301060.htm

141. Weiland, P. (2010, September 24). Biogas production: Current state and perspectives. *Applied Microbiology and Biotechnology*, Vol. 85, pp. 849–860. https://doi.org/10.1007/s00253-009-2246-7

142. Wilkie, A. C., Riedesel, K. J., & Owens, J. M. (2000, August 1). Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass and Bioenergy*, Vol. 19, pp. 63–102.

https://doi.org/10.1016/S0961-9534(00)00017-9

143. Wyman, C. E. (1994). Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. *Bioresource Technology*, *50*(1), 3–15. https://doi.org/10.1016/0960-8524(94)90214-3

144. Wyman, C. E. (1999). B IOMASS E THANOL : Technical Progress, Opportunities, and Commercial Challenges. *Annual Review of Energy and the Environment*, *24*(1), 189–226. https://doi.org/10.1146/annurev.energy.24.1.189

145. Xiao, Y., Fang, Y., Jin, Y., Zhang, G., & Zhao, H. (2013). Culturing duckweed in the field for starch accumulation. *Industrial Crops and Products*, *48*, 183–190. https://doi.org/10.1016/j.indcrop.2013.04.017

146. Xu, J., technology, G. S.-B., & 2011, undefined. (n.d.-a). Growing duckweed in swine wastewater for nutrient recovery and biomass production. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0960852410015154

147. Xu, J., technology, G. S.-B., & 2011, undefined. (n.d.-b). Growing duckweed in swine wastewater for nutrient recovery and biomass production. *Elsevier*. Retrieved from https://www.sciencedirect.com/science/article/pii/S0960852410015154

148. Zeeman, S. C., Thorneycroft, D., Schupp, N., Chapple, A., Weck, M., Dunstan, H., … Smith, S. M. (n.d.). Plastidial a-Glucan Phosphorylase Is Not Required for Starch Degradation in Arabidopsis Leaves But Has a Role in the Tolerance of Abiotic Stress 1. *Am Soc Plant Biol*. https://doi.org/10.1104/pp.103.032631

149. Zeeman, S. C., Umemoto, T., Lue, W.-L., Au-Yeung, P., Martin, C., Smith, A. M., & Chen, J. (1998). A Mutant of Arabidopsis Lacking a Chloroplastic Isoamylase Accumulates Both Starch and Phytoglycogen. In *The Plant Cell* (Vol. 10). Retrieved from www.plantcell.org

150. Zhang, L., Chen, Q., Jin, Y., Xue, H., Guan, J., Wang, Z., & Zhao, H. (2010). Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG). *Fuel Processing Technology*, *91*(12), 1845–1850. https://doi.org/10.1016/j.fuproc.2010.08.009

151. Zhao, H., Landesman, L., & Salmeán, A. (2012). *Duckweed rising at*

Chengdu: summary of the 1st International Conference on Duckweed Application and Research. https://doi.org/10.1007/s11103-012-9889-y

152. Zhu, Z.-P., Hylton, C. M., Rö Ssner, U., & Smith, A. M. (n.d.). Characterization of Starch-Debranching Enzymes in Pea Embryos 1. In *Am Soc Plant Biol*. Retrieved from http://www.plantphysiol.org/content/118/2/581.short

Chapter 3: Starved *Spirodela polyrhiza* and *Saccharomyces cerevisiae*: A Potent Combination for Sustainable Bioethanol Production

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Abstract

The depletion of non-renewable fossil fuels and the rise in their prices depicts that there is a need to fend for alternative fuel resources. Biofuels are one of these renewable alternative options. Among biofuels, bioethanol is one of the most widely used transportation fuels around the world. However, currently bioethanol is produced from food crops that is raising food versus fuel feud. *Spirodela polyrhiza* is one of the novel feedstock that can be used to produce bioethanol at large scale without causing any food and fuel competition. This study involves the collection of *Spirodela polyrhiza* from a local pond and establishment of its growth in Hoagland growth media. The plant was then given nutrient starvation stress to enhance its starch content by 78%. The high starch containing plant biomass was acid pretreated and 99.3% starch to glucose conversion was achieved. In order to ferment plant sugars, yeast strain *Saccharomyces cerevisiae* QG1 MK788210 was indigenously isolated and statistically optimized using Plackett-Burman and Central Composite Design to achieve high ethanol yield. The fermentation of plant sugar by *Saccharomyces cerevisiae* QG1 MK788210 resulted in 100% ethanol yield thus successfully achieving complete conversion of *Spirodela polyrhiza* starch to bioethanol. The study conducted demonstrates effective optimization of indigenously isolated yeast strain *Saccharomyces cerevisiae* QG1 MK788210 to deliver high ethanol yield from *Spirodela polyrhiza*. Further, this study has been successful in delivering a process for complete conversion of starch from nutrient starved *Spirodela polyrhiza* biomass into bioethanol.

3.1. Introduction

The world's resource of non-renewable fossil fuels is depleting quickly. The ever growing transportation sector and increased industrialization has resulted in the increase of world's energy demand by 30% per year and the remaining reserves of non-renewable fossil fuels are not enough to meet this escalating energy demand (ul ain Rana et al., 2019). Apart from being depleted, these non-renewable fuels also have many environmental hazards which include greenhouse gas (GHG) and carcinogenic exhaust emissions (Nilsson & Johansson, 2017). In order to alleviate all these problems world is now more inclined towards the use of environmental friendly renewable fuel options that include biofuels (Rana et al., 2019). One of the most widely used biofuel in transportation sector around the globe is bioethanol (Manochio et al., 2017). Typically, bioethanol has a higher octane number than petrol and can easily be used as vehicle fuel. Bioethanol is renewable, reduces greenhouse gas emissions and is environmental friendly fuel (Hirani et al., 2018). Majority of the ethanol currently being produced around the world use feedstocks like corn, potato, cassava and sugar beet which are food crops and are cultivated on agricultural land (Sarris & Papanikolaou, 2016). This utilization of these food crops and use of agricultural land for growth of energy crops has led to the food versus fuel competition which has resulted in high prices of food crops. This utilization of food crops and agricultural land for bioethanol production can also not be implemented in the developing due to existence of a much bigger problem i.e., food crisis (Rodionova et al., 2017). Therefore, researchers are also looking for alternative feedstock to avoid this food and fuel imbalance. *Spirodela polyrhiza* is one of the new alternative feedstock option. It is commonly known as giant duckweed and belongs to family *Lemnaceae* (Michael et al., 2017). Being a hydrophyte, it grows on the surface of water thus avoiding utilization of agricultural land for its growth and eliminating the food and fuel competition. This aquatic plant has a very high growth rate with doubling time of 16 – 24 hours and also has the ability to accumulate about 3 to 70 % of starch content in its dry weight. This starch can then be fermented into bioethanol (Cui & Cheng, 2015). Another interesting capability of this plant is that its starch content can be increased by manipulating nitrates or phosphates concentration or growth conditions like pH, temperature etc. Due to absorptive capacity of various nutrients from water duckweeds are also used as biophytoremediators for nutrient polluted wastewaters like agricultural runoff and municipal wastewater (Cheng & Stomp, 2009). The plant biomass grown and starved

on these wastewaters, with high starch content can then further be used for bioethanol production. Moreover, the plant biomass left after starch extraction can also be used for production of biomethane.

In this study *Spirodela polyrhiza* was grown under nutrient starvation condition to enhance its starch content. High starch containing plant biomass was then acid pretreated to obtain complete conversion of starch to glucose. The plant sugar was then fermented via indigenously isolated and optimized *Saccharomyces cerevisiae* QG1 MK788210 for bioethanol production.

3.2. Material and Methods

3.2.1. Isolation and Optimization of Yeast for High Bioethanol Yield

3.2.1.1. Yeast Isolation

For carrying out the fermentation process yeast was isolated from rotten grapes using WLN agar media (yeast extract 4 gL^{-1}, trypton 5 gL^{-1}, glucose 50 gL^{-1}, KH_2PO_4 0.55 gL^{-1}, KCL 0.425 gL^{-1}, $Mg_2SO_4.7H_2O$ 0.125 gL^{-1}, $CaCl_2.2H_2O$ 0.125 gL^{-1}, $FeCl_3$ 0.0025 gL^{-1}, $MnSO_4$ 0.0025 gL^{-1}, bromocresol green gL^{-1}, Agar 20 gL^{-1}). Grapes were rinsed with distilled water and then partially mashed. Mashed grapes were placed in airtight autoclaved reagent bottles for a week at room temperature. Afterwards, the grape juice from reagent bottles was serially diluted from 10^{-1} to 10^{-9} dilution and each dilution was spread on WLN agar plates supplemented with tetracycline (0.05 gL^{-1}) to avoid bacterial contamination and incubated at 30°C (Hashmi et al., 2018). Selected four morphologically different yeast isolates and further cultured them on tetracycline (0.05 gL^{-1}) supplemented YPD agar (yeast extract 10 gL^{-1}, glucose 20 gL^{-1}, peptone 20 gL^{-1}, agar 20 gL^{-1}) (Ruriani et al., 2012).

3.2.1.2. Screening for High Ethanol Producing Yeast

Fermentation experiments were carried out in order to select the yeast strain that gave maximum ethanol titer. For this purpose, yeast fermentation medium (YFM) (yeast extract 6 gL^{-1}, peptone 5 gL^{-1}, KH_2PO_4 4 gL^{-1}, $(NH_4)_2SO_4$ 2 gL^{-1}, $MgSO_4.7H_2O$ 1 gL^{-1}, glucose 150 gL^{-1}) with pH adjusted to 5.5 was used (Banat et al., 1992). Fermentation was carried out in 10 mL small fermentation reactors. About 5% v/v of 24 hours enriched yeast cultures were added to YFM and reactors were kept airtight. Anaerobic conditions were

developed by means of nitrogen flushing and syringes were attached to the reactors for capturing CO_2 produced in reactor during fermentation. The reactors were then incubated for 72 hours at 30 ℃ and 150 RPM after which ethanol concentration in media was determined. The yeast strain which gave highest ethanol yield was selected for optimization.

3.2.1.3. Determining Ethanol Content

In order to determine amount of ethanol produced by yeast, enzymatic detection of ethanol was carried out using Megazymes Ethanol Assay Kit (Bray, Ireland) in accordance with the manufacturer's instructions.

3.2.1.4. Molecular Identification of Yeast

For molecular identification of selected yeast strain, yeast DNA was isolated by means of SDS/CTAB method (Stirling, 2003). The 18S rRNA sequencing of extracted DNA was carried out by Macrogen Standard Custom DNA Sequencing Services (Macrogen Inc., Seoul, Korea) using Sanger method. The sequence obtained was then subjected to phylogenetic analysis to identify selected yeast strain.

3.2.1.5. Statistical Optimization of Selected Yeast Strain for High Ethanol Production

The selected yeast strain was optimized in order to achieve physicochemical parameters at which it gave maximum ethanol yield. Optimization studies were carried out using statistical tools that included Plackett-Burman Design and Central Composite Design through Stat Ease Design Expert Software version 7. Plackett-Burman Design was used initially to optimize 9 factors that were amount of yeast extract, peptone, KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, pH, inoculum age, inoculum size and incubation time. After entering the ranges of each parameter in the design, design gave 15 runs, each of which was carried out experimentally and the response i.e., ethanol yield was recorded in the design which was further processed by the software. Plackett-Burman Design identified three factors (KH_2PO_4, $MgSO_4.7H_2O$ and inoculum age) to significantly affect the ethanol yield. These three factors were further optimized by means of Central Composite Design in the similar way as of Plackett-Burman Design to achieve the maximum ethanol yield. Both the designs were numerically validated.

3.2.2. Production and Pretreatment of high starch containing *Spirodela polyrhiza* Biomass as Feedstock for Bioethanol production

3.2.2.1. Sampling of *Spirodela polyrhiza*

Plant Sampling was done from local garden ponds in Islamabad, Pakistan. Plant was then identified to be *Spirodela polyrhiza* which is commonly known as giant duckweed. The plant was submitted to National Herbarium of Pakistan, Quaid-i-Azam University, Islamabad and its accession number was acquired.

3.2.2.2. Culturing of *Spirodela polyrhiza*

As the plant is a hydrophyte so water based media enriched with nitrates and phosphates was used for its culturing. Plant was cultured in Hoagland Media (KNO_3 1.515 gL^{-1}, KH_2PO_4 0.68 gL^{-1}, $Ca(NO_3)_2.4H_2O$ 1.18 gL^{-1}, $MgSO_4.7H_2O$ 0.492 gL^{-1}, $ZnSO_4.7H_2O$ 0.00022 gL^{-1}, H_3BO_3 0.00285 gL^{-1}, $Na_2MoO_4.2H_2O$, 0.00012 gL^{-1}, $CuSO_4.5H_2O$ 0.00008 gL^{-1}, $MnCl_2.4H_2O$ 0.00362 gL^{-1}, $FeCl_3.6H_2O$ 0.0054 $gL^{-1}$and tartaric acid 0.003 gL^{-1}) and pH was adjusted to 5.8 (Singh et al., 2018). The *Spirodela* fronds were first washed with 30% sodium hypochlorite solution to remove any algal and bacterial concentration and then were suspended in hoagland solution contained in 10" x 10" transparent plastic boxes. The plant cultured boxes were placed in flourescent light-illuminated plant growth rack and temperature was maintained at 25 ℃.

3.2.2.3. Starch Content Determination in *Spirodela*

For the purpose of starch determination Appenorth method was used (Appenroth et al., 2010). About 200 mg fresh weight of plant was crushed and homogenized with 4 ml 18% HCL. The mixture was the shaken for 1 hour at 5 ℃. The homogenized mixture was then centrifuged at 12000 RPM for 20 minutes. An aliquot of this mixture was mixed with same amount of Lugol's solution (KI 0.5% w/v and I_2 0.25% w/v in distilled water) and its optical density was taken at 530 nm and 605 nm. The starch content was then calculated through following formulae:

$$S\% = [Cs \times Vol(extr) \times 100] \div FW$$

……. (Eq 1)

Where,

Vol (Extr) = the plant extract volume after homogenization with HCl (mL)

FW = fresh weight (mg) and

$Cs = A_{605} / (0.07757 \times P + 4.463)$

Where, A_{605} and A_{530} are the absorbances at 605 nm and 530 nm

Whereas, P was calculated using the formula:

$P= [(7.295 \times A_{605} / A_{530} – 4.463) / (7.757 – 0.729 \times A_{605}/A_{530})] \times 100$

Where, A_{605} and A_{530} are the absorbance at 605 nm and 530 nm

3.2.2.4. Enhancement of Starch Content in Plant Biomass by Nutrient Starvation

Nutrient starvation is known to enhance the starch content of duckweed (Huang et al., 2014). Starch is conserved in plant biomass at times of low nutrient availability as a storage molecule (Tao et al., 2013). In order to enhance the starch content of *Spirodela* biomass, plant was given nutrient stress. For this purpose plants were cultured in Hoagland media as described above. The plants were continuously grown in the media provided initially without any supplementation of new media as in the case of continuous growth. This resulted in a boost in plant growth at start but gradually lead to uptake of all the nutrients in media resulting in nutrient depletion. Starch content of the plants was checked after every four days. Plants were harvested when the starch content became constant and did not increased further.

3.2.2.5. Pretreatment of the High Starch Containing Plant Biomass

As the isolated yeast could only uptake glucose for fermentation, the high starch containing biomass was acid pretreated in order to convert all the starch to glucose. For this purpose, initially the high starch biomass was treated with different concentrations of

sulfuric acid ranging from 0.05% to 0.2% in order to determine the acid concentration at which maximum starch conversion to glucose can be attained. For pretreatment process feedstock loading rate was kept to be 100 $gL^{-1}$and feedstock was hydrolyzed with different concentrations of sulfuric acid solution for 1 hour at 121 ℃ (Ho et al., 2013). The glucose concentration in the solution after pretreatment was determined by DNS method (Miller, 1959). All the reactions were carried out in triplicate.

3.2.3. Fermentation of Glucose Obtained from Nutrient Starved *Spirodela* Biomass with Isolated Yeast at Optimized Parameters

The fermentation reaction was carried out at conditions previously optimized by Plackett-Burman and Central Composite Design to achieve maximum ethanol yield. The pretreated plant biomass suspension which had the highest glucose concentration was centrifuged at 14000 RPM for 20 minutes to separate the biomass from sugar containing hydrolysate. The pH of hydrolysate was adjusted to 4.8 using 1M NaOH. Fermentation was carried out by adding 15% of 72 hours yeast suspension v/v, 70% v/v acid hydrolysate containing released sugars and 15% yeast fermentation media (without glucose and containing other components at optimized conditions i.e., yeast extract 9 gL^{-1}, peptone 7 gL^{-1}, KH_2PO_4 6 gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}, $(NH_4)_2SO_4$ 1 gL^{-1}, pH 4.5, inoculum age 72 hours, inoculum size 15% and incubation time 24 hours) in 10 ml fermentation reactor. Fermentation was carried out as described above at 30 ℃ and 150 RPM. Incubation time was kept to be 48 hours. After 48 hours ethanol titer was determined by ethanol assay kit.

3.3. Results and Discussion

3.3.1. Isolation and Optimization of Yeast for High Bioethanol yield

Isolation of Yeast Strain for Ethanol Fermentation

Grape juice is used for production of wine since centuries hence, mashed grapes are the best substrate for isolation of fermentative yeast. Mashed grapes were kept under anaerobic condition to facilitate fermentation and promote yeast growth only. For the purpose of initial isolation WLN Agar was used. Countable number of colonies came on only two plates spread with 10^{-5} and 10^{-6} dilution with CFU/ml of 3.34×10^{9} and 5.6×10^{9}, respectively. On the basis of morphology four different yeast strains named QG1, QG2, QG3 and QG4 were selected for further study.

Screening for High Ethanol Producing Yeast Strain

The four selected strains were then subjected to fermentation reaction to screen out the yeast strain that gave maximum ethanol titer. The ethanol titer, % theoretical yield and glucose consumption of strains QG1, QG2, QG3 and QG4 are shown in Table 3.1. Based on these results strain QG1 was selected for optimization as it showed maximum ethanol yield with highest sugar consumption rate.

Table 3.1. Ethanol Titer, Theoretical Yield and Glucose Consumption of Initially Selected Yeast Strains

Yeast Strain	Ethanol Titer (% w/v)	Percentage of Theoretical Yield Achieved	Glucose Consumption (%)
QG1	5.9	77.12	99.65
QG 2	1.4	18.30	34.72
QG 3	3.8	49.67	62.89
QG 4	2.3	30.07	47.42

Molecular Identification of Selected Yeast Strain QG1

For molecular identification of strain QG1 18S rRNA sequencing was performed. QG1 samples were sent to MACROGEN Korea for sequencing. The obtained sequence

was subjected to phylogenetic analysis and phylogenetic tree was constructed using neighbor joining method (Fig. 3.1). The strain was identified to be *Saccharomyces cerevisiae.* Further, the sequence was submitted in NCBI Gen Bank (and its accession number was attained). The strain was thus designated as *Saccharomyces cerevisiae* QG1 MK788210.

Fig. 3.1. Phylogenetic tree based on 18S rRNA sequencing of strain QG1 showing relationship with other related strains.

Statistical Optimization of Fermentation Process Using Plackett-Burman Design

For the purpose of optimizing *S. cerevisiae* QG1 to achieve high ethanol yield by fermentation process different factors affecting the process of fermentation were statistically optimized using the Plackett-Burman Design. Nine factors that were amount of yeast extract, peptone, KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, pH, inoculum age, inoculum size and incubation time were optimized by conducting experiments in accordance with the 15 runs of the model design devised by the Design Expert Software (Table 3.2). Highest ethanol yield of 6.6 % (w/v) i.e. 85.7% theoretical yield was achieved in run 10 (yeast extract 9 gL^{-1}, peptone 7 gL^{-1}, KH_2PO_4 6 gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}, $(NH_4)_2SO_4$ 1 gL^{-1}, pH 4.5, inoculum age 72 hours, inoculum size 15% and incubation time 24 hours). Theoretical yields were calculated stoichiometrically based upon the amount of sugar initially fed to the reactor. ANOVA analysis was carried out on the model which showed that the F-value

of model is 19.97 implying that the model is significant. The software also applied F-test on each of the model factors to identify the factors that significantly affect the ethanol yield. The factors having values of "Prob>F" less than 0.0500 were indicated to be the significant model terms. In this case three factors that were KH_2PO_4, $MgSO_4.7H_2O$ and inoculum age came out to be significant with Prob>F values of 0.002, 0.0010 and 0.366, respectively (Fig. 3.2). The model shows that KH_2PO_4 effects the ethanol yield positively i.e. increase in KH_2PO_4 increased the ethanol titer in the media (Fig. 3.3a). KH_2PO_4 is used as a source of phosphorous in the fermentation media. Phosphorous is one of the significant nutrient parameter that effect the fermentation process and ethanol yield and KH_2PO_4 has been reported to be one of the best phosphorous source. Various studies conducted have also shown that enhanced KH_2PO_4 concentration has resulted in higher ethanol yield while using *S. cerevisiae* as fermenting microorganism (Yu et al., 2009). The second significant factor came out to be $MgSO_4.7H_2O$ which is used as a source of magnesium in the fermentation broth. The model indicated that $MgSO_4.7H_2O$ has a negative effect on ethanol yield as increase in $MgSO_4.7H_2O$ results in decrease in the ethanol concentration (Fig. 3.3b). Various studies have also reported that magnesium is only required in a limited concentration within the fermentation broth as it affects ethanol concentration significantly during yeast fermentation (Dombek & Ingram, 1986) (Arora et al., 2017) (Xu et al., 2018). The last significant factor, Inoculum Age had a positive effect on ethanol yield which showed that as the inoculum grew older it gave a better ethanol yield (Fig. 3c). Highest ethanol yield was obtained with 72 hr old inoculum. Different studies show that Inoculum Age is a strain specific factor whose trend is different for different strains (Benerji et al., 2010) (Manikandan & Viruthagiri, 2010).

Equation 1 gives the final equation given by the software for ethanol yield in terms of significant factors:

Ethanol Yield $= + 1.98333 + 0.33333 \times KH_2PO_4 - 0.70000 \times MgSO_4.7H_2O + 0.034722 \times$ Inoculum Age …… (Eq 2)

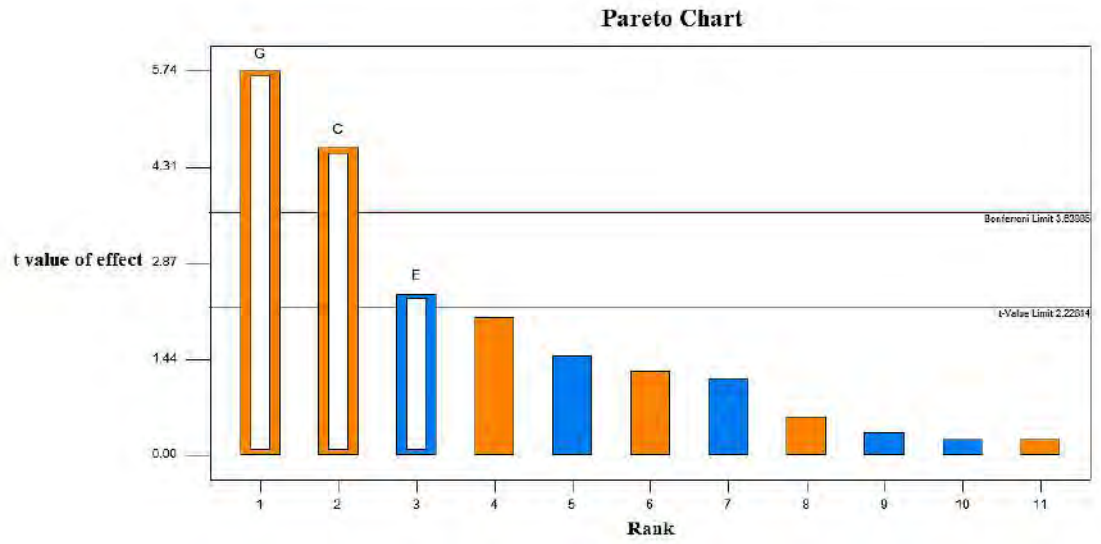
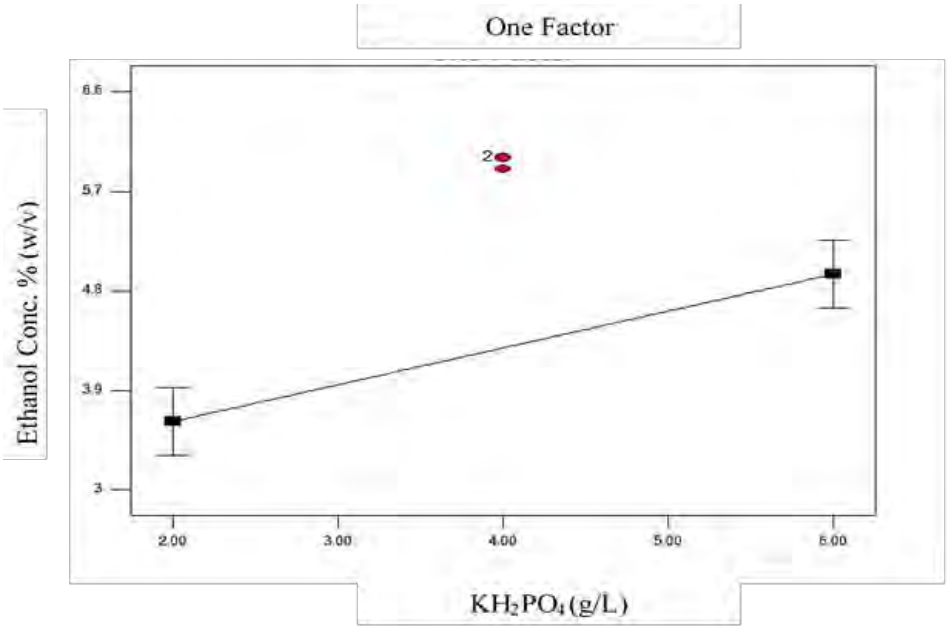
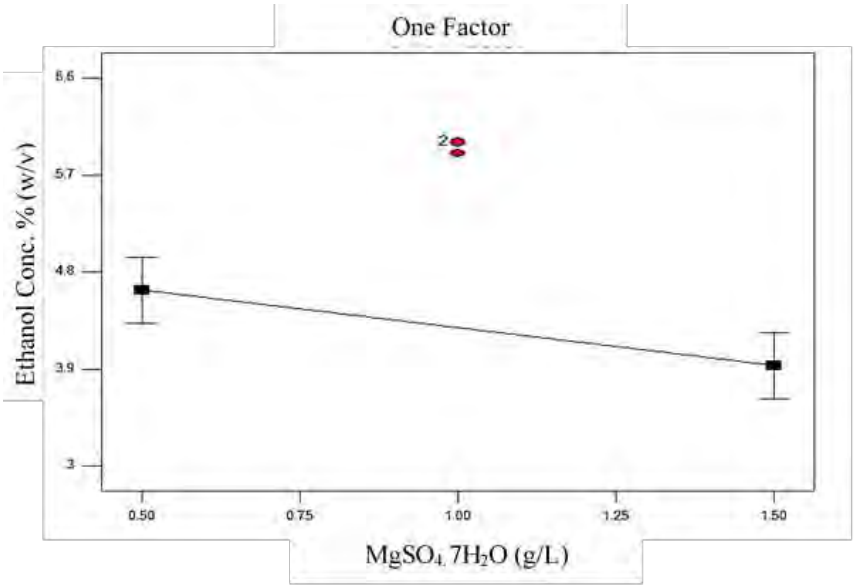


Fig. 3.2 Pareto Chart indicating the significant model terms where G corresponds to KH_2PO_4 C corresponds to $MgSO_4.7H_2O$ and E corresponds to Inoculum Age

(a)



(b)



(c)

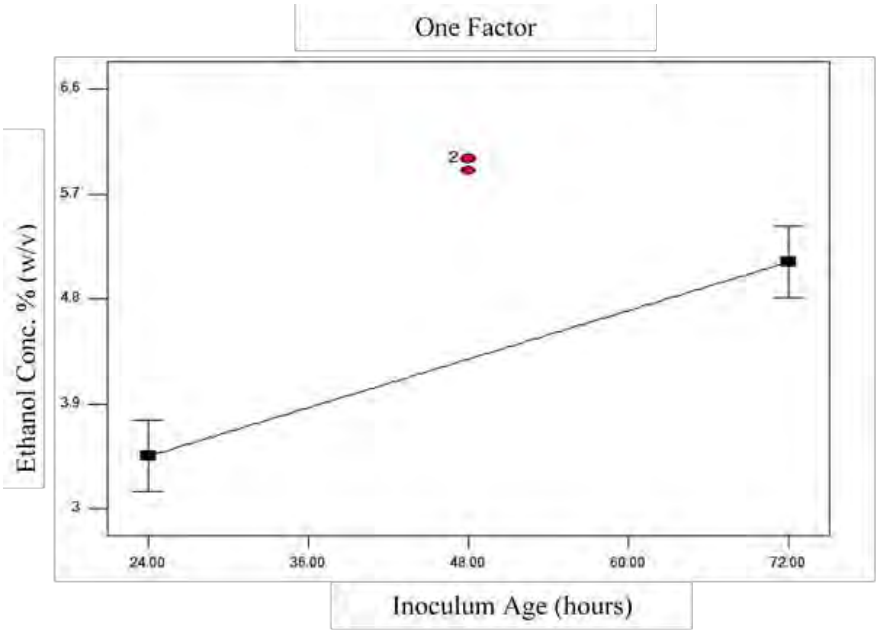


Fig. 3.3 Plot of ethanol yield as a function of (a) KH_2PO_4, (b) $MgSO_4.7H_2O$ and (c) Inoculum Age

Table 3.2 Ethanol Yield obtained in response to the conditions specified by the Plackett-Burman Design.

Runs	**Yeast Extract g/L**	**Peptone g/L**	**KH_2PO_4 g/L**	**$(NH_4)_2SO_4$ g/L**	**$MgSO_4.7H_2O$ g/L**	**pH**	**Inoculum Age hrs**	**Inoculum Size %**	**Incubation time hrs**	**Ethanol Yield % (w/v)**
1	9	3	6	3	0.5	6.5	72	5	48	5.5
2	3	7	6	3	0.5	4.5	24	5	48	4.4
3	9	3	6	3	1.5	4.5	24	15	24	3.2
4	9	7	2	3	1.5	6.5	24	5	24	3
5	6	5	4	2	1	5.5	48	10	36	6
6	6	5	4	2	1	5.5	48	10	36	5.9
7	6	5	4	2	1	5.5	48	10	36	6
8	3	3	2	1	0.5	4.5	24	5	24	3
9	3	7	2	3	1.5	4.5	72	15	48	3.3
10	9	7	6	1	0.5	4.5	72	15	24	6.6
11	3	7	6	1	1.5	6.5	72	5	24	5.9
12	3	3	2	3	0.5	6.5	72	15	24	5.3
13	3	3	6	1	1.5	6.5	24	15	48	4.1
14	9	3	2	1	1.5	4.5	72	5	48	4.1
15	9	7	2	1	0.5	6.5	24	15	48	3

Optimization of the Significant Factors by Response Surface Methodology Using Central Composite Design

The three factors, KH_2PO_4, $MgSO_4.7H_2O$ and inoculum age that were indicated to be significant by Plackett-Burman design were further optimized by using central composite design (CCD). CCD is used to find out the interactive effects of all the chosen factors to be optimized. For generating the CCD Model the ranges of each of the significant factor was kept in accordance with the results of Plackett-Burman Design. As increasing the concentration of KH_2PO_4 resulted in increased ethanol yield the range for KH_2PO_4 was further increased and kept to be 6 – 9 gL^{-1}. Same was the case with inoculum age and its range for CCD was selected to be 72 – 96 hrs. In case of, $MgSO_4.7H_2O$ as increase in its concentration decreased ethanol activity its range was further decreased and kept to be from 0.1 – 0.5 gL^{-1}. The reactions were conducted according to the conditions specified by 17 runs generated by model design (Table 3.3). For the remaining 6 out of 9 factors the conditions were kept according to run 10 of Plackett-Burman Design which gave the highest ethanol yield. Highest ethanol yield of 7.08% (w/v) i.e. 93% theoretical yield was achieved in run 11 of CCD. ANOVA analysis of the CCD gave the model F-value of 4.43 which indicated the model was significant. Two factors AB (interactive effect of KH_2PO_4 and $MgSO_4.7H_2O$) (Fig. 3.4a) and BC (interactive effect of $MgSO_4.7H_2O$ and Inoculum Age) (Fig. 3.4b) came out to be significant by having Prob>F values of 0.005 and 0.04, respectively. In case of CCD the effects of each factor were observed to be opposite to that of Plackett-Burman as further increase in the KH_2PO_4 concentration and Inoculum Age decreased the ethanol yield whereas a further decline in the $MgSO_4.7H_2O$ also decreased the ethanol yield. Both Plackett-Burman Design and Central Composite Design were numerically validated by the solutions given by software. Achieving high % theoretical yield of ethanol is demanded as in this way maximum carbohydrates in the feedstock can be converted to ethanol which can be used as biofuel.

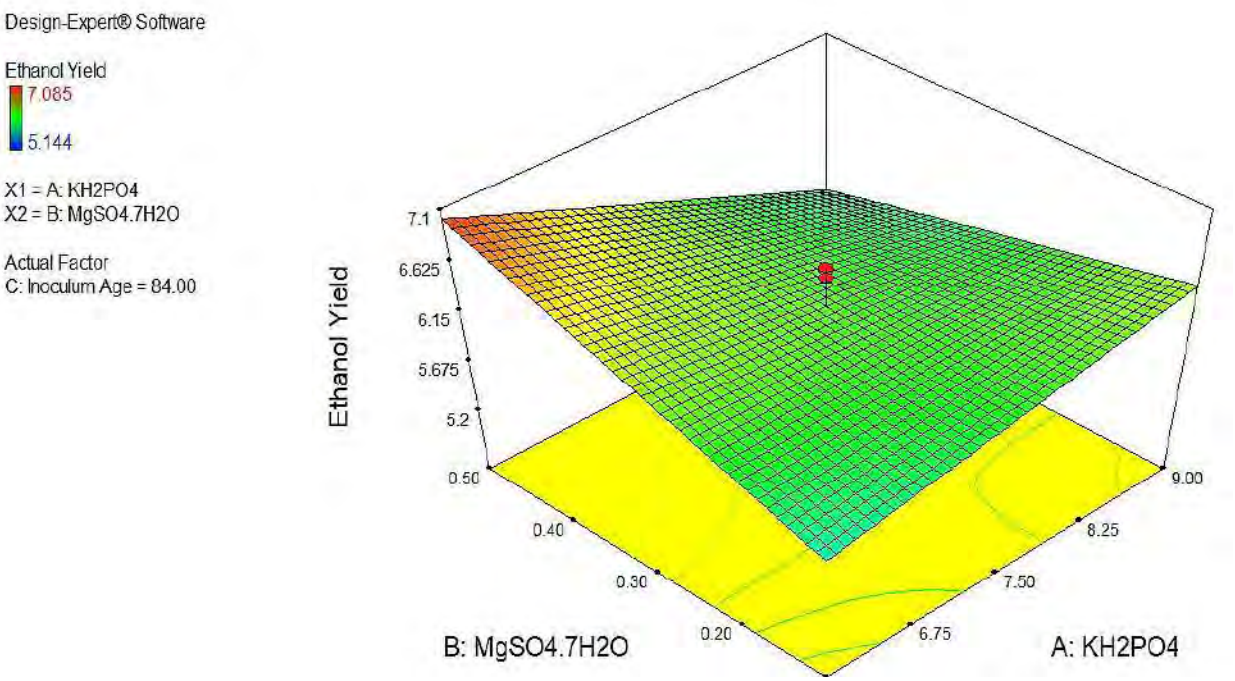
Equation 2 gives the final equation given by the software for ethanol yield in terms of significant factors:

Ethanol Yield = -10.50544 +1.72621 × KH_2PO_4 +24.22189 × $MgSO_4.7H_2O$ +0.16465 × Inoculum Age -1.64417 × KH_2PO_4 × $MgSO_4.7H_2O$ -0.016194 × KH_2PO_4 × Inoculum Age -0.13531 × $MgSO_4.7H_2O$ × Inoculum Age ….. (Eq 3)

Table 3.3 Ethanol Yield obtained in response to condition specified by Central Composite Design.

Runs	**KH_2PO_4 g/L**	**$MgSO_4.7H_2O$ g/L**	**Inoculum Age Hr**	**Ethanol Yield % (w/v)**
1	9	0.1	96	6.499
2	4.98	0.3	84	6.352
3	7.5	0.64	84	6.272
4	7.5	0.3	84	6.559
5	7.5	0.3	84	6.497
6	7.5	0.3	63	6.234
7	7.5	0.3	104	6.292
8	6	0.1	96	6.849
9	6	0.5	96	6.734
10	10.02	0.3	84	5.721
11	6	0.5	72	7.085
12	7.5	0.04	84	5.217
13	9	0.5	72	5.928
14	6	0.1	72	5.168
15	7.5	0.3	84	6.582
16	9	0.5	96	5.144
17	9	0.1	72	6.717

(a)



(b)

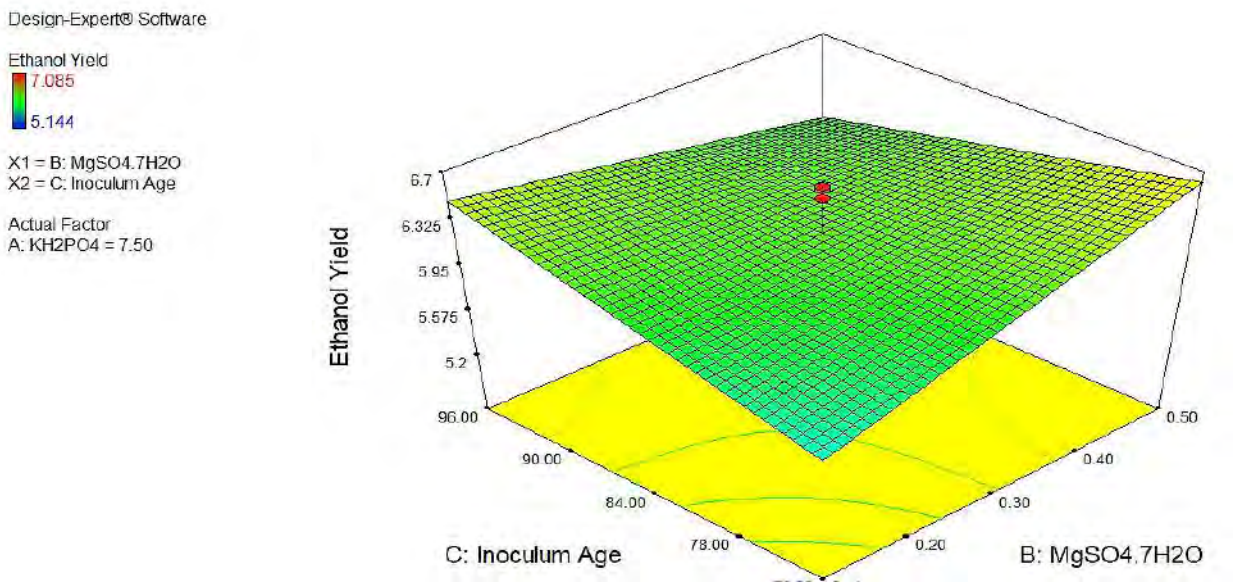


Fig. 3.4 Illustration of interactive Effect of Factor (a) AB and (b) BC by three dimensional response surface plot for ethanol yield

3.3.2. Production and Pretreatment of high starch containing *Spirodela polyrhiza* Biomass as feedstock for bioethanol production

Identification of *Spirodela polyrhiza*

The collected sample plant from garden pond was identified to be *Spirodela polyrhiza* on the basis of its morphology. The plant collected had a comparatively larger disc-like fronds with reddish underside and 12 – 16 adventitious roots which are typical of *Spirodela polyrhiza* specie (Jacobs, 1947). The identified plant was submitted to the National Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, Pakistan and was given the accession number of 129983.

Enhancement of Starch Content in Plant Biomass by Nutrient Starvation

For the purpose of increasing starch content in plant biomass various studies provided different types of stress to *Spirodela.* The most reported stress is that of heavy metals like Ni (Appenroth et al., 2010) (Xyländer et al., 1993), Cr (Appenroth et al., 2003), Fe and Cu (Xing et al., 2010) which had resulted into increase in the starch content. However, the aim of current study is to use high starch containing plant biomass for the purpose of fermentation to produce bioethanol. Heavy metals stress was not a good option in this case as it will lead to yeast toxicity. Therefore, nutrient stress was opted to enhance the starch content in plants. Nutrient starvation in different duckweed specie is known to enhance starch content in their biomass (Tao et al., 2013). For nutrient starvation plants were cultured in freshly prepared Hoagland media and were allowed to grow. New media was not replenished and as plants utilized all the nitrates and phosphates of the initially provided media they went under nutrient stress condition. Highest starch content of 1.9% Fresh weight was obtained after 20 days (Fig. 3.5). Based on starch content in plant biomass it has been calculated that *Spirodela* if cultured for purpose of starch production, can give 1870 Kg/hectar/year of starch.

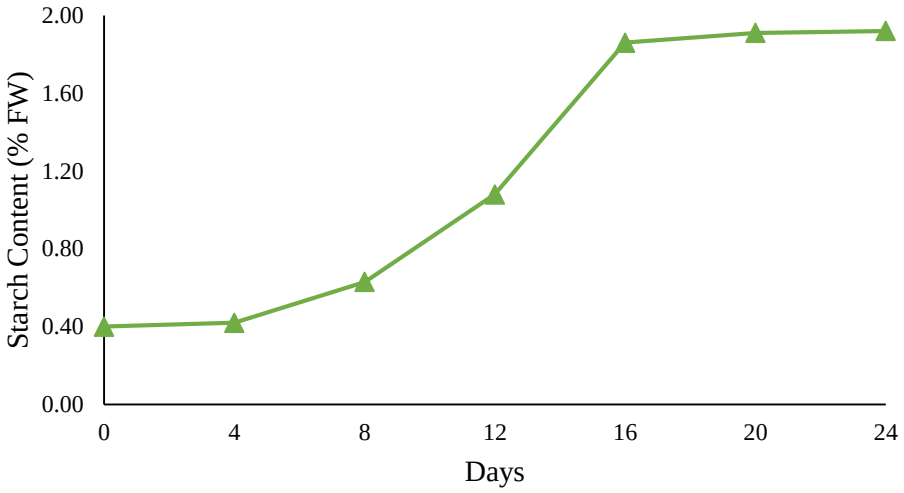


Fig. 3.5. Enhancement of *Spirodela* Starch Content under Nutrient Stress Condition

Pretreatment of the High Starch Containing Plant Biomass

The plant biomass with highest starch content of 1.9% FW was then given acidic pretreatment at high temperature to convert starch into glucose so that it can easily be fermented by *S. cerevisiae* QG1. For this purpose, initially the high starch biomass was treated with different concentrations of sulfuric acid ranging from 0.05% to 0.2% with purpose to determine the acid concentration at which maximum starch conversion to glucose can be attained. The highest starch conversion rate of 99.4% was achieved with 0.1% of sulfuric acid (Fig. 3.6).

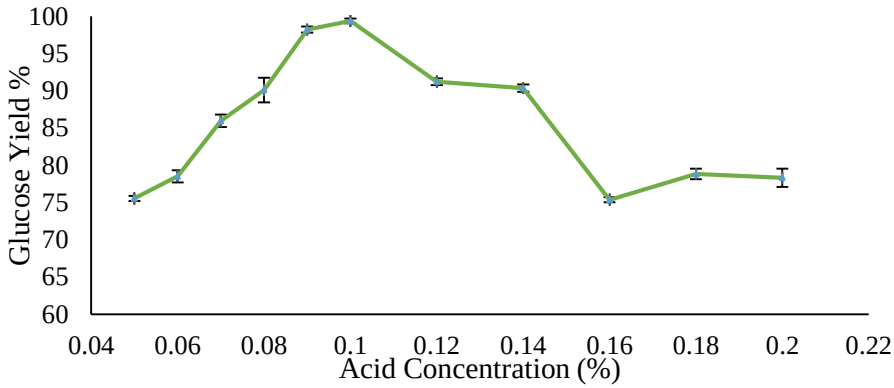


Fig. 3.6. Optimization of acidic pretreatment to achieve maximum glucose yield from high starch containing *Spirodela* biomass.

3.3.3. Fermentation of Glucose Obtained from Nutrient Starved *Spirodela* Biomass with Isolated Yeast at Optimized Parameters

The fermentation reaction was carried out using sugar containing plant hydrolysate (pretreated with 0.1% sulfuric acid and had highest glucose concentration) and isolated yeast strain *S. cerevisiae* QG1 at conditions which gave highest ethanol yield during optimization reactions performed according to Plackett-Burman and Central Composite Design. Theoretical ethanol yield of 100% was obtained in this reaction i.e., 2.2 $gL^{-1}$of glucose was converted to 1.21 $gL^{-1}$of ethanol (calculations were done stoichiometrically based on balanced chemical equation). Thus, complete conversion of starch in *Spirodela* biomass to bioethanol was successfully achieved.

3.4. Conclusions

The study conducted concludes that the isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210 shows promising potential to be utilized for obtaining high ethanol yield from feedstock like *Spirodela polyrhiza.* The enhanced starch content in *Spirodela* biomass by providing nutrient stress and successful conversion of all the plant starch to ethanol by isolated and optimized yeast strain is suggestive of the fact that this plant can potentially be used as feedstock for bioethanol production to avoid food vs. fuel feud. The high nitrate and phosphate uptaking capacity of *Spirodela* directs that in future this plant can also be utilized for treatment of nutrient rich wastewaters and then the produced biomass can be utilized for biofuel production. All the results obtained confirm that the isolated yeast strain as well as *Spirodela polyrhiza* can find promising applications in the field of biofuel production.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest

Availability of the Data The data generated and analyzed during the study is provided in the article [and its supplementary information file]

Table ESM_1. Validation of Plackett-burman and Central Composite Design.

Plackett-Burman Design			**Central Composite Design**		
Runs	**Predicted Ethanol Yield (% w/v)**	**Experimental Ethanol Yield (% w/v)**	**Runs**	**Predicted Ethanol Yield (% w/v)**	**Experimental Ethanol Yield (% w/v)**
1	4.42	4.31	1	6.19	6.13
2	4.41	4.52	2	5.35	5.42
3	5.02	5.13	3	6.36	6.32
4	4.75	4.55	4	6.11	5.95
5	3.95	4.08	5	6.37	6.31

References

1. Appenroth, K.-J., Keresztes, A., Sárvári, É., Jaglarz, A., & Fischer, W. (2003). Multiple effects of chromate on Spirodela polyrhiza: electron microscopy and biochemical investigations. *Plant Biology*, *5*(03), 315-323.
2. Appenroth, K.-J., Krech, K., Keresztes, A., Fischer, W., & Koloczek, H. (2010). Effects of nickel on the chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in biomonitoring and phytoremediation. *Chemosphere*, *78*(3), 216-223.
3. Arora, R., Behera, S., Sharma, N. K., & Kumar, S. (2017). Augmentation of ethanol production through statistically designed growth and fermentation medium using novel thermotolerant yeast isolates. *Renewable energy*, *109*, 406-421.
4. Banat, I. M., Nigam, P., & Marchant, R. (1992). Isolation of thermotolerant, fermentative yeasts growing at 52 C and producing ethanol at 45 C and 50 C. *World Journal of Microbiology and Biotechnology*, *8*(3), 259-263.
5. Benerji, D., Rajini, K., Rao, B., Banerjee, D., Rani, K., Rajkumar, G., & Ayyanna, C. (2010). Studies on physico-chemical and nutritional parameters for the production of ethanol from Mahua flower (Madhuca indica) using Saccharomyces Cerevisiae—3090 through submerged fermentation (SMF). *J Microbial Biochem Technol*, *2*, 46-50.
6. Cheng, J. J., & Stomp, A. M. (2009). Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *Clean–Soil, Air, Water*, *37*(1), 17-26.
7. Cui, W., & Cheng, J. (2015). Growing duckweed for biofuel production: a review. *Plant Biology*, *17*, 16-23.
8. Dombek, K., & Ingram, L. (1986). Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. *Appl. Environ. Microbiol.*, *52*(5), 975-981.
9. Hashmi, M., Khan, T. K., Khan, W., Hasan, F., Hameed, A., Khan, S., Badshah, M., & Shah, A. A. (2018). Comparison Between a Newly Isolated Yeast Strain and Lalvin EC-1118 for Enhanced Ethanol Yield from Sugarcane Molasses Employing Batch and Modified Fed-Batch Fermentation. *Journal of Biobased Materials and Bioenergy*, *12*(1), 134-142.

10. Hirani, A. H., Javed, N., Asif, M., Basu, S. K., & Kumar, A. (2018). A review on first-and second-generation biofuel productions. In *Biofuels: Greenhouse Gas Mitigation and Global Warming* (pp. 141-154). Springer.
11. Ho, S.-H., Huang, S.-W., Chen, C.-Y., Hasunuma, T., Kondo, A., & Chang, J.-S. (2013). Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource technology*, *135*, 191-198.
12. Huang, M., Fang, Y., Xiao, Y., Sun, J., Jin, Y., Tao, X., Ma, X., He, K., & Zhao, H. (2014). Proteomic analysis to investigate the high starch accumulation of duckweed (Landoltia punctata) under nutrient starvation. *Industrial Crops and Products*, *59*, 299-308.
13. Jacobs, D. L. (1947). An ecological life-history of Spirodela polyrhiza (Greater Duckweed) with emphasis on the turion phase. *Ecological Monographs*, *17*(4), 437-469.
14. Manikandan, K., & Viruthagiri, T. (2010). Kinetic and optimization studies on ethanol production from corn flour. *International Journal of Chemical and Biological Engineering*, *3*(2), 65-69.
15. Manochio, C., Andrade, B., Rodriguez, R., & Moraes, B. (2017). Ethanol from biomass: a comparative overview. *Renewable and Sustainable Energy Reviews*, *80*, 743-755.
16. Michael, T. P., Bryant, D., Gutierrez, R., Borisjuk, N., Chu, P., Zhang, H., Xia, J., Zhou, J., Peng, H., & El Baidouri, M. (2017). Comprehensive definition of genome features in Spirodela polyrhiza by high-depth physical mapping and short-read DNA sequencing strategies. *The Plant Journal*, *89*(3), 617-635.
17. Miller, G. (1959). Modified DNS method for reducing sugars. *Anal. Chem*, *31*(3), 426-428.
18. Nilsson, L. J., & Johansson, T. B. (2017). Environmental challenges to the energy industries. In *Sustainable Development and the Energy Industries* (pp. 47-80). Routledge.
19. Rana, Q. U. A., Irfan, M., Ahmed, S., Hasan, F., Shah, A. A., Khan, S., Rehman, F. U., Khan, H., Ju, M., & Li, W. (2019). Bio-catalytic transesterification of mustard oil for biodiesel production. *Biofuels*, 1-8.

20. Rodionova, M. V., Poudyal, R. S., Tiwari, I., Voloshin, R. A., Zharmukhamedov, S. K., Nam, H. G., Zayadan, B. K., Bruce, B. D., Hou, H., & Allakhverdiev, S. I. (2017). Biofuel production: challenges and opportunities. *International Journal of Hydrogen Energy*, *42*(12), 8450-8461.
21. Ruriani, E., SUNARTI, T. C., & Meryandini, A. (2012). Yeast isolation for bioethanol production. *HAYATI Journal of Biosciences*, *19*(3), 145-149.
22. Sarris, D., & Papanikolaou, S. (2016). Biotechnological production of ethanol: biochemistry, processes and technologies. *Engineering in life sciences*, *16*(4), 307-329.
23. Singh, V., Pandey, B., & Suthar, S. (2018). Phytotoxicity of amoxicillin to the duckweed Spirodela polyrhiza: Growth, oxidative stress, biochemical traits and antibiotic degradation. *Chemosphere*, *201*, 492-502.
24. Stirling, D. (2003). DNA extraction from fungi, yeast, and bacteria. In *PCR Protocols* (pp. 53-54). Springer.
25. Tao, X., Fang, Y., Xiao, Y., Jin, Y.-l., Ma, X.-r., Zhao, Y., He, K.-z., Zhao, H., & Wang, H.-y. (2013). Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (Landoltia punctata) under nutrient starvation. *Biotechnology for biofuels*, *6*(1), 72.
26. ul ain Rana, Q., Rehman, M. L. U., Irfan, M., Ahmed, S., Hasan, F., Shah, A. A., Khan, S., & Badshah, M. (2019). Lipolytic bacterial strains mediated transesterification of non-edible plant oils for generation of high quality biodiesel. *Journal of bioscience and bioengineering*, *127*(5), 609-617.
27. Xing, W., Huang, W., & Liu, G. (2010). Effect of excess iron and copper on physiology of aquatic plant Spirodela polyrrhiza (L.) Schleid. *Environmental Toxicology: An International Journal*, *25*(2), 103-112.
28. Xu, E., Wu, Z., Jiao, A., & Jin, Z. (2018). Effect of exogenous metal ions and mechanical stress on rice processed in thermal-solid enzymatic reaction system related to further alcoholic fermentation efficiency. *Food chemistry*, *240*, 965-973.
29. Xyländer, M., Augsten, H., & Appenroth, K.-J. (1993). Influence of nickel on the life cycle of the duckweed Spirodela polyrhiza (L.) Schleiden. *Journal of plant physiology*, *142*(2), 208-213.

30. Yu, J., Zhang, X., & Tan, T. (2009). Optimization of media conditions for the production of ethanol from sweet sorghum juice by immobilized Saccharomyces cerevisiae. *Biomass and bioenergy*, *33*(3), 521-526.

Chapter 4: Growing *Spirodela polyrhiza* for Removal of Nutrients from Agricultural Runoff and Production of Bioethanol

Abstract

The excesssive use of agrochemicals to fulfill the high demand of agricultural products has been imposing numerous negative impacts on the environment for decades worldwide. Untreated wastewater with high chemical load and unutilized nutrients of fertilizers and pesticides released from agricultural farms results in contamination of clean water bodies. *Spirodela polyrhiza*, giant duckweed, for the treatment of agriculture wastewaters was studied as an efficient and cost-effective method due to its high growth rate, less nutritional deman and excellent nutrient uptake abilities. Production of valuable biomass by *Spirodela polyrhiza*, after treatment of wastewater, also serves as an alternative feedstock for the production of biofuel (bioethanol) which is another precedence of using this method. In this study, the potential of *Spirodela polyrhiza* for taking nitrogen and phosphates from the agricultural runoff was determined by growing it in the agricultural runoff. Upto 70% and 78% removal of nitrogen and phosphates, respectively, was achieved during the treatment of agricultural runoff by *Spirodela polyrhiza*. The starch content of *Spirodela polyrhiza* was elevated 58% during 10-12 days of nutrient starvation at the end of the agricultural runoff treatment process. The biomass obtained was pretreated with dilute acid that liberated 96.4% glucose from the starch-rich biomass. In the last step, glucose was used as a substrate for the fermentation process carried out by indigenously isolated yeast strain *Saccharomyces cerevisiae* QG1 MK788210 which was previously optimized to obtain high ethanol yield. The fermentation process resulted in 100% theoretical yield of ethanol from glucose.

4.1. Introduction

Intensive use of fertilizers and pesticides in the agriculture sector to fulfill global food production demand has adversely affected and contaminated terrestrial, aquatic as well as the coastal marine environment by adding a wide range of hazardous chemicals and loads of nitrogen and phosphorus fertilizers in agricultural wastewater (Carvalho & Security, 2017). Agricultural wastewater is one of the leading cause of water pollution which not only intoxicates water but also pose serious threats to human as well as aquatic life. It also contaminates water reserves causing eutrophication that makes water unavailable for public use (Shrestha et al., 2016). Higher population growth rates along with an alarming increase in the toxic pollutants have tremendously increased the demand for clean water globally. To subside water scarcity, multiple physical and chemical purification techniques and treatments like membrane filtration, adsorption, chemical precipitation, oxidation, photocatalysis, as well as certain biological methods have been introduced and adopted to clean up wastewater and make it reusable by eliminating organic, inorganic and biological pollutants (Bolisetty et al., 2019). However, these methods methods have various constrains in terms of cost, feasibility, efficiency and environmental impact. Over the past few years, duckweeds has been investigated as a potential source of cleaning up wastewater due to its ability of up taking excessive nutrients and tolerance to high nitrogen and phosphorus load from municipal wastewaters. A few of these small floating hydrophyte duckweeds that belong to family *Lemnaceae* like *Spirodela oligorhiza, Spirodela punctate, Lemna japonica* have been previously explored for biological nutrients removal from wastewater under different conditions generating high protein biomass (Cheng & Stomp, 2009; Xu & Shen, 2011; Zhao et al., 2015). *Spirodela polyrhiza* has also been studied for its bioremediation capabilities and considered as a potent source of starch-rich biomass based on its faster growth rates along with high dry weight starch content of about 3-70% that could serve as an alternative feedstock for biofuel and bioethanol synthesis under different nutrient conditions (Cui & Cheng, 2015).

Spirodela polyrhiza, also known as giant duckweed has been studied previously under nutrient starved conditions and the acid pretreated biomass was utilized for the production of bioethanol (ul ain Rana et al., 2019). In this study, *S. polyrhiza* was grown in agricultural runoff that was synthesized at the lab by mixing NPK fertilizer in distilled water to test the capability of the plant to uptake nitrogen and phosphorus from the agricultural

runoff. The biomass synthesized by the plants during their growth in agricultural runoff was used further for the production of bioethanol. The biomass was given acidic pretreatment and bioethanol was produced via fermentation process carried out by yeast strain *Saccharomyces cerevisiae* QG1 MK788210.

4.2. Materials and Methods

4.2.1 Collection of Duckweed (*Spirodela polyrhiza*)

Plant sampling was done from local garden ponds in Islamabad, Pakistan. The plant was then identified to be *S. polyrhiza*, which is commonly known as giant duckweed. The plant was submitted to the National Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, and its accession number was acquired.

4.2.2. Culturing of *Spirodela polurhiza*

Spirodela polyrhiza being an aquatic plant needs a stable environment, adequate light, optimum pH, and temperature range for proper growth. Hoagland Media (KNO_3 1.515g/L, KH_2PO_4 0.68g/L, $Ca(NO_3)_2.4H_2O$ 1.18 g/L, $MgSO_4.7H_2O$ 0.492g/L, $ZnSO_4.7H_2O$ 0.00022g/L, H_3BO_3 0.00285g/L, $Na_2MoO_4.2H_2O$, 0.00012g/L, $CuSO_4.5H_2O$ 0.00008 g/L, $MnCl_2.4H_2O$ 0.00362g/L, $FeCl_3.6H_2O$ 0.0054 g/L and tartaric acid 0.003g/L) was used to grow plants. Hoagland Media contains all the vital micro and macronutrients needed by the plant to grow including. Healthy fronds of *Spirodela polyrhiza* were selected and washed carefully with 30% sodium hypochlorite solution to remove algal and bacterial contamination and were afterwards suspended in hoagland solution present in 10″ × 10″ sized clear plastic containers. The pH of Hoagland solution was maintained at 5.8 and plants were placed in the growth chamber under fluorescent light at room temperature (Singh et al., 2018).

4.2.3. Preparation of Agricultural Runoff

Agricultural runoff was prepared by dissolving NPK fertilizer in distilled water. About 9.8 grams of solid NPK was weighed and dissolved in 1000 ml of distilled water to form a solution similar to agricultural runoff. pH of this solution was adjusted at 5.8.

4.2.4. Culturing of *Spirodela polyrhiza* in Agricultural runoff

A solution was prepared by mixing 66% lab prepared agricultural runoff and 34% Hoagland solution and was poured into a 12″ × 6″ glass box and *S.polyrhiza* was cultured in it. The mixing of NPK and Hoagland solution was done to ensure the availability of all necessary nutrients because NPK solution lacks the other essential micronutrients required for plant growth. Instead of plastic boxes, glass trays were used in the experiment to avoid the absorption of nitrates and phosphates in plastic. Before culturing the plants in agricultural runoff, the initial nitrogen and phosphorous content present in the solution was determined. Afterward, a few healthy plants from the cultured stock and were allowed to grow in the prepared agricultural runoff. pH of the final solution i.e agricultural runoff was adjusted at 5.8 and the bioremediation was carried out in the afore mentioned growth chamber. The bioremediation was carried out for 30 days till the decline in nitrogen and phosphate concentration became constant. Nitrogen and phosphate concentration was determined every 3rd day. The plants were harvested once the bioremediation reaction was halted for further study.

4.2.5. Determination of Nitrogen and Phosphate Contents in Agricultural Runoff

For the determination of nitrogen and phosphotous content present in the agricultural runoff, Total Nitrogen Determination Kit and Phosphate Determination Kit (Merk, NY, USA) were used. The analysis was carried out using Spectroquant® Prove UV/VIS spectrophotometer (Merk, NY, USA).

4.2.6. Determination of Starch Content in *Spirodela polyrhiza* Biomass

For determination of starch content in plant biomass, Appenroth method was used (Appenroth et al., 2010). For this purpose, about 200 mg of fresh plant was ground thoroughly and homogenized in 4 ml of 18% HCL. The mixture was then stirred for 1 hour at 5 ^{o}C. The completely homogenized mixture was collected and centrifuged at 12000 RPM for 20 minutes. Later on, the supernatant was collected and mixed with Lugol's solution (KI 0.5% w/v and I_2 0.25% w/v in distilled water) in 1:1 ratio. Optical density of this solution was taken at 530 nm and 605 nm via spectrophotometer. The starch content was then calculated through following equations:

S% = [Cs x Vol (extr) x 100]/FW

Where, Vol (extr) was the plant extract volume after homogenization with HCl (mL), FW was fresh weight (mg), Cs = A_{605}/(0.07757 - P + 4.463) and A_{605} and A_{530} were the absorbances at 605 nm and 530 nm.

P was calculated by using the formula:

$P= [(7.295 \times A_{605}/A_{530} – 4.463)/(7.757 – 0.729 \times A_{605}/A_{530})] \times 100$

4.2.7. Dilute Acid Pretreatment of Plant Biomass

The plant biomass obtained after the treatment of nutrient rich wastewater was pretreated with dilute acid at previously optimized conditions in order to convert plant starch to glucose. For this purpose, the biomass was treated with 0.2% sulfuric acid for 1 hour at 121 ℃ and the feedstock loading rate was kept to be 100g/L. Pretreated plant biomass suspension was centrifuged at 14000 RPM for 20 minutes and supernatant (acid hydrolysate) was collected (ul ain Rana et al., 2019). DNS method was used for determination of the glucose concentration present in the solution after pretreatment (Miller, 1959).

4.2.8. Fermentation of Glucose obtained from *Spirodela polyrhiza* Biomass by *Saccharomyces cerevisiae* QG1 MK788210.

Saccharomyces cerevisiae QG1 MK788210 was indigenously isolated and previously optimized to give highest ethanol yield by fermenting pretreated hydrolysate of *S. polyrhiza* (ul ain Rana et al., 2019). The pH of acid hydrolysate was adjusted to 4.8. Fermentation was carried out by adding 15% v/v of 72 hours old yeast suspension, 70% v/v acid hydrolysate containing released glucose and 15% v/v yeast fermentation media (yeast extract 9 g/L, peptone 7 g/L, KH_2PO_4 6 g/L, $MgSO_4.7H_2O$ 0.5 g/L and $(NH_4)_2SO_4$ 1 g/L) to fermentation reactor. Nitrogen sparging of the fermentation reactor was carried out to develop anaerobic conditions and reactor was air sealed. Fermentation was carried out at 30 ℃ and 150 RPM and incubation time was 48 hours.

4.2.9. Ethanol Titer Determination

For determination of ethanol titer after fermentation, enzymatic ethanol detection was carried out using Megazymes Ethanol Assay Kit (Bray, Ireland) according to manufacturer’s instructions.

4.3. Results and Discussion

4.3.1. Preparation in Agricultural Runoff

Agricultural runoff was prepared in lab by dissolving commercially available NPK fertilizer is distilled water in such a way to mimic the nitrogen and phosphate concentration that is present in naturally occurring agricultural runoff (Ceschin et al., 2019). Table. 4.1 shows the chemical composition of NPK fertilizer used to prepare agricultural run-off.

Table 4.1 Composition of NPK Fertilizer used in the lab as synthetic agriculture runoff

Chemical	**w/w percentage**
Total Nitrogen (N)	20%
Potassium (K_2O)	14%
Phosphorus Pentoxide (P_2O_5)	8%
Sulphur Soluble in water	9.7%
Magnesium (Mg)	2%
Copper, Zinc, Iron, Boron, Molybdenum	Traces

4.3.2. Phytoremediation of Agricultural Runoff by *Spirodela polyrhiza*

Numerous studies has suggested and reported the utilization of duckweed species for phytoremediation of wastewaters rich in nutrients like nitrates and phosphates which includes municipal wastewater and agricultural runoff (Ceschin et al., 2019; El-Kheir et al., 2007; Kreider et al., 2019; Landesman et al., 2010). Agricultural runoff was selected in this study for analyzing the potential of *Spirodela polyrhiza* to remove nitrates and phosphates from wastewater. Plants were cultured in mixture containing 66% lab prepared agricultural runoff and 34% Hoagland media to supplement plant growth with micronutrients. The treatment was carried out for 30 days and total nitrogen and total phosphates in the agricultural runoff was measured after every 3rd day. The concentration of nitrates and phosphates in the runoff declined rapidly during the first 22 days. However, after 22nd days the removal of nitrogen and phosphate by the plant slowed down and gradually stopped by day 30th. The results indicated 70% removal of nitrogen and 78% removal of phosphates

from the wastewater by *Spirodela*, which indicates that this plant can efficiently and effectively phytoremediate agricultural runoff. Figure. 4.1 shows the obtained nitrogen and phosphate contents removed by *Spirodela polyrhiza* from Agricultural Runoff during 30 days of treatment. Previously Mohedano et al., 2012 has reported 98% and 98.8% total N and total P recovery from swine waste water using *L.punctata* in full scale duckweed ponds (Mohedano et al., 2012) whereas Xu and Shen reported removal efficiencies of 83.7% and 89.4% for total N and total P, respectively, for *S. polyrhiza* during 8 weeks of treatment (Xu & Shen, 2011). The lower removal efficiency of duckweed in this study is due to the fact that the agricultural runoff prepared in this study had far more initial nitrogen and phosphate concentration than the previously reported studies.

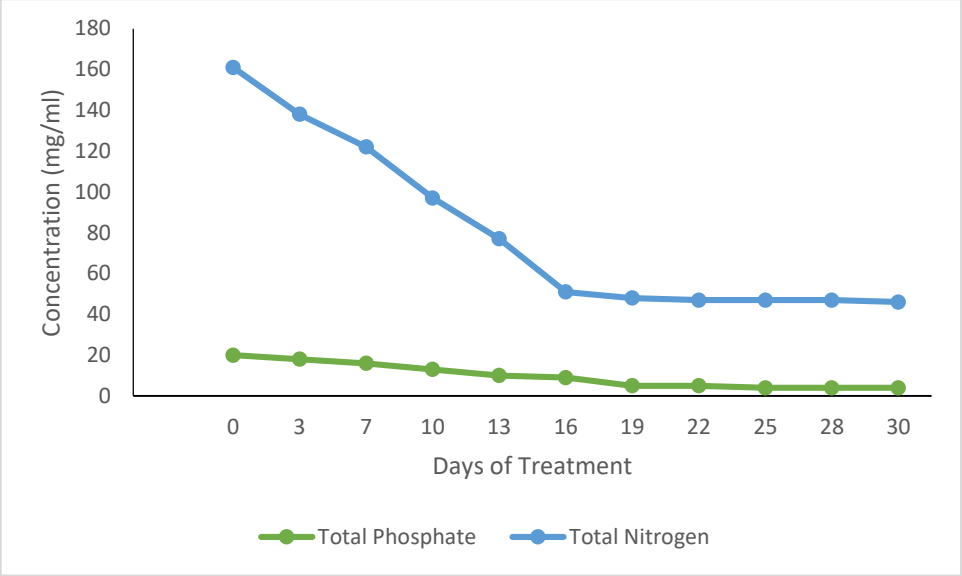


Figure 4.1. Nitrogen and phosphate contents removed by *Spirodela polyrhiza* from Agricultural Runoff during Treatment Days

4.3.3. Effect of Nutreints Stravation on Starch Content of Plant Biomass

Various studies have reported that plants of duckweed specie has the ability to acuumulate starch in their biomass at varying growth conditions (Xiao et al., 2013; Xu et al., 2011). This high starch plant biomass is then a potential feedstock for third generation bioethanol production (Ge et al., 2012). A decreased bioavailability of nitrates and phosphates in the plant growth media is one of the factors that increase starch concentration in plant biomass (Ekperusi et al., 2019). The starch content of *Spirodela polyrhiza* was continuously monitored during the phytoremediation experiment. A continuous elevation of the starch levels in the plant biomass was observed as the nitrogen and phosphate

concentration in the agricultural runoff declined. By the day 30th a 58% (Fresh Weight) of total starch content increase in plant biomass was observed (Figure 4.2).

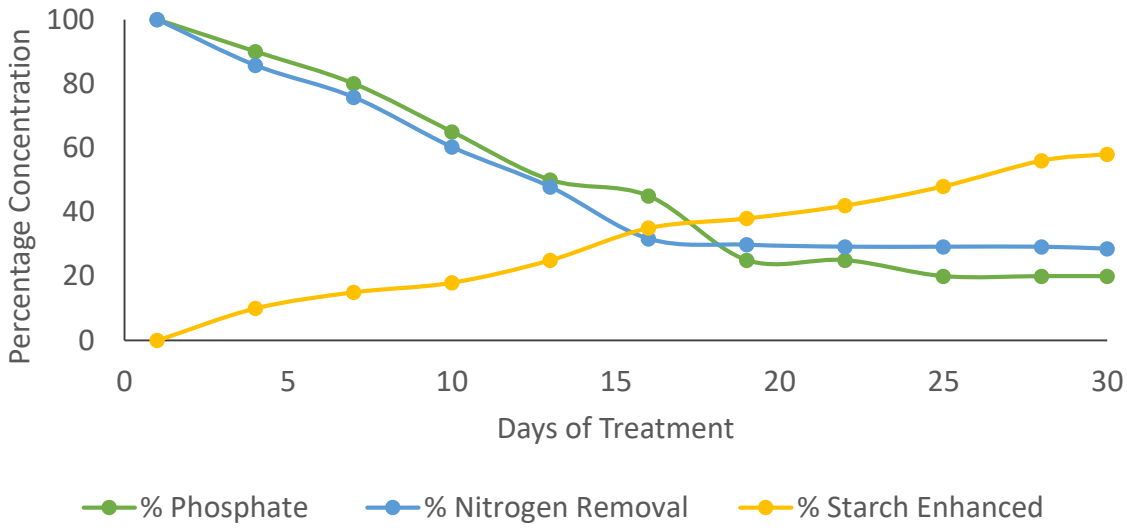


Figure 4.2. Percentage Reduction of Nitrogen and Phosphate Concentration in Agricultural Runoff with Percentage Elevation of Starch Levels in *Spirodela polyrhiza* Biomass.

4.3.4. Dilute Acid Pretreatment of *Spirodela* Biomass Harvested after Phytoremediation

The plant biomass harvested after phytoremediation of agricultural runoff had elevated levels of starch which can effectively be utilized for production of bioethanol. As the yeast used in process of fermentation for production of ethanol can only uptake glucose, thus the plant biomass with starch was pretreated with dilute acid at conditions previously optimized specifically for high starch *Spirodela* biomass, for conversion of plant starch to glucose. About 96.4% conversion rate of starch to glucose was obtained after the pretreatment.

4.3.5. Fermentation of Glucose Obtained After Pretreatment with *Saccharomyces cerevisiae* QG1 MK788210 for Bioethanol Production

Batch fermentation of plant derived glucose was carried out using yeast strain *Saccharomyces cerevisiae* QG1 MK788210 which was previously optimized to give 93% theoretical yield of ethanol while using 150 g/L of glucose. The pretreated plant hydrolysate

was fermented using afore mentioned yeast strain at previously optimized conditions and 99.8% ethanol theoretical yield was achieved**.**

4.4. Conclusion

The results obtained indicated that *Spirodela polyrhiza* was efficient in the removal of nutrients from agricultural wastewater. The consequently generated plant biomass had elevated levels of starch which was efficiently converted to glucose by dilute acid pretreatment and this glucose was successfully fermented to bioethanol by using indigenously isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210. This study concludes that the duckweed species *Spirodela polyrhiza* is a potent option to resolve two major issues simultaneously. First, it could remediate agricultural runoff by removing excessive nutrients out of it that reduces water pollution. Second, the plant biomass obtained as by-product could be used as a potential feedstock for the production of bioethanol that addresses the problem of the energy crisis in the form of production of bioethanol as biofuel. All the results obtained indicate that *Spirodela polyrhiza* has the capability of finding promising applications within the fields of bioremediation and bioenergy.

References

Appenroth, K.-J., Krech, K., Keresztes, A., Fischer, W., & Koloczek, H. J. C. (2010). Effects of nickel on the chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in biomonitoring and phytoremediation. *78*(3), 216-223.

Bolisetty, S., Peydayesh, M., & Mezzenga, R. J. C. S. R. (2019). Sustainable technologies for water purification from heavy metals: review and analysis. *48*(2), 463-487.

Carvalho, F. P. J. F., & Security, E. (2017). Pesticides, environment, and food safety. *6*(2), 48-60.

Ceschin, S., Sgambato, V., Ellwood, N. T. W., Zuccarello, V. J. E., & Botany, E. (2019). Phytoremediation performance of Lemna communities in a constructed wetland system for wastewater treatment. *162*, 67-71.

Cheng, J. J., & Stomp, A. M. J. C. S., Air, Water. (2009). Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *37*(1), 17-26.

Cui, W., & Cheng, J. (2015). Growing duckweed for biofuel production: a review. *Plant biology*, *17*, 16-23.

Ekperusi, A. O., Sikoki, F. D., & Nwachukwu, E. O. J. C. (2019). Application of common duckweed (Lemna minor) in phytoremediation of chemicals in the environment: State and future perspective. *223*, 285-309.

El-Kheir, W. A., Ismail, G., El-Nour, F., Tawfik, T., Hammad, D. J. I. J. o. A., & Biology. (2007). Assessment of the efficiency of duckweed (Lemna gibba) in wastewater treatment.

Ge, X., Zhang, N., Phillips, G. C., & Xu, J. J. B. T. (2012). Growing Lemna minor in agricultural wastewater and converting the duckweed biomass to ethanol. *124*, 485-488.

Kreider, A. N., Fernandez Pulido, C. R., Bruns, M. A., & Brennan, R. A. J. J. o. e. q. (2019). Duckweed as an Agricultural Amendment: Nitrogen Mineralization, Leaching, and Sorghum Uptake. *48*(2), 469-475.

Landesman, L., Fedler, C., & Duan, R. (2010). Plant nutrient phytoremediation using duckweed. In *Eutrophication: Causes, consequences and control* (pp. 341-354). Springer.

Miller, G. L. J. A. c. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *31*(3), 426-428.

Mohedano, R. A., Costa, R. H., Tavares, F. A., & Belli Filho, P. J. B. T. (2012). High nutrient removal rate from swine wastes and protein biomass production by full-scale duckweed ponds. *112*, 98-104.

Shrestha, S., Pandey, V. P., & Shivakoti, B. R. (2016). Water Environment in Central and East Asia: An Introduction. In *Groundwater Environment in Asian Cities* (pp. 339-343). Elsevier.

Singh, V., Pandey, B., & Suthar, S. J. C. (2018). Phytotoxicity of amoxicillin to the duckweed Spirodela polyrhiza: growth, oxidative stress, biochemical traits and antibiotic degradation. *201*, 492-502.

ul ain Rana, Q., Khan, M. A. N., Irfan, M., Shah, A. A., Hasan, F., Khan, S., Ahmed, S., Adnan, F., Li, W., Ju, M. J. B. C., & Biorefinery. (2019). Starved Spirodela polyrhiza and Saccharomyces cerevisiae: a potent combination for sustainable bioethanol production. 1-10.

Xiao, Y., Fang, Y., Jin, Y., Zhang, G., Zhao, H. J. I. c., & products. (2013). Culturing duckweed in the field for starch accumulation. *48*, 183-190.

Xu, J., Cui, W., Cheng, J. J., & Stomp, A.-M. J. B. e. (2011). Production of high-starch duckweed and its conversion to bioethanol. *110*(2), 67-72.

Xu, J., & Shen, G. J. B. t. (2011). Growing duckweed in swine wastewater for nutrient recovery and biomass production. *102*(2), 848-853.

Zhao, Y., Fang, Y., Jin, Y., Huang, J., Bao, S., Fu, T., He, Z., Wang, F., Wang, M., & Zhao, H. J. P. B. (2015). Pilot-scale comparison of four duckweed strains from different genera for potential application in nutrient recovery from wastewater and valuable biomass production. *17*, 82-90.

Chapter 5: Production of Bioethanol and Biogas from *Spirodela polyrhiza* in a Biorefinery Concept and Output Energy Analysis of the Process

Abstract

Rapid increase in the world's energy demand due to population growth and intense industrialization has resulted in the exhaustion of non-renewable fossil fuels. This depletion of fossil fuels has stimulated the search for alternative energy sources and one of the important alternative is bioenergy. Bioethanol and biogas are two of the promising biofuels that can help in minimizing the dependence on crude oil and natural gas, respectively. *Spirodela polyrhiza* is a hydrophyte with high growth rate, ability to accumulate high starch content in its biomass and high biomass yield which makes it an excellent alternative feedstock for the production of bioethanol and biogas. In this study, *Spirodela* was given nutrient stress (nitrates and phosphates) to enhance its starch content from 21% (FW) to 80% (FW) in 24 days. The plant biomass with high starch was pretreated with dilute acid and starch to glucose conversion rate of 99.6% was achieved. The plant derived glucose was fermented with indigenously isolated and optimized yeast strain and 99.8% theoretical ethanol yield was obtained. The fermentation vinasse was anaerobically digested and 0.88 NL/g VS biogas yield was obtained. Further output energy analysis of processes producing bioethanol, biogas and both bioethanol and biogas sequentially from *Spirodela* feedstock was carried out which showed that highest amount of energy i.e., 18.5 MJ/g VS is produced in the process where high starch containing *Spirodela* plant biomass was used for the production of biomethane only. The results obtained showed that high starch *Spirodela* biomass is best used in terms of energy when it is anaerobically digested for production of biogas only.

5.1 Introduction

A steady escalation in the world's demand of energy has been observed since 1900 mainly due to population growth and rapid industrialization (Rana et al., 2019). Crude oil and natural gas are two of the fossil fuels that currently dominate the energy sector (Mahdi et al., 2020). However, these two predominating fuels are limited non-renewable resources that are destined to deplete in the near future (ul ain Rana, Rehman, et al., 2019). Rapid increase in the energy demand and an inevitable exhaustion of fossil fuels has stimulated the search for alternative energy sources (Nilsson & Johansson, 2017).

One of the important alternative to the depleting fossil fuels is renewable bioenergy sources which can reduce the world's dependence upon non- renewable, notorious, fossil fuel based energy sources (AlNouss et al., 2020). Bioethanol is one of the renewable bioenergy source which is considered as one of the potential alternative to the petroleum based fossil fuels specifically in transportation sector (Manochio et al., 2017). Bioethanol is produced by sugar fermentation and its consumption as fuel comes with both social and environmental benefits that includes substantially low emission of CO_2 and generation of jobs for the local communities by the production and conversion of renewable biomass to bioethanol (Hirani et al., 2018). However, production of bioethanol poses certain challenges which include: utilization of food crops cultivated on agricultural land as feedstock like corn, cassava, sugar beet. potato, etc. which results into both scarcity and price hike of these food commodities (Sarris & Papanikolaou, 2016) (Rodionova et al., 2017). The second challenge associated with bioethanol production is fermentation vinasse which is the major contaminant of bioethanol production process and its treatment is costly (Amorim et al., 2011).

Biogas is another renewable bioenergy source which is a potential alternative to natural gas. Biogas is produced by the anaerobic digestion of various type of organic wastes including agricultural waste, municipal waste, organic industrial waste etc. (Tabatabaei et al., 2020). The main feedstock for anaerobic digestion includes larger and complex molecules like carbohydrates, proteins and lipids which are biologically converted by a consortium of anaerobic symbiotically operating microorganisms into methane and carbon dioxide (Parsaee et al., 2019). A tremendous growth in the production of biogas throughout the world has been observed in the past decade. Researchers are now inclined towards

searching for new organic sources for enhanced production of biogas (Callegari et al., 2020).

Spirodela polyrhiza has currently gained attention as potential feedstock for production of bio-based energy carriers. It is a freely floating aquatic plant belonging to family *Lemnaceace* (Michael et al., 2017). Commonly, this plant is known as giant duckweed. This hydrophyte has a doubling time of 16 to 24 hours which depicts its very high growth rate (Cui & Cheng, 2015). The ability of this plant to accumulate high starch content (up to 70% dry weight) in its biomass under varying growth conditions has captured the interest of researchers. This plant accumulated starch can be fermented for production of bioethanol. Bioethanol produced from *Spirodela* can eliminate the need of food crops and agricultural land utilization. The fermentation vinasse produced during this process can be anaerobically digested for production of biogas. The plant biomass itself can also be used directly for production of biogas.

In the current study, *Spirodela* was grown under nutrient stress condition to enhance its starch content. The plant starch was then fermented to ethanol whereas, the fermentation vinasse was anaerobically digested for production of biogas. Moreover, a comparative analysis of the energy output in four different biofuel production processes i.e., utilization of high starch *Spirodela* biomass for ethanol production only, utilization of high starch *Spirodela* biomass for ethanol production and then utilization of fermentation vinasse for biomethane production, utilization of high starch *Spirodela* biomass for biomethane production only and utilization of fresh *Spirodela* biomass for biomethane production only, was also carried out to identify the process and the biofuel which gives maximum energy output by using *Spirodela* as feedstock.

5.2 Material and Methods

5.2.1 Feedstock Production

Collection and Culturing of *Spirodela polyrhiza*

The plant sample was collected from a local garden pond in Islamabad, Pakistan and was identified to be *Spirodela polyrhiza* (commonly called giant duckweed) by National Herbarium of Pakistan, Quaid – i – Azam University, Islamabad, Pakistan. As the plant being hydrophyte, grows on water surface hence plant growth was established in

Hoagland media (KNO_3 1.515g/L, KH_2PO_4 0.68g/L, $Ca(NO_3)_2.4H_2O$ 1.18 g/L, $MgSO_4.7H_2O$ 0.492g/L, $ZnSO_4.7H_2O$ 0.00022g/L, H_3BO_3 0.00285g/L, $Na_2MoO_4.2H_2O$, 0.00012g/L, $CuSO_4.5H_2O$ 0.00008 g/L, $MnCl_2.4H_2O$ 0.00362g/L, $FeCl_3.6H_2O$ 0.0054 g/L and tartaric acid 0.003g/L) and the pH of the media was kept to be 5.8 (Singh et al., 2018). In order to remove algal, bacterial and fungal contamination from sampled plants, plants were first washed with 3% sodium hypochlorite solution. The washed and decontaminated plants were then suspended in the 3L media contained within 20" x 15" transparent glass box to facilitate light passage from all directions. The cultured plants were kept in plant growth rack illuminated by fluorescent light at 25 ℃.

Determination of Starch Content in Plant Biomass

Appenorth method was used for determination of starch content in feedstock (Appenroth et al., 2010). For this purpose, 4 ml of 18% HCl was added to 200 mg crushed fresh plant weight. The mixture was then shaken at 5 ℃ for 1 hr. Mixture was further centrifuges for 20 minutes at 12000 RPM and 1 ml of the supernatant was mixed with 1 ml of Lugol's solution (KI 0.5% w/v and I_2 0.25% w/v in distilled water). The optical density of this solution was taken at 605 nm and 530 nm. The following formulae were then used to calculate starch content.

$S\% = [Cs \times Vol(extr) \times 100] \div FW$ ….. (Eq. 1)

Where,

Vol (Extr) = the plant extract volume after homogenization with HCl (mL)

FW = fresh weight (mg) and

$Cs = A_{605} / (0.07757 \times P + 4.463)$

Where, A_{605} and A_{530} are the absorbance at 605 nm and 530 nm

Whereas, P was calculated using the formula:

$P = [(7.295 \times A_{605} / A_{530} - 4.463) / (7.757 - 0.729 \times A_{605}/A_{530})] \times 100$

Starch Content Enhancement in plant Biomass by Applying Nutrient Stress

Application of nutrient stress on duckweed is an effective way for enhancement of starch content in plant biomass (Huang et al., 2014). Plants accumulate starch as a storage molecule in their biomass when they encounter environments having low nutrient availability (Tao et al., 2013). The nutrient stress was applied to *Spirodela* as described by Rana et al., (ul ain Rana, Khan, et al., 2019). The plants were cultured in Hoagland media as described above and were grown continuously without supplementation of new media. As the plants grew they utilized all the nitrates and phosphates in the media causing nutrient depletion in media and entered into nutrient stress phase. Starch content was measured every fourth day. The plants were harvested at two stages 1) at day 12^{th} when the plant biomass reached its maximum growth and achieved highest biomass 2) at day 24^{th} where starch content was maximum because of nutrient stress. The plants which are not nutrient starved have a higher protein content rather than carbohydrates and lipids (Roman & Brennan, 2019).

5.2.2 Fermentation of High Starch *Spirodela polyrhiza* Biomass for Bioethanol Production

Dilute Acid Pretreatment of Plant Biomass with High Starch

As fermentation was carried out using previously isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210 which could only ferment glucose to ethanol hence dilute acid pretreatment was carried out to convert plant starch to glucose. For this purpose, 0.1% H_2SO_4 was used to hydrolyze feedstock with feedstock loading rate of 100 g/L. Hydrolysis was carried out for 20 min at 120 ℃ (ul ain Rana, Khan, et al., 2019). After pretreatment, the glucose concentration in hydrolysate was measured through DNS method (Miller, 1959).

Fermentation of Plant Derived Glucose with *Saccharomyces cerevisiae* QG1 MK788210

Indigenously isolated yeast strain *Saccharomyces cerevisiae* QG1 MK788210 was already optimized to obtain high ethanol yield from glucose obtained by pretreatment of high starch containing plant biomass. After pretreatment the hydrolysate pH was adjusted to 4.8. The fermentation reaction was carried out by adding 70% v/v of acid hydrolysate, 15% v/v yeast inoculum grown for 72 hours and 15% Yeast Fermentation Media (yeast

extract 9 g/L, peptone 7 g/L, KH_2PO_4 6 g/L, $MgSO_4.7H_2O$ 0.5 g/L and $(NH_4)_2SO_4$ 1 g/L). Nitrogen sparging of the fermentation reactor was carried out to develop anaerobic conditions and reactor was air sealed. Fermentation was carried out at 30 ℃ and 150 RPM and incubation time was 48 hours (Rana, et al., 2019).

Determination of Ethanol Titer

For determination of ethanol titer after fermentation, enzymatic ethanol detection was carried out using Megazymes Ethanol Assay Kit (Bray, Ireland) according to manufacturer's instructions. Ethanol was then separated from fermentation media by means of distillation whereas fermentation vinasse (unconsumed organic fraction of plant by yeast i.e., starch-less plant biomass, yeast cells and YFM) was stored for anaerobic digestion.

5.2.3 Anaerobic Digestion of Feedstock for Biogas Production

For anaerobic digestion, three different type of substrates were utilized: 1) High starch containing plant biomass harvested at day 24th 2) Plant biomass grown without nutrient stress harvested at day 12th of growth 3) Fermentation vinasse.

Inoculum Preparation

Inoculum for anaerobic digesters was prepared using cow dung obtained from a local dairy farm. Cow dung was homogenized with water in 3:7 ratio and the homogenized slurry was passed through 7 mm sieve to eliminate larger particles. The mixture was then poured into anaerobic digester connected with gas collection bag. Nitrogen sparging of the digester was carried out to develop anaerobic conditions and digester was air sealed. The anaerobic digester was then incubated at 37 ℃ for three weeks during which the inoculum started to produce biogas and was degassed.

Determination of Total and Volatile Solids of Inoculum and Feedstock

For TS and VS determination china crucibles were first heated at 550 ℃ in a muffle furnace for 2 hours. The crucibles were then removed from the furnace, cooled to room temperature and weighed on analytical balance. Grinded substrate along with inoculum was then added into separate crucibles and weighed on analytical balance again. The substrate weight was calculated by obtaining the difference between crucible weight and sample + crucible weight. The crucibles were then placed in 105 ℃ oven for 24 hours to remove all

the moisture. The crucibles were weighed again and dried sample weight was calculated by obtaining the difference between crucible weight and dried sample = crucible weight. TS was calculated using the following formula:

TS% of Sample=Weight of dried sampleWeight of initial sample*100 ….. (Eq. 2)

For determination of VS dried sample was placed in muffle furnace at 550 ℃ for 2 hours. Afterwards, crucibles were cooled to room temperature and weighed on analytical balance. The weight of as was calculated by obtaining the difference between crucible weight and ash + crucible weight (Sluiter et al., 2008). VS was calculated using the following formula:

VS% of the TS=Weight of dried sample-weight of ashweight of dried sample*100 …… (Eq. 3)

Anaerobic Digestion Reactor Setup

For the purpose of anaerobic digestion 100 ml glass reactors with air tightening rubber stoppers having outlet for gas collection were used. The reactors were attached to 60 ml gas collection vials. The working volume of the reactors was kept to be 70 ml. The inoculum to substrate ratio was kept to be 1:4 (Holliger et al., 2016). Starch was used as positive control whereas inoculum alone was used as negative control. All the reactions were carried out in triplicates. In order to calculate the amount of inoculum and substrate to be added in reactor according to 1:4 ISR following formulae were used:

Amount of Substrate=Working Volume*VS of InoculumVS of Inoculum*4*VS of Substrate ….. (Eq. 4)

Amount of Inoculum=Working Volume-Amount of Substrate ….. (Eq. 5)

The experiment was ran for 72 days. For the initial 21 days biogas and biomethane production was recorded daily whereas after day 21st the readings were noted every 3rd day. In order to determine amount of biomethane produced, biogas was passed through reactor containing scrubbing solution (3M NaOH) which effectively absorbed CO_2 and released methane (Abdeen et al., 2016). The biogas and biomethane values obtained were converted and reported in normalized liters (at standard temperature and pressure).

5.2.4 Output Energy Evaluation of Produced Biofuels

A comparative analysis of the total energy output of biofuels produced in four different processes was carried out as follow:

Utilization of high starch *Spirodela* biomass for ethanol production only where,

$E_{total} = E_{bioethanol}$ ….. (Eq. 6)

Utilization of high starch *Spirodela* biomass for ethanol production and then utilization of fermentation vinasse for biomethane production where,

$E_{total} = E_{bioethanol} + E_{biomethane}$ ….. (Eq. 7)

Utilization of high starch *Spirodela* biomass for biomethane production only where,

$E_{total} = E_{biomethane}$ ….. (Eq. 8)

Utilization of fresh *Spirodela* biomass for biomethan production only where,

$E_{total} = E_{biomethane}$ ….. (Eq. 9)

Where,

E_{total} is total energy output of the process

$E_{bioethanol}$ is energy contained in the bioethanol produced during fermentation of respective substrate

$E_{biomethane}$ is Energy contained in the biomethane produced during anaerobic digestion of respective substrate

Methane energy content is 0.051 MJ/g

Ethanol energy content is 0.025 MJ/g

5.3 Results and Discussion

5.3.1 Feedstock Production

Application of Nutrient Stress for Enhancement of Starch Content in Plant Biomass

Various studies have reported application of different stresses on the growth of *Spirodela* which has successfully enhanced its starch content. These stresses include addition of heavy metals to the growth media like Cu and Fe (Xing et al., 2010), Cr (Appenroth et al., 2003) and Ni (Xyländer et al., 1993). However, as this study aimed to use the plant biomass having high starch for purpose of fermentation and anaerobic digestion, the application of heavy metals may cause toxicity and negatively affected the physiology of microorganisms used during these processes. Therefore, nutrient stress was opted for nutrient enhancement. Studies conducted on various duckweed species depicted that nutrient starvation results in accumulation of starch in plant biomass (Tao et al., 2013). Figure 5.1 shows the plant growth pattern before and after nutrient stress. It was observed that the plant biomass increased in a regular fashion and achieved maximum biomass by the day 12th. However, no further increase in plant biomass seen during further cultivation. This could be attributed to the fact that nutrients, specifically nitrate and phosphates were stripped off from the media rapidly and was used by the plant for its growth. However, once all the nutrients diminished plant went into stress and further growth was stopped. The other side of figure shows the trend of starch content in plant biomass during its growth. The starch content in plant biomass increased slowly till day 12th during which the plant biomass was increasing rapidly. However, between day 12th and day 16th plant starch content escalated rapidly which is possibly because plant went into nutrient stress and started accumulating starch in its biomass. From day 16th to day 24th no further increase in starch content was observed. Highest Starch content of 2% Fresh Weight was obtained by the 24th. Starch content of 2% fresh weight or 31.25% dry weight was obtained by the day 24th. Based upon this increase in starch content of *Spirodela*, it was calculated that the plant biomass can yield 1870 Kg/hectar/year of starch if it is cultured for this purpose which is equivalent to the starch produced by corn per year.

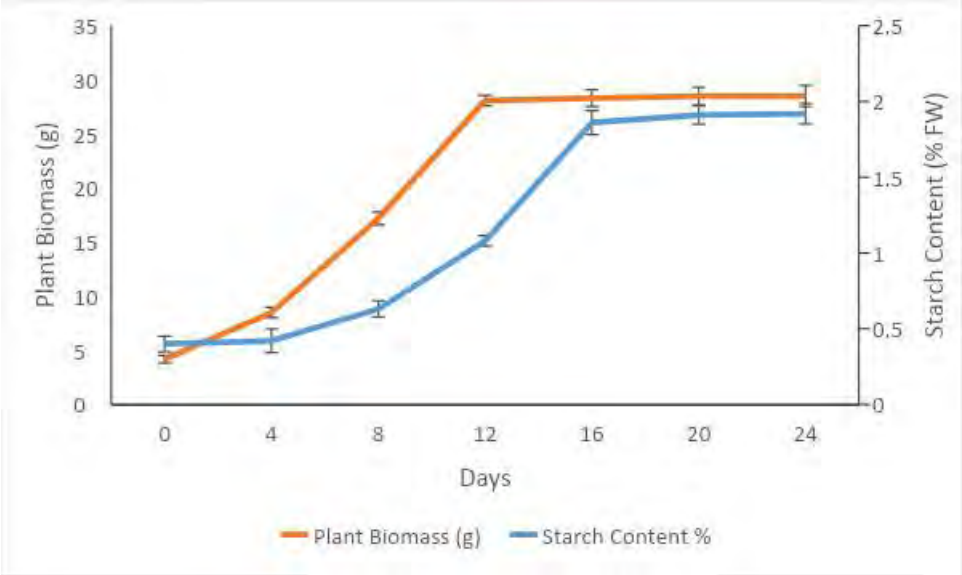


Figure 5.1 Illustration of *Spirodela* growth and starch content enhancement during application of nutrient stress

5.3.2 Fermentation of High Starch *Spirodela polyrhiza* Biomass for Bioethanol Production

Dilute Acid Pretreatment of Plant Biomass with High Starch

As the plant biomass contained starch whereas, fermentative yeast was capable of converting glucose to ethanol. Hence, plant biomass was subjected to dilute acid pretreatment for conversion of plant starch to glucose. The dilute acid pretreatment was carried out at parameters which were optimized in the previous study specifically for high starch *Spirodela* biomass. After the pretreatment 99.6% of the plant starch was converted into glucose.

Fermentation of Plant Derived Glucose with *Saccharomyces cerevisiae* QG1 MK788210

Batch fermentation of plant derived glucose was carried out using yeast strain *Saccharomyces cerevisiae* QG1 MK788210 which was previously optimized to give 93% theoretical yield of ethanol while using 150 g/L of glucose. This yeast strain was also used for fermentation of *Spirodela* derived glucose in previous study giving ethanol theoretical yield of 100%. The pretreated plant hydrolysate was fermented using afore mentioned yeast strain at previously optimized conditions and 99.8% ethanol theoretical yield was achieved which corresponded to 0.01 gram of ethanol per gram VS of *Spirodela* biomass (theoretical

yields were calculated stoichiometrically by using balanced chemical equations). Thus, we were able to achieve complete *Spirodela* starch conversion to bioethanol.

5.3.3 Anaerobic Digestion of Feedstock for Biogas Production

Determination of Total and Volatile Solids of Inoculum and Feedstock

The TS and VS of the three selected substrates for anaerobic digestion to produce biogas was carried out. Table 5.1 shows the obtained TS and VS of high starch containing plant biomass, fresh plant biomass grown without nutrient stress and fermentation vinasse.

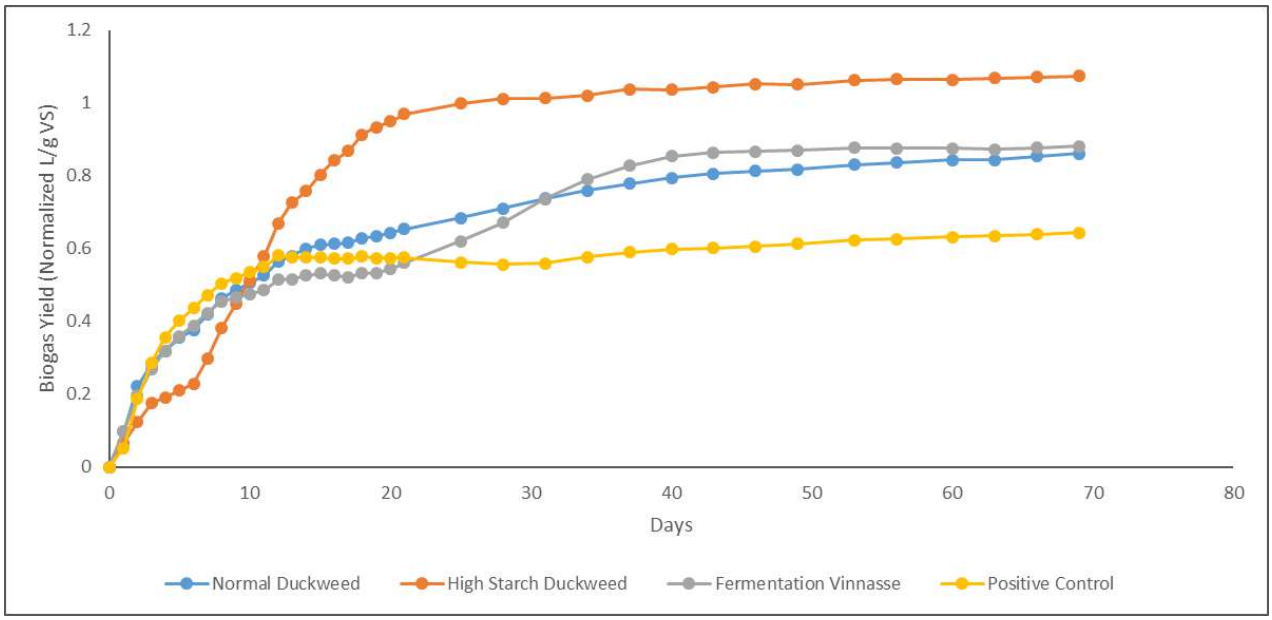
Table 5.1. Total Solids and Volatile Solids Content of Substrates Used for Anaerobic Digestion

Substrate	**Total Solids (%)**	**Volatile Solids (%)**
High Starch Containing *Spirodela* Biomass	6.54	6.13
Non-Nutrient Starved Fresh *Spiridela* biomass	10.47	8.87
Fermentation Vinasse	1.54	1.34
Inoculum for Anaerobic Digestion	2.98	2.06

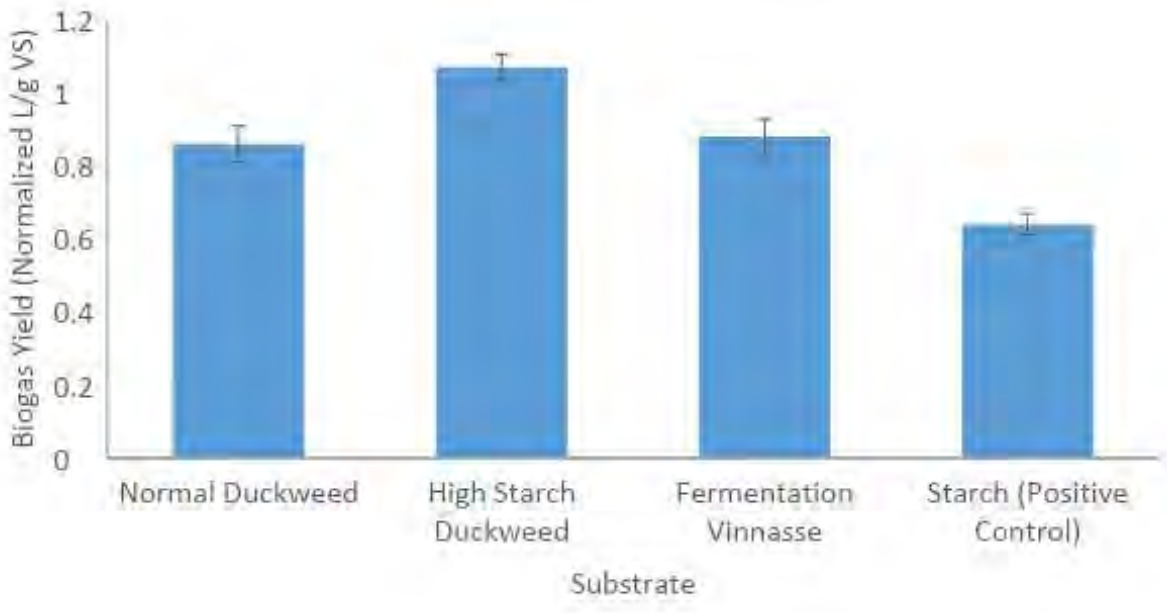
Anaerobic Digestion of Substrates for Production of Biogas and Biomethane

Anaerobic Digestion in batch mode for high starch containing plant biomass, fresh plant biomass grown without nutrient stress, fermentation vinasse, starch as positive control and inoculum as negative control was carried out for 72 days. Figure 5.2 (a) and 5.3 (a) shows that methane production in all the reactors ceased by 30th day. Figure 5.2 (b) and 5.3 (b) shows that highest biogas and biomethane yield was obtained from high starch plant biomass that was 1.07 NL/g VS and 0.795 NL/g VS respectively. Biomethane yield for fresh *Spirodela* biomass and fermentation vinasse was almost equivalent i.e., 0.68 NL/g VS and 0.71 NL/g VS respectively. Whereas, Biogas yield for fresh *Spirodela* biomass and fermentation vinasse was 0.86 NL/g VS and 0.88 NL/g VS respectively. Use of fermentation vinnase for production of biogas can help in management of distillery waste and can also be used by distillery itself instead of natural gas. According to an estimate

carried out by O'shea et al., (2020) 64% of the distillery's natural gas consumption can be reduced if anaerobic digestion of distillery byproducts is carried out and resultantly produced biogas is utilized by distillery instead of natural gas (O'Shea et al., 2020).

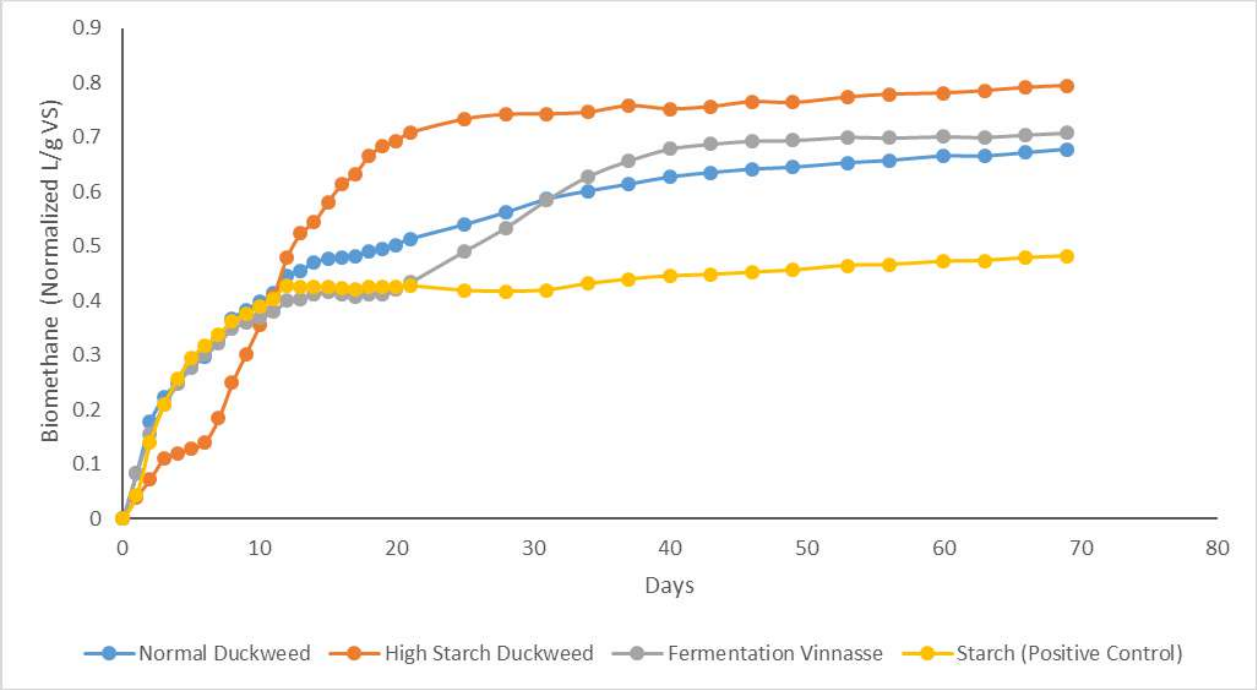


(a)

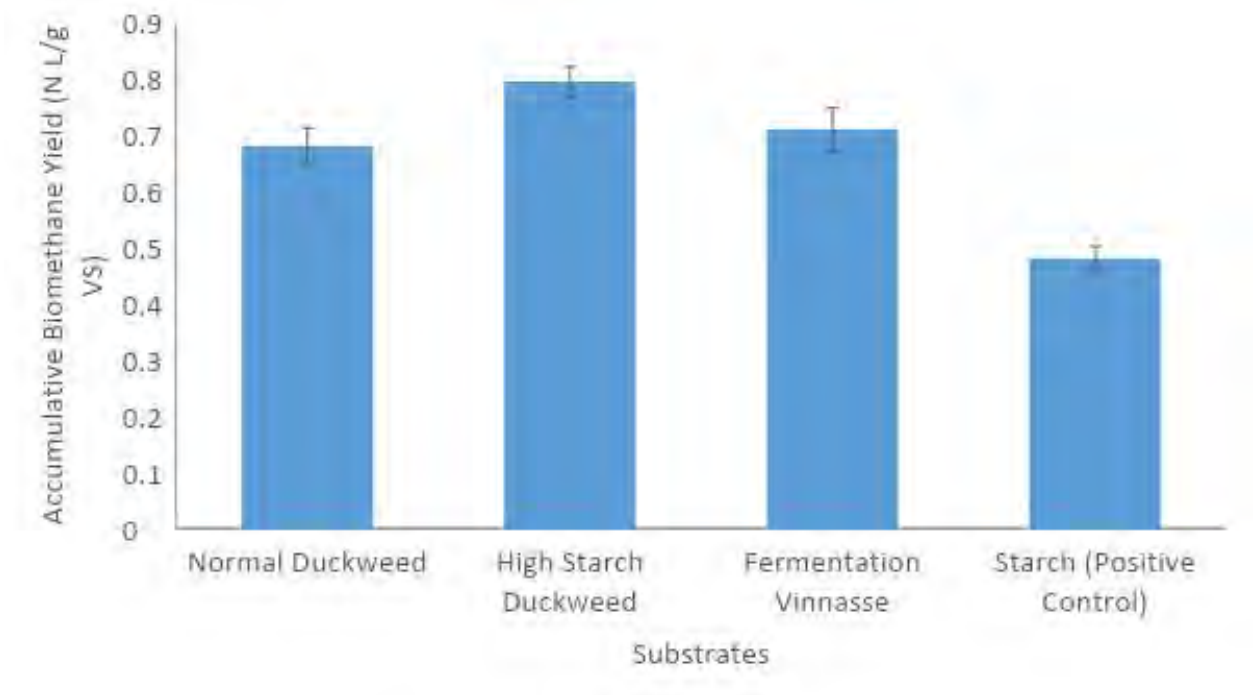


(b)

Figure 5.2 (a) (b) Biogas Yield Using Plant Biomass as Substrate



(a)



(b)

Figure 5.3 (a) (b) Biomethane Yield Using Plant Biomass as Substrate

5.3.4 Output Energy Evaluation of Produced Biofuels

Figure 5.4 shows comparative analysis of the total energy output of biofuels produced in four different processes which were utilization of high starch *Spirodela* biomass for ethanol production only, utilization of high starch *Spirodela* biomass for ethanol production and then utilization of fermentation vinasse for biomethane production, utilization of high starch *Spirodela* biomass for biomethane production only and utilization of fresh *Spirodela* biomass for biomethan production only. The results showed that highest amount of energy i.e., 18.5 MJ/g VS is produced in the process where high starch containing *Spirodela* plant biomass was used for the production of biomethane only. Second came the process in which the starch from high starch plant biomass was used for production of bioethanol whereas fermentation vinasse was used for production of biomethane. The energy output for this process was 16.5 MJ/g VS. The reason behind lower energy content of process combining the production of bioethanol and biomethane from the process involved in production of biomethane only from high starch plant biomass is the lower energy content of bioethanol itself because of its partially oxidized state. The lowest energy content was observed in the process where high starch plant biomass was only used for production of bioethanol. The results obtained showed that high starch *Spirodela* biomass is best used in terms of energy when it is anaerobically digested for production of biogas only. However, the type of fuel produced depends upon the need for which fuel production is desired.

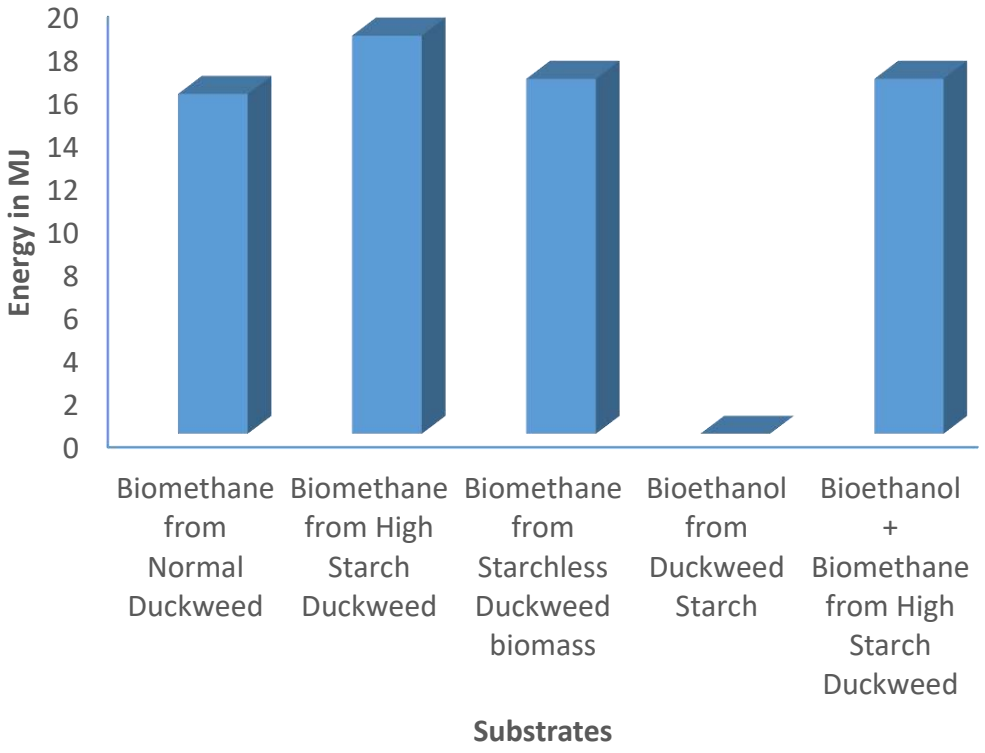


Figure 5.4 Energy Evaluation of Biofuels Produced from *Spirodela polyrhiza*

5.4 Conclusions

The study conducted concludes that *Spirodela polyrhiza* can effectively be used as a feedstock for the production of third generation biofuels specially bioethanol and biomethane. The successful conversion of *Spirodela* biomass to biofuels is also suggestive of the fact that this feedstock can replace food crops grown on agricultural land which are used for biofuel production thus effectively eliminating food versus fuel feud. The output energy analysis of the biofuels produced showed that high starch *Spirodela* biomass is when anaerobically digested for production of biomethane, yields the maximum energy content out of this plant. All the results obtained confirmed that *Spirodela polyrhiza* has the capability of finding promising applications within the field of biofuels and bioenergy.

References

Abdeen, F. R., Mel, M., Jami, M. S., Ihsan, S. I., & Ismail, A. F. (2016). A review of chemical absorption of carbon dioxide for biogas upgrading. *Chinese Journal of Chemical Engineering*, *24*(6), 693-702.

AlNouss, A., McKay, G., & Al-Ansari, T. (2020). Enhancing waste to hydrogen production through biomass feedstock blending: A techno-economic-environmental evaluation. *Applied Energy*, *266*, 114885.

Amorim, H. V., Lopes, M. L., de Castro Oliveira, J. V., Buckeridge, M. S., & Goldman, G. H. (2011). Scientific challenges of bioethanol production in Brazil. *Applied microbiology and biotechnology*, *91*(5), 1267.

Appenroth, K.-J., Keresztes, A., Sárvári, É., Jaglarz, A., & Fischer, W. (2003). Multiple effects of chromate on Spirodela polyrhiza: electron microscopy and biochemical investigations. *Plant Biology*, *5*(03), 315-323.

Appenroth, K.-J., Krech, K., Keresztes, A., Fischer, W., & Koloczek, H. (2010). Effects of nickel on the chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in biomonitoring and phytoremediation. *Chemosphere*, *78*(3), 216-223.

Callegari, A., Bolognesi, S., Cecconet, D., & Capodaglio, A. G. (2020). Production technologies, current role, and future prospects of biofuels feedstocks: a state-of-the-art review. *Critical Reviews in Environmental Science and Technology*, *50*(4), 384-436.

Cui, W., & Cheng, J. (2015). Growing duckweed for biofuel production: a review. *Plant biology*, *17*, 16-23.

Hirani, A. H., Javed, N., Asif, M., Basu, S. K., & Kumar, A. (2018). A review on first-and second-generation biofuel productions. In *Biofuels: Greenhouse Gas Mitigation and Global Warming* (pp. 141-154). Springer.

Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, C., Buffière, P., Carballa, M., & De Wilde, V. (2016). Towards a standardization of biomethane potential tests. *Water Science and Technology*, *74*(11), 2515-2522.

Huang, M., Fang, Y., Xiao, Y., Sun, J., Jin, Y., Tao, X., Ma, X., He, K., & Zhao, H. (2014). Proteomic analysis to investigate the high starch accumulation of duckweed

(Landoltia punctata) under nutrient starvation. *Industrial Crops and Products*, *59*, 299-308.

Mahdi, J. M., Mohammed, H. I., Hashim, E. T., Talebizadehsardari, P., & Nsofor, E. C. (2020). Solidification enhancement with multiple PCMs, cascaded metal foam and nanoparticles in the shell-and-tube energy storage system. *Applied Energy*, *257*, 113993.

Manochio, C., Andrade, B., Rodriguez, R., & Moraes, B. (2017). Ethanol from biomass: A comparative overview. *Renewable and Sustainable Energy Reviews*, *80*, 743-755.

Michael, T. P., Bryant, D., Gutierrez, R., Borisjuk, N., Chu, P., Zhang, H., Xia, J., Zhou, J., Peng, H., & El Baidouri, M. (2017). Comprehensive definition of genome features in Spirodela polyrhiza by high-depth physical mapping and short-read DNA sequencing strategies. *The Plant Journal*, *89*(3), 617-635.

Miller, G. (1959). Modified DNS method for reducing sugars. *Anal. Chem*, *31*(3), 426-428.

Nilsson, L. J., & Johansson, T. B. (2017). Environmental challenges to the energy industries. In *Sustainable Development and the Energy Industries* (pp. 47-80). Routledge.

O'Shea, R., Lin, R., Wall, D. M., Browne, J. D., & Murphy, J. D. J. A. E. (2020). Using biogas to reduce natural gas consumption and greenhouse gas emissions at a large distillery. *279*, 115812.

Parsaee, M., Kiani, M. K. D., & Karimi, K. (2019). A review of biogas production from sugarcane vinasse. *Biomass and bioenergy*, *122*, 117-125.

Rana, Q. U. A., Irfan, M., Ahmed, S., Hasan, F., Shah, A. A., Khan, S., Rehman, F. U., Khan, H., Ju, M., & Li, W. (2019). Bio-catalytic transesterification of mustard oil for biodiesel production. *Biofuels*, 1-8.

Rodionova, M. V., Poudyal, R. S., Tiwari, I., Voloshin, R. A., Zharmukhamedov, S. K., Nam, H. G., Zayadan, B. K., Bruce, B. D., Hou, H., & Allakhverdiev, S. I. (2017). Biofuel production: challenges and opportunities. *International Journal of Hydrogen Energy*, *42*(12), 8450-8461.

Roman, B., & Brennan, R. A. (2019). A beneficial by-product of ecological wastewater treatment: An evaluation of wastewater-grown duckweed as a protein supplement for sustainable agriculture. *Ecological Engineering: X*, *1*, 100004.

Sarris, D., & Papanikolaou, S. (2016). Biotechnological production of ethanol: biochemistry, processes and technologies. *Engineering in life sciences*, *16*(4), 307-329.

Singh, V., Pandey, B., & Suthar, S. (2018). Phytotoxicity of amoxicillin to the duckweed Spirodela polyrhiza: growth, oxidative stress, biochemical traits and antibiotic degradation. *Chemosphere*, *201*, 492-502.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Nrel, J. (2008). Determination of total solids in biomass and total dissolved solids in liquid process samples, Natl. *Renew. Energy Lab*, *1*, e6.

Tabatabaei, M., Aghbashlo, M., Valijanian, E., Panahi, H. K. S., Nizami, A.-S., Ghanavati, H., Sulaiman, A., Mirmohamadsadeghi, S., & Karimi, K. (2020). A comprehensive review on recent biological innovations to improve biogas production, part 1: upstream strategies. *Renewable energy*, *146*, 1204-1220.

Tao, X., Fang, Y., Xiao, Y., Jin, Y.-l., Ma, X.-r., Zhao, Y., He, K.-z., Zhao, H., & Wang, H.-y. (2013). Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (Landoltia punctata) under nutrient starvation. *Biotechnology for biofuels*, *6*(1), 72.

ul ain Rana, Q., Khan, M. A. N., Irfan, M., Shah, A. A., Hasan, F., Khan, S., Ahmed, S., Adnan, F., Li, W., & Ju, M. (2019). Starved Spirodela polyrhiza and Saccharomyces cerevisiae: a potent combination for sustainable bioethanol production. *Biomass Conversion and Biorefinery*, 1-10.

Qurrat ul ain Rana, Q., Rehman, M. L. U., Irfan, M., Ahmed, S., Hasan, F., Shah, A. A., Khan, S., & Badshah, M. (2019). Lipolytic bacterial strains mediated transesterification of non-edible plant oils for generation of high quality biodiesel. *Journal of bioscience and bioengineering*, *127*(5), 609-617.

Xing, W., Huang, W., & Liu, G. (2010). Effect of excess iron and copper on physiology of aquatic plant Spirodela polyrrhiza (L.) Schleid. *Environmental Toxicology: An International Journal*, *25*(2), 103-112.

Xyländer, M., Augsten, H., & Appenroth, K.-J. (1993). Influence of nickel on the life cycle of the duckweed Spirodela polyrhiza (L.) Schleiden. *Journal of plant physiology*, *142*(2), 208-213.

Chapter 6: Enhancement of Ethanol Yield from High Starch Duckweed Biomass by Utilization of Fermentable Cell Wall Monosaccharides

Abstract

Energy is the driving force for socioeconomic development. Currently, world's energy needs are fulfilled by non-renewable energy sources like fossil fuels which are depleting readily and come with the price of environmental pollution. This has led to the quest for alternative energy sources like renewable biofuels. Among biofuels, bioethanol has the potential to replace petroleum in the transport sector. However, bioethanol is produced from food crops which cause food to fuel competition. Duckweed is a promising feedstock that can alleviate this problem. High starch duckweed biomass has already been utilized for production of bioethanol but overall bioethanol yield from duckweed biomass can be enhanced by releasing and fermenting duckweed cell wall sugars as well. In this study the starch content of two duckweed species, *Spirodela polyrhiza* and *Lemna minor* was enhanced to 2% and 1.65% respectively, by applying nutrient stress. The cell wall analysis of high starch duckweeds was carried out and three fermentable sugars i.e., galactose, xylose and glucose were identified. The cell walls of these high starch duckweed biomass were subjected to two types of pretreatments: hydrothermal and ionic liquid. The hydrothermal pretreatment in case of *Lemna* and IL pretreatment in case of *Spirodela* were effective in releasing high amount of xylose and glucose from cell walls which, when fermented effectively enhance the overall bioethanol yield from high starch duckweed biomass. The pretreatments were able to increase 23.7% and 10.4% glucose yield from *Spirodela* and *Lemna*, biomass respectively. Similarly, 41% and 14.5% increase in ethanol obtained from *Spirodela* and *Lemna*, respectively was also observed.

6.1. Introduction

Energy is one of the prime factors that drives the development of a country. Energy stimulates the socioeconomic growth of developing countries. Thus, having access to energy supplies is a basic need of modern society (Moreau & Vuille, 2018). Currently, the energy demand is largely being fulfilled by means of non-renewable sources like coal, natural gas and petroleum derivatives. However, as the population and living standards of the society increases, so does the demand of energy (Schandl et al., 2016). But with the depletion of non-renewable energy resources the world will soon experience a steep hike in the price of fossil fuels and their extraction would become more environmentally damaging (Rana, et al., 2019). Moreover, the use of non-renewable energy sources causes multiple problems that include environmental pollution leading to numerous health hazards (Rana et al., 2019).

Renewable energy is the best alternative in order to overcome the multiple problems that are faced due to use of non-renewable energy sources (Akca Prill, 2020). Biofuels are among one of these alternative renewable energy sources. Biofuels are derived from biomass feedstock (Fernand et al., 2017). Bioethanol is one of the biofuels that has the potential to replace fossil fuels specifically in transport industry (Nogueira et al., 2020). Bioethanol is produced by the microbial fermentation of plant based sugars. Currently, bioethanol is produced from crops like sugar cane, cassava, sweet potato, and maize. But these feedstocks are food crops which are grown on agricultural land. Utilizing this feedstock for bioethanol production is accompanied with the challenge of food to fuel competition (Rodionova et al., 2017).

Duckweed is a tiny plant that floats on the water surface and multiply through the vegetative budding of fronds (leaf-like thallus). Duckweeds are monocotyledonous plants distributed in five different genera (*Lemna*, *Wolffia*, *Landoltia*, *Wolfiella* and *Spirodela*) and 37 diverse species. On dry basis 3-75% starch can be obtained from different species on applying different growing conditions (de Morais et al., 2019). Utilizing duckweed starch for bioethanol production can alleviate the problems associated with the afore mentioned plant feedstocks. Numerous studies have reported the effective fermentation of duckweed starch to bioethanol (J. J. Cheng & A. M. J. C. S. Stomp, Air, Water, 2009; Ge et al., 2012b; ul ain Rana, Khan, et al., 2019; Xu et al., 2011). Recently, the cell walls of duckweeds were studied and the results depicted that the duckweed cell walls have a high

proportion of three fermentable sugars that are galactose, glucose and xylose (Zhao et al., 2014). With appropriate pretreatments of the cell walls these sugars can be released and fermented to ethanol, which can effectively enhance the overall bioethanol yield from duckweed biomass rather than fermenting duckweed starch alone.

In this study two duckweed species, *Spirodela polyrhiza* and *Lemna minor* were grown in nutrient starved environment to increase their starch content. The cell wall material of high starch duckweed biomass was prepared and subjected to hydrolysis with trifluoroacetic acid (TFA) for cell wall analysis. The cell wall material was further processed using two intensive pretreatments: hydrothermal treatment and ionic liquid pretreatment to enhance the release of two fermentable sugars, i.e., glucose and xylose, from the cell walls. These sugars can easily be fermented to ethanol thereby enhancing the overall ethanol yield of high starch duckweed biomass.

6.2. Materials and Methods

6.2.1. Feedstock Production

6.2.1.1 Collection and Culturing of Duckweed

The duckweed species used in this study i.e., *Spirodela polyrhiza* and *Lemna minor* were obtained from Rutgers Duckweed Stock Cooperative, The State University of New Jersey, USA. Schenk and Hildebrandt basal salt media (Sigma) was used for plant growth and the pH of the media was kept at 5.8 for *Spirodela* and 6.5 for *Lemna* (Yu et al., 2014). About 1g fresh weight (FW) of both *Spirodela* and *Lemna* was suspended in 2.5 L SH basal salt media contained in 5 L flasks. The plants were then grown in a plant growth chamber with LED growth light, 16 hour photoperiod and 25℃ temperature. Starch content of the plants was determined every third day and plants were harvested on day 21 when starch enhancement ceased in the biomass.

6.2.1.2 Determination of Starch Content

Starch content in the plant material was determined using Appenroth method (Appenroth et al., 2010). About 200 mg FW of plant was ground and homogenized with 4 ml of 18% HCl. The homogenized mixture was continuously shaken for 1 hour at 5 ℃. After shaking, the mixture was centrifuged at 12000 RPM for 20 minutes. The supernatant procured after centrifugation was mixed with Lugol's solution (KI 0.5% w/v and I_2 0.25%

w/v in distilled water) in 1:1 ratio. The optical density of this solution was taken at 605 nm and 530 nm. For starch content determination the following formulae were used.

$S\%=[Cs\times Vol(extr)\times 100]\div FW$ ….. (Eq. 1)

Where,

Vol (Extr) = the plant extract volume after homogenization with HCl (mL)

FW = fresh weight (mg) and

$Cs = A_{605} / (0.07757 \times P + 4.463)$

Whereas, P was calculated using the formula:

$P= [(7.295 \times A_{605} / A_{530} – 4.463) / (7.757 – 0.729 \times A_{605}/A_{530})] \times 100$

A_{605} and A_{530} are the absorbance at 605 nm and 530 nm, respectively.

6.2.2. Cell Wall Monosaccharide Analysis

6.3.2.1 Preparation of Alcohol Insoluble Residue

For the preparation of AIR, plant material was harvested in 100% ethanol contained in 25 ml vials having small sized milling metal beads. The plant material was then milled for 5 minutes at 20 Hz using QIAGEN TissueLyser (Retsch, UK). The metal milling beads were removed and vials were heated at 100 ℃ for 30 minutes. After heating, the mixture was centrifuged at 10,000 RPM for 15 minutes. The supernatant was removed and pellet was washed with 70% ethanol with subsequent centrifugation until the pellet became clear of chlorophyll. A final washing with acetone was carried out. The pellet was then dried by placing in 50 ℃ oven for 24 hours (Fry, 1988).

6.2.2.2 Enzymatic Hydrolysis of Starch in AIR

Enzymatic treatment was carried out to de-starch AIR according to the method described by Harholt et al. (2006), with a few modifications. AIR was first immersed in 3 U/ml α-amylase (Megazymes) solution prepared in 50 mM MOPS (3-(*N*-morpholino) propanesulfonic acid) buffer at pH 6.5. The AIR-Enzyme mixture was then heated at 75 ℃ for 10 minutes with frequent shaking. The mixture was then cooled down to room temperature and 3 U/ml amyloglucosidase (Megazymes) and 1 U/ml Pullulanase (*Bacillus licheniformis*, Megazymes) solution prepared in 200 mM sodium acetate buffer at pH 4.5 was added to it. The mixture was heated at 40 ℃ for 2 hours with occasional shaking. Afterwards, the mixture was centrifuged for 10 minutes at 10,000 RPM, supernatant was

separated and pellet was washed with 70% ethanol and then with acetone. The de-starched AIR pellet was then oven dried at 50 ℃ overnight (Harholt et al., 2006). HPLC was used to quantify the released glucose in supernatant for estimation of starch to glucose conversion rate.

6.2.2.3 TFA Hydrolysis to Release Cell Wall Sugars

For TFA hydrolysis, about 4 mg of AIR was immersed well in 1 ml of 2 M TFA and heated at 120 ℃ for 1 hour. The AIR-TFA was then cooled down to room temperature and TFA was removed by vacuum drying in speedvac (Labconco, USA). The obtained pellet was dissolved in water by shaking at 30 ℃ for 1 hour at 1000 RPM. The solution was filtered using 0.45 µm nitrocellulose filter and diluted 4 times. The 1:4 dilution was then analyzed for cell wall monosaccharides using HPAEC-PAD (Pagliuso et al., 2018).

6.2.3 Pretreatment and Saccharification of Feedstock Cell Walls

6.2.3.1 Hydrothermal Pretreatment and Enzymatic Saccharification

De-starched AIR was first pretreated (10 mg/mL i.e., 1% w/v loading rate) with water at 120 ℃ for 20 minutes. The slurry that was produced resultantly was centrifuged at 12000 RPM for 10 minutes. The supernatant was discarded and the pellet was washed with distilled water with subsequent centrifugation.

A mixture of 0.1 M citrate buffer (pH 5.0) 0.5 ml, Ctec3 (Cellic®, Novozymes) 0.05 µl, Tetracycline 15 µl and distilled water 135 µl was added to the pellet and the mixture was incubated at 50 ℃ and 850 RPM for 48 hours. After incubation the mixture was centrifuged at 12000 RPM for 10 minutes and the supernatant was analysed using HPAEC-PAD to determine glucose and xylose concentration (Ladeira Ázar et al., 2020).

6.2.3.2 Ionic Liquid Pretreatment and Enzymatic Saccharification

Cholinium phosphate was used as an ionic liquid for pretreatment of de-starched AIR. Cholinium phosphate was prepared by the addition of phosphoric acid (85% in water) in choline hydroxide (10% w/v in water) in molar ratio of 1:3 (calculated stoichiometrically) with rigorous shaking within an ice bath. Afterwards, the solution was stirred at room temperature for 24 hours and water was removed at 55 ℃ under vaccum (Sun et al., 2018).

For the purpose of ionic liquid pretreatment, a 15% (w/v) AIR and 5% (w/v) cholinium phosphate slurry was prepared and autoclaved for 3 hours at 120 ℃. After autoclaving, the pH of the slurry was adjusted to 5 using 1 M citrate buffer and a mixture of Ctec3 and Htec3 (Cellic®) in the ratio 9:1 and loading rate of 10 mg/g of biomass was added to it. The resulting mixture was incubated at 50 ℃ for 72 hours. After incubation, the slurry was filtered using 0.45 µm nitrocellulose filters at 14000 RPM for 30 minutes and the supernatant was analyzed by HPLC at 1:20 dilution for determination of glucose and xylose concentration.

6.2.4. Fermentation of Cell Wall Sugars (Glucose and Xylose)

The hydrolysate containing cell wall sugars mainly comprising of glucose and xylose that were released by IL pretreatment in case of *Spirodela* and hydrothermal pretreatment in case of *Lemna* was lyophilized. The powdered sugars were then fermented using two indigenously isolated and optimized yeast strains i.e., *Saccharomyces cerevisiae* QG1 (MK788210) and *Clavispora lusitaniae* QG1 (MN592676). *S. cerevisiae* cannot ferment xylose and was previously optimized for fermentation of glucose obtained from *Spirodela* starch whereas *C. lusitaniae* was optimized for xylose fermentation (ul ain Rana et al., 2019). The powdered cell wall sugar mixture was dissolved in 2.5 ml sterilized distilled water and 2.5 ml optimized yeast fermentation media (9 gL-1, peptone 7 gL-1, KH2PO4 6 gL-1, MgSO4.7H2O 0.5 gL-1, (NH4)2SO4 1 gL-1) was added to it. The pH of the reaction mixture was adjusted to 4.5 and initially 15%, 72 hours old *S.cerevisiae* inoculum was added to the reaction mixture. Fermentation vials containing reaction mixture were then flushed with nitrogen, sealed airtight and incubated for 24 hours. After 24 hours ethanol titer in the reaction mixture was analyzed. Reaction mixture was then centrifuged and supernatant was decanted. The pH of supernatant was adjusted to 6 and 15%, 24 hours old *C. lusitaniae* inoculum was added to it. The fermentation vials were again flushed with nitrogen and sealed airtight. Fermentation was carried out for 48 hours and ethanol titer was analyzed.

6.2.5. Analysis

6.2.5.1. High Performance Anion Exchange Chromatography with Pulse Amperometric Detection (HPAEC-PAD)

Cell wall sugars were detected using HPAEC-PAD (Thermo Fischer Scientific, Dionex ICS 5000, Waltham, MA, USA) using PA20 column (Thermo Fischer Scientific, Dionex IC, CarboPac, Waltham, MA, USA). The oven temperature was kept at 30 ℃. NaOH was used as a mobile phase with linear gradient of 100 mM to 1 M and flow rate of 0.4 ml/min. Three Eluents were used, Eluent A (water), Eluent B (100 mM NaOH) and Eluent C (1 M NaOH). Initially, Eluent B was used for the first 30 minutes to elute reduced/neutral sugars after which there was a gradual shift to Eluent C to elute acidic sugars. Samples were always filtered through 0.45 µm nitrocellulose filters prior to analysis and their pH was kept in the range of 4.0 – 9.0 (Øbro et al., 2004).

6.2.5.2. High Performance Liquid Chromatography (HPLC)

For quantification of sugars released by saccharification, HPLC (Thermo Fischer Scientific, Ultimate 3000, Waltham, MA, USA) was carried out using the Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refractive index detector. Sulfuric acid (5 mM) was used as the mobile phase with 0.6 ml/min flow rate and the column oven temperature was kept at 65 ℃. Samples were always filtered through 0.45 µm nitrocellulose filters prior to analysis (Sundstrom et al., 2018).

6.2.5.3. Ethanol Determination

Ethanol determination was carried out using Megazymes Ethanol Assay Kit (Bay, Ireland) according to manufacturer's instructions.

6.3. Results and Discussion

6.3.1. Feedstock Production

Being a hydrophyte, duckweeds grows on water surface thus, SH basal salt media having high concentration of nitrate and phosphate salts was selected for the growth of *Spirodela* and *Lemna.* Duckweed has gathered interest as an energy crop because of its ability to accumulate high starch in its biomass under varying growth conditions. This starch can then be utilized for bioethanol production. Nutrient starvation, specifically reduced nitrates and phosphates bioavailability, is one of the growth stresses that enhances

the starch content of duckweed (Cui & Cheng, 2015). For obtaining high starch duckweed biomass, both species of duckweeds were grown in SH media with high nitrates and phosphate salt concentrations. The starch content of the plants was determined in parallel with the plant growth. Initially, for both *Spirodela* and *Lemna*, the plant biomass increased rapidly by day 12th after which the increase in plant biomass ceased whereas starch content increased rapidly (Fig. 6.1). However, the starch content in plant biomass remained constant from day 18th to day 21st so plants were harvested at day 21st. The inhibition of plant growth and increase in starch is due to the fact that plant utilized all the nutrients, specifically nitrates and phosphates, from the media by day 12th and afterwards went into starvation phase which induced metabolic synthesis of starch.

An increase in biomass (FW) from 1 g to 15.8 g for *Spirodela* and from 1 g to 19.2 g for *Lemna* was observed. In case of starch content, 80% enhancement in starch content with a final starch content of 2% FW for *Spirodela* and 72% enhancement of starch content with a final starch content of 1.65% for *Lemna minor* was observed (Figure 6.1(a) & 6.1(b)). The results we obtained were consistent with previous studies that were conducted for enhancement of starch content by manipulation of nitrates and phosphates in the media (J. J. Cheng & A. M. Stomp, 2009; Ge et al., 2012a; Iatrou et al., 2015; ul ain Rana, Khan, et al., 2019).

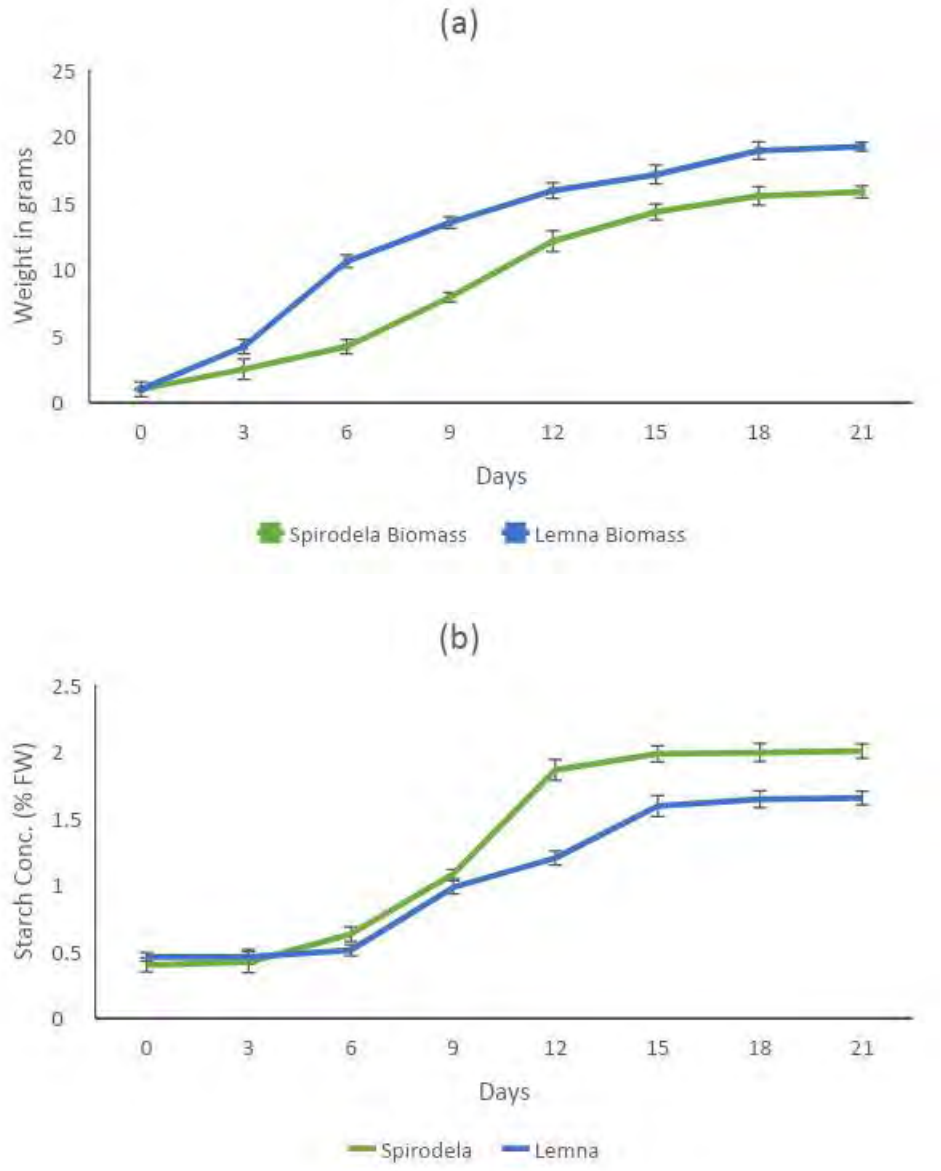


Figure 6.1 (a) Illustration of *Spirodela* and *Lemna* Growth during Application of Nutrient Stress (b) Starch Content Enhancement in *Spirodela* and *Lemna* biomass under Nutrient Starvation.

6.3.2 Cell Wall Monosaccharide Composition Analysis

The most appropriate material for bioethanol production is plant biomass that has high levels of starch, and therefore the plant biomass harvested at day 21 was selected for further study. Cell wall material i.e., AIR was prepared and de-starched enzymatically. About 312 mg of AIR from 15.8 g (FW) of *Spirodela polyrhiza* and 298 mg of AIR from 19.2 g (FW) of *Lemna minor* was obtained. The glucose obtained from enzymatic starch hydrolysis can be fermented to ethanol. The de-starched AIR was then subjected to TFA hydrolysis to

analyze the monosaccharide composition of *Spirodela* and *Lemna* cell walls as well as to identify fermentable sugars that can also be converted to bioethanol for enhancing the total bioethanol yield from duckweed biomass.

The cell walls monosaccharide analysis of both *Spirodela* and *Lemna* showed that the cell walls were mainly composed of Fucose (Fuc), Rhamnose (Rha), Arabinose (Ara), Apiose (Apo), Galactose (Gal), Glucose (Glc), Xylose (Xyl), Mannose (Man), Galacturonic Acid (GalA) and Glucuronic Acid (GlcA) (Figure 6.2). Galactose, Glucose and Xylose in both the plant cell walls were identified as fermentable sugars. In case of *Lemna* a higher amount of uronic acid and less amount of xylose indicates that its cell wall has a higher pectin content rather than hemicellulose. However, in case of *Spirodela* a significant difference between the amounts of uronic acid and xylose is not seen. A higher amount of glucose, xylose and galactose in both plants indicate that they have a considerable amount of cellulose and hemicellulose in their cell wall. Higher proportion of fermentable sugars and a relatively low lignin level in duckweed cell walls make it a useful feedstock for production of bioethanol (Zhao et al., 2014).

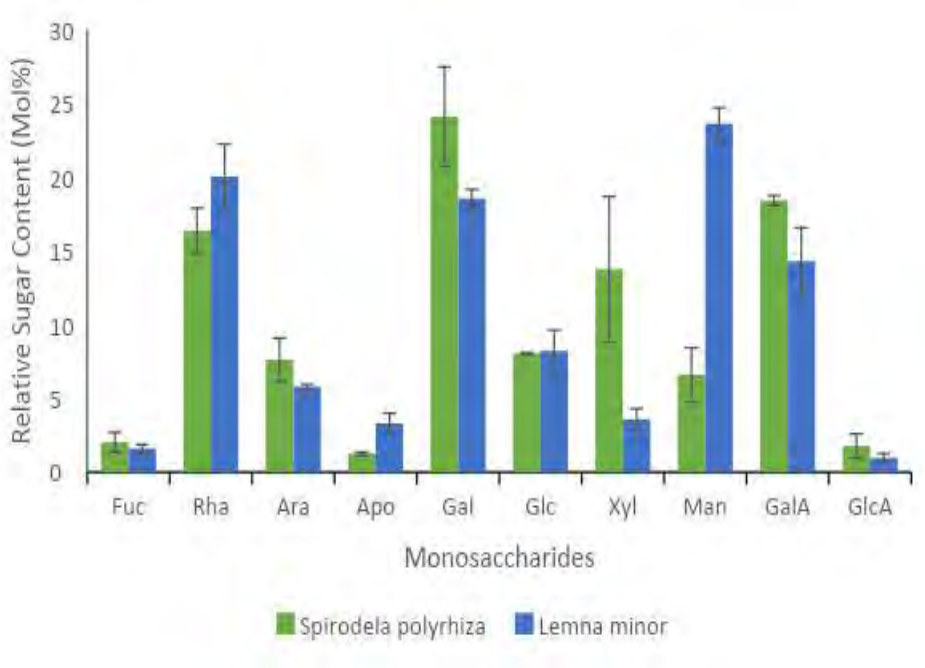


Figure 6.2 Cell Wall Monosaccharide Composition of *Spirodela polyrhiza* and *Lemna Minor*

6.3.3. Pretreatment of Duckweed Cell Wall

Glucose and xylose in the duckweed cell was targeted for fermentation to produce bioethanol. For this purpose, the cell wall material i.e., AIR was subjected to two intensive pretreatments for enhanced release of these two fermentable sugars.

In order to ease up the heterogeneous, complex and recalcitrant cell wall so that enzymes can easily access the cellulose and hemicellulose components, hydrothermal pretreatment of AIR was carried out prior to enzymatic saccharification (Saha et al., 2013). After hydrothermal pretreatment the cell wall material was subjected to enzymatic digestion and the released sugars (glucose and xylose) were analyzed using HPAEC. On the other hand, another intensive pretreatment using ionic liquid cholinium phosphate was also used on AIR to achieve high yield of glucose and xylose in hydrolysate. The hydrolysate achieved through this pretreatment was analyzed using HPLC.

The results obtained from both the pretreatments revealed that in case of *Spirodela polyrhiza* ionic liquid pretreatment released maximum amount of the glucose from cell wall material whereas the release of xylose did not have a major difference between the two treatments. The IL preteatment was successful in releasing 82.5 mg of glucose and 30 mg of xylose from the total AIR extracted. Based upon the amount of starch accumulated by plant, the glucose released from cell wall shows that 23.7% enhancement in the glucose obtained from plant starch can be achieved by utilizing cell wall glucose released through IL pretreatment. However, in case of *Lemna minor*, hydrothermal pretreatment followed by the enzymatic saccharification remained most effective in enhanced release of xylose and glucose. This treatment released 36 mg of glucose and 14.4 mg of xylose from total AIR extracted from plant fresh weight. Based upon amount of starch accumulated in the plants, the glucose released from cell walls shows that 10.4% enhancement in the total glucose obtained from plant starch can be achieved by utilizing cell wall glucose released through hydrothermal pretreatment. The results also showed that *Spirodela* cell wall comprise of higher content of glucose and xylose as compared to *Lemna*. The different effect of these two pretreatments on two different species of duckweed can be attributed to the difference in the cell wall structure and composition in these two duckweed species. The released sugars can be fermented to bioethanol and can effectively enhance the ethanol

yield from duckweed, compared to previous studies using only the starch present in duckweed.

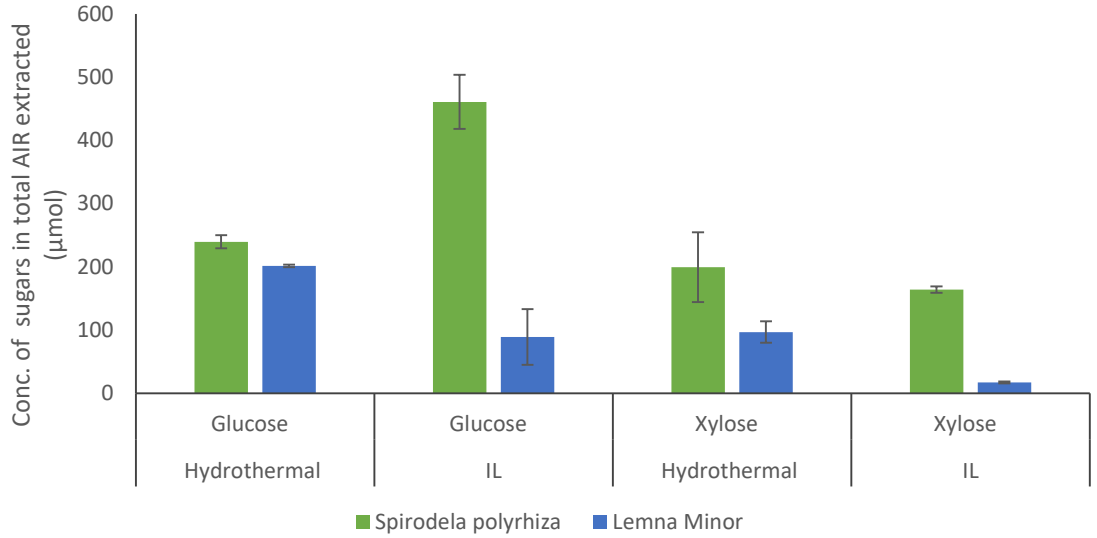


Figure 6.3. Comparative analysis of amount of glucose and xylose released by enzymatic saccharification from *Spirodela* and *Lemna* cell walls after hydrothermal pretreatment and ionic liquid pretreatment.

6.3.4. Fermentation of Cell Wall Sugars (Glucose and Xylose)

As IL pretreatment in case of *Spirodela* and hydrothermal pretreatment in case of *Lemna* released maximum amount of sugars from AIR, the hydrolysates of these pretreatments were lyophilised and selected for fermentation using two indigenously isolated yeast strains i.e., *Saccharomyces cerevisiae* QG1 (MK788210) and *Clavispora lusitaniae* QG1 (MN592676) which were previously optimized to give highest ethanol yield from glucose and xylose, respectively (ul ain Rana et al., 2019). Fermentation was carried out in two phases. As *S. cerevisiae* can only utilize glucose it was added first to the fermentation media. *S. cerevisiae* yielded 100% theoretical ethanol yield in case of both *Spirodela* and *Lemna.* After complete conversion of glucose to ethanol *S. cerevisiae* cells were removed and *C. lusitaniae* inoculum was added for conversion of xylose to ethanol. *C. lusitaniae* achieved 99.2% and 97% theoretical yield with *Spirodela* and *Lemna*, respectively. The results depicted that the lyophilised hydrolysate does not contain anything that was inhibited the microorganisms. Theoretical yields were calculated stoichiometricaly. These results also suggested that about 41% and 14.5% increase in ethanol yield can be achieved by utilizing cell wall sugars (glucose and xylose) together with plant starch instead of using

plant starch alone from *Spirodela* and *Lemna*, respectively. A proper lifecycle analysis and technoeconomical analysis can be conducted from these findings to assess the production and cost feasibility of this process at large scale.

6.4. Conclusions

The study conducted concluded that high starch duckweed can effectively be used for production of third generation bioethanol. However, the ethanol yield can also be increased by utilizing the cell wall fermentable sugars as well. The duckweed cell walls contain a high proportion of glucose and xylose which, by using appropriate pretreatment can be released and fermented. All the results obtained indicated the sequential hydrothermal pretreatment in case of *Lemna* and IL pretreatment in case of *Spirodela* were effective in releasing high amount of xylose and glucose from cell wall which, when fermented effectively enhanced the overall bioethanol yield from high starch duckweed biomass.

References

1. Akca Prill, M. (2020). *Energy dependency, the potential supply of renewable energies and the political responses in Turkey in the decades since the oil-crisis* Technische Universität].
2. Appenroth, K.-J., Krech, K., Keresztes, A., Fischer, W., & Koloczek, H. (2010). Effects of nickel on the chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in biomonitoring and phytoremediation. *Chemosphere*, *78*(3), 216-223.
3. Cheng, J. J., & Stomp, A. M. (2009). Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *Clean–Soil, Air, Water*, *37*(1), 17-26.
4. Cheng, J. J., & Stomp, A. M. J. C. S., Air, Water. (2009). Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *37*(1), 17-26.
5. Cui, W., & Cheng, J. (2015). Growing duckweed for biofuel production: a review. *Plant biology*, *17*, 16-23.
6. de Morais, M. B., Barbosa-Neto, A. G., Willadino, L., Ulisses, C., & Junior, T. C. J. J. o. P. G. R. (2019). Salt stress induces increase in starch accumulation in duckweed (Lemna aequinoctialis, Lemnaceae): biochemical and physiological aspects. *38*(2), 683-700.
7. Fernand, F., Israel, A., Skjermo, J., Wichard, T., Timmermans, K. R., & Golberg, A. (2017). Offshore macroalgae biomass for bioenergy production: Environmental aspects, technological achievements and challenges. *Renewable and Sustainable Energy Reviews*, *75*, 35-45.
8. Fry, S. C. (1988). *The growing plant cell wall: chemical and metabolic analysis*. Longman Group Limited.
9. Ge, X., Zhang, N., Phillips, G. C., & Xu, J. (2012a). Growing Lemna minor in agricultural wastewater and converting the duckweed biomass to ethanol. *Bioresource Technology*, *124*, 485-488.

10. Ge, X., Zhang, N., Phillips, G. C., & Xu, J. J. B. T. (2012b). Growing Lemna minor in agricultural wastewater and converting the duckweed biomass to ethanol. *124*, 485-488.
11. Harholt, J., Jensen, J. K., Sørensen, S. O., Orfila, C., Pauly, M., & Scheller, H. V. (2006). ARABINAN DEFICIENT 1 is a putative arabinosyltransferase involved in biosynthesis of pectic arabinan in Arabidopsis. *Plant physiology*, *140*(1), 49-58.
12. Iatrou, E. I., Stasinakis, A. S., & Aloupi, M. (2015). Cultivating duckweed Lemna minor in urine and treated domestic wastewater for simultaneous biomass production and removal of nutrients and antimicrobials. *Ecological Engineering*, *84*, 632-639.
13. Ladeira Ázar, R. I., Bordignon-Junior, S. E., Laufer, C., Specht, J., Ferrier, D., & Kim, D. (2020). Effect of Lignin Content on Cellulolytic Saccharification of Liquid Hot Water Pretreated Sugarcane Bagasse. *Molecules*, *25*(3), 623.
14. Moreau, V., & Vuille, F. (2018). Decoupling energy use and economic growth: Counter evidence from structural effects and embodied energy in trade. *Applied energy*, *215*, 54-62.
15. Nogueira, L. A. H., Souza, G. M., Cortez, L. A. B., & de Brito Cruz, C. H. (2020). Biofuels for Transport. In *Future Energy* (pp. 173-197). Elsevier.
16. Øbro, J., Harholt, J., Scheller, H. V., & Orfila, C. (2004). Rhamnogalacturonan I in Solanum tuberosum tubers contains complex arabinogalactan structures. *Phytochemistry*, *65*(10), 1429-1438.
17. Pagliuso, D., Grandis, A., Igarashi, E. S., Lam, E., & Buckeridge, M. S. (2018). Correlation of apiose levels and growth rates in Duckweeds. *Frontiers in chemistry*, *6*, 291.
18. Rana, Q. U. A., Irfan, M., Ahmed, S., Hasan, F., Shah, A. A., Khan, S., Rehman, F. U., Khan, H., Ju, M., & Li, W. J. B. (2019). Bio-catalytic transesterification of mustard oil for biodiesel production. 1-8.
19. Rodionova, M. V., Poudyal, R. S., Tiwari, I., Voloshin, R. A., Zharmukhamedov, S. K., Nam, H. G., Zayadan, B. K., Bruce, B. D., Hou, H., & Allakhverdiev, S. I. J. I. J. o. H. E. (2017). Biofuel production: challenges and opportunities. *42*(12), 8450-8461.
20. Saha, B. C., Yoshida, T., Cotta, M. A., Sonomoto, K. J. I. C., & Products. (2013). Hydrothermal pretreatment and enzymatic saccharification of corn stover for efficient ethanol production. *44*, 367-372.

21. Schandl, H., Hatfield-Dodds, S., Wiedmann, T., Geschke, A., Cai, Y., West, J., Newth, D., Baynes, T., Lenzen, M., & Owen, A. (2016). Decoupling global environmental pressure and economic growth: scenarios for energy use, materials use and carbon emissions. *Journal of cleaner production*, *132*, 45-56.
22. Sun, J., Liu, D., Young, R. P., Cruz, A. G., Isern, N. G., Schuerg, T., Cort, J. R., Simmons, B. A., & Singh, S. (2018). Solubilization and Upgrading of High Polyethylene Terephthalate Loadings in a Low-Costing Bifunctional Ionic Liquid. *ChemSusChem*, *11*(4), 781-792.
23. Sundstrom, E., Yaegashi, J., Yan, J., Masson, F., Papa, G., Rodriguez, A., Mirsiaghi, M., Liang, L., He, Q., & Tanjore, D. (2018). Demonstrating a separation-free process coupling ionic liquid pretreatment, saccharification, and fermentation with Rhodosporidium toruloides to produce advanced biofuels. *Green chemistry*, *20*(12), 2870-2879.
24. ul ain Rana, Q., Khan, M. A. N., Irfan, M., Shah, A. A., Hasan, F., Khan, S., Ahmed, S., Adnan, F., Li, W., & Ju, M. (2019). Starved Spirodela polyrhiza and Saccharomyces cerevisiae: a potent combination for sustainable bioethanol production. *Biomass Conversion and Biorefinery*, 1-10.
25. ul ain Rana, Q., Rehman, M. L. U., Irfan, M., Ahmed, S., Hasan, F., Shah, A. A., Khan, S., Badshah, M. J. J. o. b., & bioengineering. (2019). Lipolytic bacterial strains mediated transesterification of non-edible plant oils for generation of high quality biodiesel. *127*(5), 609-617.
26. Xu, J., Cui, W., Cheng, J. J., & Stomp, A.-M. J. B. e. (2011). Production of high-starch duckweed and its conversion to bioethanol. *110*(2), 67-72.
27. Yu, C., Sun, C., Yu, L., Zhu, M., Xu, H., Zhao, J., Ma, Y., & Zhou, G. (2014). Comparative analysis of duckweed cultivation with sewage water and SH media for production of fuel ethanol. *PLoS One*, *9*(12), e115023.
28. Zhao, X., Moates, G., Wellner, N., Collins, S., Coleman, M., & Waldron, K. (2014). Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (Lemna minor). *Carbohydrate polymers*, *111*, 410-418.
29. Qurat ul ain Rana, Q., Khan, M. A. N., Irfan, M., Shah, A. A., Hasan, F., Khan, S., Ahmed, S., Adnan, F., Li, W., & Ju, M. (2019). Starved Spirodela polyrhiza and

Saccharomyces cerevisiae: a potent combination for sustainable bioethanol production. *Biomass Conversion and Biorefinery*, 1-10.

Conclusions

The study conducted concludes that the isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210 shows promising potential to be utilized for obtaining high ethanol yield from feedstock like *Spirodela polyrhiza.* The enhanced starch content in *Spirodela* biomass by providing nutrient stress and successful conversion of all the plant starch to ethanol by isolated and optimized yeast strain is suggestive of the fact that this plant can potentially be used as feedstock for bioethanol production. The high nitrate and phosphate uptaking capacity of *Spirodela* directs that this plant can also be utilized for treatment of nutrient rich wastewaters. All the results obtained confirm that the isolated yeast strain as well as *Spirodela polyrhiza* can find promising applications in the field of biofuel production.

Spirodela polyrhiza was also found to be efficient in the removal of nutrients from agricultural wastewater. The consequently generated plant biomass had elevated levels of starch which was efficiently converted to glucose by dilute acid pretreatment and this glucose was successfully fermented to bioethanol by using indigenously isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210. This study depicted that the duckweed species *Spirodela polyrhiza* is a potent option to resolve two major issues simultaneously. First, it could remediate agricultural runoff by removing excessive nutrients out of it that reduces water pollution. Second, the plant biomass obtained as by-product could be used as a potential feedstock for the production of bioethanol that addresses the problem of the energy crisis in the form of production of bioethanol as biofuel.

The study conducted also showed that *Spirodela polyrhiza* can effectively be used as a feedstock for the sequential production of third generation bioethanol and biomethane. The high starch plant biomass can be fermented into ethanol whereas the fermentation vinnasse along with starchless plant biomass can be used for biogas production. The successful conversion of *Spirodela* biomass to biofuels is also suggestive of the fact that this feedstock can replace food crops grown on agricultural land which are used for biofuel production thus effectively eliminating food versus fuel feud. The output energy analysis of the biofuels produced showed that high starch *Spirodela* biomass is when anaerobically digested for production of biomethane, yields the maximum energy content out of this plant.

The study also analysed the ways by which ethanol yield from high starch duckweed biomass can be enhanced. The ethanol yield can be increased by utilizing the cell wall fermentable sugars. The duckweed cell walls contain a high proportion of glucose and xylose which, by using appropriate pretreatment can be released and fermented. All the results obtained indicated the sequential hydrothermal pretreatment and enzymatic saccharification in case of *Lemna* and IL pretreatment in case of *Spirodela* were effective in releasing high amount of xylose and glucose from cell wall which, when fermented can effectively enhance the overall bioethanol yield from high starch duckweed biomass.

All the results obtained indicate that *Spirodela polyrhiza* has the capability of finding promising applications within the fields of bioremediation and bioenergy.

Future Prospects

- As the research conducted shows that by growing *Spirodela polyrhiza* under varying growth conditions, increases the starch content in its biomass, this starch apart for being used for bioethanol production can also be used for production of useful bio-based polymers like starch derived bioplastics for e.g. Polylactic acid (PLA).

- Apart from agricultural wastewater, *Spirodela polyrhiza* can also be employed for the treatment of other wastewater streams like municipal wastewater, domestic wastewater, sewerage etc. and the consequently generated biomass can be used for production of biofuels.

- Evaluation of *Spirodela polyrhiza*'s cell wall showed that apart from fermentable sugars, the cell wall was also constituted of various other monosaccharides. Isolation and Identification of microbial strains that can utilize other monosaccharide sugars present in plant cell wall and plant biomass to produce biofuels or other bioproducts can be performed as a follow up study.

- This study focused on fermentation of glucose and xylose present in *Spirodela polyrhiza* cell wall only for fermentation whereas galactose has the highest percentage of fermentable sugars present in cell wall. Release of galactose using various pretreatments and its fermentation to ethanol can also be studied for enhancing the overall ethanol yield from *Spirodela* biomass.

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ORIGINAL ARTICLE

Starved *Spirodela polyrhiza* and *Saccharomyces cerevisiae*: a potent combination for sustainable bioethanol production

Qurrat ul ain Rana[1] · Muhammad Adil Nawaz Khan[1] · Muhammad Irfan[1,2] · Aamer Ali Shah[1] · Fariha Hasan[1] · Samiullah Khan[1] · Safia Ahmed[1] · Fazal Adnan[3] · Weizun Li[4] · Meiting Ju[4] · Malik Badshah[1]

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Abstract
The depletion of nonrenewable fossil fuels and the rise in their prices depicts that there is a need to fend for alternative fuel resources. Biofuels are one of these renewable alternative options. Among biofuels, bioethanol is one of the most widely used transportation fuels around the world. However, currently, bioethanol is produced from food crops, which is raising food versus fuel feud. *Spirodela polyrhiza* is one of the novel feedstock that can be used to produce bioethanol at large scale without causing any food and fuel competition. This study involves the collection of *S. polyrhiza* from a local pond and establishment of its growth in Hoagland growth media. The plant was then given nutrient starvation stress to enhance its starch content by 78%. The high-starch-containing plant biomass was acid-pretreated, and 99.3% starch-to-glucose conversion was achieved. In order to ferment plant sugars, yeast strain *Saccharomyces cerevisiae* QG1 MK788210 was indigenously isolated and statistically optimized using Plackett–Burman and central composite design to achieve high ethanol yield. The fermentation of plant sugar by *Saccharomyces cerevisiae* QG1 MK788210 resulted in 100% ethanol yield thus successfully achieving complete conversion of *S. polyrhiza* starch to bioethanol. The study conducted demonstrates effective optimization of indigenously isolated yeast strain *S. cerevisiae* QG1 MK788210 to deliver high ethanol yield from *S. polyrhiza*. Further, this study has been successful in delivering a process for complete conversion of starch from nutrient-starved *S. polyrhiza* biomass into bioethanol.

Keywords *Spirodela polyrhiza* · Duckweed · *Saccharomyces cerevisiae* · Bioethanol · Fermentation · Pretreatment

1 Introduction

The world's resource of nonrenewable fossil fuels is depleting quickly. The ever growing transportation sector and increased industrialization has resulted in the increase of world's energy demand by 30% per year and the remaining reserves of nonrenewable fossil fuels are not enough to meet this escalating energy demand [1]. Apart from being depleted, these nonrenewable fuels also have many environmental hazards, which include greenhouse gas (GHG) and carcinogenic exhaust emissions [2]. In order to alleviate all these problems, the world is now more inclined towards the use of environmental friendly renewable fuel options that include biofuels [3]. One of the most widely used biofuel in transportation sector around the globe is bioethanol [4]. Typically, bioethanol has a higher octane number than petrol and can easily be used as vehicle fuel. Bioethanol is renewable, reduces greenhouse gas emissions and is environmentally friendly fuel [5]. Majority of the ethanol currently being produced around the world use feedstocks like corn, potato, cassava and sugar beet, which are food crops and are cultivated on agricultural land [6]. This utilization of these food crops and use of agricultural land for growth of energy crops has led to the food versus fuel competition, which has resulted in high prices of food crops.

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✉ Malik Badshah
malikbadshah@qau.edu.pk

[1] Department of Microbiology, Quaid-i-Azam University, Islamabad 45320, Pakistan

[2] Department of Microbiology and Cell Science, Genetics Institute and Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32610, USA

[3] Atta-ur-Rahman School of Applied Biosciences, National University of Sciences & Technology, Islamabad, Pakistan

[4] College of Environmental Science and Engineering, Nankai University, Tijan 300350, China

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This utilization of food crops and agricultural land for bioethanol production can also not be implemented in the developing countries due to existence of a much bigger problem, i.e., food crisis [7]. Therefore, researchers are also looking for alternative feedstock to avoid this food and fuel imbalance. *Spirodela polyrhiza* is one of the new alternative feedstock options. It is commonly known as giant duckweed and belongs to family *Lemnaceae* [8]. Being a hydrophyte, it grows on the surface of water thus avoiding utilization of agricultural land for its growth and eliminating the food and fuel competition. This aquatic plant has a very high growth rate with doubling time of 16–24 h and also has the ability to accumulate about 3 to 70% of starch content in its dry weight. This starch can then be fermented into bioethanol [9]. Another interesting capability of this plant is that its starch content can be increased by manipulating nitrates or phosphates concentration or growth conditions like pH and temperature. Due to absorptive capacity of various nutrients from water, duckweeds are also used as biophytoremediators for nutrient-polluted wastewaters like agricultural runoff and municipal wastewater [10]. The plant biomass grown and starved on these wastewaters, with high starch content can then further be used for bioethanol production. Moreover, the plant biomass left after starch extraction can also be used for production of biomethane.

In this study, *S. polyrhiza* was grown under nutrient starvation condition to enhance its starch content. High-starch-containing plant biomass was then acid-pretreated to obtain complete conversion of starch to glucose. The plant sugar was then fermented via indigenously isolated and optimized *Saccharomyces cerevisiae* QG1 MK788210 for bioethanol production.

2 Material and methods

2.1 Isolation and optimization of yeast for high bioethanol yield

2.1.1 Yeast isolation

For performing the fermentation process, yeast was isolated from rotten grapes using WLN agar media (yeast extract 4 g L^{-1}, trypton 5 g L^{-1}, glucose 50 g L^{-1}, KH_2PO_4 0.55 g L^{-1}, KCL 0.425 g L^{-1}, $Mg_2SO_4.7H_2O$ 0.125 g L^{-1}, $CaCl_2.2H_2O$ 0.125 g L^{-1}, $FeCl_3$ 0.0025 g L^{-1}, $MnSO_4$ 0.0025 g L^{-1}, bromocresol green g L^{-1}, agar 20 g L^{-1}). Grapes were rinsed with distilled water and then partially mashed. Mashed grapes were placed in airtight autoclaved reagent bottles for a week at room temperature. Afterwards, the grape juice from reagent bottles was serially diluted from 10^{-1} to 10^{-9} dilution, and each dilution was spread on WLN agar plates supplemented with tetracycline (0.05 g L^{-1}) to avoid bacterial contamination and incubated at 30 °C [11]. Four of the morpholgically different isolates were then selected from all the isolated colonies and further cultured on tetracycline (0.05 g L^{-1})-supplemented YPD agar (yeast extract 10 g L^{-1}, glucose 20 g L^{-1}, peptone 20 g L^{-1} and agar 20 g L^{-1}) [12].

2.1.2 Screening for high-ethanol-producing yeast

Fermentation experiments were carried out in order to select the yeast strain that gave maximum ethanol titer. For this purpose, yeast fermentation medium (YFM) (yeast extract 6 g L^{-1}, peptone 5 g L^{-1}, KH_2PO_4 4 g L^{-1}, $(NH_4)_2SO_4$ 2 g L^{-1}, $MgSO_4.7H_2O$ 1 g L^{-1} and glucose 150 g L^{-1}) with pH adjusted to 5.5 was used [13]. Fermentation was carried out in 10-mL small fermentation reactors. About 5% v/v of 24-h enriched yeast cultures were added to YFM, and reactors were kept airtight. Anaerobic conditions were developed by means of nitrogen flushing, and syringes were attached to the reactors for capturing CO_2 produced in reactors during fermentation. The reactors were then incubated for 72 h at 30 °C and 150 RPM after which ethanol concentration in media was determined. The yeast strain that gave highest ethanol yield was selected for optimization.

2.1.3 Determining ethanol content

In order to determine amount of ethanol produced by yeast, enzymatic detection of ethanol was carried out using Megazymes Ethanol Assay Kit (Bray, Ireland) in accordance with the manufacturer's instructions.

2.1.4 Molecular identification of yeast

For molecular identification of selected yeast strain, yeast DNA was isolated by means of SDS/CTAB method [14]. The 18S rRNA sequencing of extracted DNA was carried out by Macrogen Standard Custom DNA Sequencing Services (Macrogen Inc., Seoul, Korea) using Sanger method. The sequence obtained was then subjected to phylogenetic analysis to identify selected yeast strain.

2.1.5 Statistical optimization of selected yeast strain for high ethanol production

The selected yeast strain was optimized in order to achieve physicochemical parameters at which it gave maximum ethanol yield. Optimization studies were carried out using statistical tools that included Plackett–Burman design and central composite design through Stat Ease Design Expert Software version 7. Plackett–Burman design was used initially to optimize nine factors, which are the amount of yeast extract, peptone, KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, pH, inoculum age, inoculum size and incubation time. After entering the ranges of each parameter in the design, design gave 15 runs, each of

which was carried out experimentally, and the response, i.e., ethanol yield was recorded in the design, which was further processed by the software. Plackett–Burman design identified three factors (KH_2PO_4, $MgSO_4.7H_2O$ and inoculum age) to significantly affect the ethanol yield. These three factors were further optimized by means of central composite design in the similar way as of Plackett–Burman design to achieve the maximum ethanol yield. Both the designs were numerically validated.

2.2 Production and pretreatment of high-starch containing *S. polyrhiza* biomass as feedstock for bioethanol production

2.2.1 Sampling of *S. polyrhiza*

Plant sampling was done from local garden ponds in Islamabad, Pakistan. The plant was then identified to be *Spirodela polyrhiza*, which is commonly known as giant duckweed. The plant was submitted to the National Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, and its accession number was acquired.

2.2.2 Culturing of *S. polyrhiza*

The plant is a hydrophyte, so water-based media enriched with nitrates and phosphates was used for its culturing. The plant was cultured in Hoagland media (KNO_3 1.515 g L^{-1}, KH_2PO_4 0.68 g L^{-1}, $Ca(NO_3)_2.4H_2O$ 1.18 g L^{-1}, $MgSO_4.7H_2O$ 0.492 g L^{-1}, $ZnSO_4.7H_2O$ 0.00022 g L^{-1}, H_3BO_3 0.00285 g L^{-1}, $Na_2MoO_4.2H_2O$, 0.00012 g L^{-1}, $CuSO_4.5H_2O$ 0.00008 g L^{-1}, $MnCl_2.4H_2O$ 0.00362 g L^{-1}, $FeCl_3.6H_2O$ 0.0054 g $L^{-1}$and tartaric acid 0.003 g L^{-1}), and pH was adjusted to 5.8 [15]. The *Spirodela* fronds were first washed with 30% sodium hypochlorite solution to remove any algal and bacterial concentration and then were suspended in Hoagland solution contained in 10" × 10" transparent plastic boxes. The plant cultured boxes were placed in fluorescent light-illuminated plant growth rack, and temperature was maintained at 25 °C.

2.2.3 Starch content determination in *Spirodela biomass*

For the purpose of starch determination Appenorth method was used [16]. About 200 mg fresh weight of plant was crushed and homogenized with 4 mL 18% HCL. The mixture was then shaken for 1 h at 5 °C. The homogenized mixture was then centrifuged at 12,000 RPM for 20 min. An aliquot of this mixture was mixed with same amount of Lugol's solution (KI 0.5% w/v and I_2 0.25% w/v in distilled water), and its optical density was taken at 530 nm and 605 nm. The starch content was then calculated through following formulae:

$$S\% = [Cs \times \mathrm{Vol(extr)} \times 100] \div \mathrm{FW} \quad (1)$$

where Vol (extr) = the plant extract volume after homogenization with HCl (mL), FW = fresh weight (mg) and $Cs = A_{605} / (0.07757 \times P + 4.463)$, where A_{605} and A_{530} are the absorbances at 605 nm and 530 nm.

Whereas, P was calculated using the formula

$$pP = [(7.295 \times A_{605}/A_{530} - 4.463)/(7.757 - 0.729 \times A_{605}/A_{530})] \times 100$$

where A_{605} and A_{530} are the absorbances at 605 nm and 530 nm.

2.2.4 Enhancement of starch content in plant biomass by nutrient starvation

Nutrient starvation is known to enhance the starch content of duckweed [17]. Starch is conserved in plant biomass at times of low nutrient availability as a storage molecule [18]. In order to enhance the starch content of *Spirodela* biomass, plant was given nutrient stress. For this purpose, plants were cultured in Hoagland media as described above. The plants were continuously grown in the media provided initially without any supplementation of new media as in the case of continuous growth. This resulted in a boost in plant growth at start but gradually lead to uptake of all the nutrients in media resulting in nutrient depletion. Starch content of the plants was determined after every 4 days. Plants were harvested when the starch content became constant and did not increase further.

2.2.5 Pretreatment of the high-starch-containing plant biomass

As the isolated yeast could only uptake glucose for fermentation, the high-starch-containing biomass was acid-pretreated in order to convert all the starch to glucose. For this purpose, initially, the high-starch biomass was treated with different concentrations of sulfuric acid ranging from 0.05 to 0.2% in order to determine the acid concentration at which maximum starch conversion to glucose can be attained. For pretreatment process, feedstock loading rate was kept to be 100 g L^{-1}, and feedstock was hydrolyzed with different concentrations of sulfuric acid solution for 1 h at 121 °C [19]. The glucose concentration in the solution after pretreatment was determined by DNS method [20]. All the reactions were carried out in triplicate.

2.3 Fermentation of glucose obtained from nutrient-starved *Spirodela* biomass with isolated yeast at optimized parameters

The fermentation reaction was carried out at conditions previously optimized by Plackett–Burman and central composite design to achieve maximum ethanol yield. The pretreated plant biomass suspension that had the highest glucose

concentration was centrifuged at 14,000 RPM for 20 min to separate the biomass from sugar-containing hydrolysate. The pH of hydrolysate was adjusted to 4.8 using 1M NaOH. Fermentation was carried out by adding 15% of 72-h yeast suspension v/v, 70% v/v acid hydrolysate containing released sugars and 15% yeast fermentation media (without glucose and containing other components at optimized conditions, i.e., yeast extract 9 g L^{-1}, peptone 7 g L^{-1}, KH_2PO_4 6 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, $(NH_4)_2SO_4$ 1 g L^{-1}, pH 4.5, inoculum age 72 h, inoculum size 15% and incubation time 24 h) in a 10-mL fermentation reactor. Fermentation was carried out as described above at 30 °C and 150 RPM. Incubation time was kept to be 48 h. After 48 h, ethanol titer was determined by ethanol assay kit.

3 Results and Discussion

3.1 Isolation and optimization of yeast for high bioethanol yield

3.1.1 Isolation of yeast strain for ethanol fermentation

Grape juice is used for production of wine since centuries; hence, mashed grapes are the best substrate for isolation of fermentative yeast. Mashed grapes were kept under anaerobic condition to facilitate fermentation and promote yeast growth only. For the purpose of initial isolation, WLN agar was used. Countable number of colonies came on only two plates spread with 10^{-5} and 10^{-6} dilution with CFU/mL of 3.34×10^9 and 5.6×10^9, respectively. On the basis of morphology, four different yeast strains named QG1, QG2, QG3 and QG4 were selected for further study.

3.1.2 Screening for high-ethanol-producing yeast strain

The four selected strains were then subjected to fermentation reaction to screen out the yeast strain that gave maximum ethanol titer. The ethanol titer, % theoretical yield and glucose consumption of strains QG1, QG2, QG3 and QG4 are shown in Table1. Based on these results, strain QG1 was selected for optimization as it showed maximum ethanol yield with highest sugar consumption rate.

Table 1 Ethanol titer, percent of theoretical yield achieved and glucose consumption of initially selected yeast strains

Yeast strain	Ethanol titer (% w/v)	Percentage of theoretical yield achieved (%)	Glucose consumption (%)
QG 1	5.9	77.12	99.65
QG 2	1.4	18.30	34.72
QG 3	3.8	49.67	62.89
QG 4	2.3	30.07	47.42

3.1.3 Molecular identification of selected yeast strain QG1

For molecular identification of strain, QG1 18S rRNA sequencing was performed. QG1 samples were sent to MACROGEN Korea for sequencing. The obtained sequence was subjected to phylogenetic analysis and phylogenetic tree was constructed using neighbor-joining method (Fig. 1). The strain was identified to be *Saccharomyces cerevisiae*. Further, the sequence was submitted in NCBI Gen Bank (and its accession number was attained). The strain was thus designated as *Saccharomyces cerevisiae* QG1 MK788210.

3.1.4 Statistical optimization of fermentation process using Plackett–Burman design

For the purpose of optimizing *S. cerevisiae* QG1 to achieve high ethanol yield by fermentation process, different factors affecting the process of fermentation were statistically optimized using the Plackett–Burman design. Nine factors, which are the amount of yeast extract, peptone, KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, pH, inoculum age, inoculum size and incubation time, were optimized by conducting experiments in accordance with the 15 runs of the model design devised by the Design Expert Software (Table 2). Highest ethanol yield of 6.6% (w/v), i.e., 85.7% theoretical yield was achieved in run 10 (yeast extract 9 g L^{-1}, peptone 7 g L^{-1}, KH_2PO_4 6 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, $(NH_4)_2SO_4$ 1 g L^{-1}, pH 4.5, inoculum age 72 h, inoculum size 15% and incubation time 24 h). Theoretical yields were calculated stoichiometrically based upon the amount of sugar initially fed to the reactor. ANOVA was used on the model, which showed that the F-value of the model is 19.97 implying that the model is significant. The software also applied F-test on each of the model factors to identify the factors that significantly affect the ethanol yield. The factors having values of "Prob>F" less than 0.0500 were indicated to be the significant model terms. In this case, three factors, which are KH_2PO_4, $MgSO_4.7H_2O$ and inoculum age, came out to be significant with Prob>F values of 0.002, 0.0010 and 0.366, respectively (Fig. 2). The model shows that KH_2PO_4 affects the ethanol yield positively, i.e., the increase in KH_2PO_4 increased the ethanol titer in the media (Fig. 3a). KH_2PO_4 is used as a source of phosphorous in the fermentation media. Phosphorous is one of the significant nutrient parameter that affect the fermentation process and ethanol yield, and KH_2PO_4 has been reported to be one

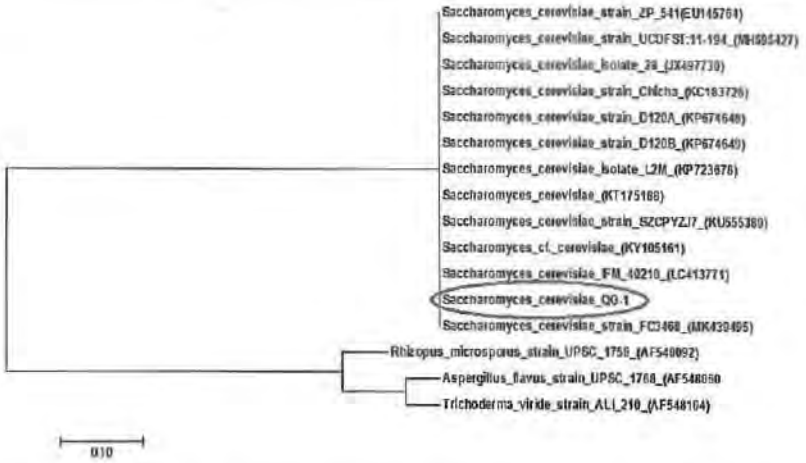

Fig. 1 Phylogenetic tree based on 18S rRNA sequencing of strain QG1 showing relationship with other related strains

of the best phosphorous source. Various studies conducted have also shown that enhanced KH_2PO_4 concentration has resulted in higher ethanol yield while using *S. cerevisiae* as fermenting microorganism [21]. The second significant factor came out to be $MgSO_4.7H_2O$, which is used as a source of magnesium in the fermentation broth. The model indicated that $MgSO_4.7H_2O$ has a negative effect on ethanol yield as increase in $MgSO_4.7H_2O$ results in decrease in the ethanol concentration (Fig. 3b). Various studies have also reported that magnesium is only required in a limited concentration within the fermentation broth as it affects ethanol concentration significantly during yeast fermentation [22–24]. The last significant factor, inoculum age had a positive effect on ethanol yield, which showed that, as the inoculum grew older, it gave a better ethanol yield (Fig. 3c). Highest ethanol yield was obtained with 72-h-old inoculum. Different studies show that inoculum age is a strain-specific factor whose trend is different for different strains [25, 26].

Equation 1 gives the final equation given by the software for ethanol yield in terms of significant factors:

$$\begin{aligned}\text{Ethanol yield} = {} & +1.98333 + 0.33333 \\ & \times KH_2PO_4 - 0.70000 \times MgSO_4.7H_2O \\ & + 0.034722 \times \text{Inoculum age} \end{aligned} \quad (2)$$

3.1.5 Optimization of the significant factors by response surface methodology using central composite design

The three factors, KH_2PO_4, $MgSO_4.7H_2O$ and inoculum age, that were indicated to be significant by Plackett–Burman

Table 2 Ethanol yield obtained in response to the conditions specified by the Plackett–Burman design

Runs	Yeast g/L	Peptone g/L	KH_2PO_4 g/L	$(NH_4)_2SO_4$ g/L	$MgSO_4.7H_2O$ g/L	pH	Inoculum Age hrs	Inoculum Size %	Incubation time hrs	Ethanol yield % (w/v)
1	9	3	6	3	0.5	6.5	72	5	48	5.5
2	3	7	6	3	0.5	4.5	24	5	48	4.4
3	9	3	6	3	1.5	4.5	24	15	24	3.2
4	9	7	2	3	1.5	6.5	24	5	24	3
5	6	5	4	2	1	5.5	48	10	36	6
6	6	5	4	2	1	5.5	48	10	36	5.9
7	6	5	4	2	1	5.5	48	10	36	6
8	3	3	2	1	0.5	4.5	24	5	24	3
9	3	7	2	3	1.5	4.5	72	15	48	3.3
10	9	7	6	1	0.5	4.5	72	15	24	6.6
11	3	7	6	1	1.5	6.5	72	5	24	5.9
12	3	3	2	3	0.5	6.5	72	15	24	5.3
13	3	3	6	1	1.5	6.5	24	15	48	4.1
14	9	3	2	1	1.5	4.5	72	5	48	4.1
15	9	7	2	1	0.5	6.5	24	15	48	3

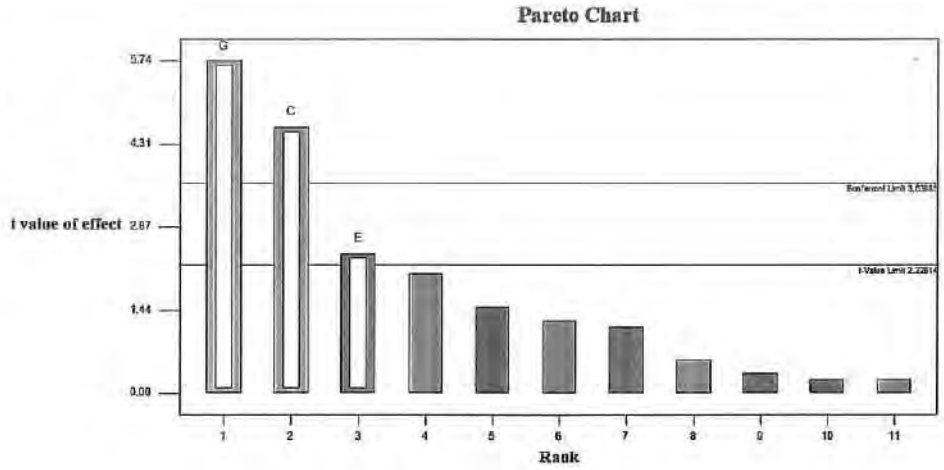

Fig. 2 Pareto chart indicating the significant model terms where G corresponds to KH_2PO_4, C corresponds to $MgSO_4.7H_2O$ and E corresponds to inoculum age

design were further optimized by using central composite design (CCD). CCD is used to find out the interactive effects of all the chosen factors to be optimized. For generating the CCD model, the ranges of each of the significant factors were kept in accordance with the results of Plackett–Burman design. As increasing the concentration of KH_2PO_4 resulted in increased ethanol yield, the range for KH_2PO_4 was further increased and kept to be 6–9 g L^{-1}. Same was the case with inoculum age, and its range for CCD was selected to be 72–96 h. In case of $MgSO_4.7H_2O$, as increase in its concentration decreased ethanol activity, its range was further decreased and kept to be from 0.1 to 0.5 g L^{-1}. The reactions were conducted according to the conditions specified by 17 runs generated by model design (Table 3). For the remaining six out of nine factors, the conditions were kept according to run 10 of Plackett–Burman design that gave the highest ethanol yield. Highest ethanol yield of 7.08% (w/v), i.e., 93% theoretical yield was achieved in run 11 of CCD. ANOVA of the CCD gave the model F-value of 4.43, which indicated that the model was significant. Two factors AB (interactive effect of KH_2PO_4 and $MgSO_4.7H_2O$) (Fig. 4a) and BC (interactive effect of $MgSO_4.7H_2O$ and inoculum age) (Fig. 4b) came out to be significant by having Prob>F values of 0.005 and 0.04, respectively. In case of CCD, the effects of each factor were observed to be opposite to that of Plackett–Burman as further increase in the KH_2PO_4 concentration and inoculum age decreased the ethanol yield, whereas a further decline in the $MgSO_4.7H_2O$ also decreased the ethanol yield. Both Plackett–Burman design and central composite design were numerically validated by the solutions given by software. The results for validation are given in Online Resource 1. Achieving high % theoretical yield of ethanol is demanded as in this way maximum carbohydrates in the feedstock can be converted to ethanol, which can be used as biofuel.

Equation 2 gives the final equation given by the software for ethanol yield in terms of significant factors:

$$\begin{aligned}\text{Ethanol yield} = {} & -10.50544 + 1.72621 \times KH_2PO_4 \\ & + 24.22189 \times MgSO_4.7H_2O \\ & + 0.16465 \times \text{Inoculum age} - 1.64417 \\ & \times KH_2PO_4 \times MgSO_4.7H_2O - 0.016194 \\ & \times KH_2PO_4 \times \text{Inoculum age} - 0.13531 \\ & \times MgSO_4.7H_2O \times \text{Inoculum age} \end{aligned} \quad (3)$$

3.2 Production and pretreatment of high-starch-containing *S. polyrhiza* biomass as feedstock for bioethanol production

3.2.1 Identification of *S. polyrhiza*

The collected sample plant from garden pond was identified to be *S. polyrhiza* on the basis of its morphology. The plant collected had a comparatively larger disc-like fronds with reddish underside and 12–16 adventitious roots, which are typical of *S. polyrhiza* species [27]. The identified plant was submitted to the National Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, Pakistan, and was given the accession number of 129983.

3.2.2 Enhancement of starch content in plant biomass by nutrient starvation

For the purpose of increasing starch content in plant biomass, various studies provided different types of stress to *Spirodela*. The most reported stress is that of heavy metals like Ni [16, 28], Cr [29], Fe and Cu [30], which had resulted into increase in the starch content. However, the aim of current study is to use high-starch-containing plant biomass for the purpose of fermentation to produce bioethanol. Heavy metals stress was

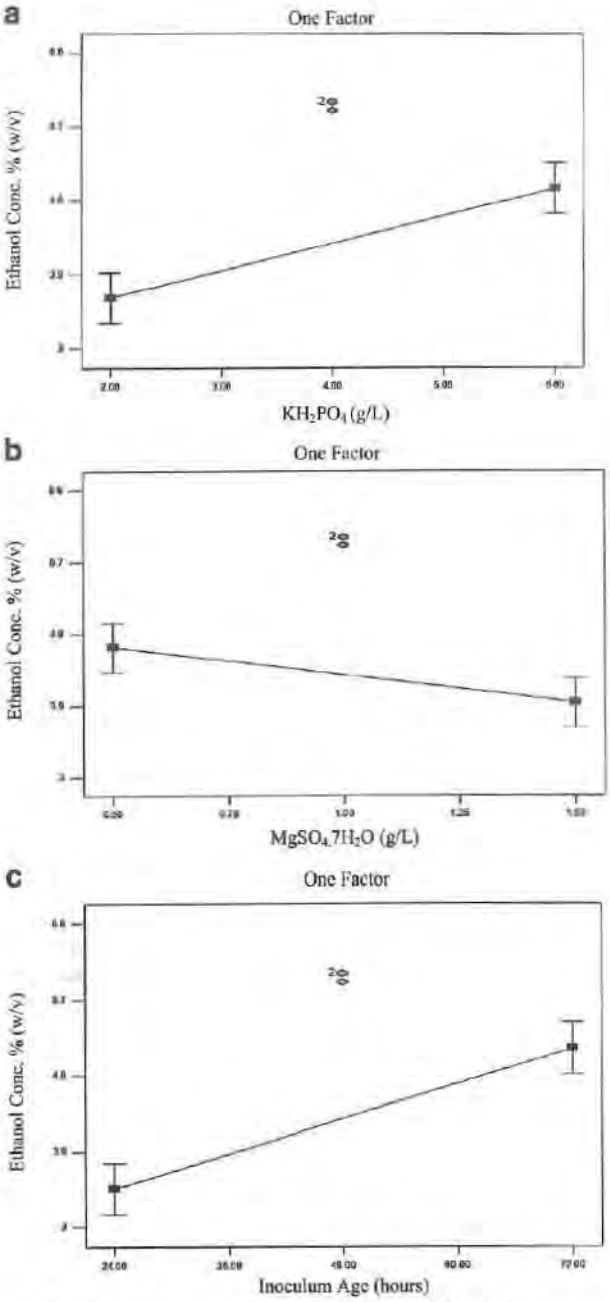

Fig. 3 Plot of ethanol yield as a function of a KH_2PO_4, b $MgSO_4.7H_2O$ and c inoculum age

not a good option in this case as it will lead to yeast toxicity. Therefore, nutrient stress was opted to enhance the starch content in plants. Nutrient starvation in different duckweed species is known to enhance starch content in their biomass [18]. For nutrient starvation, plants were cultured in freshly prepared Hoagland media and were allowed to grow. New media was not replenished and, as plants utilized all the nitrates and phosphates of the initially provided media, they went under nutrient stress condition. Highest starch content of 1.9% fresh weight was obtained after 20 days (Fig. 5). Based on starch content in plant biomass, it has been calculated that *Spirodela*, if cultured for purpose of starch production, can give 1870 Kg/hectar/year of starch.

Table 3 Ethanol yield obtained in response to conditions specified by central composite design

Runs	KH_2PO_4 g/L	$MgSO_4.7H_2O$ g/L	Inoculum age Hr	Ethanol yield % (w/v)
1	9	0.1	96	6.499
2	4.98	0.3	84	6.352
3	7.5	0.64	84	6.272
4	7.5	0.3	84	6.559
5	7.5	0.3	84	6.497
6	7.5	0.3	63	6.234
7	7.5	0.3	104	6.292
8	6	0.1	96	6.849
9	6	0.5	96	6.734
10	10.02	0.3	84	5.721
11	6	0.5	72	7.085
12	7.5	0.04	84	5.217
13	9	0.5	72	5.928
14	6	0.1	72	5.168
15	7.5	0.3	84	6.582
16	9	0.5	96	5.144
17	9	0.1	72	6.717

3.2.3 Pretreatment of the high-starch-containing plant biomass

The plant biomass with highest starch content of 1.9% FW was then given acidic pretreatment at high temperature to convert starch into glucose so that it can easily be fermented by *S. cerevisiae* QG1. For this purpose, initially, the high-starch biomass was treated with different concentrations of sulfuric acid ranging from 0.05 to 0.2% with purpose to determine the acid concentration at which maximum starch conversion to glucose can be attained. The highest starch conversion rate of 99.4% was achieved with 0.1% of sulfuric acid (Fig. 6).

3.3 Fermentation of glucose obtained from nutrient-starved *Spirodela* biomass with isolated yeast at optimized parameters

The fermentation reaction was carried out using sugar-containing plant hydrolysate (pretreated with 0.1% sulfuric acid and had highest glucose concentration) and isolated yeast strain *S. cerevisiae* QG1 at conditions that gave highest ethanol yield during optimization reactions performed according to Plackett–Burman and central

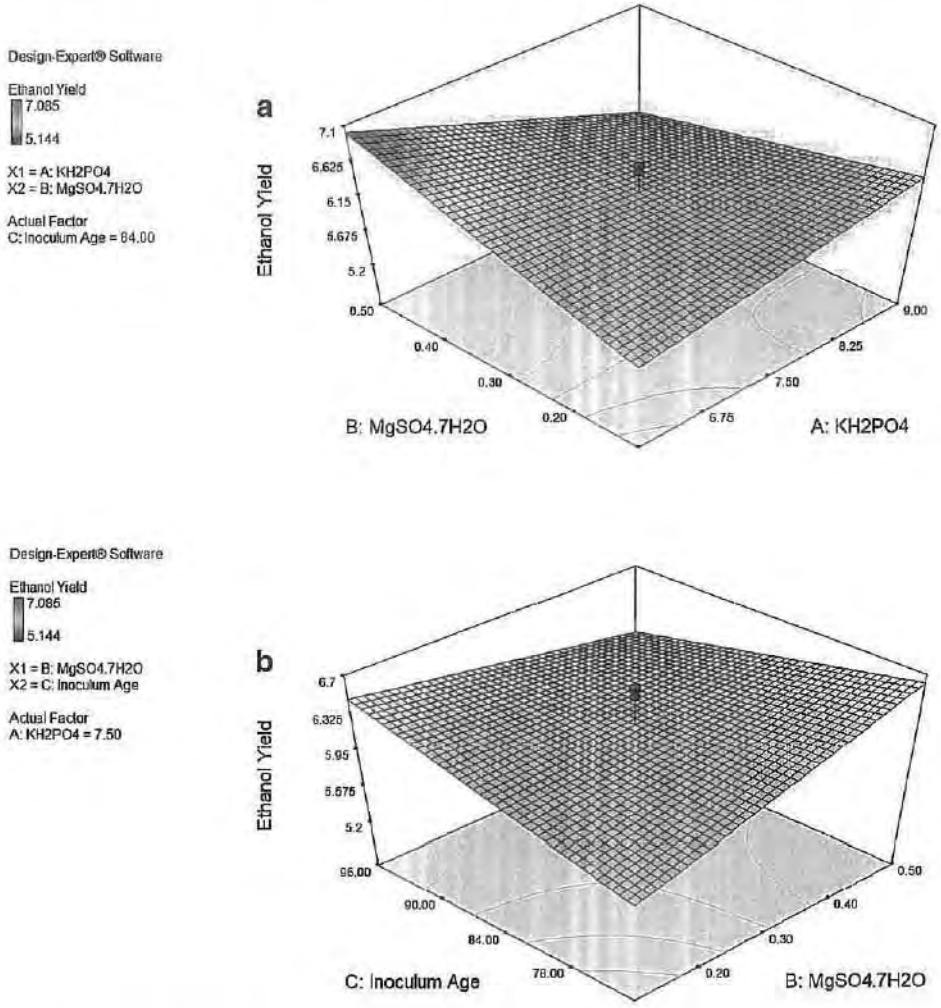

Fig. 4 Illustration of interactive effect of factor a AB and b BC by three-dimensional response surface plot for ethanol yield

composite design. Theoretical ethanol yield of 100% was obtained in this reaction, i.e., 2.2 g $L^{-1}$of glucose was converted to 1.21 g $L^{-1}$of ethanol (calculations were done stoichiometrically based on balanced chemical equation). Thus, complete conversion of starch in *S.* biomass to bioethanol was successfully achieved.

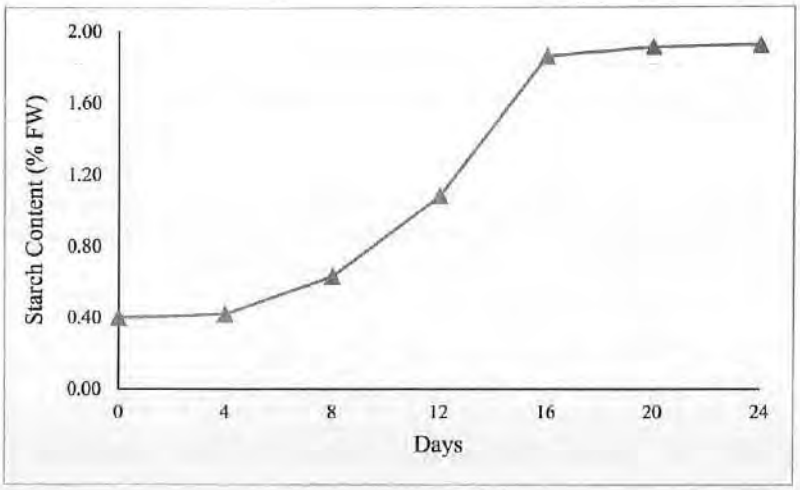

Fig. 5 Enhancement of *Spirodela* starch content under nutrient stress condition

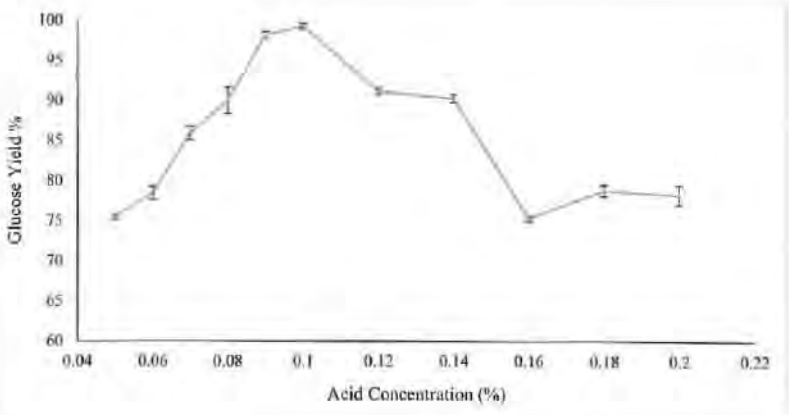

Fig. 6 Optimization of acidic pretreatment to achieve maximum glucose yield from high-starch-containing *Spirodela* biomass

4 Conclusions

The study conducted concludes that the isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210 shows promising potential to be utilized for obtaining high ethanol yield from feedstock like *S. polyrhiza*. The enhanced starch content in *Spirodela* biomass by providing nutrient stress and successful conversion of all the plant starch to ethanol by isolated and optimized yeast strain is suggestive of the fact that this plant can potentially be used as feedstock for bioethanol production to avoid food versus fuel feud. The high nitrate and phosphate uptaking capacity of *Spirodela* directs that, in the future, this plant can also be utilized for treatment of nutrient-rich wastewaters and then the produced biomass can be utilized for biofuel production. All the results obtained confirm that the isolated yeast strain as well as *S. polyrhiza* can find promising applications in the field of biofuel production.

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Availability of the data The data generated and analyzed during the study is provided in the article [and its supplementary information file].

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rana Q, Rehman MLU, Irfan M, Ahmed S, Hasan F, Shah AA, Khan S, Badshah M (2019) Lipolytic bacterial strains mediated transesterification of non-edible plant oils for generation of high quality biodiesel. J Biosci Bioeng 127(5):609–617
2. Nilsson LJ, Johansson TB (2017) Environmental challenges to the energy industries. In: Sustainable development and the energy industries. Routledge, pp 47–80
3. Rana QUA, Irfan M, Ahmed S, Hasan F, Shah AA, Khan S, Rehman FU, Khan H, Ju M, Li W, Badshah M (2019) Biocatalytic transesterification of mustard oil for biodiesel production. Biofuels 1–8
4. Manochio C, Andrade B, Rodriguez R, Moraes B (2017) Ethanol from biomass: a comparative overview. Renew Sust Energ Rev 80: 743–755
5. Hirani AH, Javed N, Asif M, Basu SK, Kumar A (2018) A review on first-and second-generation biofuel productions. In: Biofuels: greenhouse gas mitigation and global warming. Springer, pp 141–154
6. Sarris D, Papanikolaou S (2016) Biotechnological production of ethanol: biochemistry, processes and technologies. Eng Life Sci 16(4):307–329
7. Rodionova MV, Poudyal RS, Tiwari I, Voloshin RA, Zharmukhamedov SK, Nam HG, Zayadan BK, Bruce BD, Hou H, Allakhverdiev SI (2017) Biofuel production: challenges and opportunities. Int J Hydrog Energy 42(12):8450–8461
8. Michael TP, Bryant D, Gutierrez R, Borisjuk N, Chu P, Zhang H, Xia J, Zhou J, Peng H, El Baidouri M (2017) Comprehensive definition of genome features in *Spirodela polyrhiza* by high-depth physical mapping and short-read DNA sequencing strategies. Plant J 89(3):617–635
9. Cui W, Cheng J (2015) Growing duckweed for biofuel production: a review. Plant J 17:16–23
10. Cheng JJ, Stomp AM (2009) Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. Clean–Soil Air Water 37(1):17–26
11. Hashmi M, Khan TK, Khan W, Hasan F, Hameed A, Khan S, Badshah M, Shah AA (2018) Comparison between a newly isolated yeast strain and Lalvin EC-1118 for enhanced ethanol yield from sugarcane molasses employing batch and modified fed-batch fermentation. J Biobased Mater 12(1):134–142
12. Ruriani E, SUNARTI TC, Meryandini A (2012) Yeast isolation for bioethanol production. HAYATI J Biosci 19(3):145–149
13. Banat IM, Nigam P, Marchant R (1992) Isolation of thermotolerant, fermentative yeasts growing at 52 C and producing ethanol at 45 C and 50 C. World J Microbiol Biotechnol 8(3):259–263
14. Stirling D (2003) DNA extraction from fungi, yeast, and bacteria. In: PCR protocols. Springer, pp 53–54
15. Singh V, Pandey B, Suthar S (2018) Phytotoxicity of amoxicillin to the duckweed *Spirodela polyrhiza*: growth, oxidative stress,

biochemical traits and antibiotic degradation. Chemosphere 201: 492–502
16. Appenroth K-J, Krech K, Keresztes A, Fischer W, Koloczek H (2010) Effects of nickel on the chloroplasts of the duckweeds *Spirodela polyrhiza and Lemna minor* and their possible use in biomonitoring and phytoremediation. Chemosphere 78(3):216–223
17. Huang M, Fang Y, Xiao Y, Sun J, Jin Y, Tao X, Ma X, He K, Zhao H (2014) Proteomic analysis to investigate the high starch accumulation of duckweed (*Landoltia punctata*) under nutrient starvation. Ind Crop Prod 59:299–308
18. Tao X, Fang Y, Xiao Y, Y-l J, Ma X-r, Zhao Y, He K-z, Zhao H, Wang H-y (2013) Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (Landoltia punctata) under nutrient starvation. Biotechnol Biofuel 6(1):72
19. Ho S-H, Huang S-W, Chen C-Y, Hasunuma T, Kondo A, Chang J-S (2013) Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. Bioresour Technol 135:191–198
20. Miller G (1959) Modified DNS method for reducing sugars. Anal Chem 31(3):426–428
21. Yu J, Zhang X, Tan T (2009) Optimization of media conditions for the production of ethanol from sweet sorghum juice by immobilized *Saccharomyces cerevisiae*. Biomass Bioenergy 33(3):521–526
22. Dombek K, Ingram L (1986) Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. Appl Environ Microbiol 52(5):975–981
23. Arora R, Behera S, Sharma NK, Kumar S (2017) Augmentation of ethanol production through statistically designed growth and fermentation medium using novel thermotolerant yeast isolates. Renew Energy 109:406–421
24. Xu E, Wu Z, Jiao A, Jin Z (2018) Effect of exogenous metal ions and mechanical stress on rice processed in thermal-solid enzymatic reaction system related to further alcoholic fermentation efficiency. Food Chem 240:965–973
25. Benerji D, Rajini K, Rao B, Banerjee D, Rani K, Rajkumar G, Ayyanna C (2010) Studies on physico-chemical and nutritional parameters for the production of ethanol from Mahua flower (*Madhuca indica*) using *Saccharomyces Cerevisiae*—3090 through submerged fermentation (SMF). J Microbial Biochem Technol 2: 46–50
26. Manikandan K, Viruthagiri T (2010) Kinetic and optimization studies on ethanol production from corn flour. Intl J Chem Biol Eng 3(2):65–69
27. Jacobs DL (1947) An ecological life-history of *Spirodela polyrhiza* (Greater Duckweed) with emphasis on the turion phase. Ecol Monogr 17(4):437–469
28. Xyländer M, Augsten H, Appenroth K-J (1993) Influence of nickel on the life cycle of the duckweed Spirodela polyrhiza (L.) Schleiden. J. Plant Physiol 142(2):208–213
29. Appenroth K-J, Keresztes A, Sárvári É, Jaglarz A, Fischer W (2003) Multiple effects of chromate on *Spirodela polyrhiza*: electron microscopy and biochemical investigations. Plant Biol 5(03): 315–323
30. Xing W, Huang W, Liu G (2010) Effect of excess iron and copper on physiology of aquatic plant *Spirodela polyrhiza* (L.) Schleid. Schleid Environ Toxicol 25(2):103–112