PRODUCTION OF BIOETHANOL USING LIGNOCELLULOSIC BIOMASS

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CANDIDATE'S DECLARATION

I Manjary Vyas hereby certify that the work which is being presented in the thesis entitled **"Production of bioethanol using lignocellulosic biomass"** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period from July, 2019 to June, 2025 under the supervision of Prof. Jai Gopal Sharma, Department of Biotechnology, Delhi Technological University, Delhi and Co Supervision of Dr. Kriti Bhandari, Department of Biotechnology, Delhi Technological University, Delhi, India.

The matter presented in the thesis has not been submitted by me for the award of any other degree.

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This is to certify that the student has incorporated all the corrections suggested by the examiners in the thesis and statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

Fast declining fossil fuels, acute energy crisis energy, a spike in greenhouse gas emissions, and various environmental problems had led to a search for sustainable biofuels production. Extensive research is done in the field of biofuels such as bioethanol due to energy security, low environmental pollution effects, and cost benefits. The bioethanol production process has some necessary steps: pretreatment process, enzymatic hydrolysis, fermentation, distillation, and drying. The conventional ethanol-producing substances are consumable food-based material. Nowadays, attention has been searched for a new alternative to raw material (i.e., nonfood based material) due to the world food crisis. Microorganisms have received much attention for biofuel production through lignocellulosic feedstocks. They secrete collaborative enzymes for digesting the lignocellulosic biomass to simple sugars and afterward fermenting them to alcohol. Other fuels are produced from the same feedstocks as bioethanol. Still, it faces challenges compared to its production processes, such as the price of raw material, pretreatment methods, enzymatic hydrolysis, and low tolerance of the fermenting strain leading to its fewer yields, downstream processing, production of undesired solvents, and fermentation inhibitors. So a new promising biofuel as bioethanol has taken a great attraction of the researchers due to its competitive properties compared to gasoline. The research and development on bioethanol as a gasoline substitute indicates that it is a representative renewable energy source, able to elevate engine performance, combustion and also reduce greenhouse gas emissions.

In present research, the duckweed (*Lemna minor*, Family-Lemnaceae) has been highlighted as a feasible feedstock due to its rapid growth rate and adaptability in various aquatic environments. Its biochemical makeup, characterized by high starch content and low lignin content, allows for effective fermentation and saccharification. Duckweed offers numerous advantages, including minimal land use, considerable biomass output, and wastewater treatment. The paper highlights the potential of duckweed, specifically Lemna minor, as a sustainable source of bioethanol. Its advantageous characteristics like non-food feedstock, fastest growing angiosperm,

global adaptability across the world climates, assimilation of high starch with some nutrient modification, and less or no lignin content, make it a suitable candidate for bioethanol production. This research includes the production of starch-enhanced *Lemna minor* in nutrition starvation conditions for bioethanol production from it. The starch enhancement technique was standardized by performing experiments sequentially with organic manure, nitrogen-free Hoagland media and full-strength Hoagland media. Initially, the starch quantity was found to be 7% but it has been observed that during nitrogen stress, high starch accumulation was observed in *Lemna minor*, and on the 9^{th} day it was found to be maximum, which was 26 % with standard deviation± 0.3. It was also observed that protein, glucose, and fructose composition were dropped during this experiment. One-way ANOVA analysis was performed for statistical analysis. It has been found that during the starch enhancement experiment, the starch level was significantly ($P < 0.05$) higher in nitrogen-free Hoagland media compared to the organic manure and full-strength Hoagland media. So *Lemna minor* may be used to replace commercial food feedstock's for bioethanol production.

During ethanol production amylase is commonly used enzyme to saccharify the raw plant biomass. This research mainly entailed the collection and preparation of raw materials, maintenance of microorganisms, and optimization of physical and chemical variables for the production of amylolytic cocktail and ethanol. OFAT determined the significant components that influenced the yield of enzymatic activity and ethanol. RSM with CCD was used to determine the influences between factors to obtain the highest yields of enzymes and ethanol. ANOVA was done to examine the significance of factor interactions while response surface plots showed how different factors affected enzyme reaction. *Aspergillus niger* MTCC-12987, *Saccharomyces cereviceae*MTCC-171, and *Candida shehatae* MTCC-12913 were used for amylolytic cocktail and ethanol production respectively. Minitab 22 software was employed and response surface regression with Central composite design was used to examined an optimized process for maximum yield of enzyme activity and ethanol production. The highest enzyme activity value predicted by the software is 1063.81U/ml, which was closer to the experimental value of 1072.4 U/ml. Again the maximum ethanol production predicted by software is 11.83 % was closer to the experimental value of 10.77 %. Thus, the experimental performance under optimal conditions fitted the model's predictions fairly close. For the purpose to evaluate the combined effects of all independent variables in a fermentation process that may have developed from their interaction with one another, the RSM methodology has been employed as an alternative tool for statistical analysis.

Dedicated to

My Family

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LIST OF SYMBOLS & ABBREVIATIONS

Symbol or abbreviations	Description
LB	Lignocellulosic biomass
C. shehatae	*Candida shehatae*
S. cereviceae	*Saccharomyces cereviceae*
A. niger	*Aspergillus niger*
RSM	Response surface methodology
sp.	Species
$CaSO_4$	Calcium sulphate
$ZnCl_2$	Zinc chloride
K_2HPO_4	Di potassium hydrogen sulphate
KH_2PO_4	Potassium di hydrogen sulphate
$MgSO_4$	Magnesium sulphate
KCl	Potassium chloride
$NaNO_3$	Sodium nitrate
$(NH_4)_2SO_4$	Ammonium sulphate
NH_4Cl	Ammonium chloride
KNO_3	Potassium nitrate
GC-MS	Gas chromatography -Mass spectrometry
CCD	Central composite design
DNS	Di-nitrosalicyclic acid
O.D.	Optical density
HCl	Hydrochloric acid
NaCl	Sodiumchloride
NH4SO4	Ammonium sulphate
H2SO4	Sulfuricacid
CaCO3	Calcium carbonate
NaOH	Sodium hydroxide
SSF	Simultaneous saccharification and fermentation
SmF	Submerged fermentation
mM	Millimeter

TRS	Total reducing sugars
MTCC	Microbial type culture collection
ºC	Degree Celsius
A	Alpha
µ	Micron
U	Enzymeunits
Ml	Milli-litre
G	Gram
Mg	Microgram
RPM	Rotation perminute
%	Percentage
Nm	Nanometer
DW	Dryweight
CMC	Carboxy methyl cellulase
Cm	Centimeter
BG	Beta glucosidase
UV	Ultraviolet
Kg	Kilogram
L	Litre
B	Beta

Chapter 1

Introduction and Review of Literature

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 General

Fossil fuels are forms of energy that evolved over millions of years from the remains of animals and plants which were buried below rocks and sediments. In the 21st century, natural gas, coal, and petroleum have become known as crucial sources of energy. The worldwide crude oil utilization is expected to be at 100.6 million barrels per day (USEIA, 2019). Furthermore, it is believed that global usage of energy risenby 33.5% between 2010 and 2030 (Abdelaziz et al., 2011). Fossil fuels negatively impact the natural world by discharging greenhouse gases, such as CO_2, which lead to global warming. The rising temperature driven the global warming is destructive to nature and causes the disappearance of millions of creatures. It has been pointed out that CO_2 emissions have raised approximately 1.6 times in the recent three decades (Hosseini et al., 2013). The poisonous gases are discharged, and they are extremely injurious to humans. Also, acid rain is happened due to unlimited fuel utilization (Hosseiniet et al., 2013)

Energy utilization is increasing fastly due to large economic growth, continuing industrialization, and raising populations, causing the depletion of fossil fuels. The draining of fossil fuels is driving the hunt for new and alternative renewable feedstocks to produce renewable biofuels, replacing petroleum-based fuels.The seek for sustainable manufacture of biofuels has been encouraged by the fast depletion, serious energy scarcity, rising releases of greenhouse gases, along with various sustainability concerns. Thereby, supplying energy alternatives from environmentally friendly sources is crucial to addressing the power requirements of generations to come. A further environmentally friendly energy source that utilizes materials derived from biological sources is biofuel. To address this problem, researchers have been investigating making bioethanol as another source of energy. Bioethanol is gaining prominence in the seekfor sources of clean energy owing to itsability for preventing global warming and helps minimize rely on fossil fuels.On a global scale, this has been among the most widely utilized biofuels (Ruan et al., 2019).

Alvira et al., 2010, explained that why ethanol is an excellent substitute to fuels made from oil are outlined below: (i) ethanol is capable of being manufactured using renewable resources; (iii) it is capable of being utilized straight away in cars when blended using various percentages of fuel; (iv) it releases fewer contaminants than natural gas and petroleum given that oxygen facilitates it burn more effective; and (ii) it is not as toxic in comparison to other alcoholic fuels; (v) the byproducts from inadequate combustion become less harmful.

1.2 Classifying bioethanol on the basis of feedstock utilized for their manufacturing

According to the conversion approach and feedstock applied, there are four major generations of bioethanol (Naik et al., 2010). First-generation bioethanol is made up from food crops and fruits like corn, sugarcane, wheat, and grapes. Second-generation bioethanol is manufactured from lignocellulosic biomass, which contains plant waste leftover after the harvesting of crops, as well as other non-food sources (Ayodele et al., 2020). Bioethanol is made up from microalgae, classified as the third generation of bioethanol (Devi et al., 2023). Fourth generation of bioethanol manufactured from gene-modified or engineered crops (Devi et al., 2023).

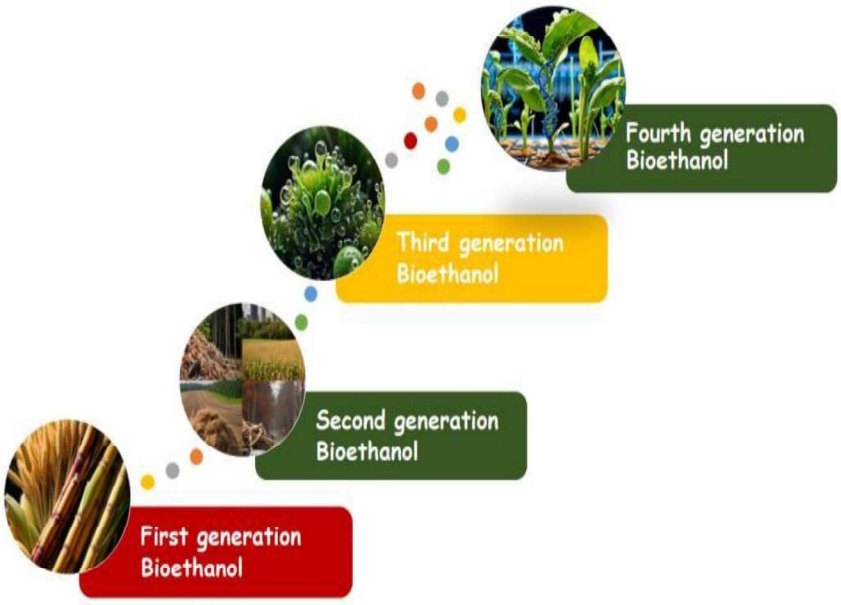


Figure 1.1: Generation of Bioethanol

1.2.1 First generation bioethanol

A common raw substrate for the production of first-generation bioethanol is sugarcane and maize. First-generation bioethanol is produced from direct sugar feedstocks, including maize and sugarcane (Pavlecic et al., 2017). Potato waste, barley, sugar beets and Whey are other less significant feedstocks. Yeast fermentation converts the crop's fermentable carbohydrates into ethanol. Even though there are many drawbacks, first-generation bioethanol improves communities of farmers and agricultural enterprises by increasing the demand for crops. The increase in worldwide feed prices for both humans and animals has been in line with the manufacture of them. Furthermore, they disrupt biodiversity and compete for water in particular regions. In addition, a significant part of land is needed for the crops that are used to produce first-generation bioethanol (Ben et al., 2016).

1.2.2 Second generation bioethanol

Non-edible biomass made from lignocellulose is used to make second-generation bioethanol. The three kind of biomass mainly used to manufacture second-generation bioethanol are non-homogeneous (e.g. municipal solid wastes), quasi-homogeneous (e.g. agricultural and forest residues), and homogeneous waste (e.g. white wood chips) (Taherzadeheet al., 2008).

The cost of biomass is considerably less compared to that of corn, sugarcane, and vegetable oil. But this biomass is normally hard to obtained fermentable sugars. Several problems with first-generation biofuels have been addressed by second-generation biofuels. Considering they derive from non-food biomass, they cannot compete with food crops. Corn Stover (corn leaves, stalks, and stems) is one example of a cellulose-based substance that is capable of being handled for biomass.

Still, an excessive amount of soil nutrients might be required to accomplish this. Additionally, the biomass needs to be pretreated with the aim to release the sugars required for manufacturing bioethanol. The method for producing second-generation fuel require pretreatment compared to that of first-generation biofuels (Kucera et al., 2017).

1.2.3 Third generation bioethanol

Algal biomass is utilized to make third-generation biofuel. The amount of lipids decides the amount of bioethanol produced from algae. In colder regions, it fails to circulate entirely and deteriorates more rapidly, and also demands fertilizer and energy. In contrast with first and second-generation bioethanol, third-generation bioethanol provide a higher quantity of energy per acre. Algae can flourish in places that are restricted for first- and second-generation crops. Sewage, wastewater, and saltwater from salt lakes or oceans can be utilized to grow it efficiently. To make the extraction process feasible, more research is required (Li et al., 2014).

1.3 Pros and cons of second generation bioetahnol

Second generation bioethanol produced by fermenting many different kinds of carbohydrates obtained from lignocellulosic biomass. According to Xu et al., 2010, second-generation biomass consists of basically inedible lignocellulosic biomasses, have a range of raw materials, and require minimal agricultural inputs for cultivation.

The issue of food versus fuel has been resolved by second-generation feedstocks, although lignocellulosic ethanol have some financially feasibility issues. Since lignocellulose has a recalcitrant structure, which demands extra, expensive pretreatment methods for enhancing its digestibility before its hydrolysis using enzymes (Mosier et al., 2005). Additionally, the process requires a lot of land for cultivation, fresh water, and particular agricultural inputs for producing second-generation feedstocks, and long-term biomass harvesting could impact soil quality and the management of nutrients (Sander and Murthy, 2010). As a result, researchers are interested in studying feedstocks that are economically and environmentally suitable.

At present, the primary providers of sugar and starch required for producing bioethanol are crops (first-generation biomass) having high sugar amounts, such as corn, wheat, and sugar cane (Naik et al., 2010, Sarkar et al., 2012). However, the manufacturing of bioethanol through such crops may result in a significant impact on the costs of food and food availability, considering that they serve as crucial sources of food (Sarkar et al., 2012). On the contrary, biomass made from lignocellulosic

material, wood wastes, or second-generation renewable energy resources makes an attractive alternative when they are significantly cheaper than first-generation biomass and not competent along the manufacture of food for living world (McAloon et al. 2000, Cherubini et al., 2010). Additionally, lignocellulosic biomass may reduced the emissions of greenhouse gases whenever used as liquid fuels like bioethanol (Galbe et al., 2002, Wyman et al., 1990, Balat et al., 2008).

The three types of naturally occurring polymers that make up lignocellulosic biomass —cellulose (30–60%), hemicellulose (20–40%), and lignin (15–25%)—constitute the fundamental components of the second generation bioethanol production materials. The composition of cellulose in lignocellulosic biomass is an important problem in the manufacturing of bioethanol by the second-generation lignocellulosic materials, in comparison with the first-generation biomass. Fermentation could produce 651.1 L of bioethanol from one tonne of glucan from cellulose and 666.2 L of bioethanol from one tonne of xylan from hemicellulose (Calderon & Arantes, 2019). Lignin prevents the cellulose and hemicellulose constituents from chemical and biological degradation. When attempting to manufacture bioethanol from second generation substrates, pretreatments thatdigest lignin from lignocellulosic biomass and convert cellulose and hemicellulose into digestible carbohydrates are important.

Regarding this, many kinds of chemicals have been used, including ionic liquids, deep eutectic solvents, organic solvents, oxidizing agents, acids, and alkalis. Pretreatment, however, could increase the cost of bioethanol by up to 40%. The release of inhibitory chemicals during processing of lignocellulosic biomass, as well as the proportion of primary energy consumed per mass of treated biomass, have significant aspects when establishing a pretreatment method. Since the present understanding of environmentally friendly and clean procedures, it has become essential to analyze pretreatment not simply on the basis of pricing, but also in terms of environmentalfavored, chemical recyclability, and total environmental impact reduction.

Table 1.1 summerized some of the compositional analysis of lignocellulose biomass and ethanol yield in different raw substrates used as feedstocks (Kumar et al., 2017)

Table 1.1: The total content of lignocellulose materials and ethanol yield in different feed stocks (Kumar 2017)

Compounds	Cellulose%	Lignin%	Pentosan%	Ash %	Silica%	Theoretical Ethanol yield (Gallons/ton)
Wheat stalk fiber	29-51	16-21	26-32	4.5-9	3-7	96.1-144.3
Rice stalk fiber	28-48	13-16	23-28	15-20	9-14	89-132
Barley Stalk fiber	31-45	14-15	24-19	5-7	3-6	128
Oat stalk fiber	31-48	16-19	27-38	6-8	4-6.5	101.3-150
Rye stalk fiber	33-50	16-19	27-30	2-5	0.5-4	104.7-139
Coniferous wood fiber	40-45	26-34	7-14	< 1	-	80.1-101.7
Deciduous wood fiber	38-49	23-30	19-26	< 1	-	98.8-130.1

1.4 Duckweed: a non food based feed stock for second generation bioethanol production

Recently, one of the plant-based biomass, duckweed, emerged as a lignocellulosic feedstock for sustainable bioethanol production (Yang, 2022). Duckweed is a small, floating aquatic plant that can be adapted for various water bodies, including ponds, lakes, and wastewater treatment systems. *Growing* duckweed needs minimal land and water compared to terrestrial plants. There are many commercial and environmentally friendly benefits when utilizing duckweed (*Lemna minor*) as a raw material for making bioethanol. Duckweed is an aquatic plant that proliferates rapidly and is recognized for its substantial biomass production as well as its ability to survive in wastewater that is loaded with nutrients (Ziegler et al., 2023). The potential of duckweed to soak up phosphate and nitrogen additionally serves to minimize eutrophication and contamination of water. In light of its particular quality, it is frequently leveraged to produce biomass and in wastewater treatment. Its fast expansion allows frequent harvesting throughout the whole year, increasing bioethanol productivity. Faizal et al., 2021 found the suitability of duckweed species such as *Spirodelapolyrhiza*, *Lemna minor*, *Landoltia punctata*, *Lemna aequinoctialis*,*Spirodelapolyrrhiza*, *Wolffia arrhizal*,and*Wolffia globose* for bioethanol production. A substantial amount of carbohydrates (19.8 to 38% of dry weight) can be identified in duckweed, including starch, cellulose, hemicellulose,

glucose, galactose, xylose, mannose, fructose, ribose, arabinose, and rhamnose. Starch, a major compositional carbohydrate element of the plant matter, is rapidly processed into fermentable sugars for the synthesis of bioethanol. Further complex sugars, such as cellulose and hemicellulose, are hydrolyzed by enzymes, resulting in simple sugars that are capable of being fermented. Additionally, it becomes easier to saccharify and ferment duckweed biomass due to its lignin content compared to terrestrial plant biomass. Its soft cell walls make it easier to process enzymatically (Pagliuso et al., 2021). Duckweed is rich in protein and its total amount varies from 19.8% to 48.2% of dry weight. It incorporates both essential and non-essential amino acids, such as valine, arginine, alanine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine. Amino acid derivatives that include citrulline, cystathionine, hydroxyproline, gamma-aminobutyric acid, and taurine have been additionally discovered in duckweed. Following enzymatic hydrolysis and fermentation, leftover biomass, which is high in protein and fiber, can be utilized for high-quality feed for animals, specifically for fish, poultry, and cattle. The commercial potential for manufacturing bioethanol using duckweed improves through the inclusion of this byproduct. The biorefinery approach has been followed by the dual-use, i.e., bioethanol and animal feed production, and this boosts the effectiveness of resources through the manufacturing of bioethanol from starch and animal feed from protein (Leng et al., 1995). 11.4% of the overall dry weight of duckweed constitutes total fatty acids. Fatty acids and phytosterols, comprising stigmasterol, sitosterol, and campesterol, make up the lipid fraction of this plant.Duckweed is known for having a variety of secondary metabolites, like tannins, terpenoids, phenolic compounds, and flavonoids. By maintaining the structural integrity of cellular membranes in microbial structures, fatty acids positively assist in the production of bioethanol. Enhancing microbial tolerance to ethanol toxicity leads to greater fermentation performance. While synthesizing bioethanol, fatty acid composition present in duckweed help in growth for microbial species (Baek et al., 2021).Duckweed's rich biochemical constitution leads it to possible applications in the culinary, pharmaceutical, and cosmetic industries, alongside serving as a promising feedstock for the production of bioethanol. It is used in reducing global warming and achieving the goal of sustainable energy from renewable sources (Appenroth et al., 2021, Bergmann et al., 2000).

1.5 About *Lemna Minor* (a kind of duckweed)

Bioethanol produced from lignocellulosic biomass comes under category of second-generation bioethanol. The major challenge associated with this class of bioethanol production is the processing of non-food biomass.Manufacturing of nonfood biomass to bioethanol consisting of four stages: pretreatment, fermentation, distillation, and drying. These steps must be eco-friendly and inexpensive. So, if the biomass has more sugar content, this gives additional benefits for its pretreatment and saccharification because it require less and dilute chemicals to avail sugars for fermentation (Sree et al., 2016). The aquatic duckweed plant is a suitable candidate for cheap bioethanol production because it contains less lignin and more starch. *Lemnaceae* is a member of the monocotyledonous family of five genera (*Lemna*, *Landoltia*, *Spirodela*, *Wolffia,* and *Wolfiella*) (Chen et al., 2012 &Sembada et al., 2019). Among all these weed plants, one of the plant*s Lemna minor*, a small flowering plant that floats on water bodies, is a good candidate for bioethanol production. When suitable growth conditions were applied, duckweed proliferates its biomass in 1–3 days and supplied continued biomass for 9–12 months in a year. Under starvation conditions, the starch contents of duckweed can accumulate from 3% to 75%of dry weight. In comparison to agricultural waste, duckweed has high carbohydrate and low lignin content so its saccharification is relatively easier (Faizal et al., 2021). Its doubling time makes it an ideal candidate for bioethanol production due to high biomass production. The tiny size of duckweed plant has a high surface area-to-volume ratio, so it is fastly dried. Various pretreatments have been developed to make the duckweed biomass more accessible to obtain a higher sugar yield (Gönen et al., 2018). Acid, alkali, thermal, biological, and combination approaches are usually applied for the pretreatment of plant biomass but due to duckweed's less lignin content, instead of acid and alkali pretreatment, thermal pretreatment using water and biological pretreatment is preferred (Kumar et al., 2017 & Lee et al. , 2015). One of the pretreatment technologies applied to duckweed is a steam explosion (Zhao et al., 2015). It (with or without catalysis) requires less power and chemical consumption. This technique involved, saturated steam with high pressure into a bioreactor containing biomass (Agbor et al., 2011 & Chiaramonti et al., 2012) . Another technique is wet oxidation

technology which is used for oxidation of plant biomass in water using oxygen as a catalyst (Talebnia et al., 2010). Biological approaches use microorganisms which produce enzymes for the degradation of many kind of plant biomass. The advantage of this method is that it requires minimal energy and fewer chemicals in comparison with other methods (Taherzadeh et al., 2008). During pretreatment, various kinds of sugars are released which mainly involve pentose and hexose sugars. So, an important task is the fermentation process of all kinds of sugars simultaneously which includes both pentoses and hexoses. These sugars are released during the saccharification and fermentation process. There are some other barriers in the field of bioethanol production such as distillation, and drying. (Sindhu et al., 2016).

*Lemna minor*can be modified for high starch accumulation and high biomass production, which makes it an excellent feedstock for the production of bioethanol. Several investigations have been done to study *Lemna minor's* potential for the manufacturing of bioethanol, refining the cultivation and fermentation methods. Weihua et al., 2010, found that *Lemna minor* can be cultured on a wide range of media, including nutrient-loaded solutions like Schenk & Hildebrandt medium and agricultural wastewater, together with natural or artificial reservoirs that have enough light and temperature. *Lemna minor's* expansion rate is 3.5 to 14.1 gm^{-2} day^{-1} during cultivation in Schenk & Hildebrandt medium and swine lagoon sewage (Weihua et al., 2010).When cultivated in low nutrient solution or grown in the dark (suppressing photosynthesis) with the supplement of glucose, the starch quantity leveled up to 10–36% (w/w). The enzymatic hydrolysis of starch provides around 96.2% of the glucose content (Xu et al., 2011).

When compared with traditional biofuel sugar crops, *Lemna minor* needs a minimum area and fertilizer for cultivation, therefore providing sustainability to the environment with less competitiveness to these crops. Muradov *et al.* 2012 studied the cultivation and characterization of *Lemna minor*bio-char and its catalytic capability for biogas formation. They obtained 25% methane and 82% carbon dioxide when treated with duckweed biochar. Kuznetsova et al., 2019, found that *Lemna minor* produced more lipids at optimal temperature (19-26°C °C), pH (6.3-8.0), and daily light integral (405 to 9000 lx). *Lemna gibba* can be cultivated at relative growth

rates (RGRs) of 7.47 g/m²d, 10.6 g/m²d, and 13.63 g/m²d during the 1st, 2nd, and 3rd weeks with yield (g/m² d) of starch,protein, and lipid of 4.96, 7.68, and 1.90, respectively by using wastewater(Vermaet al., 2016). After harvesting, *Lemna minor* residues can be transformed into biochar, which increases soil fertility and carbon fixation (Reddy et al., 1985). *Lemna minor* cannot have the same invasive properties as other aquatic weeds, ensuring environmental security (Culley et al., 1973). It can also inhibit the growth of algal blooms and aquatic weeds, enhancing water quality and biodiversity (Zhao et al., 2014). *Lemna minor* plays an important role in carbon restoration by absorbing environmental CO_2 and storing it in its biomass. Thus, this plant helps to reduce global warming (Zha0 et al., 2014). Due to the protein richness of *Lemna minor*, it can be used instead of soybean meal and fishmeal, reducing reliance on conventional feed sources and lowering costs for livestock and aquaculture industries (Iqbal et al., 1999).

Present strategies to enhance starch accumulation in duckweed include nutrient starvation, phytohormone treatment, and genetic modification. Zhao et al 2015 found that the starch content during Nitrogen deficiency conditions increased from 2.4 % to 24.5 %. Shiet al., 2023 investigated that the Calvin-Benson-Bassham pathaway and the oxidative pentose-phosphate cycle were operative during metabolic growth conditions, leading to a futile cycle with net ATP utilization, in accordance with the 13C-metabolic flux studies during photomixotrophic growth. Genetic modification focused on overexpressing starch-modulating enzymes in the plant metabolic pathway, such as ADP-glucose pyrophosphorylase (AGPase), but stability and large-scale implementation are main concerns (Kleczkowskiet al., 1991). The duckweed plant body comprises metabolically functional non-structural tissue (Wolverton et al., 1981). The starch value and low fiber content of the cultured plant were used to determine its ability as a possible feedstock for the brewing industry (Carnovale et al., 2022). Sustainable production of starch-enhanced plants need study of their nutritional requirements and environmental conditions **(**Meussdoerffer et al., 2009).Throughout the year, they can flourish in an assortment of challenging circumstances, such as high temperatures, extremes in light intensity and pH, inadequate amounts of nutrients, and drought conditions (Ziegler et al., 2023).

The present study aimed to make a culture system for the production of starch-enhanced *Lemna minor*. So, developing an optimization method to enhance starch biosynthesis in *Lemna minor* is important for its commercial use as a bioethanol feedstock. For large-scale biomass production, controlled nitrogen modulation is essential. This research paper provides sequential steps of a nutrient management approach, alternating nitrogen-sufficient (Organic manure) and nitrogen-deficient (nitrogen-free Hoagland media) phases, which helps to provide sustainable growth while enhancing starch production.

1.6 Review of Literature

Bioethanol can be used in countless ways for commercial purposes. Since bioethanol reduces air pollution and the heavy reliance on fossil fuels, it is widely accepted as a fuel for transportation, either by itself or mixed with gasoline (Aggarwal et al., 2022). Bioethanol can be applied as a feedstock for the chemical production of ethylene, butanol, and acetic acid (Ayyanna et al., 2023). Spirits and liquors are among the alcoholic beverages that can be created by further purifying bioethanol (Dahiya et al., 2018). Bioethanol may be blended with other biofuels, such as biodiesel, to create various biofuel combinations that can be used to make electricity (Niculescu et al., 2019 & Cheng et al. 2009). Paints, varnishes, and supplies for cleaning are among the most common everyday goods that can be used with bioethanol. Thus, an adequate supply of bioethanol is required by appropriate process optimization.

1.6.1 Bioethanol production process

For the production of bioethanol, biomass feedstock (agricultural residues, energy crops, or lignocellulosic materials) is utilized through a variety of approaches, including size reduction, pretreatment, and hydrolysis, to transform the complex carbohydrates into fermentable sugars (Lange et al., 2007). Following that, microorganisms were employed to ferment the sugars in the supplied feedstock. Different microorganisms ferment a range of carbohydrates. For example, *Pichia stipitis* ferments xylose, whereas *Escherichia coli*, *Kluyveromyces marxianus*, *Zymomonas mobilis*, *Saccharomyces cerevisiae*, and *Clostridium thermocellum* can break down cellulose (Saddler et al, 1982, Dien et al., 2003, Arora et al., 2015

&Gonçalves et al., 2016). Furthermore, lignin may be broken down by the white rot fungi (Pérez-Contreras et al., 2025).

Many researchers have recently worked to improve the yield and production of ethanol by employing various methods during the fermentation process.[29] Later, the fermented broth undergoes distillation and drying to extract the ethanol and enhance its purity, respectively (Yang et al., 2012 & Park et al., 2021). Further, pure ethanol is blended with petroleumto operate automobiles (Saikia et al., 2024). Figure 1.1shows the steps involved in the bioethanol production process.

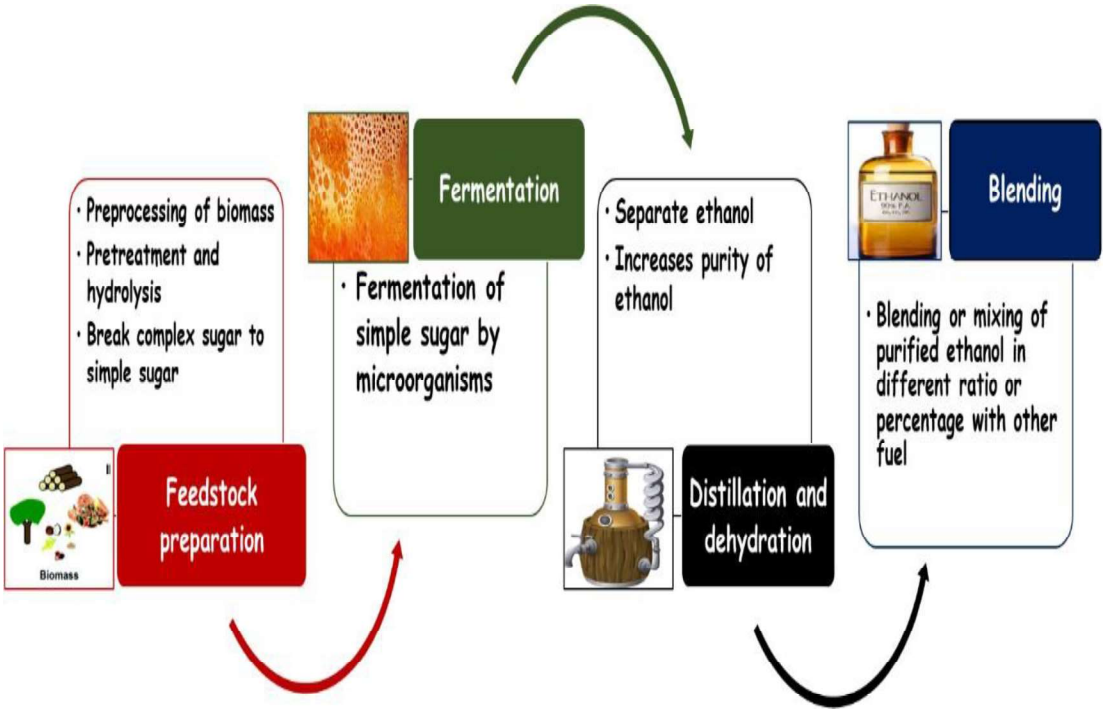


Figure 1.2: Bioethanol production process

1.6.1.1 Pretreatment strategy

Appropriate pretreatment is important for maximizing the release of fermentable sugars and boosting bioethanol yields from lignocellulosic plant biomass. Pretreatment raises the success rate of enzymatic hydrolysis, breaks up the cellular structure of the plant, including *Lemna minor*, and eliminates non-fermentable materials (O'Mahoney et al., 2022).Operational steps of pretreatment methods during

the fermentation process are illustrated in Figure 1.2-

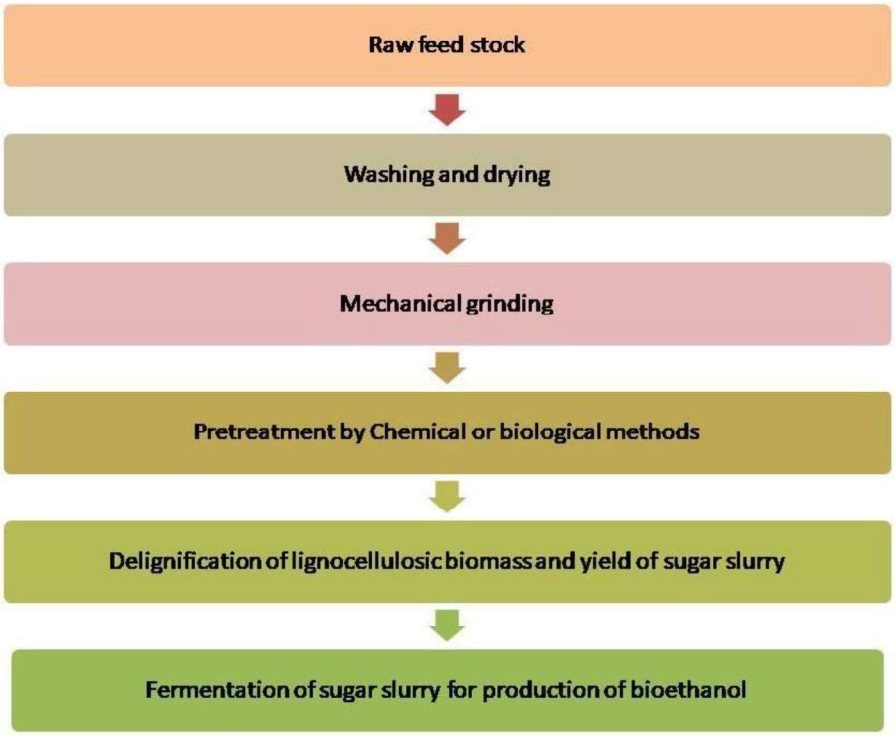


Figure 1.3: Steps involved in the pretreatment process

a) Mechanical pretreatment

Grinding and milling are generalized mechanical pretreatment techniques. These methods minimize the particle size of lignocellulosic biomass, increasing the surface area and allowing greater enzyme accessibility. By decreasing the particle size of the lignocellulosic biomass, mechanical pretreatment provides better diffusion of enzymes, allowing the hydrolysis of cellulose and hemicellulose into fermentable sugars (Phojaroen et al., 2022). Another benefit of smaller particle sizes is to enhance chemical pretreatment effectiveness with better solvent invasion. However, mechanical methods can better deconstruct the lignocellulose with hydrothermal, chemical, or biological pretreatment (Phojaroen et al., 2022). So the overall biomass digestibility can be enhanced, sugar release increased, and thus, bioethanol production efficiency has been improved. Different types of biomass require different types of mechanical pretreatment (Mood et al., 2016).

b) Chemical pretreatment

Acid and alkaline pretreatment by employing dilute or concentrated chemicals comes under this category. For acid treatment, acids such as sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) are commonly used to hydrolyze lignocellulosic plant biomass, digest lignin, and increase cellulose accessibility, facilitating enzymaticsaccharification (Yáñez et al., 2017). Acid pretreatment with dilute concentration is cost-effective and effective at moderate temperatures (Kumar et al., 2017).On the other hand, concentrated acid methods are corrosive and require careful acid recovery and handling (Jönsson et al. 2016). Major parameters during this pretreatment process are acid concentration, reaction time, temperature, and inhibitor formation, such as furfural and hydroxymethylfurfural (HMF). Inhibitors can hinder microbial fermentation, necessitating detoxification steps (Gönen *et al*.2018). Gönen *et al*., 2018 studied optimizing acid loading and reaction parameters to improve sugar recovery from duckweedswhile minimizing byproduct formation, were making acid pretreatment a key step in duckweed's bioethanol production. On the other hand, in comparison to acid pretreatment, alkaline pretreatment is performed at lower temperatures and pressures, minimizing sugar degradation and inhibitor formation. It enhanced the disruption of lignocellulosic biomass and improved its digestibility to enzymes by efficiently removing the lignin. Alkaline reagents such as sodium hydroxide (NaOH), potassium hydroxide (KOH), ammonia, or calcium hydroxide $Ca(OH)_2$, etc., are used in dilute and concentrated form for this kind of pretreatment (Xu et al., 2010, Kng et al., 2015 & Zhang et al., 2023). Ammonia-based methods, such as ammonia fiber explosion (AFEX), are advantageous in the context of improved sugar accessibility and more biomass exposure for enzymatic saccharification (Maedeh et al., 2024). Still, some challenges such as chemical recovery, cost-effectiveness, reaction time adjustment, and alkali disposal are there. According to Zhao et al, 2012, alkaline treatments are good for optimizing duckweed's starch accessibility and generate fewer inhibitors.

c) Pretreatment by heat

Pretreatment by heat comprises hot water pretreatment and steam explosion methods. Wang et al, 2016, found that the lignin becomes porous and irregular, increasing surface area and pore volume, which enhances enzyme accessibility, followed by

liquid hot water pretreatment. It is a mild pretreatment procedure that doesn't influence corrosion and does not require any catalysts. Water penetrates the plant biomass at high pressure, hydrates the cellulose, and recovers the majority of the hemicelluloses and a small amount of lignin. Water auto-ionization releases hydronium ions, which dissolve hemicelluloses. By setting the pH close to neutral, fewer fermentation inhibitors are developed (Kululo et al., 2025). With limited negative impact on the environment and efficient energy efficiency, steam explosion pretreatment seems to be a good method for boosting the digestibility of lignocellulosic biomass (Bhuka et al., 2022).

d) Biological pretreatment

Biological pretreatment is an effective, eco-friendly method for the treatment of lignocellulosic biomass, utilizing microorganisms such as fungi and bacteria to digest lignin, cellulose, and hemicellulose. Some white-rot fungi like *Phanerochaete chrysosporium, Trametes versicolor, Pleurotus ostreatus* produce different types of ligninolytic enzymes likelaccases, manganese peroxidase, and lignin peroxidase, which make cellulose more accessible (Hermosilla et al., 2018). Some Brown-Rotand Soft-Rot Fungi, such as *Gloeophyllum trabeum* and *Chaetomium globosum*, target cellulose, hemicelluloses, and lignin modification (Hermosilla et al., 2018). Also, some bacterial strains like *Pseudomonas putida, Bacillus subtilis, and Actinomycetes* (*Streptomyces spp.*) digest lignin effectively, with some fungal species (Devendra et al., 2023). This approach is advantageous over chemical and physical pretreatments, including lower energy requirements, mild reaction conditions, and minimal environmental impact. While being less harmful to the environment compared to other methods, this approach requires precise control of the conditions along with incubation times to accomplish its maximum efficiency (Devendra et al., 2023).

e) New approaches to pretreatment

Microwave-Assisted Pretreatment: This technique uses a conductive heating process, minimizing treatment time and energy consumption compared to traditional methods (Zhu et al., 2006, Ehaib et al. 2015 &Shuguang et al., 2016). This method breaks lignin and hemicellulose and exposes cellulose for enzymatic digestion (Khatri et al.,

2015). The key parameters during this procedure are power, irradiation time, and moisture content (Cahanti et al., 2023). Sharma *et al. 2025,* Investigated that the method improved sugar yield and enhanced bioethanol production. This method further improves biomass digestibility when combined with acid or alkali chemicals (Bussemaker et al., 2013). It has been observed that microwave-assisted pretreatment is a suitable approach for sustainable bioethanol manufacturing (Erico et al., 2021).

Pretreatment with Ultrasound: Ultrasound pretreatment involves high-frequency sound waves that degrade the complex structure of lignocellulosic biomass, enhancing enzymatic accessibility and fermentation efficacy (Victor et al., 2021 & Zumar et al., 2018). Ramirez et al., 2024*,* found that it can reduce processing time and chemicals required in comparison to traditional techniques. It is environmentally friendly due to less energy utilization and fewer chemical residues (Subhedar et al., 2016). When combined with alkali or acid hydrolysis techniques, it can increase sugar yield.[58] Still, equipment cost and process optimization are required for large-scale production (Kininge et al., 2022).

1.6.2 Engineered microbes and enzymes to optimize bioethanol fermentation

By releasing various enzymes that break down sugar and starch, microorganisms such as bacteria and fungi ferment biomass broth. Mohd Azhar et al., 2017, investigated *Saccharomyces cerevisiae* and *Pichia stipitis* ability to yield large amounts of ethanol. An essential part of enhancing efficiency and productivity is the selection and improvement of the microorganisms for the fermentation of different kinds of sugar and starch. They also investigated the capacity of *Pichia stipitis* to naturally ferment xylose along with additional sugars apart from pentoses. Lin et al.*,* 2012, found that *Saccharomyces cerevisiae* BY4742 is affordable and resistant to a range of pH values during the ethanol fermentation process. However, there are certain limitations, which include bacterial contamination, high ethanol concentration, temperature, osmotic pressure, and wild-type yeast contamination that can affect fermentation (Basso et al., 2008). Brandt et al., 2019*,* discussed the ability of wild yeast and bacteria to ferment lignocellulose and have greater resistance to inhibitors than standard strains.The detrimental impact of high temperature and ethanol concentration on the

manufacturing of ethanol can be avoided by employing thermotolerant yeast and ethanol. Yanase et al., 2010, determined thatthe thermotolerant yeast *Kluyveromyces marxianus* can withstand high temperatures and ferment both hexose and pentose.

Several other microorganisms, including *Candida*, *Pichia stipitis*, *Schizo saccharomyces*, and *Pachyso lentannophilus,* have been examined as possible replacements for traditional yeast since *Saccharomyces cerevisiae* was unable to ferment it (Mussatto et al., 2012). Enzymatic or chemical hydrolysis of cellulose and hemicellulose produces simple fermentable sugars such as mannose, hexoses, arabinose, glucose, and xylose (Mosier et al., 2005).To reduce dependence on non-renewable energy, researchers are working to improve ethanol production from lignocellulose, a renewable resource consisting of lignin, cellulose, and hemicellulose, through different processes involving pretreatment (Wonago et al., 2015).Coculturing the *Saccharomyces cerevisiae* strain with an additional bacterial or fungal strain or genetically modifying it were further possibilities investigated to induce the yeast to ferment xylose. The profitable fermentation of lignocellulose has been accomplished by employing the coculture of yeast and *Zymomonasmobilis* (Nielsen et al., 2016 & Badenr et al., 2010). One of the suggested strategies is co-culturing because this method enables the cultivation of two or more microorganisms in a single reactor and provides more ethanol than a single culture (Joshi et al., 2022). Fermentation of either pentose or hexose by microorganisms can be handled by co-culturing. For instance, coculturing *Saccharomyces cerevisiae* with *Pichia fermentans* and *Pichia stipitis* increased ethanol production many times by fermenting both hexose and pentose (Singh et al., 2014 & Karagöz et al., 2014).

Enzymes are crucial for the manufacturing of bioethanol at each phase, including hydrolysis and pretreatment. Given their capacity to dissolve lignin and diminish cellulose's polymerization and crystallinity, enzymes such as laccase, manganese peroxidase, lignin peroxidase, or lytic polysaccharide monooxygenase are commonly used in the pretreatment of biomass, consequently leading to the biomass's increased porosity de Araújo et al., 2023). Hydrolyzing cellulose and hemicellulose into sugar, which is usable when fermentation proceeds by cellulases and hemicellulases. *Aspergillus niger* and *Trichoderma reesei* are two popular fungal species applied for

large-scale manufacturing of enzymes in optimum circumstances since both yield hemicellulase and cellulose (Vasić et al., 2021 & Alabdalall et al., 2023). Enzymes can be deemed more efficient, healthy, and reusable by immobilizing them on supports such as magnetic nanoparticles, which in turn serves to lower expenses (Dutta et al., 2023). To bring together pretreatment and hydrolysis methods followed by optimization of the bioethanol process of manufacture, Sheldn et al., 2007investigated the implementation of co-immobilized or a combination of immobilized enzymes.Geet al., 2012, efficiently fermented *Lemna minor* hydrolysates by two yeast strains (self-flocculating yeast SPSC01 and conventional yeast ATCC 24859) with an elevated ethanol quantity of 0.485 g/g (glucose). Su et al.,2015, bioengineered *Corynebacterium crenatum* by incorporating genes from *Saccharomyces cerevisiae*, so the bacterium was able to ferment duckweed into higher alcohols. Chen *et al., 2012,* studied pectinase pretreatment for duckweed (*Landoltia punctata*) with pectinase, so this efficiently enhanced ethanol yield.

1.6.3 Amylase-driven optimization of lignocellulosic bioethanol production

For ideal glucose yield and, therefore, optimum bioethanol synthesis, amylase enzyme dosage, temperature, and duration of time are maintained. Optimizing the synthesis of amylase enzymes through multiple microbial sources and feedstocks has become the object of numerous investigations. Shah et al., 2014, found that the most appropriate conditions to synthesize α-amylase by *Bacillus subtilis* using a medium containing 10 g/l peptone and 0.5% starch, for 48 hours at 45°C and pH 8.5. He also observed that the partially purified amylase enzyme attained optimum activity at pH 6, when loaded with 1.5% of substrate for 15 mins at 50°C.

Combining the One Factor at a Time (OFAT) approache and Response surface methodology (RSM), Kahlouche et al., 2022, observed that the optimized parameters yield maximum reducingsugars, were at a bread ratio of 0.03 g/mL, alpha-amylase concentration of 0.2 ml/l, temperature of 100 °C, and 5.3 pH, when using *Saccharomyces cereviceae*. Under these conditions, the yield of RS reached 90%, with bioethanol concentration of about 85.8 g/l after 72 h of batch fermentationMicroalgaerepresent another starch content option that can be applied to

create bioethanol. Yerizamet al.,2023, found that *Chlorella pyrenoidosa*, a microalga cultured in medium with 40% (v/w) α-amylase derived from *Aspergillus niger*, yielded the highest amount of glucose (0.675 mg/ml) during nine days of fermentation and the optimal Bioethanol content of 28.07%.The suitable carbon source for maximum amylase production, primarily from *Bacillus* and *Aspergillus* species, is starch at a concentration of 0.5 to 1% (w/v) (Bakri et al.,2021 & Ghosh et al., 2008) Along with starch, agricultural byproducts such as rice straw, wheat bran, soybean, pomegranate, banana, orange, and potato peels can also function as inexpensive and effective feedstock for the synthesis of amylase. Suitable feedstocks were found to include wheat bran and pomegranate peel for this purpose (Mahmood et al., 2018, Liaqat et al., 2024, Ahmed et al., 2020 & Novia et al., 2025). To induce maximum amylase activity, the sources of carbon and nitrogen can be optimized, along with their ratios (Lal et al., 2016). Additionally, enhancing the amylase product can be achieved with a medium that incorporates nitrogen sources such as peptone, yeast extract, or soybean meal with carbon sources such as starch (Lal et al., 2016).

1.6.4 Lignocellulosic biomass forbioethanol: leveraging novel feedstocks for renewable energy

The economics and efficiency of making lignocellulosic bioethanol rely primarily on the careful selection of the biomass feedstock and the optimization of each of the steps in the process. To create bioethanol, various kinds of lignocellulosic biomass have been explored; the selection of these feedstocks is contingent upon their composition and availability. Since they are highly abundant and have more cellulose and hemicellulose content, agricultural wastes, including sugarcane bagasse, wheat straw, rice straw, and maize stover, provide suitable alternatives (Kumar et al., 2009). Other possible substrates are forestry wastes like sawdust. Better biomass-yielding non-food energy crops that can be planted on marginal soils include switchgrass andmiscanthus (Oliveira et al., 2017). Given that lignocellulosic biomass is more widely available worldwide, possesses more energy security, and is more affordable than sugar or starch-based feedstock, it does not compete with the production of food or feed. A more involved pretreatment and conversion strategy must be undertaken to handle the heterogeneous and complicated structure of lignocellulose, which is composed of cellulose, hemicellulose, and lignin.

For continuous year-round biorefinery operations, lignocellulosic biomass harvesting is frequently seasonal and involves stabilization and storage. When it comes to changing lignocellulose into fermentable sugars, pretreatment and hydrolysis processes might be expensive and release inhibitory byproducts (Broda et al., 2022, Nongthombam et al., 2017 &Beluhan et al. 2023). Methods such as the multi-criteria decision-making (MCDM) algorithm can be used to acquire the appropriate biomass for the best possible output of bioethanol (Taherdoostet al. 2023). The practice of valuing biomass to create biofuels has captured the curiosity of numerous scientists. Pagliuso et al., 2022, reported that Duckweed biomass contains less lignin in comparison to terrestrial plant biomass, so it becomes easier to saccharify and ferment it for the production of bioethanol.

Additionally, it is easy to digest enzymatically because of its soft cell walls. Further, for the production of bioethanol, starch, an essential component of the duckweed plant matter's composition, provides advancement due to quickly transforming into fermentable sugars.

Xumeng et al. reported that 20.3% (w/w) total glucan, 32.3% (w/w) proteins, trace hemicellulose, and undetectable lignin were all detected in the collected duckweed (Lemna minor) biomass. Up to 96.2% (w/w) of glucose was enzymatically released from the cellulose and starch portions of duckweed. Both the self-flocculating yeast SPSC01 and the conventional yeast ATCC 24859 fermented the enzymatic hydrolysates, producing a high ethanol yield of 0.485 g/g (glucose) (Xumeng et al., 2012).

Table 1.2 lists many different sources, in addition to the positive aspects and pitfalls of each, which are utilized for the efficient manufacture of bioethanol. Also, this table highlights the approaches that have been assessed to investigate the impact and production of biofuels. This table provides information about different sources used for bioethanol production.

Table 1.2: Different sources used for bioethanol production with their advantages and disadvantages

Biomass Source	Author Name (Reference No.)	Ethanol Yield	Methodology	Advantages	Disadvantages
Duckweed (*Lemna minor*)	(Gusain et al., 2017)	0.218g/g	Simultaneous saccharification and fermentation	High growth rate, optimal starch, and naturally lower lignin content	Needs immediate processing
Sugarcane molasses	(Jiang et al., 2024)	-	Metabolic pathway analysis, MAP kinase signaling pathway study	Explores the dual roles of amino acids in ethanol production	Focuses on amino acid metabolism, not directly on ethanol yield
Lignin (from plant material)	(Wang et al., 2023)	-	Isolation of lignin with a native-like structure	Preservation of native lignin structure	Not directly related to ethanol production
Spirodelapolyrrhiza biomass	(Romanowska-Duda et al., 2023)	-	Valorization for biofuel production	Utilizes aquatic biomass for biofuels	Efficiency and scalability challenges
Plant biomass	(Bernacki et al., 2023)	-	Study of stress response and cell death regulators proteins	Potential biotechnological applications	Focus on stress response rather than ethanol yield
Corn, Cellulosic Biomass	(Qiao et al., 2023)	5.7-10.1% increase	InSiReP 2.0 protein evolution strategy	Improved ethanol yield and tolerance	Limited to specific cellulase variants
Sweet Sorghum Silage	(Zhu et al., 2023)	28.42 g/L	Synergistic microbial consortia with lactic acid bacteria	Enhanced biomass preservation and ethanol yield	Ensiling challenges for coarse materials
Rice Straw, Sugarcane Bagasse, Sweet Sorghum Bagasse	(Nunta et al., 2023)	15.3 g /L (RS)	Yeast fermentation: comparison of strains	Effective valorization of residues; high PAC yield	Variation in efficiency among different residues and yeasts
Wheat Straw	(Ziaei-Rad et al., 2023)	38.9-42.0 g /L	Ultrasound-assisted ionic liquid pretreatment	Cost-effective, high delignification rates	Requires optimization of pretreatment conditions
Pineapple Waste Hydrolysate	(Pilap et al., 2022)	38.02 g /L	High-temperature fermentation using *Saccharomyces ludwigii*	Effective at high temperatures and inhibitor tolerance	High-temperature requirements may limit scalability
Rice straw (green pretreatment)	(Kumari et al., 2022)	-	Use of petha wastewater for pretreatment, analysis of cellulose content, and sugar yield.	Green, environmentally friendly pretreatment with improved sugar yield.	Limited focus on ethanol production efficiency post-pretreatment.
Anabaenavariabilis (waste-utilized medium)	(Deb et al., 2022)	7.3 g/L (27% yield)	Use of wastewater and solid waste for cyanobacterial cultivation, bioethanol, and co-product production.	Effective waste bioremediation, high biomass valorization, and multiple co-products.	High production costs, scalability challenges, and waste management issues.

Biomass Source	Author Name (Reference No.)	Ethanol Yield	Methodology	Advantages	Disadvantages
Wheat bran (psychrohalophilic xylanase)	(Rajabi et al., 2022)	7.3 g/L (26.8% yield)	Characterization of xylanase from camel rumen, application in bioethanol production.	Effective hydrolysis of agricultural wastes increases ethanol yield.	Potential scalability issues, enzyme activity under specific conditions.
Chestnut shell (NaOH pretreatment)	(Lee et al., 2022)	98% yield	Optimization of NaOH pretreatment at room temperature, glucose recovery, and fermentation.	Energy-efficient, low-temperature pretreatment, high glucose recovery.	Optimization complexity, potential scaling issues.
Oil palm empty fruit bunches	(Sukhang et al., 2020)	0.258–0.281 g/g LCB (~25.8–28.1 g/L)	H_2SO_4 pretreatment + cellulase SSF/SHF	Moderate yield; uses non-edible agricultural residue	Requires acid pretreatment; moderate loadings
Arthrospira platensis biomass	(Esquivel-Hernández et al., 2021)	3.02 g/L (bioethanol), 9.67 g/L (lactic acid)	Multi-product biorefinery, fermentation with *Saccharomyces cerevisiae* and *Lactobacillus acidophilus*	Multi-product recovery, economic feasibility, and reduced waste generation	Economic analysis of the fermentation scale and costs, optimization of fermentation processes
Stinging nettle stems	(Smuga-Kogut et al., 2021)	7.3 g/L	Ionic liquid pretreatment, enzymatic hydrolysis, and fermentation	High ethanol concentrations, effective use of agricultural waste	Efficiency of ionic liquid pretreatment, comparison with other biomass sources
Panicum prionitis biomass	(Gauna et al., 2021)	-	Secretome characterization of lignocellulose-degrading fungi, proteomic analysis	Identification of efficient lignocellulolytic enzymes, the potential for improved biomass degradation	Specificity of fungal enzymes for lignin degradation, variability in enzyme activities
Potatoes (*Bacillus subtilis* strains)	(Maleki et al., 2021)	16.3 g/L (NZS), 21.5 g/L (NS:Z)	Used metabolically engineered *Bacillus subtilis* with *Zygomonas mobilis* and *Saccharomyces cerevisiae* genes in consolidated bioprocessing (CBP) for ethanol production.	High ethanol production with affordable CBP using renewable biomass.	Requires further metabolic and process engineering for higher yields.
Corn stover	(Chu et al., 2013)	52.3 g/L (71 %)	Tween40 + SSF (fed-batch)	Good yield, high conversion efficiency	Uses surfactants; multi-step prep
Lignocellulose residues (Amaranthus green proteins)	(Madadi et al., 2021)	Estimated 6400-12,400 tons	Extracted green proteins from Amaranthus as surfactant and absorbant applications; increased enzymatic saccharification and trace metal bioremediation.	Significant increase in bioethanol yield; effective trace metal remediation.	High cost and complexity of green protein extraction and application.
Napier grass and rice straw (*Kluyveromyces marxianus*)	(Ha-tran et al., 2021)	-	Engineered *Kluyveromyces marxianus* for bioethanol production using pretreated biomass from *Hungatei clostridium thermocellum*.	Enhanced ethanol production from biomass feedstocks; dual microbial system efficiency.	Needs optimization for maximum efficiency and scalability.

Biomass Source	Author Name (Reference No.)	Ethanol Yield	Methodology	Advantages	Disadvantages
Decaying oil palm empty fruit bunch (OPEFB)	(Riyadi et al., 2020)	-	Enzymatic and genetic characterization, GPC, GC-MS, genome analysis	Effective lignin degradation, identification of lignin-degrading genes	Lack of direct ethanol yield data
Birch wood	(Liao et al., 2020)	-	Integrated biorefinery, reductive catalytic fractionation	Lower CO2 footprint, economically competitive	Complexity in the biorefinery process, not directly focused on ethanol yield
Cotton plant biomass	(Ibarra et al., 2019)	-	Screening and characterization of β-1,4-endoglucanase from Cotton Boll Weevil	Higher efficiency of the enzyme compared to the commercial cellulase	Limited application to second-generation ethanol, lack of direct yield data
Agave americana L.	(Niechayev et a., 2018)	-	Modelling growth responses to environmental factors	Potential for growth in water-limited regions, cold tolerance	Requires further validation for practical biofuel crop use
Spirulina platensis	(Pereira et al., 2019)	Max yield: 2.98 ±0.07 g/L (with 5.0% whey)	Mixotrophic cultivation using dairy wastewater (cheese whey)	High biomass productivity and carbohydrate content, cost reduction by using waste	Lower antioxidant capacity, protein, and lipid content compared to autotrophic culture
Ulvan (from Ulva species)	(Reisky et al., 2019)	-	Metabolic pathway analysis of marine flavobacterium *Formosa agariphila* to degrade ulvan	Converts algal polysaccharides into fermentable monosaccharides, turning a renewable resource into a valuable bioresource	Limited knowledge of microbial pathways and enzyme functions for broader applications
Cyanobacteria (*Synechocystis sp.* PCC 6803)	(Lasryet al., 2019)	In silico yield: 1.054 mmol·g/DW/h	Genome-scale metabolic network model for ethanol production, in silico analysis of gene knockouts	Identification of mutants coupling growth and ethanol production, efficient in photoautotrophic conditions	Requires in silico validation and potential experimental confirmation
Chlorella vulgaris	(Pszczółkowska et al., 2019)	Up to 2 g/L dry biomass	Cultivation of methane waste from anaerobic biomass digestion, waste treatment analysis	Effective waste treatment and biomass production reduce chemical oxygen demand.	Limited to methane waste treatment, further optimization is required for broader applications.
Molasses	(Zentou et al., 2019)	-	Comparison of Monod and Andrews models for fermentation kinetics	Identifies suitable mathematical models for different substrate	Model accuracy varies with substrate concentration and

Biomass Source	Author Name (Reference No.)	Ethanol Yield	Methodology	Advantages	Disadvantages
				concentrations and improves the fermentation process	requires specific adjustments
Biofuel-producingmicroorganisms (*Pichiastipites*)	(Concu et al., 2019)	-	PTML model and artificial neural network for enzyme function prediction	Accurate enzyme function prediction improves understanding of biofuel production mechanisms	Limited to enzyme function prediction, not directly reporting ethanol yields
Banana pseudo-stem	(Linzmeyer et al., 2019)	-	Pervaporation for ethanol recovery from fermentation broth	Lower-energy-cost alternative to distillation, better permeance with by-products	Effects of by-products on ethanol recovery, varying permeation results
Sudan grass	(Acevedo et al., 2019)	-	Analysis of biomass composition and saccharification after pretreatment	High lignocellulosic content suitable for bioethanol, effective alkaline pretreatment	Variability in biomass composition and stress response among accessions
Oil Palm Frond (OPF)	(Tai et al., 2019)	-	Solid-state fermentation; Optimization using Response Surface Methodology (RSM)	Increased CMCase and xylanase production; Utilizes agricultural waste as substrate	High cost of cellulase production; Need for further optimization for commercial application
Chloris barbata	(Obeng et al., 2019)	30.7% glucose recovery	Autoclaving combined with a mild alkaline solution; Different NaOH concentrations and temperatures.	Significant increase in glucose recovery; Efficient pretreatment for bioethanol production	Limited information on ethanol yield; Need for scaling up processes
Spruce S (1-2) transition layer	(Reza et al., 2019)	-	Single-axis electron tomography; 3D structure analysis of cellulose elementary fibrils	Improved understanding of cellulose structure; Better insight into biomass conversion processes	Technical challenges in obtaining structural data

1.6.5 Exploring duckweed's benefits and addressing the challenges for industrial feasibility during the bioethanol production process

When given favorable climatic conditions, duckweed grows very quickly—it can double in size in two to three days (G-Yu et al., 2011 & Pagliuso et al., 2022). It has the capability of flourishing in many different kinds of water environments, especially nutrient-rich wastewater and agricultural runoff, and offers the twin advantages of detoxifying wastewater and yielding biomass simultaneously (Borisjuk et al., 2018). Duckweed production has a lower environmental impact and less competition with food crops than established biofuel crops like maize or sugarcane, since it requires fewer cultivation spaces and the addition of fertilizer (Zhao et al., 2014). Despite the possibility of duckweed producing bioethanol, there are still a lot of barriers. The manufacture of ethanol needs to be made economically feasible by refining large-scale growing and harvesting systems. Improved strains of duckweed that have greater biomass productivity, stress tolerance, and ethanol yield are the ultimate objective of ongoing breeding and genetic engineering studies. It is imperative to conduct a thorough assessment of the commercial feasibility of producing bioethanol from duckweed, addressing each stage from cultivation to ethanol recovery. Realizing duckweed's assurance as a trustworthy and effective means to produce bioethanol will require significant effort in these areas (Zhao et al., 2014).

1.6.6 *Lemna Minor* as a leading species for bioethanol: insights into its starch enhancement strategy and production potential

Lemna minor, or ubiquitous duckweed, has a high percentage of starch and a rapid growth rate, which renders it an excellent feedstock to produce bioethanol. Several studies have investigated *Lemna minor's* potential for producing bioethanol, refining the growing and fermentation techniques. According to Weihua et al.2010, *Lemna minor* can be produced on a range of media, including nutrient-rich solutions like Schenk & Hildebrandt medium and agricultural effluent, along with natural or artificial reservoirs that have sufficient light and temperature. *Lemna minor's* biomass or growth rate is 3.5 to 14.1 gm^{-2} day^{-1} when it was cultivated in Schenk & Hildebrandt medium and swine lagoon effluent. Duckweed, after being cultivated in media low in nutrients or when

maintained in the dark (suppressing photosynthesis) with the addition of glucose, the starch content rose to 10–36% (w/w). The enzyme-driven hydrolysis of starch liberated around 96.2% of the bound glucose (Weihua et al., 2010).Comparing *Lemna minor* cultivation with conventional biofuel crops, it requires less area and fertilizer, therefore minimizing its influence on the environment and lowering its competitiveness with food crops.*Lemna minor* does not need to be pretreated chemically or thermally.Zhao et al., 2015, found that when an enzyme cocktail of CellicCTec 2 (0.87 FPU/g substrate) together with Novozyme 188 (2 U g1 substrate) were used so 94.7% (w/w) of the theoretical yield of ethanol was gained from steam-exploded duckweed biomass (1% w/w). While *Lemna minor* shows great potential for Bioethanol production, challenges remain in optimizing large-scale cultivation, harvesting, and processing methods to achieve economically viable ethanol yields. Research aiming to enhance *Lemna minor* strains through genetic engineering and breeding for improved biomass productivity and starch yield is ongoing.

1.6.7 Advancing *Lemna Minor* through genetic engineering: strategies for increased biomass and bioethanol yield

Applying *Agrobacterium tumefaciens*-mediated transformation, transgenic plants of an Indian isolate of *Lemna minor* have been created. Chhabra et al. transformed the hard nodular cell masses ('nodular calli') that were developed on BAP-pretreated daughter frond explants in B5 medium containing sucrose, 2,4-D, and 2-iP or TDZ under light conditions (Chhabra et al., 2011). These calli were then co-cultured with *Agrobacterium tumefaciens* strain EHA105 harbouring a binary vector with genes for β-glucuronidase and neomycin phosphotransferase. Bioremediation of pharmaceutically important proteins can be achieved by synthesizing them from the modified *Lemna minor* (Chhabra et al., 2011). Yamamoto et al., 2001, successfully genetically transformed *Lemna minor* by using Agrobacterium-mediated methods and improved biomass features. Further, Liu et al., 2020, applied heavy-ion irradiation mutagenesis to build up *Lemna aequinoctialis* mutants with rich starch accumulation, improving bioethanol synthesis. To enhance *Lemna minor's* biomass productivity and starch yield, which are desirable traits for bioethanol production, ongoing research also attempts to strengthen it through genetic engineering and breeding.

Chapter 2

Materials and Methods

CHAPTER 2

MATERIALS AND METHODS

Present study focused on producing bioethanol from *Lemna minor* through simultaneous saccharification and fermentation procedures. *Lemna minor* was cultivated under nitrogen starvation conditions to enhance starch production. These experiments has been performed by sequential steps of nutrient management approach, alternating nitrogen-sufficient (Organic manure) and nitrogen-deficient (Nitrogen free Hoagland media) phases, helps to provide sustainable growth while enhancing starch production. For large-scale biomass production, controlled nitrogen modulation is essential.

For fermentation of *Lemna minor* biomass, the amylolytic enzymatic cocktail was derived from *Aspergillus niger* MTCC-12975 cultured in media containing copra meal as substrate. In the simultaneous saccharification and fermentation process, an amylolytic enzymatic cocktail was used to saccharify the Lemna minor starch with the mono-culturing and coculturing *Saccharomyces cereviceae* MTCC-171 and *Candida shehatae* MTCC-12913 and was evaluated for maximum fermentation and optimized ethanol production. One factor at a time (OFAT) and Response surface methodology (RSM) were applied to screen and optimize factors to produce maximum enzyme activity and ethanol production. RSM requires less time than OFAT and studies interaction between the different factors like temperature, pH, time, substrate concentration, and many other additives factor during amylolytic cocktail and ethanol production.

2.1 Materials and chemicals:

Chemicals and Glass wares: All the chemicals used in this study was taken from Thermo- fisher, sigma Aldrich, Hi media and glasswares from Bororsil.

Different Instruments Used in the Analysis: Instruments used in the analysis are: weighing balance (Danver scales), hot air oven (Matrix) centrifuge (Remi),

Centerifuge for falcon tubes (Remi R8C), BOD incubator (Thermotech), UV- visible spectrophotometer (Shimadzu), kjeldjal (Foss), GCMS (Agilent GC-6890 MS-5973)

2.2 Collection and characterization of raw substrates

Dried processed coconut meal was procured from a coconut oil processing factory in Shabad village, New Delhi. Copra pulp was ground to fine powder and sieved which was further used for amylolytic cocktail production. *Lemna minor* was collected from Department of Zoology, University of Delhi, and grown in starvation conditions for starch enhancement and dried under the shade. It was then used for ethanol production. Components in copra meal and *Lemna minor* biomass assayed by DNS method (Miller et al., 1959) for Glucose and fructose. The protein content was estimated by Kjeldahl, 1883, method. Crystalline cellulose constituent was analysed by using Updegraff, 1969, reagent method. The starch component was determined by the megazyme total starch assay method (amyloglucosidase/ α-amylase method; AA/AMG 11/01, AOAC Method 996.11-2002).

2.3 Starch enhancement in Duckweed (*Lemna minor*) and its compositional analysis

2.3.1 Experimental layout for production of starch enhanced lemna minor biomass

There are two sequential steps done for high starch duckweed production. Step 1: High biomass production in organic manure and Step 2: starch accumulation of plant biomass in nitrogen-free Hoagland media. To prepare Hoagland media without nitrogen 1.34 grams of the following salt mixture have been dissolved in 600 ml of distilled water under gentle stirring: 136.03 mg/l potassium phosphate monobasic, 372.70 mg/l potassium chloride, 554.90 mg/l calcium chloride dihydrate, 240.33 mg/l magnesium sulfate, 1.81 mg/l manganese chloride tetrahydrate, 2.86 mg/l boric acid, 0.025 mg/l molybdic acid (sodium salt) dihydrate, 0.045 mg/l copper chloride, 0.11 mg/l zinc chloride, and 33.00 mg/l EDTA ferric monosodium salt. The pH was adjusted to the desired level using 1N hydrochloric acid or sodium hydroxide, and

then the final volume was made to 1 liter with distilled water. The medium was sterilized by autoclaving at 15 psi (121°C) for 15 minutes.

For control group plants were grown on full strength solution of Hoagland media. To prepare the full strength Hoagland media, 1.63 gm of the following salt mixture in 600 ml of distilled water have been dissolved under gentle stirring until: 606.60 mg/l potassium nitrate, 656.40 mg/l calcium nitrate, 240.76 mg/l magnesium sulfate, 115.03 mg/l ammonium phosphate monobasic, 1.81 mg/l manganese chloride tetrahydrate, 2.86 mg/l boric acid, 0.016 mg/l molybdenum trioxide, 0.22 mg/l zinc sulfate heptahydrate, 0.08 mg/l copper sulfate pentahydrate, and 5.00 mg/l ferric tartrate. The pH was adjusted to the desired level using 1N hydrochloric acid or sodium hydroxide, then the final volume was made up to 1 liter with distilled water. The medium was sterilized by autoclaving at 15 psi (121°C) for 15 minutes.

Each step was performed in duplicate. Organic manure was in dry condition which further dissolved in tap water for biomass production of plants. After each day, separated plant biomass was sampled for 25 consecutive days and analyzed for starch content. Further protein, glucose, and fructose were also examined for the highest starch enhancement day.

2.3.2 Plant Biomass Collection and Production

Lemna minor was collected from cemented tanks of the Department of Zoology, University of Delhi, India. In-house production of duckweed plants was performed in plastic pots of 0.5 m in diameter and 0.6 m in depth (7-liter tap water) at Delhi NCR in August. 75 gm of organic manure was dissolved in it. This organic manure is made up of cow dung, leaves of margosa, mustard, gum Arabic, and other trees. The tap water contains traces of bleaching powder and potash alum. The plant biomass has a doubling capacity in approximately 3 days. The growth rate was determined by Bergmann's method with some modification. Fresh plant biomass was weighed and Dry weight was obtained after sun-drying biomass of *Lemna minor*.

Growth rate = increased dry weight area $^{-1}$ time^{-1} (gm m^{-2} week^{-1})

Figure 2.1: Lemna minor plant initially grown for starch enhancement experiments in A: organic manure, B: Nitrogen free hoagland media, and C: full strength Hoagland media

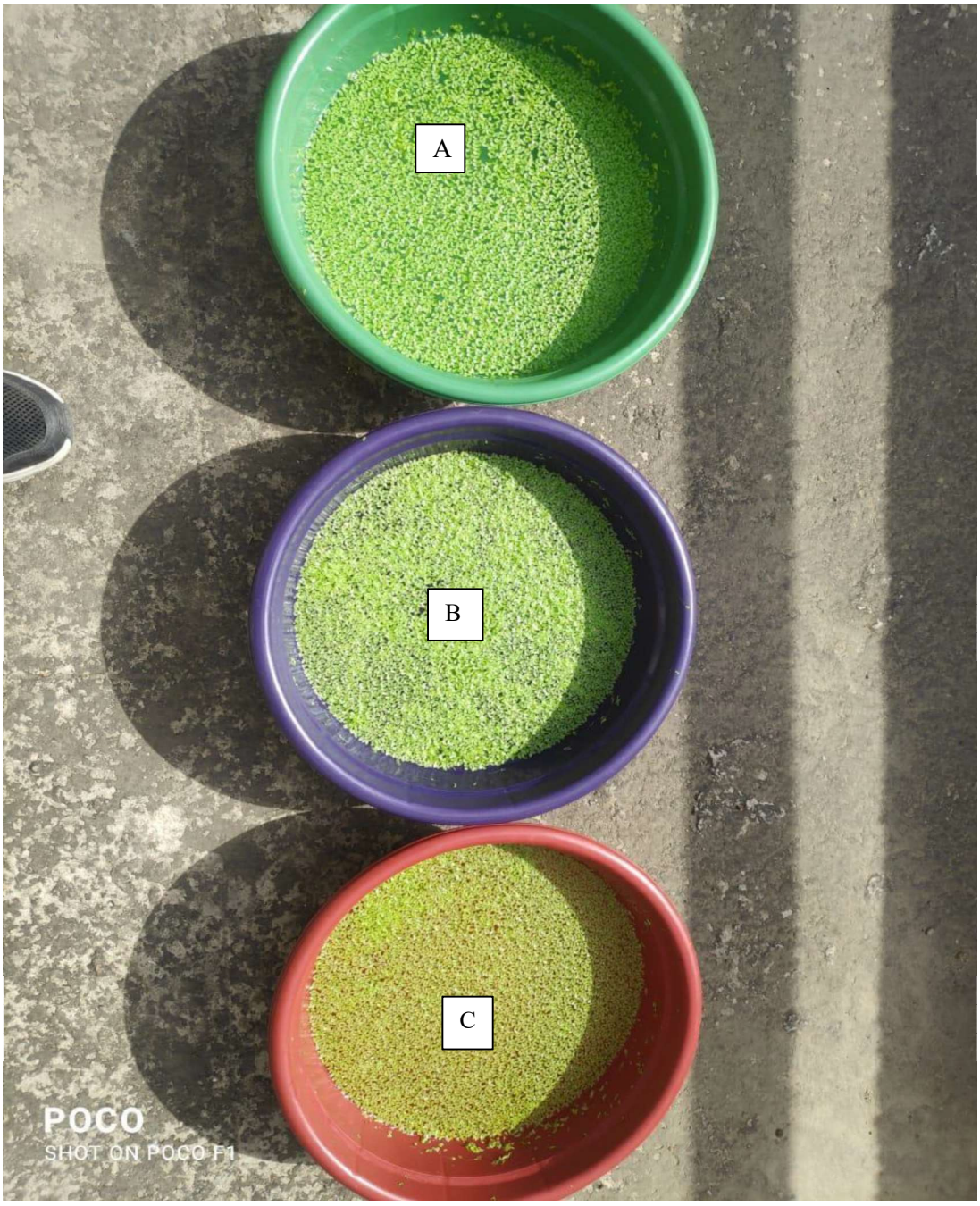


Figure 2.2: Lemna minor plant growth on 9th day in A: organic manure, B: Nitrogen free hoagland media, and C: full strength Hoagland media

2.3.3 Starch enhancement experiment

After sufficient biomass production in organic manure, 125 gm plant biomass was collected and transferred in 5.4 gm of nitrogen-free Hoagland media dissolved in 4.5 liters of tap water. The experiment was conducted for 25 consecutive days. On each day plant biomass was collected by using a strainer and dried in sunlight. The duckweed biomass was monitored every day for starch composition until the starch quantity became stabilized. The water lost due to evaporation was replaced with tap water every day throughout the experimental time. When the starch content of the duckweed grown in tap water was dropped and became stable, the experiment was stopped. This experiment was again performed for starch-enhanced plant production. On the 9^{th} day, the duckweed biomass was collected using a strainer. It was scattered onto a concrete board and sun-dried for two days. The dried biomass was collected in plastic bags, sealed, and stored in the refrigerator.

2.3.4 Compositional analysis of plant biomass

The starch analysis protocol was taken from the magazyme total starch assay procedure (amyloglucosidase/α-amylase method; AA/AMG 11/01, AOAC Method 996.11-2002). The starch quantity of *Lemna minor* was found on a dry basis.

To determine total starch content b AOAC method, approximately 90–100 mg of the sample was grinded to a fine powder. For samples containing free sugars or dextrins, extracted twice using 10 ml of 80% ethanol at 80°C, centrifuge, and discard the supernatant. Wet the sample with 0.2 ml of 80% ethanol, add 3.0 ml of thermo stable α-amylase solution, and boil for a total of 5 minutes, vortexes intermittently. Cooled to 50°C and then add 4.0 ml of sodium acetate buffer (pH 4.5) and 0.1 ml of amyloglucosidase (AMG) solution. It was then incubated at 50°C for 30 minutes with vortex mixing. Transferred the hydrolyze solution into a 100 ml flask, diluted with the help of distilled water, and mixed well. Centrifuge aliquots as needed. For color development, pipette 0.1 or 1.0 ml of the supernatant into test tubes, add GOPOD reagent, and incubate at 50°C for approximately 20 minutes. The absorbance was measured at 510 nm. Prepare glucose standards (50 and 100 µg in 100 µL), run blanks, and calculate glucose concentration. Convert to starch content using a factor

of 0.9 (glucose to starch). For resistant starch analysis, pre-treat samples with cold 1.7–2 M NaOH or hot dimethyl sulfoxide (DMSO), neutralize, and proceed with α-amylase hydrolysis.

Starch (%) = (Absorbance of sample− Blank /Slope of glucose calibration) × (Dilution Volume/ Sample Mass) × 0.9 × 100

Crystalline cellulose content was estimated using Updegraff reagent method (Bauer C Ibá, 2014). In this method dry plant biomass sample has been grinded. Soluble sugars and lipids were removed by washing with 85% ethanol, centrifuged, and the alcohol-insoluble residue (AIR) has been collected. The Updegraff reagent, [mixture of acetic acid, nitric acid, and water in a ratio of 8:1:2 (v/v/v)] has been added 1.0 ml per sample tube.The tubes were sealed and heated in a boiling water bath at approximately 98–100°C for about 30 minutes to dissolve hemicelluloses and lignin. Then the tubes were cooled and centrifuged (e.g., 10 minutes at ~12000 × g), remove the supernatant was removed and pellet was washed several times with water, then with acetone to remove residual reagents. The pellet was dried and collected.

Cellulose (%) = ([Glucose] assay (µg/mL) × V hydrolysate (mL)) × 0.90× 100/1000 × m sample (mg)

Glucose and fructose estimation were done by the DNS method at 540 nm for the same plant biomass. Reducing sugar analysed by the Dinitro salicylic acid method (Miller et al., 1959) briefly 1 ml of extracted hydrolysate with an equal amount of DNS solution (3,5 dinitro salicylic acid 1 gm, Na2SO3 0.05 gm, sodium potassium tartrate 18.2 gm, NaOH 1 gm, and phenol 0.2 gm per 100 mL of H2O) was heated for 10 min at 100 °C and optical density at 540 nm was recorded upon cooling using standard glucose curve (1mg/ml). Total reducing sugars (glucose and fructose) were calculated with the help of following formula:

mg Sugar recovered (ml) = Volume of liquid hydrolysate ÷ Amount of sample taken for hydrolysis × 100

The graph was prepared by plotting O.D. against the concentration gradient of glucose and fructose solutions. The quantitative estimation of glucose and fructose in an

unknown solution was determined by a calibration curve.

Nitrogen and protein content were estimated by method given by (kjeldahl 1883). The process was completed in three different steps: Digestion: in this step 1 gm sample, 7gm potassium sulphate, 0.8 gm cupric sulphate, 4 drop hydrogen peroxide and 12 ml sulfuric acid was taken in 170 ml test tube and heated at 420 °C for one hour. Distillation: after digestion sample was subjected to distillation by 40 gm/l boric acid and 400 gm/l sodium hydroxide. Methyl red (7 ml) and bromo cynol green (10 mL) were used as an indicator dye (Foss). Titration: titration was done with 0.1 N HCl. After 3 steps the protein content is determined by the Kjeldahl method by using the following formula-

Calculation of Nitrogen content (N) = 1.4 X (A-B)X Normality of HCl

A= volume of HCl used in the sample titration

B= volume of HCl used in the blank titration

Calculation of total protein

= N X 6.25

Whereas 6.25 is the dextrose factor as observed in the table.

Statics: Each level of treatment for starch enhancement was performed in duplicate. The one-way Analysis of Variance (ANOVA) can be utilized for the case of a quantitative outcome with categorical explanatory starch percentage variable levels. Statistical significance was accepted at the $P < 0.05$ level.

2.4 Microorganism and Maintenance:

Saccharomyces cereviceae MTCC-171, *Candida shehatae* MTCC-12913 and *Aspergillus niger*MTCC-12987 were procured from the Institute of Microbial Technology, Chandigarh (MTCC), India. Lyophilized cultures were sub-cultured in their respective media. *Aspergillusniger*weresubcultured in potato dextrose agar (PDA) slant

1 L of PDA was made by dissolving 4 gm potato extract, 20 gm dextrose, and 15 gm agar in 900 ml of distilled water, heated and stirred until fully dissolved, then adjust the volume to 1 L and autoclaved at 121 °C (~15 psi) for 15 minutes. After cooling to about 45–50 °C, 0.1 mg/ml solution of chlorteteracyclin was added to inhibit bacteria, mixed gently, and poured ~20–25 ml into sterile Petri dishes, allowing the medium to solidify. While *Saccharomyces cereviceae* and *Candida shehatae* were cultured in yeast peptone dextrose agar (YPDA) slant and incubated at 37°C for five days.

For preparing Yeast Peptone Dextrose (YPD) 1 l of medium, dissolve 20 gm peptone, 10 gm yeast extract, 20 gm dextrose, and 15 gm agar in 900 ml distilled water, heat and stir until all components are fully dissolved, cool slightly and adjust the volume to 1 l, check that the pH is about 6.5 ± 0.2 (at 25 °C), sterilize by autoclaving at 121 °C for 15 minutes. After cooling to about 45–50 °C, 0.1 mg/ml solution of chlorteteracyclin was added to inhibit bacteria, mixed gently, and poured ~20–25 ml into sterile Petri dishes, allowing the medium to solidify.

Preliminary screening of amylase production of *Aspergillus niger* was performed by standard starch agar plate method. Starch agar was prepared by dissolving the required nutrients (Peptone: 5 gm/ l, Yeast extract: 1.5 gm/ l, Soluble starch: 2.0 gm/ l, NaCl: 5.0 gm/ l, Agar: 15 gm/ l, Distilled water to 1 l, Final pH ≈ 7.2–7.4). It was autoclaved at 121 °C (~15 psi) for 15 minutes. After cooling to about 45–50 °C, 0.1 mg/ml solution of chlorteteracyclin was added to inhibit bacteria, mixed gently, and poured ~20–25 ml into sterile Petri dishes, allowing the medium to solidify. The test microorganisms were inoculated by streaking cultures on a starch agar plate and incubated at 37°C for two days followed by pouring gram's iodine solution over the plate. After flooded the plate with iodine solution: unhydrolyze starch turns dark blue with iodine, while zones where starch has been broken down by microbial amylase remain clear, indicating positive starch hydrolysis. Clear zone formation around the microbial colonies is an indication of the amylolytic ability of microbes.

Figure 2.3: Amylase production of *Aspergillus niger*determination by standard starch agar plate method, yellow zone indicate the hydrolysis of starch

All the fungal cultures were sub-cultured in their respective broth for two to three days while incubating at 30°C in an orbital shaker rotating at 230 rpm. For culturing of *Aspergillus niger* 1 L PD broth made by dissolve 4 gm potato extract, 20 gm dextrose in 900 mL of distilled water, heated and stirredupto full dissolution, then finally made the volume to 1 L and autoclave at 121 °C (~15 psi) for 15 minutes. When cooled to about 45–50 °C, sterile chlortetracycline (~40 mg/l) added to inhibit bacteria, mix gently.

Saccharomyces cereviceae and *Candida shehatae*were cultured in yeast peptone dextrose agar (YPDA) slant and incubated at 37°C for five days. For preparing 1 L of yeast peptone dextrose broth, dissolve 20 gm peptone, 10 gm yeast extract, 20 gm dextrose, and in 900 ml distilled water, heated and stirred until all components were fully dissolved, cooled slightly and made the volume to 1 L, sterilized by autoclaving at 121 °C for 15 minutes.

2.5 Optimization of various physical and chemical variables for the production of amylase enzyme

Optimization of cultural conditions for maximum amylase production was done by using classical approach of one factor at a time. All the batch experiments were carried out by putting 1ml inoculum of *Aspergillus niger* MTCC-12987 in each of 250 ml conical flask containing modified PDY (Potato Dextrose Yeast) broth. Baseline media has been maintained atparameters: Copra meal 3 % w/v, pH: 4, Time for incubation : 8 day, Temperature: 30°C, Minerals (.02% w/v): KH_2PO_4, Nitrogen source (0.025% w/v): Yeast extract, Carbon source (4% w/v): Potato starch .

Maximum enzymatic activity was determined by varying various parameters such as substrate concentration, pH, temperature, incubation time, metal ions, nitrogen, and carbon sources. The effect of different variables on enzyme activity was monitored separately by adjusting it systematically withall other variables constant. All the experiments were performed in replicates.

2.5.1 Effect of substrate concentration

The optimization of substrate concentration was done with supplementation Tris HCl buffer-soaked copra meal concentration ranging from 1%-5% (w/v). 50 ml of baseline liquid medium kept at different concentrations of copra meal within 250 ml Erlenmeyer flasks. Each flask was autoclaved for 15 minutes at 121°C and 15 psi pressure. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.5.2 Effect of pH variation

pH optimization involves analysis of enzyme activity across a range of pH 3-5. 50 ml of baseline liquid medium kept at different pH ranges within 250 ml Erlenmeyer flasks. Each flask was autoclaved for 15 minutes at 121°C and 15 psi pressure. After cooling of medium, 0.1mg/ml solution of chloramphenicol has been addedto inhibit any undesirable bacterial growth. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant as in base media except (pH and optimized parameter: 3% (w/v) copra meal). All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by

putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.5.3 Effect of time variation

The time of incubation was optimized by measuring enzyme activity on the 4^{th}, 6^{th}, 8^{th}, 10^{th}, and 12^{th} day of incubation. 50 ml of baseline liquid medium kept at different time variation within 250 ml Erlenmeyer flasks. Each flask was autoclaved for 15 minutes at 121°C and 15 psi pressure. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant in base media except the time for incubation with (optimized parameters: copra meal 3% and pH 4). All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.5.4 Effect of Temperature variation

Temperature optimizations were done by varying temperature between 20°C-40°C temperature. 50 ml of baseline liquid medium kept at different temperatures within 250 ml Erlenmeyer flasks. Each flask was autoclaved for 15 minutes at 121°C and 15 psi pressure. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant as in base media except temperature with (optimized parameters: copra meal 3%, pH 4

and samples were collected on 8th day). All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.5.5 Effect of metal ions

The effect of each metal ion was done separately on the activity of the amlolyticenzymatic cocktail, by adding 0.02% concentration of the $CaSO_4$, $ZnCl_2$, K_2HPO_4, KH_2PO_4, $MnCl_2$, $MgSO_4$, and KCl. 50 ml of baseline liquid medium kept at different metal ions with (0.02% w/v) concentrations within 250 ml Erlenmeyer flasks. Each flask was autoclaved for 15 minutes at 121°C and 15 psi pressure. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant as in base media except the previously optimized substrate concentration (Optimized parameters: copra meal 3%, pH 4, samples were collected on 8th day at 30°C). All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.5.6 Effect of different nitrogen sources:

For determining the effect of different nitrogen sources, yeast extract was replaced with 0.025% w/v peptone, $NaNO_3$, $(NH)_4SO_4$, NH_4Cl, and $KNO_3$to study the effect

on enzyme activity. 50 ml of baseline liquid medium kept at different nitrogen sources (0.025% w/v) within 250 ml Erlenmeyer flask. Each flask was autoclaved for 15 minutes at 121°C and 15 psi pressure. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant as in base media except different nitrogen sources (0.025% w/v) with (optimized parameters: copra meal 3%, pH 4, samples were collected on 8^{th} day at 30°C with metal ion concentration 0.02% w/vKH_2PO_4). All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.5.7 Effect of different carbon sources:

For the optimization of carbon sources, different substrates like cellulose, pectin, guar gum, cellobiose, and carboxymethyl cellulose (CMC) were used at 0.025% concentration. 50 ml of baseline liquid medium kept at different carbon sources (0.025%) with in every 250 ml Erlenmeyer flask. Each flask was autoclaved for 20 minutes at 121°C and 15 psi pressure. After cooling, 0.1mg/ml solution of chloramphenicol was added to the medium to inhibit any undesirable bacterial growth. All variables remain constant as in base media except Different carbon sources at (optimized parameters: copra meal 3% (w/v), pH 4, time duration 8^{th} day at 30°C with metal ion 0.02% (w/v)KH_2PO_4, 4% (w/v) Potato starch). All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by putting 1 ml inoculum of *Aspergillus niger* MTCC-12987. It was incubated in an orbital shaker rotating at 230 rpm.To estimate the enzyme activity 1 ml of each sample was centrifuged at 8000 rpm for 10 minutes and crude supernatant was used for analysis.The amylolytic activity of enzymes was assessed after 24 hours of incubation with soluble starch. The released maltose sugar was quantified using the dinitrosalicylic acid (DNS) method; a maltose standard curve was used for quantification.All the experiments were performed in replicates.

2.6 Optimization of various physical and chemical variables for the manufacturing of maximum ethanol production:

Optimization of maximum ethanol production was performed with four factors namely pH, temperature, *Lemna minor* biomass, and enzymatic cocktail concentration by using monoculture and coculture of *Saccharomyces cereviceae* MTCC-171 and *Candida shehatae*MTCC-12913 fungal culture. Each experimental set was performed for 72 hours followed by estimation of ethanol production at different time intervals. The method used for ethanol estimation is potassium dichromate colorimetric assay at the wavelength 580 nm. Later the optimization conditions for ethanol production analyzed by gas chromatography. Baseline media: (For 25 ml media composition) composition as follows- Substrate concentration (Lemna minor biomass): 1.75 g, Amylolytic cocktail: 6ml, pH: 5, Temperature: 35 °C, Yeast extract: 75 mg, Peptone: 75 mg, $(NH_4)_2SO_4$: 30 mg, KH_2PO_4: 30 mg, $MgSO_4 \cdot 7H_2O$: 7.5 mg, $CaCl_2$:1.5 mg

2.6.1 Effect of substrate concentration (Lemna minor biomass):

The optimization of substrate concentration was done with supplementation of Tris HCl buffer-soaked Lemna minor ranging from 0.5 g- 3 g. Different concentration of *Lemna minor* biomass was taken in 25 ml of baseline liquid medium in 200 ml Erlenmeyer flaskswereautoclaved at 121°C and 15 psi pressure for 15 minutes. After cooling of medium, 0.1mg/ml solution of chloramphenicol was added to inhibit any undesirable bacterial growth.All variables remain constant as in base media except the substrate concentration. All the batch experiments were carried out by using monoculturing and coculturing of *Saccharomyces cereviceae* MTCC-171 and *Candida shehatae* MTCC-12913 fungal culture. Culture media was then incubated for 72 hours at 30°C and 180 rpm in an orbital shaker. Samples were taken to track the synthesis of bioethanol at various time intervals (0, 3, 18, 24, 48 and 72 hours).A series of ethanol standards were prepared in deionized water (0.2–2 % v/v). Each unknown sample were mixed in a test tube with 5 ml of acidified $K_2Cr_2O_7$ reagent, 5 ml of acetate buffer (pH ~4.3–4.5), and 1 ml of sample or standard, and incubated at room temperature for 120 minutes. After incubation, the absorbance of the solution was measured at 580 nm against a blank solution. Then the absorbance values were measured using standards to plot a calibration curve (absorbance vs. ethanol concentration), which determined the ethanol concentration of samples. All the experiments were performed in replicates.

2.6.2 Effect of Amylolytic cocktail concentration:

The optimization of amylolytic cocktail concentration was done by supplementating its concentration ranging from 4-8 ml. Different concentration of Amylolytic cocktail were put in 25 ml. of baseline liquid medium after cooling, which were previously autoclaved at 121°C and 15 psi pressure for 15 minutes. Also after cooling of medium, 0.1mg/ml solution of chloramphenicol has been added to inhibit any undesirable bacterial growth. All variables remain constant as in base media except the amylolytic cocktail concentration with (optimized parameter: *Lemna minor* biomass 1g). All the batch experiments were carried out by using monoculture and coculture of *Saccharomyces cereviceae*MTCC-171 and *Candida shehatae*MTCC-12913 fungal culture. Culture media was then incubated for 72 hours at 30°C and 180 rpm in an orbital shaker. Samples were taken to track the synthesis of bioethanol at various time intervals (0, 3, 18, 24, 48 and 72 hours).A series of ethanol standards were prepared in deionized water (0.2–2 % v/v). Each unknown sample were mixed in a test tube with 5 ml of acidified $K_2Cr_2O_7$ reagent, 5 ml of acetate buffer (pH ~4.3–4.5), and 1 ml of sample or standard, and incubated at room temperature for 120 minutes. After incubation, the absorbance of the solution was measured at 580 nm against a blank solution. Then the absorbance values were measured using standards to plot a calibration curve (absorbance vs. ethanol concentration), which determined the ethanol concentration of samples. All the experiments were performed in replicates.

2.6.3 Effect of pH variation:

pH optimization involves analysis of ethanol production across a range of pH 4-6. Different pH adjusted in 25 ml. of baseline liquid medium in 200 ml Erlenmeyer flasks. Each flask was autoclaved at 121°C and 15 psi pressure for 15 minutes. After cooling of medium, 0.1mg/ml solution of chloramphenicol has been added to inhibit any undesirable bacterial growth. All variables remain constant as in base media except the previously at (optimized parameters: *Lemna minor* biomass 1g, amylolytic cocktail 7 ml). All the batch experiments were carried out by using monoculture and coculture of *Saccharomyces cereviceae* MTCC-171 and *Candida shehatae* MTCC-12913 fungal culture. Culture media was then incubated for 72 hours at 30°C and 180 rpm in an orbital shaker. Samples were taken to track the synthesis of bioethanol at various time intervals (0, 3, 18, 24, 48 and 72 hours).A series of ethanol standards were prepared in deionized water (0.2–2 % v/v). Each unknown sample were mixed in a test tube with 5 ml of

acidified $K_2Cr_2O_7$ reagent, 5 ml of acetate buffer (pH ~4.3–4.5), and 1 ml of sample or standard, and incubated at room temperature for 120 minutes. After incubation, the absorbance of the solution was measured at 580 nm against a blank solution. Then the absorbance values were measured using standards to plot a calibration curve (absorbance vs. ethanol concentration), which determined the ethanol concentration of samples. All the experiments were performed in replicates.

2.6.4 Effect of Temperature variation:

Temperature optimizations were done by varying temerature 25-45°C temperatures. Different temperature ranges adjusted in 25 ml. of baseline liquid medium in 200 ml Erlenmeyer flask. Each flask was autoclaved at 121°C and 15 psi pressure for 20 minutes. After cooling of medium, 0.1mg/ml solution of chloramphenicol has been added to inhibit any undesirable bacterial growth. All variables remain constant as in base media except the previously optimized temperature. All the batch experiments were carried out by using monoculture and coculture of *Saccharomyces cereviceae* MTCC-171 and *Candida shehatae* MTCC-12913 fungal culture. Culture media was then incubated for 72 hours at 30°C and 180 rpm in an orbital shaker. Samples were taken to track thesynthesisof bioethanol at various time intervals (0, 3, 18, 24, 48 and 72 hours).A series of ethanol standards were prepared in deionized water (0.2–2 % v/v). Each unknown sample were mixed in a test tube with 5 ml of acidified $K_2Cr_2O_7$ reagent, 5 ml of acetate buffer (pH ~4.3–4.5), and 1 ml of sample or standard, and incubated at room temperature for 120 minutes. After incubation, the absorbance of the solution was measured at 580 nm against a blank solution. Then the absorbance values were measured using standards to plot a calibration curve (absorbance vs. ethanol concentration), which determined the ethanol concentration of samples. All the experiments were performed in replicates.

2.7 Ethanol estimation in monoculturing and coculturing of *Candida shehatae* and *Saccharomyces cerevisiae*

Ethanol was estimated in different sets of performed experiments with microbial strains after 72 hours of incubation by GCMS (Agilent GC-6890 MS-5973). The GC system having an FID detector and a column (30 m ×250µm) was used for detection. Column temperature was initially maintained at 40°C; for 5min, heated up to a final temperature of 260°C at a rate of 12°C/min, and it has been maintained for 10 min.

Helium (He) was the carrier gas with a source temperature of 180 °C. The bioethanol concentration was quantified through the following formula where

Unknown concentration = Concentration of ethanol in the sample to be analyzed

Known concentration = standard ethanol concentration

Conc. Unknown (%) = Area unknown/ Area known X Conc. known

2.8 Statistical analysis:

RSM analysis was performed using Minitab. Probability ($p < 0.05$) values have been utilized for demonstrating the difference in outcomes.

2.9 Screening of main components using OFAT and response surface methodology (RSM):

The main component of each factor was screened by OFAT and their interactions were studied through RSM. Central composite design (CCD) was used to optimize conditions on seven factors or variables: pH (A), temperature (B), substrate (copra) concentration (C), KH_2PO_4 (%) (D), peptone (E), time of incubation (F) and carboxy methyl cellulose (G) and their effect on amylase activity. Similarly, four factors namely pH, temperature, *Lemna minor* biomass, and enzyme volume were evaluated for their effect on ethanol production by CCD. CCD runs in three points namely corner or factorial (2^n), axial (2n), and center (nc). Applying Equations (1), the total number of experimental runs was calculated.

$$N=2^n+2n+nc \qquad (1)$$

Where n is the independent variable or factor, nc is the total number of center points and total N is the total number of experimental runs.

A total of 88 runs comprising 64 factorial, 14 axial and 10 centers were designed to model and optimize amylase production while 31 experimental runs having 16 factorial, 8 axial and 7 centers were designed for ethanol production.

Table 2.1: Implementing RSM with Central Composite Design (CCD) for amylase an ethanol production with different parameters

For amylase production			
Total runs (N)	Factorial runs (n)	Axial runs (n)	Central runs (nc)
88	64	14	10
For ethanol production			
Total runs (N)	Factorial runs (n)	Axial runs (n)	Central runs (nc)
31	16	8	7

Minitab statistical software 22 was used to calculate the experimental run and data. The level of the independent variables and their experimental ranges for amylase activity and ethanol percentage (Y) are presented in Table 2.2

Table 2.2: Levels of independent variables considered for amylase and ethanol optimization

Amylase activity(U/ml)							
Description	**Variables**	**Unit**	**Levels**				
			-2	**-1**	**0**	**1**	**2**
pH	A	-	3	3.5	4	4.5	5
Temperature	B	°C	20	25	30	35	40
Coconut oil cake concentration	C	%(w/v)	1	2	3	4	5
KH_2PO_4	D	%(w/v)	0.001	0.01	0.02	0.03	0.04
Peptone	E	%(w/v)	0.0625	0.125	0.25	0.5	1
Time of incubation	F	Days	4	6	8	10	12
Carboxy Methyl Cellulose	G	%(w/v)	0.001	0.5	1	1.5	2
Ethanol production (%)							
pH	A	-	4	4.5	5	5.5	6
Temperature	B	°C	25	30	35	40	45
Lemna minor biomass	C	%	0.25	0.5	1	2	3
Amylase enzyme extract	D	ml	4	5	6	7	8

Table 2.3: A three-factor central composite design (CCD) model having coded and non-coded data created with Minitab software for amylolytic cocktail production

Run	Space type	Coded factor							Non coded factor							
		pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	Enzyme activity (U/ml)
1	Corner	-1	-1	-1	-1	-1	-1	1	3.5	25	2	0.01	0.125	6	2	842.86
2	Corner	1	-1	-1	-1	-1	-1	-1	4.5	25	2	0.01	0.125	6	0.5	740.71
3	Corner	-1	1	-1	-1	-1	-1	-1	3.5	35	2	0.01	0.125	6	0.5	880.86
4	Corner	1	1	-1	-1	-1	-1	1	4.5	35	2	0.01	0.125	6	2	741.43
5	Corner	-1	-1	1	-1	-1	-1	-1	3.5	25	4	0.01	0.125	6	0.5	903.86
6	Corner	1	-1	1	-1	-1	-1	1	4.5	25	4	0.01	0.125	6	2	764.43
7	Corner	-1	1	1	-1	-1	-1	1	3.5	35	4	0.01	0.125	6	2	809.14
8	Corner	1	1	1	-1	-1	-1	-1	4.5	35	4	0.01	0.125	6	0.5	850.71
9	Corner	-1	-1	-1	1	-1	-1	-1	3.5	25	2	0.03	0.125	6	0.5	920.71
10	Corner	1	-1	-1	1	-1	-1	1	4.5	25	2	0.03	0.125	6	2	826.57
11	Corner	-1	1	-1	1	-1	-1	1	3.5	35	2	0.03	0.125	6	2	826
12	Corner	1	1	-1	1	-1	-1	-1	4.5	35	2	0.03	0.125	6	0.5	867.57
13	Corner	-1	-1	1	1	-1	-1	1	3.5	25	4	0.03	0.125	6	2	849
14	Corner	1	-1	1	1	-1	-1	-1	4.5	25	4	0.03	0.125	6	0.5	890.57
15	Corner	-1	1	1	1	-1	-1	-1	3.5	35	4	0.03	0.125	6	0.5	890
16	Corner	1	1	1	1	-1	-1	1	4.5	35	4	0.03	0.125	6	2	795.86
17	Corner	-1	-1	-1	-1	1	-1	-1	3.5	25	2	0.01	0.5	6	0.5	920
18	Corner	1	-1	-1	-1	1	-1	1	4.5	25	2	0.01	0.5	6	2	825.86
19	Corner	-1	1	-1	-1	1	-1	1	3.5	35	2	0.01	0.5	6	2	825.29
20	Corner	1	1	-1	-1	1	-1	-1	4.5	35	2	0.01	0.5	6	0.5	866.86
21	Corner	-1	-1	1	-1	1	-1	1	3.5	25	4	0.01	0.5	6	2	848.29
22	Corner	1	-1	1	-1	1	-1	-1	4.5	25	4	0.01	0.5	6	0.5	889.86

Run	Space type	Coded factor							Non coded factor							
		pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	Enzyme activity (U/ml)
23	Corner	-1	1	1	-1	1	-1	-1	3.5	35	4	0.01	0.5	6	0.5	889.29
24	Corner	1	1	1	-1	1	-1	1	4.5	35	4	0.01	0.5	6	2	795.14
25	Corner	-1	-1	-1	1	1	-1	1	3.5	25	2	0.03	0.5	6	2	865.14
26	Corner	1	-1	-1	1	1	-1	-1	4.5	25	2	0.03	0.5	6	0.5	906.71
27	Corner	-1	1	-1	1	1	-1	-1	3.5	35	2	0.03	0.5	6	0.5	906.14
28	Corner	1	1	-1	1	1	-1	1	4.5	35	2	0.03	0.5	6	2	812
29	Corner	-1	-1	1	1	1	-1	-1	3.5	25	4	0.03	0.5	6	0.5	929.14
30	Corner	1	-1	1	1	1	-1	1	4.5	25	4	0.03	0.5	6	2	835
31	Corner	-1	1	1	1	1	-1	1	3.5	35	4	0.03	0.5	6	0.5	902.29
32	Corner	1	1	1	1	1	-1	-1	4.5	35	4	0.03	0.5	6	0.5	876
33	Corner	-1	-1	-1	-1	-1	1	-1	3.5	25	2	0.01	0.125	10	0.5	917.43
34	Corner	1	-1	-1	-1	-1	1	1	4.5	25	2	0.01	0.125	10	2	823.29
35	Corner	-1	1	-1	-1	-1	1	1	3.5	35	2	0.01	0.125	10	2	822.71
36	Corner	1	1	-1	-1	-1	1	-1	4.5	35	2	0.01	0.125	10	0.5	864.29
37	Corner	-1	-1	1	-1	-1	1	1	3.5	25	4	0.01	0.125	10	2	845.71
38	Corner	1	-1	1	-1	-1	1	-1	4.5	25	4	0.01	0.125	10	0.5	887.29
39	Corner	-1	1	1	-1	-1	1	-1	3.5	35	4	0.01	0.125	10	0.5	886.71
40	Corner	1	1	1	-1	-1	1	1	4.5	35	4	0.01	0.125	10	2	792.57
41	Corner	-1	-1	-1	1	-1	1	1	3.5	25	2	0.03	0.125	10	2	862.57
42	Corner	1	-1	-1	1	-1	1	-1	4.5	25	2	0.03	0.125	10	0.5	904.14
43	Corner	-1	1	-1	1	-1	1	-1	3.5	35	2	0.03	0.125	10	0.5	903.57
44	Corner	1	1	-1	1	-1	1	1	4.5	35	2	0.03	0.125	10	2	809.43
45	Corner	-1	-1	1	1	-1	1	-1	3.5	25	4	0.03	0.125	10	0.5	926.57
46	Corner	1	-1	1	1	-1	1	1	4.5	25	4	0.03	0.125	10	2	832.43

Run	Space type	Coded factor							Non coded factor							
		pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	Enzyme activity (U/ml)
47	Corner	-1	1	1	1	-1	1	1	3.5	35	4	0.03	0.125	10	2	831.86
48	Corner	1	1	1	1	-1	1	-1	4.5	35	4	0.03	0.125	10	0.5	873.43
49	Corner	-1	-1	-1	-1	1	1	1	3.5	25	2	0.01	0.5	10	2	861.86
50	Corner	1	-1	-1	-1	1	1	-1	4.5	25	2	0.01	0.5	10	0.5	903.43
51	Corner	-1	1	-1	-1	1	1	-1	3.5	35	2	0.01	0.5	10	0.5	902.86
52	Corner	1	1	-1	-1	1	1	1	4.5	35	2	0.01	0.5	10	2	808.71
53	Corner	-1	-1	1	-1	1	1	-1	3.5	25	4	0.01	0.5	10	0.5	925.86
54	Corner	1	-1	1	-1	1	1	1	4.5	25	4	0.01	0.5	10	2	831.71
55	Corner	-1	1	1	-1	1	1	1	3.5	35	4	0.01	0.5	10	2	831.14
56	Corner	1	1	1	-1	1	1	-1	4.5	35	4	0.01	0.5	10	0.5	872.71
57	Corner	-1	-1	-1	1	1	1	-1	3.5	25	2	0.03	0.5	10	0.5	942.71
58	Corner	1	-1	-1	1	1	1	1	4.5	25	2	0.03	0.5	10	2	848.57
59	Corner	-1	1	-1	1	1	1	1	3.5	35	2	0.03	0.5	10	2	848
60	Corner	1	1	-1	1	1	1	-1	4.5	35	2	0.03	0.5	10	0.5	889.57
61	Corner	-1	-1	1	1	1	1	1	3.5	25	4	0.03	0.5	10	2	871
62	Corner	1	-1	1	1	1	1	-1	4.5	25	4	0.03	0.5	10	0.5	912.57
63	Corner	-1	1	1	1	1	1	-1	3.5	35	4	0.03	0.5	10	0.5	912
64	Corner	1	1	1	1	1	1	1	4.5	35	4	0.03	0.5	10	2	817.86
65	Axial	-2.8	0	0	0	0	0	0	3	30	3	0.02	0.25	8	1	1086.86
66	Axial	2.8	0	0	0	0	0	0	5	30	3	0.02	0.25	8	1	907
67	Axial	0	-2.828	0	0	0	0	0	4	20	3	0.02	0.25	8	1	1109.57
68	Axial	0	2.828	0	0	0	0	0	4	40	3	0.02	0.25	8	1	1074.14
69	Axial	0	0	-2.828	0	0	0	0	4	30	1	0.02	0.25	8	1	1053
70	Axial	0	0	2.8284	0	0	0	0	4	30	5	0.02	0.25	8	1	1067.71

Run	Space type	Coded factor							Non coded factor							
		pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	pH	temp (°C)	Copra (%)	KH2PO4 (%)	Peptone (%)	Time of incubation (days)	CMC (%)	Enzyme activity (U/ml)
71	Axial	0	0	0	-2.82843	0	0	0	4	30	3	0.001	0.25	8	1	1076.71
72	Axial	0	0	0	2.828427	0	0	0	4	30	3	0.04	0.25	8	1	945.57
73	Axial	0	0	0	0	-2.8284271	0	0	4	30	3	0.02	0.0625	8	1	935
74	Axial	0	0	0	0	2.8284271	0	0	4	30	3	0.02	1	8	1	1030
75	Axial	0	0	0	0	0	-2.8284271	0	4	30	3	0.02	0.25	4	1	1049.29
76	Axial	0	0	0	0	0	2.8284271	0	4	30	3	0.02	0.25	12	1	1047.29
77	Axial	0	0	0	0	0	0	-2.828	4	30	3	0.02	0.25	8	0.001	1036.57
78	Axial	0	0	0	0	0	0	2.828	4	30	3	0.02	0.25	8	2	1014.14
79	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
80	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
81	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
82	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
83	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
84	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
85	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
86	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
87	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005
88	Centre	0	0	0	0	0	0	0	4	30	3	0.02	0.25	8	1	1005

Table 2.4: Model analysis for amylase production from *Aspergillus niger* sp. using ANOVA test.

Source	DF	Seq SS	Contribution	F-Value	P-Value	Remark
Model	14	485036	76.81%	17.27	0.000	Significant
Linear	7	174631	27.66%	3.42	0.003	
pH	1	31375	4.97%	14.14	0.000	
temperature (°C)	1	7295	1.16%	4.41	0.039	
Coconut oil cake concentration	1	173	0.03%	0.01	0.920	
KH2PO4 (%)	1	3047	0.48%	0.31	0.582	
Peptone (%)	1	666	0.11%	0.72	0.400	
Time of incubation (days)	1	3033	0.48%	2.02	0.159	
Carboxy Methyl Cellulose (%)	1	129042	20.44%	2.26	0.137	
Square	7	310405	49.16%	22.11	0.000	
pH*pH	1	97485	15.44%	13.33	0.000	
temperature (°C)*temperature (°C)	1	21646	3.43%	0.35	0.557	
Coconutoil cake concentration* Coconut oil cake conc.	1	29918	4.74%	2.58	0.113	
KH2PO4 (%)*KH2PO4 (%)	1	56411	8.93%	12.00	0.001	
Peptone (%)*Peptone (%)	1	9497	1.50%	4.09	0.047	
Time of incubation (days)*Time of incubation (days)	1	22644	3.59%	3.98	0.050	
Carboxy Methyl Cellulose (%)* Carboxy Methyl Cellulose (%)	1	72802	11.53%	36.30	0.000	
Error	73	146422	23.19%			
Lack-of-Fit	64	146422	23.19%	*	*	
Pure Error	9	0	0.00%			
Total	87	631458	100.00%			

Table 2.5: Model summary statistics for optimal amylase production (response Y)

Source	S	R-sq	R-sq(adj)	Press	R-sq(pred)	Remarks
Linear	75.5667	27.66%	21.33%	530208	16.03%	
Linear + Square	44.7859	76.81%	72.37%	2995016	0.00%	Suggested
Linear + Interaction	87.1609	29.02%	0.00%	754047	0.00%	
Full quadratic	51.5231	78.14%	63.43%	3031637	0.00%	

Table 2.6: A three-factor central composite design (CCD) model having coded and non-coded data created with Minitab software for bioethanol production

Run	Space type	Coded factor				Non coded factor				
		pH	Temp (^{0}C)	Lemna minor biomass (%)	Amylase enzyme extract (ml)	pH	Temp (^{0}C)	Lemna minor biomass (%)	Amylase enzyme extract (ml)	Ethanol (%)
1	Corner	-0.5	-0.5	-0.5	-0.5	4.5	30	0.5	5	9.35
2	Corner	0.5	-0.5	-0.5	-0.5	5.5	30	0.5	5	9.725
3	Corner	-0.5	0.5	-0.5	-0.5	4.5	40	0.5	5	9.975
4	Corner	0.5	0.5	-0.5	-0.5	5.5	40	0.5	5	10.35
5	Corner	-0.5	-0.5	0.5	-0.5	4.5	30	2	5	9.7
6	Corner	0.5	-0.5	0.5	-0.5	5.5	30	2	5	10.075
7	Corner	-0.5	0.5	0.5	-0.5	4.5	40	2	5	10.325
8	Corner	0.5	0.5	0.5	-0.5	5.5	40	2	5	10.7
9	Corner	-0.5	-0.5	-0.5	0.5	4.5	30	0.5	7	9.775
10	Corner	0.5	-0.5	-0.5	0.5	5.5	30	0.5	7	10.15
11	Corner	-0.5	0.5	-0.5	0.5	4.5	40	0.5	7	10.4
12	Corner	0.5	0.5	-0.5	0.5	5.5	40	0.5	7	10.775
13	Corner	-0.5	-0.5	0.5	0.5	4.5	30	2	7	10.125
14	Corner	0.5	-0.5	0.5	0.5	5.5	30	2	7	10.5
15	Corner	-0.5	0.5	0.5	0.5	4.5	40	2	7	10.75
16	Corner	0.5	0.5	0.5	0.5	5.5	40	2	7	11.125
17	Axial	-1	0	0	0	4	35	1	6	10.95
18	Axial	1	0	0	0	6	35	1	6	11.5
19	Axial	0	-1	0	0	5	25	1	6	10.225
20	Axial	0	1	0	0	5	45	1	6	11.025
21	Axial	0	0	-1	0	5	35	0.25	6	10.375
22	Axial	0	0	1	0	5	35	3	6	10.825
23	Axial	0	0	0	-1	5	35	1	4	11.225

Run	Space type	Coded factor				Non coded factor				
		pH	Temp (^{0}C)	Lemna minor biomass (%)	Amylase enzyme extract (ml)	pH	Temp (^{0}C)	Lemna minor biomass (%)	Amylase enzyme extract (ml)	Ethanol (%)
24	Axial	0	0	0	1	5	35	1	8	11.4
25	Centre	0	0	0	0	5	35	1	6	11.625
26	Centre	0	0	0	0	5	35	1	6	11.625
27	Centre	0	0	0	0	5	35	1	6	11.625
28	Centre	0	0	0	0	5	35	1	6	11.625
29	Centre	0	0	0	0	5	35	1	6	11.625
30	Centre	0	0	0	0	5	35	1	6	11.625
31	Centre	0	0	0	0	5	35	1	6	11.625

Table 2.7: Model summary statistics for optimal ethanol production (response Y)

Source	S	R-sq	R-sq(adj)	Press	R-sq(pred)	Remarks
Linear	0.66542	0.2135	0.0925	15.0852	0	
Linear + Square	0.41735	0.7382	0.643	29.1425	0	Suggested
Linear + Interaction	0.75842	0.2141	0	30.0066	0	
Full quadratic	0.48885	0.7388	0.5102	35.8634	0	

Table 2.8: Model analysis for ethanol production from *Lemna minor* sp. using ANOVA test

Source	DF	Seq SS	Contribution	F-Value	P-Value
Model	8	10.8058	73.82%	7.75	0.000
Linear	4	3.1255	21.35%	5.22	0.004
pH	1	0.7004	4.79%	4.02	0.057
Temperature	1	1.8150	12.40%	10.42	0.004
Lemna minor biomass (%)	1	0.0241	0.16%	3.06	0.094
Amylase enzyme extract (ml)	1	0.5859	4.00%	3.36	0.080
Square	4	7.6803	52.47%	11.02	0.000
pH*pH	1	0.5361	3.66%	7.47	0.012
Temperature*Temperature	1	2.8491	19.46%	21.68	0.000
Lemna minor biomass (%)*Lemna minor biomass (%)	1	3.2477	22.19%	20.64	0.000
Amylase enzyme extract (ml)*Amylase enzyme extract (ml)	1	1.0474	7.16%	6.01	0.023
Error	22	3.8319	26.18%		
Lack-of-Fit	16	3.8319	26.18%	*	*
Pure Error	6	0.0000	0.00%		
Total	30	14.6377	100.00%		

Chapter 3

Result and Discussions

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Starch enhancement experiments result in *Lemna minor*

3.1.1 Environmental conditions and biomass production of duckweed (*Lemna Minor*)

The starch-enhanced experiment was performed in early September in the year 2023. The temperature variations in the overall experiment are shown in Figure 3.1. Throughout the four-week biomass cultivation period, the temperature of the duckweed water varied between 79°F to 95°F.

The pH remains slightly acidic during the whole experiment, as given in Figure 3.2. 200 g of fresh duckweed was harvested after four weeks when cultured in organic manure. The growth rate observed was 6.5gm/m^2/week (dry weight) in organic manure.

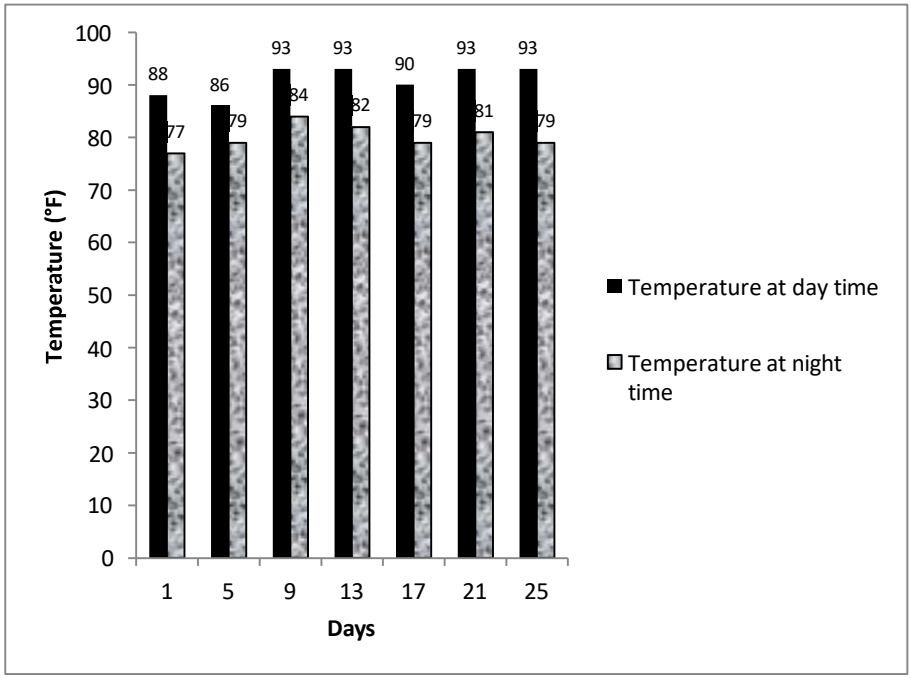


Figure 3.1: Temperature (°F) during starch Enhancement

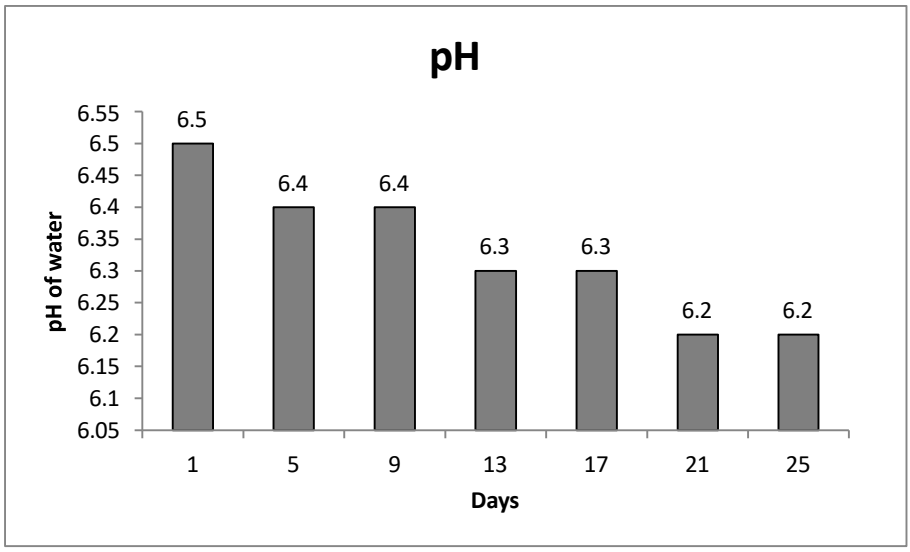


Figure 3.2: pH of water during starch enhancement

Optimal temperature and pH levels suggest that the experimental setup maintained stable conditions suitable for *Lemna minor* growth. Extreme variation could point to environmental stress affecting biomass production and starch accumulation. Temperature affects enzyme activity involved in the starch biosynthetic pathway, while pH affects nutrient absorption. There are no significant differences in pH or temperature trends found during treatments. By observing temperature and pH trends, it has been concluded that the increase in starch content is due to nitrogen deprivation and not due to the environmental shifts.

3.1.2 Starch accumulation triggering

Lemna minor starch enhancement experiment exhibits that this duckweed could have emerged as a helpful industrial feedstock in bioethanol production. The starch and protein contents of the duckweed before starch accumulation were 7% and 20% respectively. Starch was enhanced in *Lemna minor* very rapidly in nitrogen-free Hoagland media during the first couple of days, as shown in Figure 3.3. It was found that starch content occurred at its highest concentration on the 9th day. It was estimated up to 26 % with a standard deviation of ± 0.3, which was more than three times as high as the starch content of untreated *Lemna minor*. From the 9th day, it declined up to the 21st day. The starch was quantified up to the 25th day. From the 21st

day, starch reduction was stabilized at 5.5 % ± 0.2. During the starch enhancement experiment, the starch level was significantly ($P < 0.05$) higher in nitrogen-free Hoagland media compared to the organic manure.

The reduction in starch content after the 9th day in *Lemna minor* under nitrogen-free Hoagland media enhanced starch accumulation by altering carbon fixation from the protein synthesis metabolic pathway (Xu et al., 2011 & Appenroth et al., 2010).

At the end of the experiment, the stabilization of 5.5% starch quantity suggests that *Lemna minor* reached a physiological equilibrium, where starch degradation and synthesis balanced each other. This is possibly due to the initiation of alternative metabolic pathways or increased respiration demand, which can lower overall starch accumulation (Sree et al., 2015).

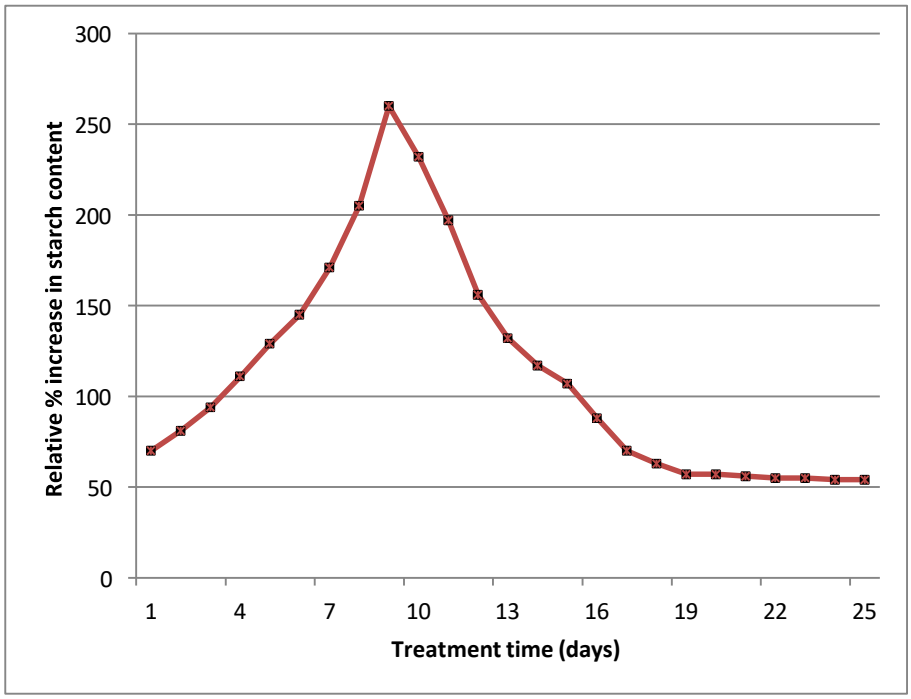


Figure 3.3: Relative percentage increase in starch concentration v/s time required for treatment with nitrogen-free Hoagland media

The values on the vertical axis of Figure 3.3 represent a percentage increase relative to a baseline value, the initial concentration of a specific biochemical component, starch. If 260% is shown, it suggests that the measured parameter increased 2.6 times (or

160% more) compared to its original or control value. If the baseline is 100%, then 260% means the value has increased 2.6-fold from its initial or reference level. The percentage scale helps visualize relative changes across different treatments rather than absolute concentrations.

During nitrogen starvation conditions, starch biosynthesis in *Lemna minor* is mainly regulated by ADP-Glucose Pyrophosphorylase (AGPase), Granule-Bound Starch Synthase (GBSS), Soluble Starch Synthase (SSS), and Starch Branching Enzyme (SBE). ADP-Glucose Pyrophosphorylase (AGPase) is a rate-limiting enzyme, converting glucose-1-phosphate into ADP-glucose, the precursor for starch synthesis. Due to nitrogen starvation, AGPase activity is upregulated, which provides more starch accumulation (Appenroth et al., 2010).

Granule-Bound Starch Synthase (GBSS) and Soluble Starch Synthase (SSS) enzymes polymerize ADP-glucose into amylose and amylopectin, respectively. Under nitrogen-deficient conditions, their expression increases, leading to higher starch deposition [14]. Another enzyme is Starch Branching Enzyme (SBE), which can form the α-1,6-glycosidic bonds in amylopectin, thus contributing to starch enhancement (Sree et al., 2015).

The bioethanol production from duckweed is notably credited to its high amount of carbohydrates, which are attributed to starch production. Starch content on the 9^{th} day in organic manure was found to be (8.5 % ± 0.4), in control media (6.2 % ± 0.4), and in nitrogen-free Hoagland media (26 % ± 0.3). It was observed that the starch content was drastically enhanced, and the protein content of the duckweed leveled off in nitrogen-free Hoagland media as given in Table 1. As given in the table, the concentration of glucose and fructose has shown moderate variation when compared to plant biomass grown in nitrogen-free Hoagland media, with plant biomass grown in organic manure and full-strength Hoagland media (Table 3.1)

Table 3.1: Percentage of Protein, Starch, Fructose, and Glucose in organic manure, nitrogen-free Hoagland and full-strength Hoagland Media in *Lemna minor* plant biomass

Constituent (%)	Organic Manure (±SD)	Nitrogen-Free Hoagland (±SD)	Full-Strength Hoagland (Control) (±SD)
Protein	20.28 ± 0.6	14.38 ± 0.4	22.5 ± 0.3
Starch	8.5 ± 0.4	26.0 ± 0.3	6.2 ± 0.4
Fructose	2.0 ± 0.2	1.4 ± 0.1	1.8 ± 0.2
Glucose	4.0 ± 0.3	2.6 ± 0.2	3.8 ± 0.3

Table 3.1 compares the percentage composition of protein, fructose, and glucose in *Lemna minor* cultured under three distinct nutrient conditions: organic manure and nitrogen-free Hoagland media with a control media (full-strength Hoagland media). The standard deviations (SD) represent variability in the measurements. The percentage of protein content was considerably lower in plants cultivated with nitrogen-free Hoagland media (14.38 % ± 0.4) in comparison to organic manure (20.28 % ± 0.6) and full-strength Hoagland media (22.5 % ± 0.3), signifying that nitrogen starvation leads to carbon fixation towards starch synthesis. The percentage of fructose content was also slightly elevated in organic manure-treated plants (2.0% ± 0.2) in comparison with full-strength Hoagland media (1.8 % ± 0.2) and nitrogen-free Hoagland media (1.4% ± 0.1). Also, similar findings were observed for glucose percentage, for plants grown with organic manure (4.0% ± 0.3), full-strength Hoagland media (3.8 % ± 0.3), and in nitrogen-free Hoagland media (2.6% ± 0.2). It has been assumed that the concentration of glucose and fructose percentage is decreased due to consumption by the plant during starvation conditions. This data highlights the importance of nitrogen availability in relocating carbohydrate metabolism. The findings conclude that nitrogen deprivation may be a feasible method for optimizing starch accumulation in *Lemna minor*.

Due to less lignin and high starch content, aquatic duckweed (*Lemna minor)* has been selected as a suitable candidate for starch enhancement and further bioethanol production (Liu et al., 2020). When the nutrition starvation condition was applied to duckweed, the enzymes expressed in starch metabolic pathway were up-regulated, whereas the starch degradation cycle showed no significant changes. These

consequences resulted in the flux of carbon source into starch and thus enhanced starch composition in duckweed.

In this research work, Nitrogen-free Hoagland media triggers more starch accumulation in *Lemna minor*, mainly due to the plant's metabolic adaptation to nitrogen deficiency (Humphrey et al., 1977 & Yu et al., 2017). In nitrogen-deprivation conditions, plants modify the carbon flux pathway from protein production to initiating carbohydrate storage, which enhances starch accumulation (Kruger et al., 2020). In a nitrogen-starvation condition, minimum nitrogen availability restricted the synthesis of amino acids and thus proteins. So, the alternate metabolic pathway provides an excess of photosynthetically fixed carbon, fixing which promotes starch biosynthesis and storage (Appenroth et al., 2016). Hoagland media provides other crucial micronutrients, ensuring that starch biosynthesis is not controlled by deficiencies due to other essential elements. Thus, optimal carbon assimilation and further storage of it in the form of starch occurred in the plant (Xu et al., 2011 & Landolt et al., 1987)

When compared to organic manure, which is a rich source of nutrients (inorganic or organic form of nitrogen), this nitrogen can support protein synthesis (Geisseler et al., 2021 & Adame et al., 2024), thus dropping the metabolic synthesis of the starch accumulation mechanism (Ullah et al., 2022). Opiyo *et al.* 2023 reported that when compared to the regulated environment of Hoagland medium, the organic manure's heterogeneous makeup and changing accessibility to nutrients may control the dynamics of nitrogen release and more nutrient absorption by the plant, which results in less starch development.

Appenroth et al. 2010 studied the impact of nickel on the chloroplasts of two different duckweed species, *Spirodelapolyrhiza* and *Lemna minor*, where an enhancement in starch content of the plant was observed. Similar results of heavy metal stress were reported by Appenroth*et al.* 2001 for chromate and Sree *et al.* 2014 for cobalt. In contrast to heavy metal exposure, nitrogen starvation is an excellent method for enhancing starch accumulation in *Lemna minor*, enhancing starch quantity to over 40% of dry weight by redirecting carbon flux from protein synthesis (Xu et al., 2011).

Heavy metal stress copper (Cu>10 mg l (-1) and cadmium (Cd>0.5 mg l (-1) deteriorate the antioxidant system in duckweed and thus minimize overall biomass yield because of their toxicity (Hou et al., 2007). Nitrogen starvation is a suitable method for industrial-scale bioethanol manufacturing because it does not leave any toxic residues (Xu et al., 2011). Also, heavy metal exposure needs an expensive detoxification procedure before processing, making it unfeasible for large-scale production (Hou et al., 2007). Nitrogen starvation is an environmentally sustainable method and can be combined with wastewater treatment (Sree et al., 2015). In spite of this, heavy metal stress poses contamination risks, reducing its practical applicability. Thus, nitrogen starvation and starch enhancement are the preferred methods for large-scale bioethanol production (Chen et al., 2018).

For large-scale biomass production of starch to enhance *Lemna minor*, the use of heavy metals is not feasible as these toxic heavy metals, when they enter the environment, may lead to bioaccumulation and biomagnification, which means long-term persistence in animals or plant bodies, including humans, with increasing concentration (Gupta et al., 2024 & Chormare et al., 2022).

The present paper demonstrates a method that is eco-friendly and less expensive. The use of nitrogen-free media for the production of starch-enhanced *Lemna minor* is a promising approach for bulk starch production. By using nitrogen-free Hoagland media, starch was enhanced up to 26% which is 3 times more than the initial *Lemna minor* biomass (7%).

The protein content of duckweed has a positive correlation with the quantity of nitrogen provided, as reported by Chaiprapat et al. 2005 and Leng et al.1995. In the present study, Protein, Glucose, and fructose content were found to decrease (nitrogen-free Hoagland Media) during the starch enhancement in comparison to the other two growing media used for plant culturing (organic manure and full-strength Hoagland media). It is assumed that sugars were consumed during the starch-enhancement because the plant needs sugars to survive in starvation conditions (Van dyac et al., 2023). For large-scale bioethanol production, controlled nitrogen modulation is essential (Opiyo et al., 2023). This research paper provides insight into

a cyclic nutrient management approach, alternating nitrogen-sufficient (Organic manure) and nitrogen-deficient (Nitrogen-free Hoagland media) phases, which helps to provide sustainable growth while enhancing starch production (Tu et al., 2012).

3.2 Composition of Copra meal and Lemna minor biomass before and after starch enhancement

Copra meal and *Lemna minor* biomass was characterized for composition as mentioned in Table 3.2.

Table 3.2: Composition of Copra meal and *Lemna minor* biomass (Before and after starch enhancement)

Chemical constituents	Lemna minor (Before starch enhancement) (mg/100mg)	Lemna minor (After starch enahancement) (mg/100mg)	Copra meal (mg/100mg)
Glucose	4.6 ± 0.3	2 ± 0.3	0.2± 0.1
Fructose	2.3± 0.2	1± 0.2	0.3± 0.1
Cellulose	23 ± 0.3	23 ± 0.3	42 ± 0.5
Starch	7 ± 0.4	26 ± 0.4	1.6± 0.2
Protein	17.28 ± 0.6	14.60 ± 0.6	21± 0.4

By the analysis of chemical composition, It was found that *Lemna minor* was high in starch (26 ± 0.4) which makes it suitable for saccharifiaction and fermentation during ethanol production while protein rich value (21± 0.4) of copra meal makes its suitable for amylolytic enzymatic cocktail production. Copra meal is also rich in multi-minerals (Owolabi et al., 2018) which worked as additives during enzyme formation.

3.3 Classical optimization or one factor at one time (OFAT) to screen important components to optimize amylase production

For *Aspergillus niger* MTCC-12987 to grow and produce amylase there must be a substrate that will act as physical support as well as provide it with some form of energy. Hence the use of coconut meal substrate, which has good porosity and is therefore appropriate for filamentous fungus adhesion. The growth of amylase increased gradually with increased concentrations of substrates, up to a maximum value recorded at 3% concentration of coconut meal, giving amylase activity of 1178

U/ml. These findings showed that submerged-stage fermentation is a good strategy for amylase enzyme production (Baysal et al., 2003). The maximum amylase activity was found to be 1168 U/ml on the 8th day of incubation. After the 8th day of incubation, the activity of the enzyme was decreased. This could be due to less water involved or because amylase got destroyed by other conditions that were in the culture. The pH might have been altered due to excretion products (Kaliappan, et al.,2013 & Chugh et al., 2016) Increased amylase activity was found with KH_2PO_4(1178 U/ml), $CaSO_4$, and $MgSO_4$, while decreased amylase enzyme production was found with KCl, $MnCl_2$, and K_2HPO_4, which showed similar results as reported by Arshdeep *et al.*, 2020. Certain nitrogen sources, for example, peptone (1175U/ml), $NaNO_3$, $(NH)_4SO_4$, and NH_4Cl, significantly increased the level of amylase produced. However, amylase production decreased with $NaNO_3$, NH_4Cl, NH_4NO_3, and KNO_3 as nitrogen sources. Suganthi et al., 2011 reported enhanced amylase production with some organic and inorganic nitrogen supplements like peptone, sodium nitrate, casein hydrolysate, ammonium chloride, ammonium nitrate, and potassium nitrate. Maximum amylase activity of 1028 U/ml was found at 30°C, which is by the findings of Alva et al., 2007. Higher temperatures cause cell death due to alterations in cell metabolism. Varalakshmi et al., 2009 reported 22°C for optimal amylase activity by *Aspergillus niger* JGI 24. The pH of the fermentation medium is adjusted to 4, as the maximum enzyme activity of 1053 U/ml was observed, to support fungal culture and enzyme formation. Maximum amylase activity was reported within the range of 3 to 5 pH (Alva et al., 2007, Haq et al., 2002, Kaneko et al., 1996, Shafique et al., 2010) and subsequently decreased at pH 7. Some other researchers also reported that an acidic pH is required for amylase production from *Aspergillus niger* (Hernández et al., 2006, Mitidieri et al., 2006, Uguru et al., 1997). Carboxymethyl cellulose (1007 U/ml) was also found to be an important carbon source (Figure 3.4). Coconut meal, at 3%, is identified as the best enzyme producer. Optimized parameters for amylase production are pH 4, temperature 30°C, peptone 0.25%, carbon source (0.025%), and metal ions KH_2PO_4at 0.02% for amylase production, which produced 1187U/ml amylase enzyme by *Aspergillus niger*12987.

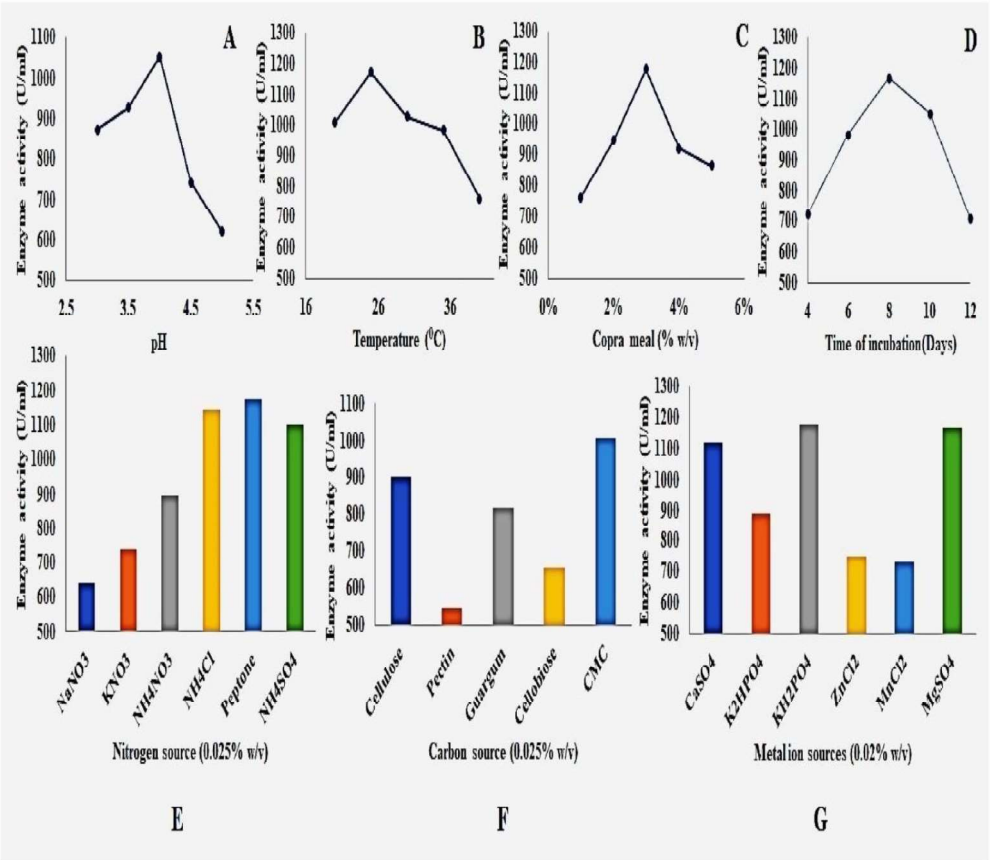


Figure 3.4: One factor at a time (OFAT) or classical optimization to screen important components of each factor for amylase production. Different factors are; A: pH, B: Temperature, C: Copra meal conc., D: Time of incubation, E: Nitrogen source, F: Carbon source, G: Metal ion source.

3.4 Classical optimization or one factor at one time (OFAT) to screen important components to optimize ethanol production

The production of ethanol were optimized concerning four parameters, namely the pH, temperature, *Lemna minor* biomass concentration, and the volume of enzyme used with monoculturing and coculturing of *Saccharomces cerevisiae* and *Candida shehatae.* In each group, ethanol concentration was also determined at different time intervals up to 72 hours.

Table 3.3: One factor at a time (OFAT) approach to screen important parameters for ethanol production. Different factors are; pH, Temperature, *Lemna minor* biomass, Amylase enzyme crude extract.

Optimization Parameters	**Saccharomces cerevisiae**	**Ethanol Yield (%)**	**Candida shehatae**	**Ethanol Yield (%)**	**Saccharomyces cerevisiae + Candida shehatae**	**Ethanol Yield (%)**
pH	5.3	9.79	5. 6	2.26	5.5	10.64
Temperature °C	37	10.27	33	1.78	35	10.89
Lemna minor Biomass (gm)	1	10.04	1	2.30	1	10.42
Amylase extract (ml.)	6.9	10.35	7.2	2.44	7	10.98

The production of ethanol was optimized concerning four parameters, namely the pH, temperature, *Lemna minor* biomass concentration, and the volume of enzyme used. In each group, ethanol concentration was also determined at different time intervals up to 72 hours. When subjected to monoculturing, The optimized parameters for ethanol yield were 5.3 pH, 37°C, 1% *Lemna minor* biomass, and 6.9 ml crude enzyme with monoculturing of *Saccharomces cerevisiae* and 5.6 pH, 33°C, 1% *Lemna minor* biomass, and 7.2 ml crude enzyme with monoculturing of *Candida shehatae.*The maximum ethanol production was reported at 5 pH, 35°C, 1% *Lemna minor* biomass, and 7 ml crude enzyme with coculturing of *Saccharomces cerevisiae* and *Candida shehatae* (Table 3.3).

Lin et al. 2012, also observed that 30°C –40°C was optimal for *Saccharomyces cerevisiae* BY4742, and at 50°C showed a decline production of ethanol. This might be due to the denaturation of membranous transport system enzymes and ribosomes at higher temperatures. Hu et al., 2012, reported that the optimum pH range of (4.0–8.0) for *Saccharomyces cerevisiae* JZ1C is good for bioethanol production which was similar to the findings of the present study. At lower pH cytoplasm of microorganisms is acidified and thus decreases in biomass yield (Hu et al., 2012).Optimizing and controlling these factors or parameters are crucial to achieving optimal ethanol yield in the fermentation process.

3.5 Response surface methodology (RSM) by Central composite design (CCD) model and data analysis for optimal amylase production

A three-factor central composite design (CCD) model created with Minitab software, which includes experimental data on amylase enzyme activity by *Aspergillus niger*issummarized in Table 2.3.

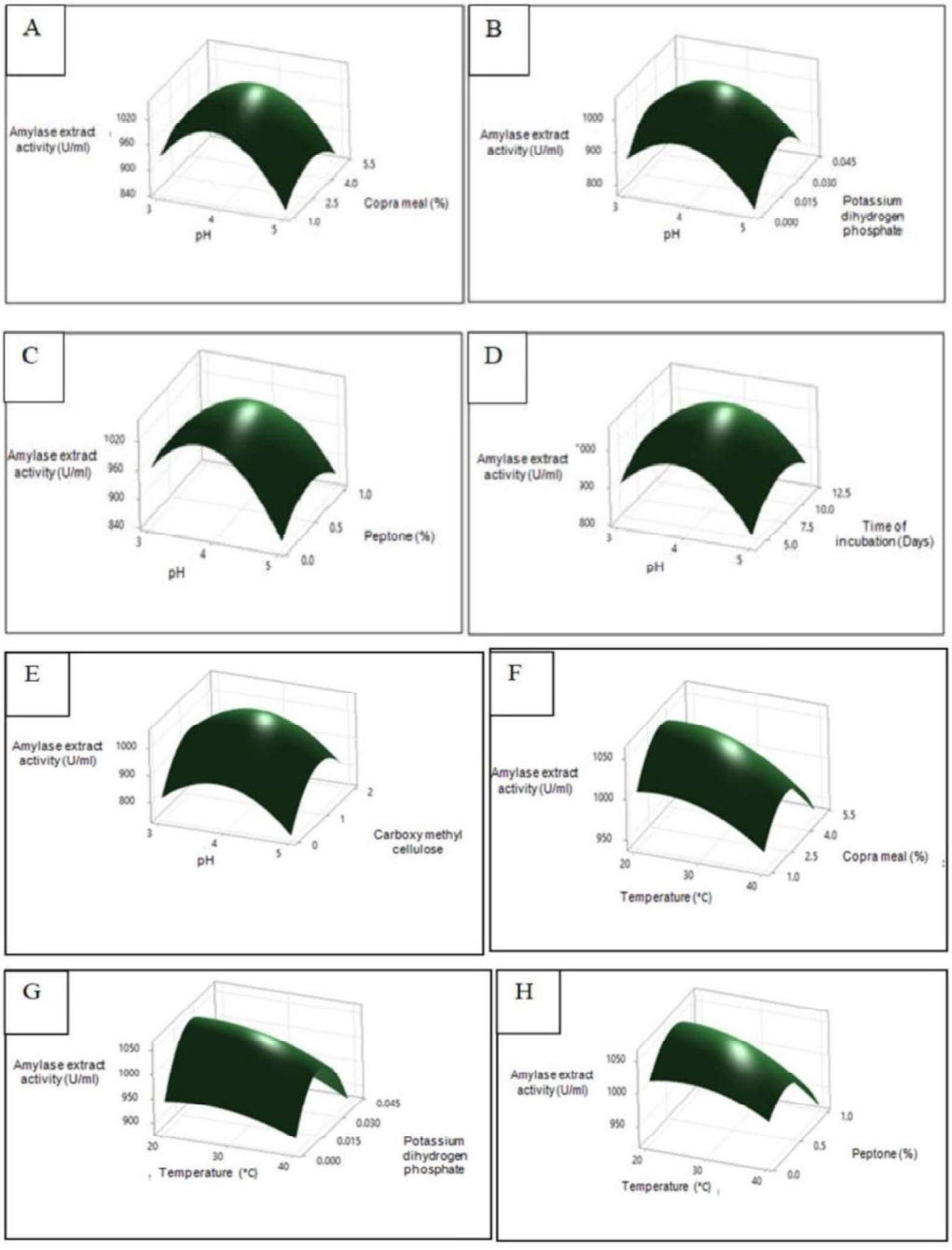
A Linear square model relating amylase activity in U/ml (response) was chosen by CCD (Table 2.5) and was represented as coded parameters by the linear equation as given in following Equation-

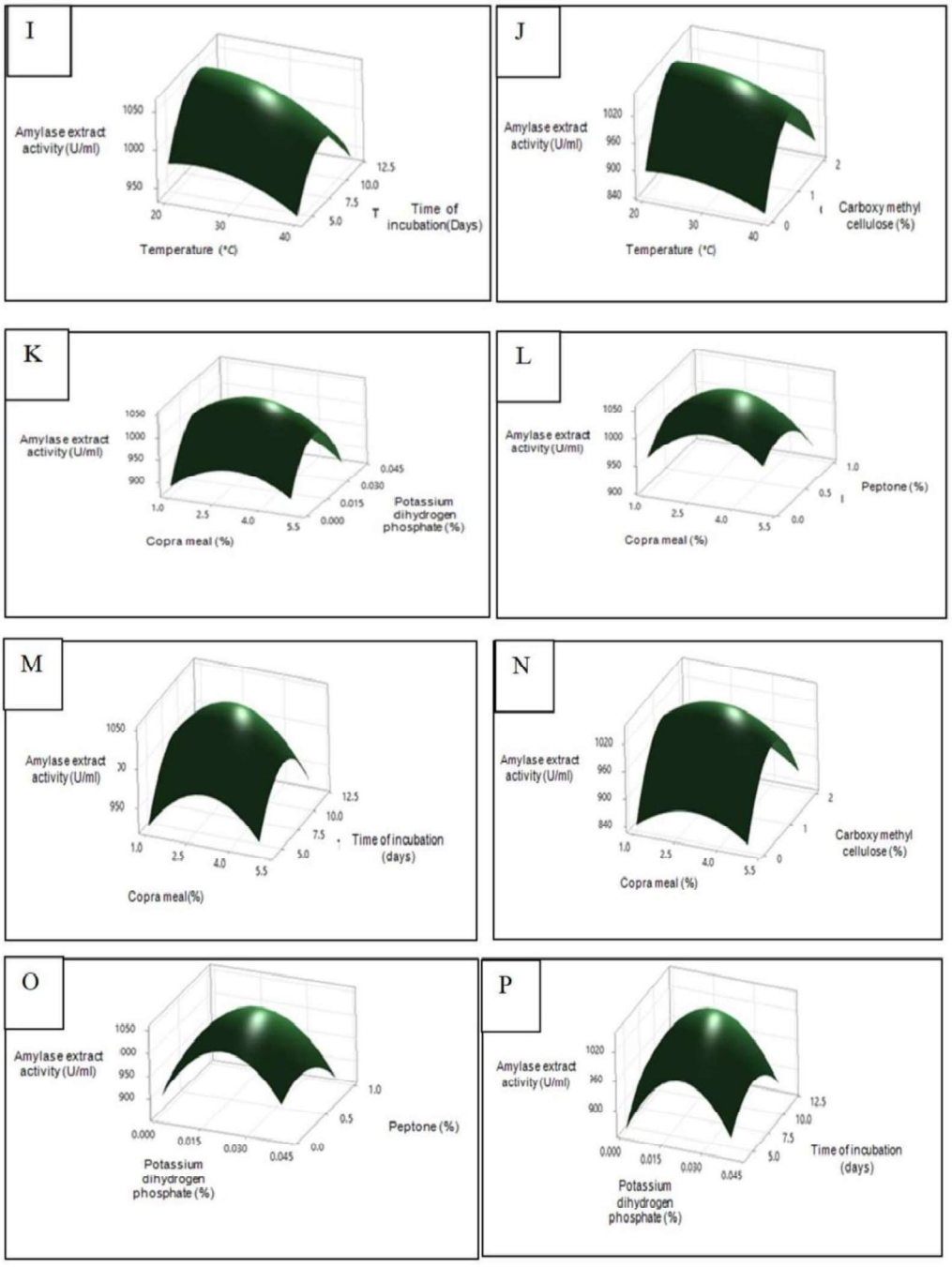
Enzyme activity(U/ml) = -1419+ 866pH + 8.8temperature(°C)
+75.3 Coconut oil cake concentration + 11772 KH2PO4 (%)
+ 194.8 Peptone (%) + 65.6Time of incubation (days)
+297.2 Carboxy Methyl Cellulose (%) - 113.2pH * pH
- 0.183 temperature (°C)*temperature (°C)
- 12.46 Coconut oil cake concentration * Coconut oil cake concentration
-279931 KH2PO4 (%) * KH2PO4 (%) – 218 Peptone (%) * Peptone (%)
- 3.87 Time of incubation (days) * Time of incubation (days)
- 138.5 Carboxy Methyl Cellulose (%) * Carboxy Methyl Cellulose (%)

An Analysis is of variance (ANOVA) was conducted to determine the significance of each factor interaction, and no significant interactions were discovered between the factors. The results indicated that the factors were independent of each other. ANOVA demonstrated the model's adequacy, with an F-value of 17.27 and a P-value of <0.0001(Table 2.4), indicating that it was adequate and very acceptable. The linear square process was proposed because the R^2 value of 0.768 and the smallest standard deviation (44.78) indicated that this model could explain 77% of the variability in the response and that the model was proved to be acceptable (Table 2.5).

The Lack of fit value rises as an outcome of the regression equation's inability fitting the experimental data since of its complex nature of natural variables and unpredictable interactions in between different biological ingredients that presentin coconut oil cake and peptone. Further, considering KH_2PO_4 has been demonstrated to

reduce pH stability and cause transition metabolism in fermentation, it led to an increase in lack of fit.




I
Amylase extract activity (U/ml)
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1000
950
20
30
40
Temperature (°C)
12.5
10.0
7.5
5.0
Time of incubation(Days)
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Amylase extract activity (U/ml)
1020
960
900
840
20
30
40
Temperature (°C)
2
1
0
Carboxy methyl cellulose (%)
K
Amylase extract activity (U/ml)
1050
1000
950
900
1.0
2.5
4.0
5.5
Copra meal (%)
0.045
0.030
0.015
0.000
Potassium dihydrogen phosphate (%)
L
Amylase extract activity (U/ml)
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950
900
1.0
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4.0
5.5
Copra meal (%)
1.0
0.5
0.0
Peptone (%)
M
Amylase extract activity (U/ml)
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1.0
2.5
4.0
5.5
Copra meal(%)
12.5
10.0
7.5
5.0
Time of incubation (days)
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Amylase extract activity (U/ml)
1020
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900
840
1.0
2.5
4.0
5.5
Copra meal (%)
2
1
0
Carboxy methyl cellulose (%)
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Amylase extract activity (U/ml)
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Potassium dihydrogen phosphate (%)
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Peptone (%)
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Amylase extract activity (U/ml)
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Potassium dihydrogen phosphate (%)
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10.0
7.5
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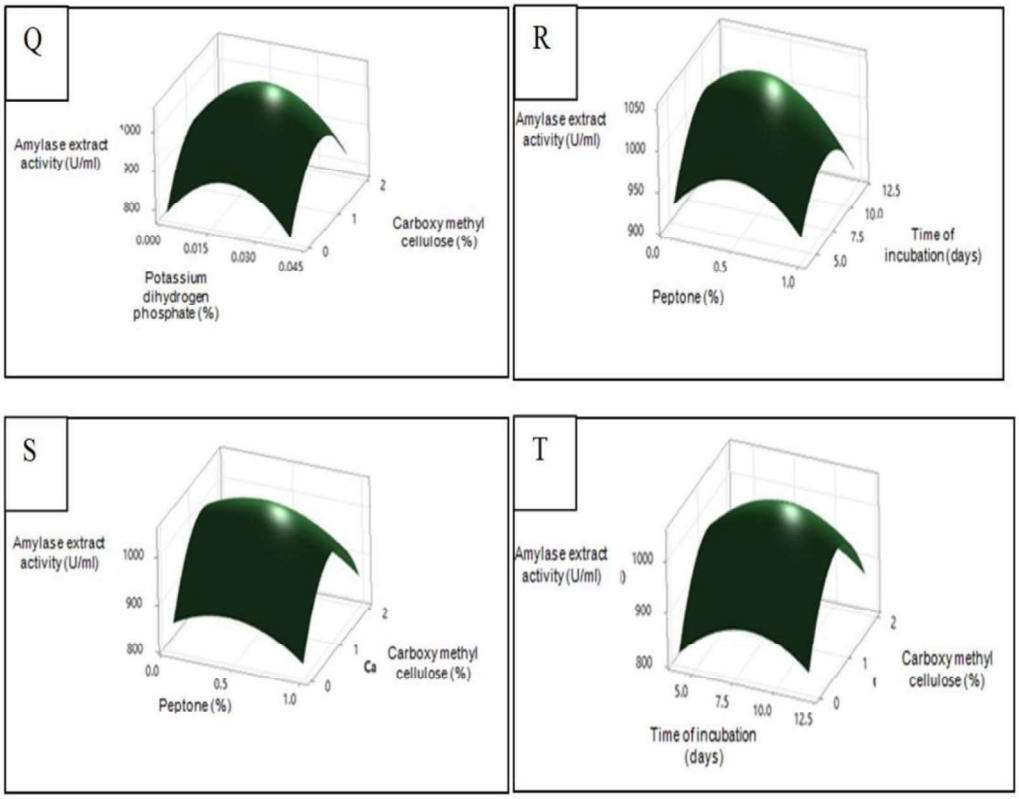
Time of incubation (days)



Hold Values	
pH	4
temperature (°C)	30
Coconut oil cake concentration	3
KH2PO4 (%)	0.0205
Peptone (%)	0.53125
Time of incubation (days)	8
Carboxy Methyl Cellulose (%)	1.0005

Figure 3.5: 3D surface plot of various combinations of each factor for Amylase cocktail preparation (A-T)

The model was found to be significant, and three-dimensional graphs were generated for regression analysis of the Central Composite Design, representing the response surfaces for the interaction effects of pH, temperature, copra meal, and time of incubation. These plots depicted the influence of several factors and the interaction effect of each parameter on the response (Figure 3.5 & 3.6).

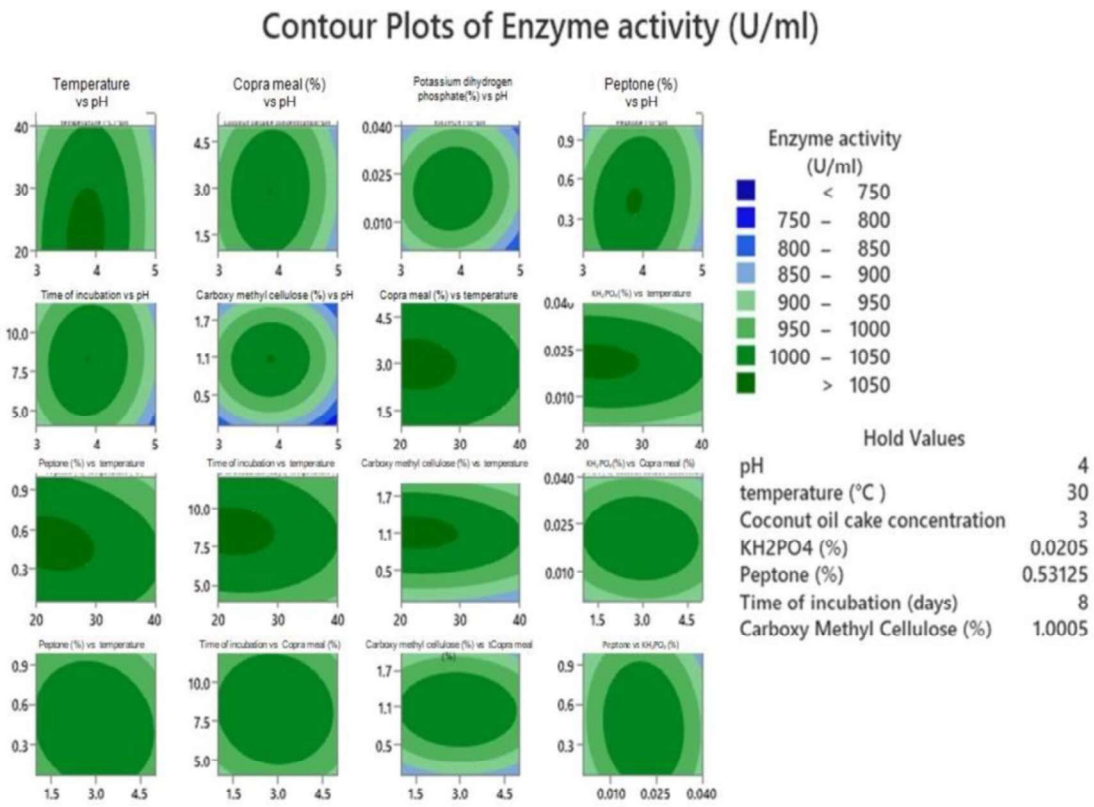


Figure 3.6: 3D Contour plot of various combinations of each factor for amylolytic cocktail production

The response optimizer program assessed the optimized medium with the highest amylase activity. For best enzyme synthesis, the media should have a pH of 3.83, a temperature of 22.2^{0}C, 2.94% copra, 0.021% potassium dihydrogen orthophosphate, 0.460% peptone, and 1.091% CMC, followed by 8.5 days of incubation with a projected activity of 1063.81U/ml (Figure 3.7). The highest enzyme activity value predicted by the software was closer to the experimental value of 1072.4 U/ml (at pH of 4, a temperature of 24, 3 copra, 0.02% potassium dihydrogen orthophosphate, 0.025% peptone, and 0.025% CMC indicating the applicability and accuracy of RSM in optimizing the enzyme optimization process.

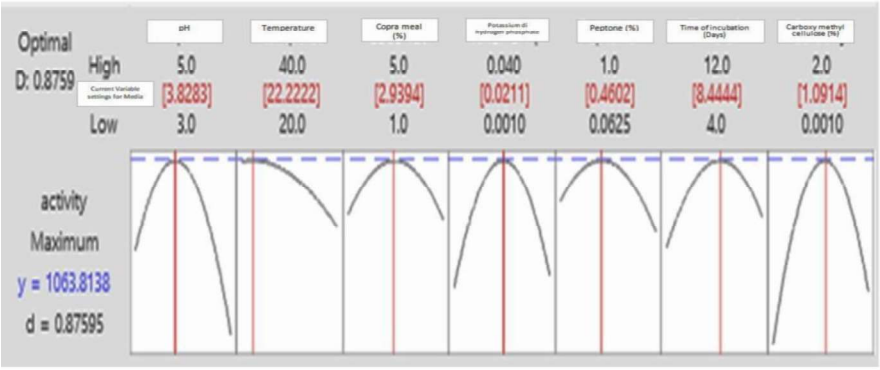


Figure 3.7: Optimized media for maximum amylase enzyme production produced by response optimizer.

3.6 Response surface methodology (RSM) by Central composite design (CCD) model and data analysis for optimal ethanol production

A three-factor central composite design (CCD) model created with Minitab software, which includes experimental data on ethanol production by *Lemna minor* is summarized in Table 2.6.

A Linear square model relating production in% (response) was chosen by CCD (Table 2.7) and was represented as coded parameters by the linear equation as given in the following Equation -

Regression Equation in Uncoded Units

Ethanol (%) = -40.40 + 8.87pH + 1.072Temperature + 2.097*Lemna minor*biomass (%)
+ 2.452 Amylase enzyme extract (ml) - 0.853 pH * pH
- 0.01453 Temperature * Temperature
- 0.713*Lemna minor*biomass(%)**Lemnaminor*biomass(%)
- 0.1913Amylase enzyme extract (ml) * Amylase enzyme extract (ml)

An Analysis of variance (ANOVA) was conducted to determine the significance of each factor interaction, and significant interactions were discovered between the factors. The results indicated that the factors were dependent on each other (Figure 3.8). ANOVA demonstrated the model's adequacy, with an F-value of 7.75 and a P-value of < 0.05 (Table 2.8), indicating that it was adequate and very acceptable. The

linear square process was proposed because the R^2 value of 0.738 and the smallest standard deviation (0.417) indicated that this model could explain 74% of the variability in the response and that the model was accurate (Table 2.7).

Both the amylase enzyme extract and the biomass of *Lemna minor* consist of several kinds of physiologically active compounds which might interact during the experiment and are difficult to predict statistically. When complex interactions occurred by fibers, secondary metabolites, and changing enzyme activity, it may become more challenging for the statistical model to produce a perfect fit. The pH at one point in time has an immediate impact on subsequent pH in continuous processes like simultaneous fermentation and saccharification. The pH is a logarithmic measurement of hydrogen ion concentration ($[H^+]$), even small numerical variations produce large variations in ionic concentration. A high Lack of Fit (LOF) value might have been a consequence of the model being unable to properly represent pH variations when the real relationship reflects a logarithmic trend. AdditionallyF-Value for the Model = 7.75, P-Value < 0.05, indicating the overall model is statistically significant and $R^2 = 0.738$, means the model captures most of the data variability.

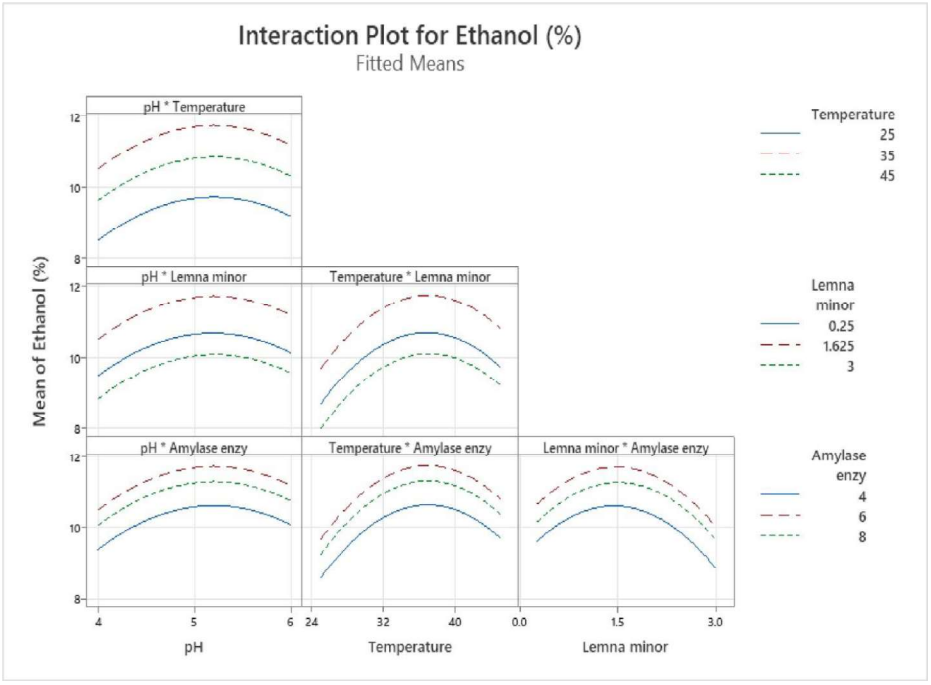


Figure 3.8: Interaction plot between the factors.

Three-dimensional graphics demonstrating the response surfaces for the interaction effects of pH, temperature, *Lemna minor* biomass, and crude enzyme volume have been developed for regression analysis of the Central Composite Design following the model had been established to be significant. These plots depicted the influence of several factors and the interaction effect of each parameter on the response (Figure 7 A-B).

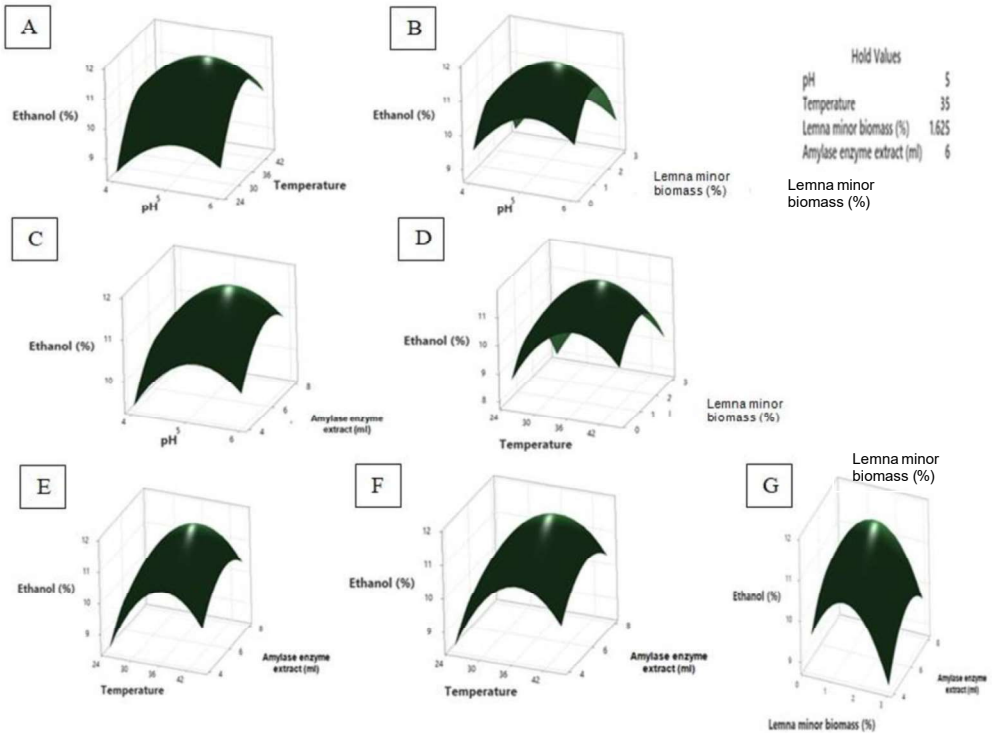


Figure 3.9: 3D surface plot of various combinations of each factor for ethanol production

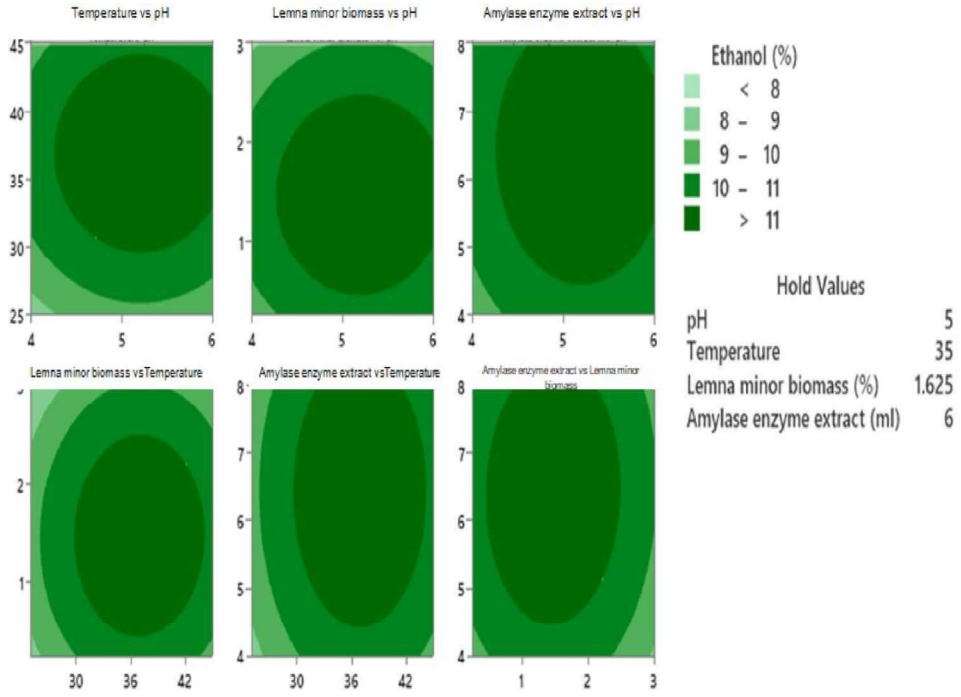


Figure 3.10: 3D Contour plot of various combinations of each factor for ethanol production

.The response optimizer programmer assessed the optimized medium with the highest ethanol production. For maximum ethanol production, the media should have a pH of 5.21, a temperature of 37°C, 1.47% *Lemna minor* biomass, and 6.42 ml of crude enzyme with a projected ethanol production of 11.83 % (Figure 3.11). The highest ethanol production predicted by the software was closer to the experimental value of 10.77% (at pH 5.5, Temp 35 °C, *Lemna minor* biomass 1gm, crude enzyme extract 7ml.) indicating the applicability and accuracy of RSM in optimizing enzyme optimization process.

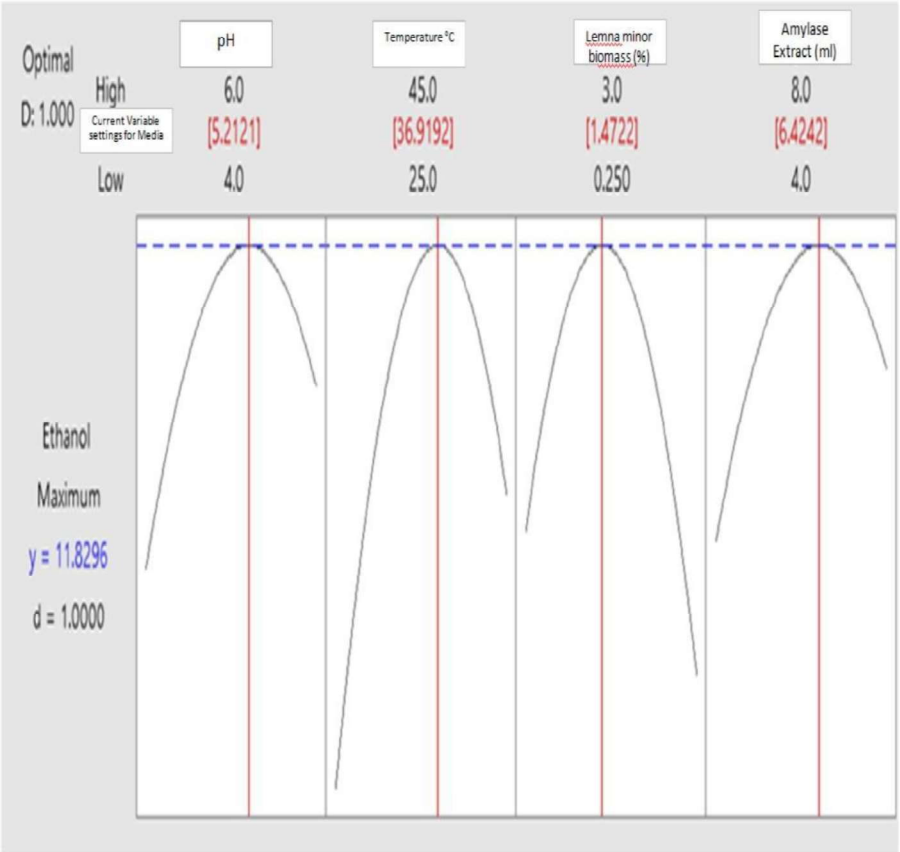


Figure 3.11: Optimized media for maximum ethanol production produced by response optimizer.

3.7 Ethanol estimation in monoculturing and coculturing of *Candida shehatae* and *Saccharomyces cerevisiae*:

The present work shows the efficient production of amylolytic cocktails and bioethanol formation. For this purpose, coconut meal and starch-enhanced duckweed (*Lemna minor*) were used as a substrate respectively. Apart from the selection of the substrates, in-house production of the amylolytic cocktail was done for the saccharification of *Lemna minor* biomass. After saccharification monoculturing and coculturing of *Saccharomyces cerevisiae* and *Candida shehatae* were done for efficient ethanol production. The idea behind this strategy is that monoculture of *Saccharomyces cerevisiae* ferments hexose sugars and monoculture of *Candida shehatae* ferments pentose sugars and when these microorganisms are cultured together, both hexoses and pentoses sugar fermentation can be achieved and in

present work, this strategy showed enhanced ethanol production in comparison to monoculturing. The GCMS analysis results of ethanol concentrations in monoculture and co-cultured *Saccharomyces cerevisiae* and *Candida shehatae* provide important information on their respective abilities to produce ethanol while using *Lemna minor* biomass as a raw substrate. Alcohol percentage with *Saccharomyces cerevisiae* was found to be 10.19% which is much higher than that of *Candida shehatae* generating only 2.32%. On the contrary, when both species were grown together the concentration of ethanol production was increased to 10.775% as shown in (Table 3.4, Figure 3.12 A-D). Hawaz et al., 2024, used cocultured *Saccharomyces cerevisiae* and *Wickerhamomyces anomalous* in RSM-optimized fermentation conditions using sugarcane molasses as an initial substrate for optimal ethanol production[44] but sugarcane molasses is a well-known food material used by around the world. The present study used nonfood biomass for bioethanol production. Hickert et al., 2013, found that coculturing of *Candida shehatae* HM 52.2 with *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate effectively utilized glucose and xylose simultaneously and increased ethanol yield. Similar results were also observed with the present research work with the advantage of that there is no use of synthetic medium.

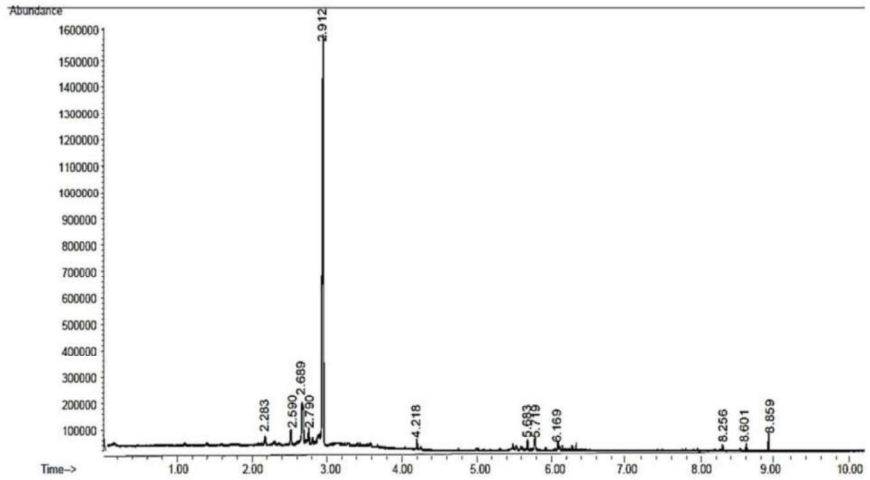


Figure 3.12: (A): GCMS of standard ethanol 95% (749.778 g/L ethanol)

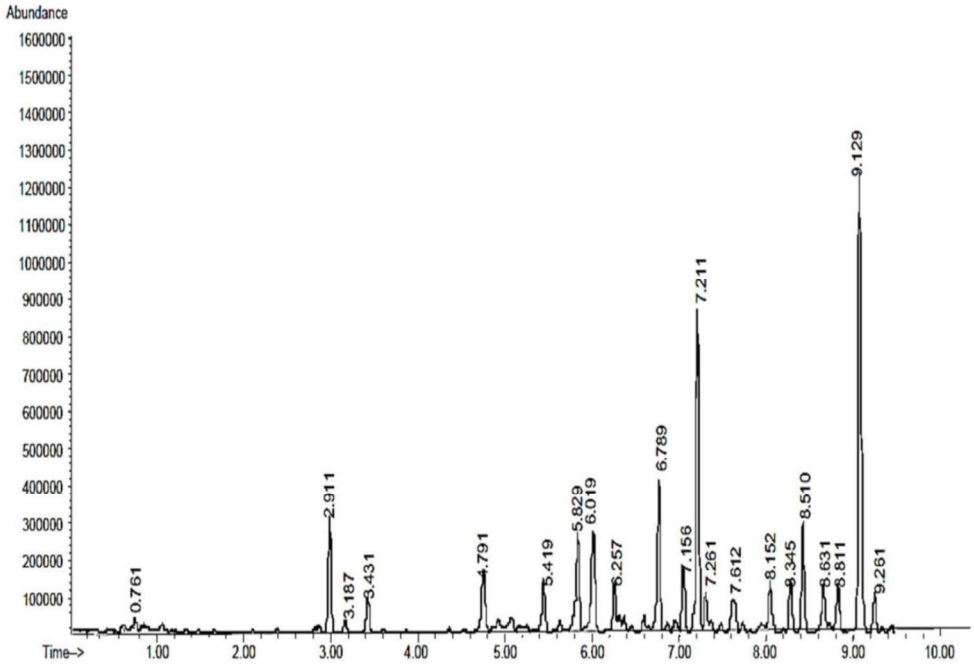


Figure 3.12 (B): GCMS of *Saccharomyces cerevisiae* cultured in optimized ethanol production media

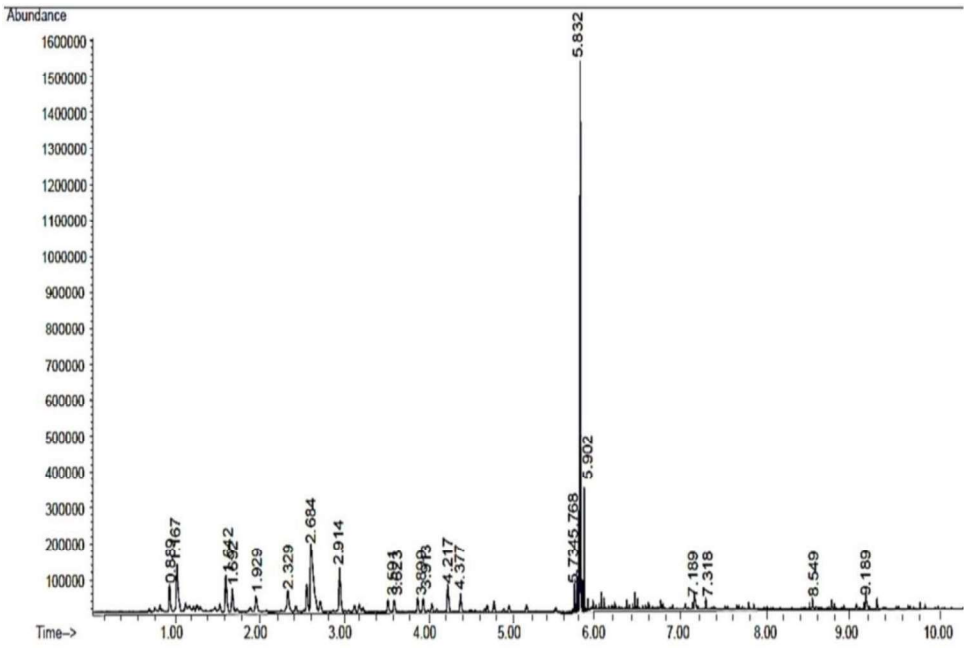


Figure 3.12 (C): GCMS of *Candida shehatae*cultured in optimized ethanol production media

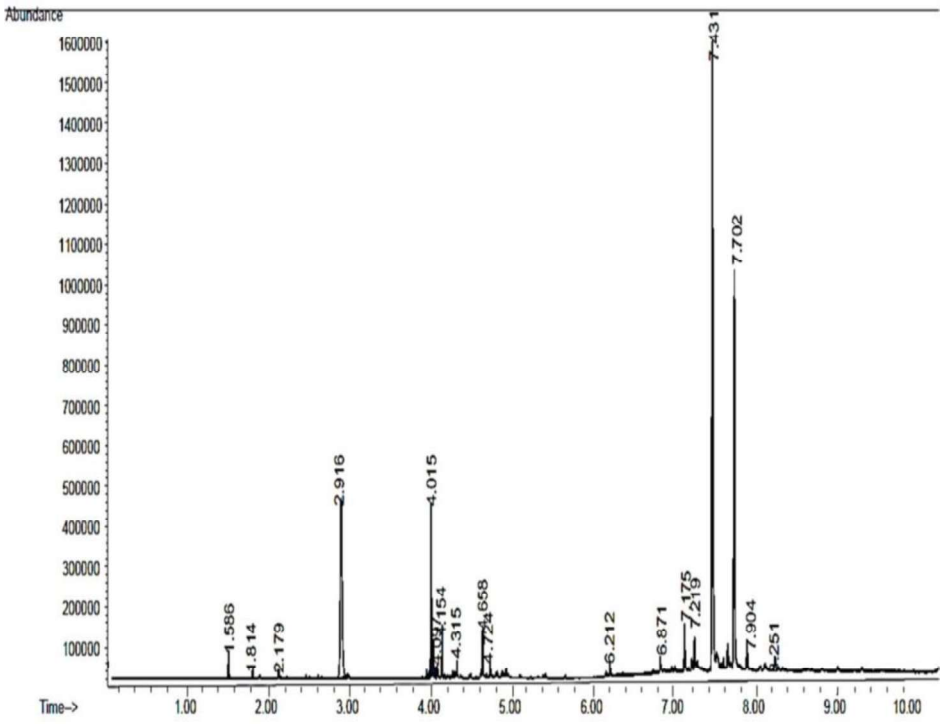


Figure 3.12 (D): GCMS of cocultured media.

The bioethanol concentration was quantified through the following formula where

Unknown concentration = Concentration of ethanol in the sample to be analyzed

Known concentration = standard ethanol concentration

Conc. Unknown (%) = Area unknown/ Area known X conc. known

Here area known- 849597895

Conc known- 4 ng/ µl

Now the percent of ethanol was quantified by the following formula:

Ethanol % (v/v) = Ethanol volume (µl)/ Total sample volume (µl) X 100

Density of ethanol is 0.78924 g/mL, which is used to convert ethanol percentage during ethanol production as given in following equation:

Concentration in g/L=10x X 0.78924

Where x is ethanol %.

Table 3.4: Ethanol production by mono and cocultured fungal strains estimated by GCMS data

Microorganisms	Retentiontime(min)	Area	Ethanol(µl)	Ethanol%	Ethanol (g/L)
Saccharomyces cerevisiae	2.914	94886773	0.40760314	10.1900784	80.42417476416
Candida shehatae	2.911	21577730	0.09269101	2.31727514	18.288862314936
Saccharomyces cerevisiae + *Candida shehatae*	2.916	100332040	0.43099426	10.7748564	85.03947665136

After saccharification monoculturing and coculturing of *Saccharomyces cerevisiae* and *Candida shehatae* were done for efficient ethanol production. The idea behind this strategy is that monoculture of *Saccharomyces cerevisiae* ferments hexose sugars and monoculture of *Candida shehatae* ferments pentose sugars and when these microorganisms are cultured together, both hexoses and pentoses sugar fermentation can be achieved and in present work, this strategy showed enhanced ethanol production in comparison to monoculturing.Alcohol percentage with *Saccharomyces cerevisiae* was found to be 10.19% which is much higher than that of *Candida shehatae* generating only 2.32%. On the contrary, when both species were grown together the concentration of ethanol production was increased to 10.775% and *Lemna minor* has been found to be an excellent starch source making it perfect for the synthesis of bioethanol.

Chapter 4

Conclusion, Future Scope & Social Impact

CHAPTER 4

CONCLUSION, FUTURE SCOPE & SOCIAL IMPACT

4.1 Conclusion

Present research work demonstrates the significance of nitrogen-free Hoagland media for starch content enhancement in*Lemna minor, when* compared to full strength Hoagland media and organic manure, highlighting the metabolic alteration induced by nitrogen starvation conditions. Following a series of experiments combining organic manure, nitrogen-free Hoagland medium, and full-strength Hoagland media, the starch enhancement technique was standardised. The total amount of starch was originally estimated to be 7%, but it was eventually noted that Lemna minor produced considerable amounts of starch during nitrogen stress, attaining its optimum level on the ninth day at 26% with a standard deviation of ± 0.3. Furthermore, it has been discovered that during this research study, the proportions of protein, glucose, and fructose dropped. For statistical analysis, a one-way ANOVA analysis was applied. The starch level was notably ($P < 0.05$) much higher in nitrogen-free Hoagland media than in full strength Hoagland media (controle group) andorganic manure through the starch enhancement experiment.

The decrease in protein content and elevation in carbohydrate content under nitrogen-deprived conditions indicate a redirection of carbon flux toward starch biosynthesis. In comparison to other abiotic stress methods, such as heavy metal exposure, nitrogen starvation provides a sustainable approach for maximizing starch yield with no compromise with plant biomass availability. This method is cost-effective, suitable for large-scale production, and has a sustainable approach**,** presenting the most promising strategy for sustainable bioethanol production. Future research should work on optimizing cultivation parameters and large-scale implementation for industrial bioethanol production.

The key objective of this study is to enhance ethanol production by starch using Lemna minor. Lemna minor has been found to be an excellent starch source making it perfect for the generation of bioethanol; still additional investigations are needed to

develop more efficient starch conversion strategies. The investigation also relies on producing an amylolytic enzyme cocktail for the saccharification of Lemna minor starch into sugars that are fermentable, leveraging copra meal as the raw material for enzyme synthesis.

Response Surface Methodology (RSM) was implemented together with conventional optimization techniques to systematically improve the production of enzymes and ethanol yield. By considering parameters like pH, temperature, time, substrate concentration, and One Factor at a Time (OFAT) assays were utilized for identifying the optimum conditions for enzyme and ethanol production. Subsequently, RSM with Central Composite Design (CCD) was applied for examining the interaction impacts between the various components.

Candida shehatae MTCC-12913, *Saccharomyces cereviceae* MTCC-171, and *Aspergillus niger* MTCC-12987 have been used for manufacturing ethanol and amylolytic cocktail, respectively. An optimal approach for the best yield of enzyme activity and ethanol production has been evaluated using Minitab 22 software and response surface regression with Central composite design. The software's greatest estimated value for enzyme activity was 1063.81 U/ml, which was nearer the experimental result of 1072.4 U/ml. The response optimizer program assessed the optimized medium with the highest amylase activity. For best enzyme synthesis, the media should have a pH of 3.83, a temperature of 22.2^0C, 2.94% copra, 0.021% potassium dihydrogen orthophosphate, 0.460% peptone, and 1.091% CMC, followed by 8.5 days of incubation with a projected activity of 1063.81U/ml (Figure 6). The highest enzyme activity value predicted by the software was closer to the experimental value of 1072.4 U/ml (at pH of 4, a temperature of 24, 3 copra, 0.02% potassium dihydrogen orthophosphate, 0.025% peptone, and 0.025% CMC indicating the applicability and accuracy of RSM in optimizing the enzyme optimization process. By ANOVA analysis, F-value of 17.27 with by a p-value < 0.0001 shown the overall model are statistically significant.

Amylolytic cocktail activity was significantly increased by manipulating variables like pH, temperature, substrate composition, and nitrogen sources, as per the study.

Carboxy methy cellulose stimulates the formation of cellulase, lowers the chances of contamination and provides an ongoing carbon supply.

Once again, the software-predicted maximum ethanol production of 11.83% was closer to the experimental outcomes of 10.77 %. The response optimizer programmer assessed the optimized medium with the highest ethanol production. For maximum ethanol production, the media should have a pH of 5.21, a temperature of 37°C, 1.47% *Lemna minor* biomass, and 6.42 ml of crude enzyme with a projected ethanol production of 11.83 % (Figure 8). By ANOVA analysis, F-Value (7.75) + P-Value < 0.05 shown the overall model are statistically significant. The highest ethanol production predicted by the software was closer to the experimental value of 10.77% (at pH 5.5, Temp 35 °C, *Lemna minor* biomass 1gm, crude enzyme extract 7ml.) indicating the applicability and accuracy of RSM in optimizing enzyme optimization process. Co-cultivation of *Candida shehatae* and *Saccharomyces cerevisiae was* better than monoculturingduring ethanol production.As an outcome, the performance of the conformation experiment under optimal conditions precisely resembled the projected results of the model. The model was found to be significant, and three-dimensional graphs were generated for regression analysis of the Central Composite Design, representing the response surfaces for the interaction effects of different parameters for amylolytic cocktail and ethanol production.Results provide insights into biofuel, bioremediation, and enzyme applications. Future research should target ensuring improvement in process parameters and also on new microbes for increasing sustainability as well as efficiency in ethanol as well as enzyme production.

4.2 Future Scope:

4.2.1 Pond systems or pilot-scale bioreactors for continuous biomass production:

Between lab-scale and large industrial setups, pilot-scale systems permit cultivation testing in more realistic circumstances. Pond or circulating bioreactor systems ensure continual development, harvesting, and fertiliser delivery for Lemna minor (duckweed) or other aquatic plants, which brings about the rise in biomass yield periodically. Even before scaling, these systems serve with recognising issues like as light limitation, fertiliser consumption, contamination, and harvesting issues. A regular supply of feedstock for future procedures of bioethanol production can be accomplished with constant cultivation as rather than batch.

4.2.2 Integrating the manufacturing of bioethanol with wastewater treatment makes possible a biorefinery model that maximises both the environment and the economy:

As a means for a single system to clean the water (removing nutrients like nitrogen, phosphorus, COD, and BOD) while producing biomass which can then be processed into biofuel, wastewater should be applied as a growth medium for biomass (such as Lemna minor). Costs have been reduced by the dual use, which minimises wastewater treatment prices and fertilizer/nutrient expense. During growth, the biomass goes through processing to make bioethanol by fermentation and saccharification.

4.2.3 Breeding or picking strains of Lemna minor which are high in starch

Some strains or geographic subtypes exhibit a greater natural accumulation of starch amount, which is advantageous for the production of bioethanol. Through selection or breeding strains can be developed with enhanced starch content. Following saccharification, high starch boosts the sugar production, which increases the ethanol yield. Productivity can be further improved through adopting strains that are appropriate for their local environment, light, temperature, or nutrient requirements.

4.2.4 For advantages of enhanced starch content, improved metabolism, or resilience to environmental stress, genetic engineering or marker-assisted selection may be utilized:

For the purpose to boost starch accumulation, genetic engineering provides the direct modification of genes involved in starch production, storage and transport. Through the addition of molecular markers that correspond to desirable attributes (such as high starch or stress tolerance), promotes selection to be quicker and accurate results. Tolerance to environmental stressors (heat, cold, salt, and pollution) plays an important role in development of plant biomass for outdoor and industrial environments. These changes have the possibility of raising biomass yield, starch content, and ultimately the production of bioethanol.

4.2.5 Evaluating leftover biomass for purposes such as biochar, fertiliser, and animal feed

After the extraction of bioethanol, which frequently utilises a significant quantity of starch or carbohydrates, "residual biomass" (fibres, proteins, and minerals) gets left. Leveraging leftovers lowers trash disposal expenses and adds economic benefit. It additionally increases sustainability.

4.2.6 Aligning with policy frameworks which encourage sustainable energy and the worldwide bioeconomy

Systems that utilise sustainable bioethanol should be aligned with government incentives and policies (carbon credits, renewable fuel standards, subsidies). Aligning with policy, the schemes gain financial or regulatory support so will participating in the worldwide bioeconomy (reuse, recycling, and resource recovery). During bioethanol manufacturing process, it is making sure that environmental, safety, land, water, and greenhouse gas accounting standards are met. Coordinated projects have an increased probability to get finance, securing permission, and being appreciated by the public.

4.3 Social Impact

Bioethanol has gained popularity in the search for sustainable and renewable energy resources, notably due to its potential to help mitigate global warming and reduce our dependence on fossil fuels. Duckweed, particularly*Lemna minor*, is gaining consideration as a feedstock for bioethanol production. This small aquatic plant grows at a surprising rate, doubling its biomass in just 24 to 48 hours in optimum conditions. Duckweed is easily acclimated to several water conditions, such as lakes, ponds, and treatment facilities for wastewater. So, it is not only a part of bioethanol production but also cleans up water by absorbing many metallic (Pb, Cr, As, etc.) and nonmetallic ions (N, P, etc). Thus, it provides an inexpensive farming process with low-priced input because it does not demand fertilizers and can grow on organic waste. Along with this, *Lemna minor* can be harvested many times, devoid of the need for replanting, which further minimizes manufacturing costs. These features make it an appealing substrate for bioethanol as well as a source of dual positive aspects through the cleanup of wastewater and subsequently biomass production, benefiting environmental sustainability. In comparison to sugar crops, duckweed needs fewer resources, such as land and water, to develop. Research indicates that, when optimized, it can yield bioethanol amounts that compete with traditional biofuel crops like corn and sugarcane, with a less harmful impact on the environment. Thus, provide this plant as an alternative option in areas where agricultural land is necessary and competition for food is involved. Introducing duckweed (Lemna minor) as a base plant for the making of bioethanol has various commercial and ecological benefits in the view of bioethanol production. This plant produces considerable quantities of biomass and survives in nutrient-rich effluent.

During the synthesis of bioethanol, starch, which is the main compositional carbohydrate in duckweed, rapidly transforms into fermentable sugars. Its complex carbohydrates like cellulose and hemicelluloses can also break down into simple sugars that can ferment. Additionally, since duckweed biomass has less lignin compared to sugar crops, it can easily saccharify and ferment. Also, it takes little effort to process enzymatically due to its soft cell walls.

After fermentation and enzymatic hydrolysis, the rest of the biomass, which is abundant in fiber and protein, can be employed for creating excellent-quality animal feed, specifically for fish, poultry, and cattle. By incorporating this byproduct, the commercial viability of leveraging duckweed for manufacturing bioethanol is raised. By the dual-use nature of the biorefinery approach, which generates bioethanol from starch and animal feed from protein, resources become more profitable?

It contains many types of fatty acids, which significantly assist in the formation of bioethanol by maintaining the firmness of the membranes of cells in microbial structures. It enhances fermentation efficiency through boosting bacterial resistance to ethanol toxicity. Fatty acid content in duckweed assists in determining the most appropriate fermentation conditions and microbial species during bioethanol formation.

Future research and development in genetic engineering technology, breeding methods, and process optimization are crucial in realizing the potential of *Lemna minor's* ability to produce sustainable bioethanol. More research and innovation are required in this area to meet environmental issues as well as the world's energy needs.

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Title of the Thesis Production of bioethanol using lignocellulosic biomass

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LIST OF CONFERENCES ATTENDED

- Participated in the conference on ―Recent Advances in Biotechnology (icRAB-2022)‖ organized by Dr B R Ambedkar National Institute of technology, Jalandhar on 2-4 Dec. 2022.

- Participated in the conference on ―Exploring the Frontiers of Biological and Chemical Sciences: A Sustainable Pathway to the Future‖ organized by Shri Vaishnav Institute of Management and Science, Indore on 5-6 June, 2025.

- Participated in the conference on ―Role of biotechnology in biological sciences: striving for a sustainable future, RBBS- 2025‖ organized by Delhi University, Miranda house, Delhi, on 6-8 Feb, 2025.

CERTIFICATE OF PARTICIPATION

This certificate is awarded to Manjary Vyas

from Delhi Technological University

for participating in the International Conference *"Role of Biotechnology in Biological Sciences: Striving for a Sustainable Future"* (RBBS-2025) organized by the Department of Zoology, Miranda House from February 6-8, 2025.

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With pleasure it is to certify that Prof. / Dr. / Ms. / Mr. Manjary Vyas representing the institution Delhi Technological University has participated/ presented (poster/oral) titled Bioethanol Production by using lignocellulosic feedstock in the **National Conference** on **Exploring the Frontiers of Biological and Chemical Sciences : A Sustainable Pathway to the Future** held at Shri Vaishnav Institute of Management & Science, Indore.

We wish him/her a bright future ahead.

Dr. Kshama Paithankar
Head, Department of Computer Science
Coordinator, IQAC Conference Co-chairperson

Dr. George Thomas
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Conference Chairperson

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CERTIFICATE OF PARTICIPATION

Is Presented to

Manjary Vyas

For Oral presentation on the title

"**Potential of duckweed for production of bioethanol**" with Co-author/s: Jaigopal Sharma, Kriti Bhandari

at International Conference on **Recent Advances in Biotechnology (icRAB -2022)**
on 2nd-4th December 2022

Dr. Sumer Singh Meena
(Conference Chair)

Dr. Nikhil G N
(Organizing Secretary)

Publications

PUBLICATIONS

- Manjary Vyas, Kriti Bhandari & Jai Gopal Sharma. Optimization, simultaneous saccharification and fermentation of Lemna minor using amylolytic cocktail produced from copra meal. Indian Journal of Chemical Technology, Vol. 32, May 2025, pp. 343–354. DOI: 10.56042/ijct.v32i3. 14310.

- Vyas, M., Bhandari, K., & Sharma, J. G. (2025). Harnessing green synthesis for bioethanol production: insights from duckweed (*Lemna minor*). Biofuels, 1–18. https://doi.org/10.1080/17597269.2025.2545034.

- Manjary Vyas, Kriti Bhandari & Jaigopal Sharma. Revolutionizing Sugar Feedstocks: Starch Enhancement in Duckweed (Lemna minor) with Nitrogen-Free Hoagland Media. Bentham publication, Letters in organic chemistry, August 2025. DOI: 10.2174/011570178638 5014250630070914.

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RESEARCH ARTICLE

Revolutionizing Sugar Feedstocks: Starch Enhancement in Duckweed (*Lemna minor*) Using Nitrogen-Free Hoagland Media

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Abstract: Bioethanol production from duckweed (*Lemna minor*) plants is a promising approach to producing renewable energy. Due to its advantageous characteristics, such as being a non-food feedstock, the fastest-growing angiosperm, globally adaptable to various climates, capable of high starch accumulation under nutrient modifications, and possessing low to no lignin content, Lemna minor is a promising candidate for bioethanol production. This research aimed to produce starch-enhanced *Lemna minor* under conditions of nutritional starvation. The starch enhancement technique was standardized by performing experiments sequentially with organic manure, nitrogen-free Hoagland media, and full-strength Hoagland media. Initially, the starch quantity was found to be 7%. However, it was observed that during nitrogen stress, high starch accumulation occurred in *Lemna minor*, and on the 9th day, it reached its maximum, at 26% with a standard deviation of ±0.3. It was also observed that the protein, glucose, and fructose composition dropped during this experiment. One-way ANOVA analysis was performed for statistical analysis. It was found that during the starch enhancement experiment, the starch level was significantly ($P < 0.05$) higher in nitrogen-free Hoagland media compared to the organic manure. Therefore, *Lemna minor* may be used as a substitute for commercial food feedstocks in bioethanol production.

Keywords: Lignocellulosic biomass, Duckweed (*Lemna minor*), starch enhancement, nitrogen-free Hoagland media, bioethanol, ANOVA analysis.

1. INTRODUCTION

Petroleum is currently a major source of energy for the transportation sector worldwide, including in India [1]. Its price is increasing daily. One of the major challenges is the search for eco-friendly, affordable, renewable energy sources that can replace fossil fuels, given the growing demand for energy [2]. At present, the production process of bioethanol is under investigation to overcome the finite fuel crisis related to the eco-social environment [3, 4]

Bioethanol is a modern and renewable biofuel and is used instead of petroleum-based fossil fuels. It has lower emissions after complete combustion, and any CO_2 released by a vehicle is captured during crop growth [5, 6]. It will reduce dependence on petroleum-based fuels as an energy source. Due to the rapid depletion of petroleum and its increasing price, many countries worldwide have begun blending ethanol with gasoline. However, there is a clear-cut shortage of ethanol for blending with petrol for the transport sector. Currently, we require more ethanol to blend with petrol. A major global source of ethanol production is food-based crops, such as corn and sugarcane. Therefore, a careful assessment must be made to ensure that growing these crops for fuel does not divert land away from actual food production. This drives a search for eco-friendly, inexpensive, and renewable energy sources, such as second-generation ethanol derived from non-food-based plant biomass [7, 8].

It is manufactured through a fermentation process using microorganisms based on the raw material, known as the biological fermentation method [9,10]. Several other methods have also been employed for bioethanol production, including cellulose hydrogenation, gasification, and hydrothermal liquefaction. Cellulose hydrogenation degrades cellulose into ethanol using metal catalysts under high-temperature and pressure conditions [11]. Gasification produces gas (syngas), composed of CO, H_2, and CH_4, from biomass, which can be catalytically converted into bioethanol [12]. Hydrothermal liquefaction can digest biomass into bio-oil and bioethanol under high-pressure, subcritical water conditions [13]. When compared to these thermochemical methods, biological fermentation is more environmentally friendly, cost-effective, and energy-efficient because it operates at mild conditions without requiring any catalysts [14].

According to the applied feedstock conversion style, bioethanol can be classified into four categories [15]. First-generation bioethanol is produced from direct starch and sugar-rich food crops and fruits, such as corn, sugarcane, wheat, potatoes, and grapes [16]. Second-generation bioeth-

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Optimization, simultaneous saccharification and fermentation of Lemna minor using amylolytic cocktail produce from copra meal

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Ethanol, is a renewable liquid fuel derived from the fermentation of sugars or starches in biomass. Amylase is commonly used enzyme to saccharify the raw plant biomass. Optimization of saccharification and fermentation of duckweed (*Lemna minor*) plays a crucial role in harnessing maximum fermentable sugars through enzymatic hydrolysis of this plant biomass. This research mainly entailed the collection and preparation of raw materials, maintenance of microorganisms, and optimization of physical and chemical variables for the production of amylolytic cocktail and ethanol. OFAT determined the significant components that influenced the yield of enzymatic activity and ethanol. RSM with CCD was used to determine the influences between factors to obtain the highest yields of enzymes and ethanol. ANOVA was done to examine the significance of factor interactions while response surface plots showed how different factors affected enzyme reaction. *Aspergillus niger* MTCC-12987, *Saccharomyces cereviceae* MTCC-171, and *Candida shehatae* MTCC-12913 were used for amylolytic cocktail and ethanol production, respectively. Minitab 22 software was employed and response surface regression with Central composite design was used to exmined an optimized process for maximum yield of enzyme activity and ethanol production. The highest enzyme activity value predicted by the software is 1063.81 U/mL, which was closer to the experimental value of 1072.4 U/mL. Again the maximum ethanol production predicted by software is 11.83% was closer to the experimental value of 12.1% . Thus, the conformation experiment's performance under optimal conditions fitted the model's predictions fairly closely. For the purpose of to evaluate the combined effects of all independent variables in a fermentation process that may have developed from their interaction with one another, the RSM methodology has been employed as an alternative tool for statistical analysis.

Keywords: Amylase optimization, Enzyme yield, Ethanol production, One Factor at aTime (OFAT), Response Surface Methodology (RSM)

Introduction

Fossil fuels are the dominant form of energy throughout the world, Still, they emit polluting gases and particles and contribute to global warming by using up reserves of fossil fuels and giving off greenhouse gases[1]. Researchers have been working on developing bioethanol for use as an alternative source of energy to address this problem. A variety of plants, which include grains, tubers, leaves, leaf stems, roots, fruits, and fruit peels, can be used to produce alcohol fuel[2-4]. The first stage of the bioethanol process involved ethanol production from starch (1G), but present-day biofuel production is based on cellulose, hemicellulose, and lignin because they tend to decrease greenhouse gas emissions[5]. Bioethanol production from food sources can result in competition with food production and consequently pose challenges to food security[6]. However, switching to non-food biomass, for instance, lignocellulosic biomass, is now taking place to produce new ethanol, e.g., the next generation of ethanol[7]. Since lignocellulosic biomass is available and cheap globally, it is extensively used in the production of ethanol[7].

Considering lignocellulosic biomass, Bioethanol production from aquatic plants is a very promising way to do so, as they have a higher starch content and lower lignin content than most other plants, hence serving as a cheap source of bioethanol that requires very little or no pretreatment. There are several benefits from extracting bioethanol using *Lemna minor* in comparison to food and lignocellulosic feedstocks. Being a member of the Lemnaceae family with high starch accumulation and a fast-expanding rate, *Lemna minor* also suits well for biofuel generation. *Lemna minor* cannot compete with food production, unlike food-based feedstocks like cornor sugarcane, thus helping to avoid potential challenges with food security. *Lemna minor* serves as a green and environmentally friendly solution for the

Review Article

Harnessing green synthesis for bioethanol production: insights from duckweed (*Lemna minor*)

Manjary Vyas, **Kriti Bhandari** & Jai Gopal Sharma

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Abstract

Bioethanol is a renewable liquid fuel derived from the fermentation of sugars obtained from plants or