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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 generation of bioethanol owing to its successful cultivation in a wide range of water resources[8]. *Lemna minor* is a more efficient option for producing bioethanol compared to using lignocellulosic feedstocks like plant waste biomass[9]. Due to its high starch content, its processing is simple and cheap since it is easy to transform it into bioethanol[10]. Additionally, *Lemna minor* is an extremely appealing feedstock owing to its ability to adjust to a wide range of environmental circumstances as well as low land needs, thereby helping establish a more efficient and sustainable bioethanol synthesis. Under starvation conditions, the starch content of *Lemna minor* can be increased, and its biomass doubles within a span of one to three days, giving an endless supply of biomass for nine totwelve months per year[11]. Non-food biomass can be converted to bioethanol through four stages i.e., pretreatment, fermentation, distillation, and drying[12]. *Lemna minor* starch can be converted to fermentable sugar through enzymatic saccharification without thermophysical pretreatments[13]. By using microorganisms that secrete amylase, bioethanol production can be enhanced. Amylase converts starch into glucose and maltose during saccharification[14] and can be isolated from plants, fungi, bacteria, and animals. This enzyme can digest and regenerate biomass obtained from plants and activity in a wide range of pH and temperatures with limited resources[15]. Also, this enzyme can be extensively used in the brewing industry to digest malt[16] and in desizing textiles during textile finishing[17]. There has been an increasing desire for an accessible and effective strategy for boosting the generation of amylase enzymes due to the demand for sustainable enzymes globally[18-19]. In comparison with enzymes derived from bacteria, fungal amylase is more robust. *Aspergillus* species have different amylases, which are useful in many industries and are capable of producing a large variety of enzymes[20]. Saccharification of *Lemna minor* generates pentose and hexose sugars. *Saccharomyces cerevisiae* can ferment only hexose,while the pentose sugar remains sun-utilized[21]. The benefit of yeast co-culturing and immobilizing yeast cells is to produce ethanol using both types of sugars[22-23].

This study focused on producing bioethanol from *Lemna minor* through simultaneous saccharification and fermentation procedures. *Lemna minor* was cultivated under starvation conditions to enhance starch production. The amylolytic enzymatic cocktail was derived from *Aspergillus niger* MTCC-12975 cultured in media containing copra meal as substrate. One factor at a time (OFAT) and Response surface methodology (RSM) were employed to screen and optimize factors to produce maximum enzyme activity. RSM requires less time than OFAT and studies interaction between the different factors like temperature, pH, time, substrate concentration, and many other additives factor. 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.

Experimental Section

Collection and preparation of raw material

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 Botany, 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[24] for Glucose and fructose and their Protein content estimated by Kjeldahl method[25]. The starch analysis protocol was taken from the megazyme total starch assay procedure (amyloglucosidase/α-amylase method; AA/AMG 11/01, AOAC Method 996.11) [26].

Microorganism and Maintenance

Saccharomyce scereviceae 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. *Aspergillus niger* were subcultured in potato dextrose agar (PDA) slant while *Saccharomyces cereviceae* and *Candida shehatae* were cultured in yeast peptone dextro seagar (YPDA) slant and incubated at 37°C for five days. Preliminary screening of amylase production of *Aspergillus niger* was performed by streaking cultures on a starch agar plate incubated at 37°C for two days followed by pouring gram's iodine solution over the plate. Clear zone formation around the microbial colonies is an indication of the amylolytic ability of microbes. 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.

Optimization of variables for the manufacturing of amylase enzyme and maximum ethanol production

Enzymatic activity was optimized by studying substrate concentration, pH, temperature, incubation time, metal ions, nitrogen, and carbon sources. Optimization was done via one factor at a time technique (OFAT) throughout. The optimization of substrate concentration was done by using Tris HCl buffer-soaked copra meal ranging from 1%-5% (w/v). pH optimization involves analysis of enzyme activity across a range of pH 3-5. The time of incubation was optimized by measuring enzyme activity on the 4th, 6th, 8th, 10th, and 12th day of incubation. Temperature optimization varied between 20°C-40°C temperature. The effect of each metal ion was done separately on the activity of the amlolytic enzymatic cocktail, by adding 0.02% concentration of the $CaSO_4$, $ZnCl_2$, K2HPO_4, KH_2PO_4, $MnCl_2$, $MgSO_4$, and KCl. Yeast extract was replaced with 0.025% peptone, $NaNO_3$, $(NH)_4SO_4$, NH_4Cl, and KNO_3 to study the effect of nitrogen sources on enzyme activity. For the optimization of carbon sources, different substrates like cellulose, pectin, guar gum, cellobiose, and carboxymethyl cellulose (CMC) were used at 0.025% concentration. To estimate the enzyme activity 1 mL of each sample was centrifuged at 8000 rpm for 10 min Crude supernatant was used for analysis. The activity of enzymes was assessed after 24 h of incubation except the time for incubation. The effect of its different variables on enzyme activity was monitored separately by adjusting it systematically with all other variables constant.

Optimization of maximum ethanol production was performed on 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 h 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. Maximum ethanol production was achieved at a pH of 5.5, 1% *Lemna minor biomass*, and 7 mL of crude enzymatic concentration at 48 h.

Simultaneous saccharification and fermentation for ethanol production

By monoculture and coculturing of *Candida shehatae* and *Saccharomyces cerevisiae, an* investigation of maximum saccharification and ethanol production was done. Optimized media of 5.5 pH, 1% *Lemna minor,* and 7 mL of crude enzymatic concentration were used. Culture media was then incubated for 72 hours at 30°C and 180 rpm in an orbital shaker. Samples were taken to track the generation of bioethanol at various time intervals (0, 3, 18, 24, 48 and 72 h).

Enzyme activity estimation

Enzyme activity was measured by the 3,5-dinitrosalicylic acid (DNS) method of reducing sugar (Maltose) following the protocol of Miller, 1959[24]. Briefly, 0.25 mL of 0.05 M tris HCl buffer (7.5pH) was added to 0.25 mL of 1% starch solution and 0.5 mL of crude sample followed by 10 min incubation at 50°C. 3 mL of DNS reagent was added and boiled for 3 min to stop the reaction. Rochelle salt is also added to increase the shelf life of DNS reagents. The reaction mixture was cooled and absorbance was noted at 540 nm. Enzyme activity was calculated as the amount of enzyme required to produce 1 µM of maltose per (U/min) min under the assay condition. The amount of maltose generated was calculated from the standard curve of maltose plotted between absorbance at 540 nm and concentration of maltose in µM.

Screening of main components using OFAT and 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). Total number of experiment run was calculated by following Eq. (1)[27].

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

Table 1 — Composition of Copra meal and Lemna minor biomass

Chemical constituents	Lemna minor (% w/w)	Copra meal (% w/w)
Glucose	2	0.2
Fructose	1	0.3
Starch	26	1.6
Protein	14.60	21

Where n is the independent variable or factor, nc is the number of center points and total N is 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. 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 1.

Ethanol estimation in monoculture and coculturing of *Candida shehatae* and *Saccharomyces cerevisiae*

Ethanol was estimated in different sets of performed experiments with microbial strains after 72 h of incubation by GCMS. The GC system having an FID detector and a column (φ30 m ×250 μm) was used for detection. Column temperature was initially maintained at 40°C; for 5 min, heated up to a final temperature of 260°C at a rate of 12°C/min, and 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,

$$\text{Conc. unknown (\%)} = (\text{Area unknown}/\ \text{Area known}) \times \text{conc. known} \quad \ldots (2)$$

Where, unknown concentration = Concentration of ethanol in the sample to be analyzed and known concentration = standard ethanol concentration

Statistical analysis

RSM analysis was performed using Minitab. The difference in values was indicated in the form of probability ($p < 0.05$) values.

Results and Discussion

Composition of Copra meal and Lemna minor biomass

Copra meal and *Lemna minor* biomass was characterized for composition as mentioned in Table 1. By analysis of chemical composition, It was found that *Lemna minor* is high in starch which makes it suitable for saccharifiaction and fermentation during ethanol production while protein rich value of copra meal makes its suitable for amylolytic enzymatic cocktail production. Copra meal is also rich in multi minerals[28] which worked as additives during enzyme formation.

OFAT for screening important components to optimize amylase and ethanol 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[29]. 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[30-31]. 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[32]. 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[33]. Maximum amylase activity of 1028 U/mL was found at 30°C, which is by the findings of Alva *et al.*, 2007[34]. 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[35]. The pH of the fermentation medium is adjusted to 4, as the maximum enzyme activity of 1053 U/mL was

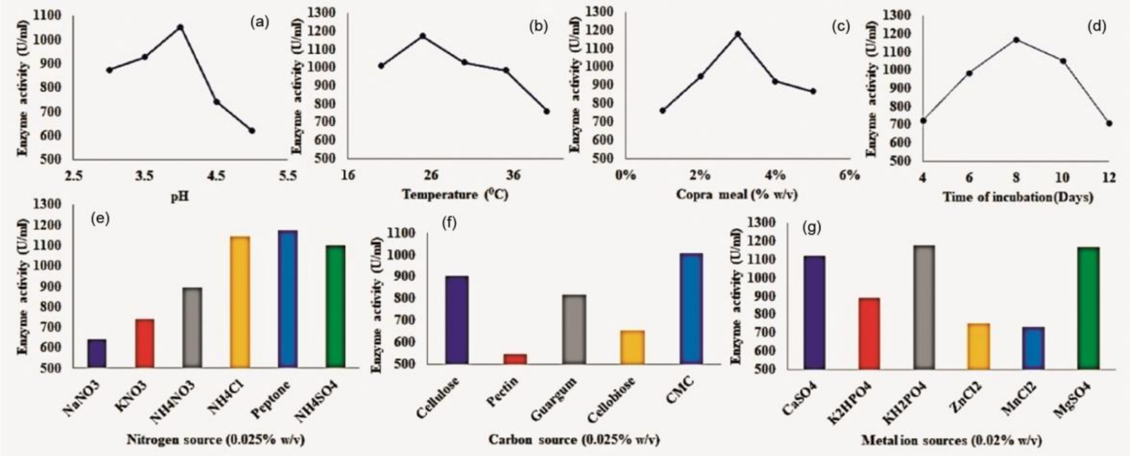


Fig. 1 — One factor at a time (OFAT) or classical optimization to screen important components of each factor for amylase production. Different factors are: (a) pH, (b) temperature, (c) copra meal conc., (d) time of incubation, (e) nitrogen source, (f) carbon source and (g) metal ion source

observed, to support fungal culture and enzyme formation. Maximum amylase activity was reported within the range of 3 to 5 pH[34, 36-38] and subsequently decreased at pH 7. Some other researchers also reported that an acidic pH is required for amylase production from *Aspergillus niger*[39-41]. Carboxymethyl cellulose (1007 U/mL) was also found to be an important carbon source (Fig. 1). 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_4 at 0.02% for amylase production, which produced 1187 U/mL amylase enzyme by *Aspergillus niger* 12987.

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 h. The present study showed that optimal ethanol production was achieved at 35°C. Lin *et al*. also observed that 30-40°C was optimal for *Saccharomyces cerevisiae* BY4742, and at 50°C showed a decline production of ethanol[42]. This might be due to the denaturation of membranous transport system enzymes and ribosomes at higher temperatures. Hu et al 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[43]. The maximum ethanol production was reported at 5 pH (11.8%), 35°C (11.9%), 1% *Lemna minor* biomass (11.7%), and 6 mL crude enzyme (11.1%) as shown in Fig. 2. Optimizing and controlling these factors or parameters are crucial to achieving optimal ethanol yield in the fermentation process.

Methodology, 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* is summarised in Supplementary information (Table S1).

A Linear square model relating amylase activity in U/mL (response) was chosen by CCD and was represented as coded parameters by the linear equation as given in Eq. (3).

Enzyme activity(U/mL) = -1419+ 866 pH + 8.8 temperature(°C)
+75.3 Coconut oil cake concentration + 11772 KH_2PO_4 (%)
+ 194.8 Peptone (%) + 65.6 Time of incubation (days)
+297.2 Carboxy Methyl Cellulose (%) - 113.2 pH * pH
- 0.183 temperature (°C)*temperature (°C)
- 12.46 Coconut oil cake concentration * Coconut oil cake concentration
-279931 KH_2PO_4 (%) * KH_2PO_4 (%) – 218 Peptone (%) * Peptone (%)
- 3.87 Time of incubation (days) * Time of incubation (days)
- 138.5 Carboxy Methyl Cellulose (%) * Carboxy Methyl Cellulose(%) ….(3)

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

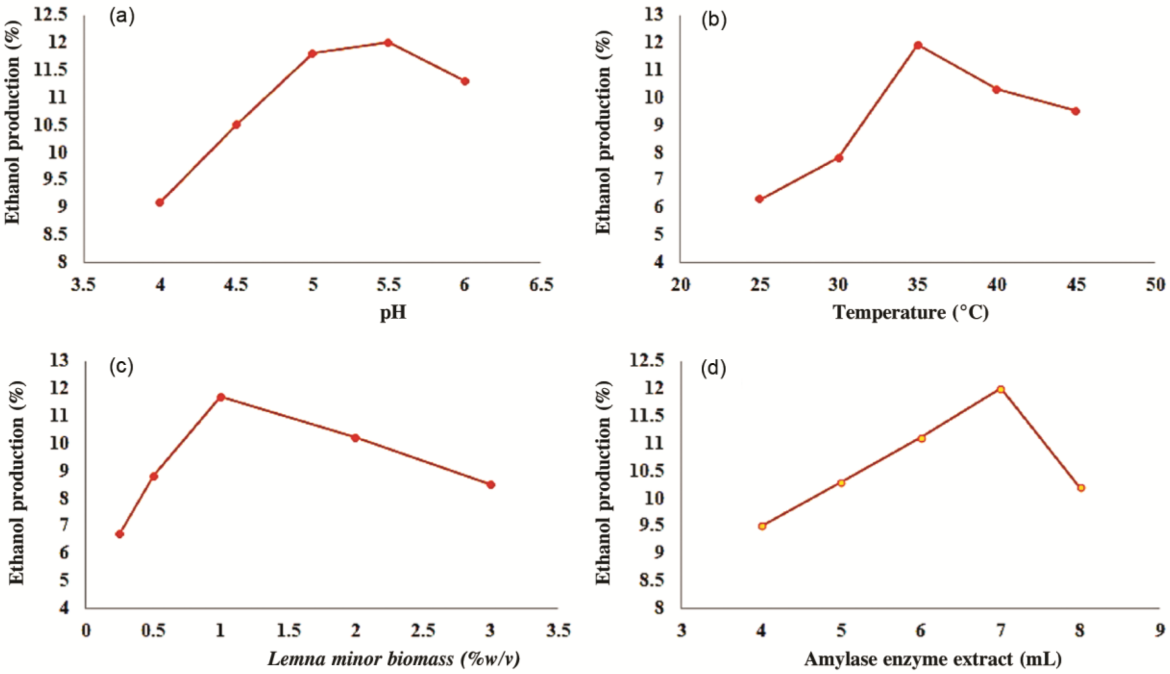
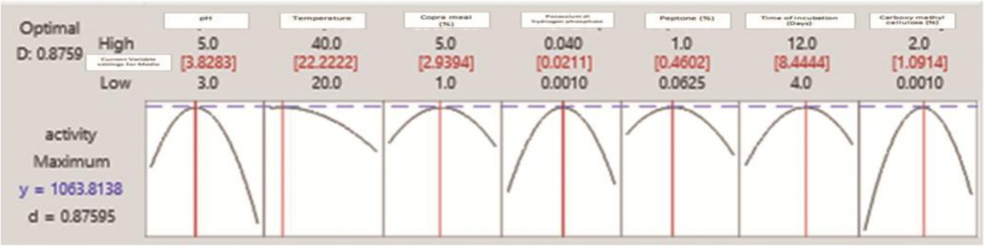


Fig. 2 — One factor at a time (OFAT) or classical optimization to screen important components of each factor for ethanol production. Different factors are: (a) pH, (b) temperature, (c) *Lemna minor* biomass, (d) amylase enzyme crude extract



Optimal D: 0.8759		pH	Temperature	Copra meal (%)	Potassium di hydrogen phosphate	Peptone (%)	Time of incubation (Days)	Carboxy methyl cellulose (%)
	High	5.0	40.0	5.0	0.040	1.0	12.0	2.0
	Current Variable settings for Media	[3.8283]	[22.2222]	[2.9394]	[0.0211]	[0.4602]	[8.4444]	[1.0914]
	Low	3.0	20.0	1.0	0.0010	0.0625	4.0	0.0010

activity
Maximum
y = 1063.8138
d = 0.87595

Fig. 3 — Optimized media for maximum amylase enzyme production produced by response optimizer

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 S2), 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 S3).

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 present in coconut oil cake and peptone. Further, considering KH_2PO_4 has been demonstrated to reduce pH stability and cause transition metabolism in fermentation, it leads to an increase in lack of fit.

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 are shown in Fig. S1(a)-(b) in Supplementary Information..

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°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 1063.81 U/mL (Fig. 3). The highest enzyme activity value predicted by the software was closer to the

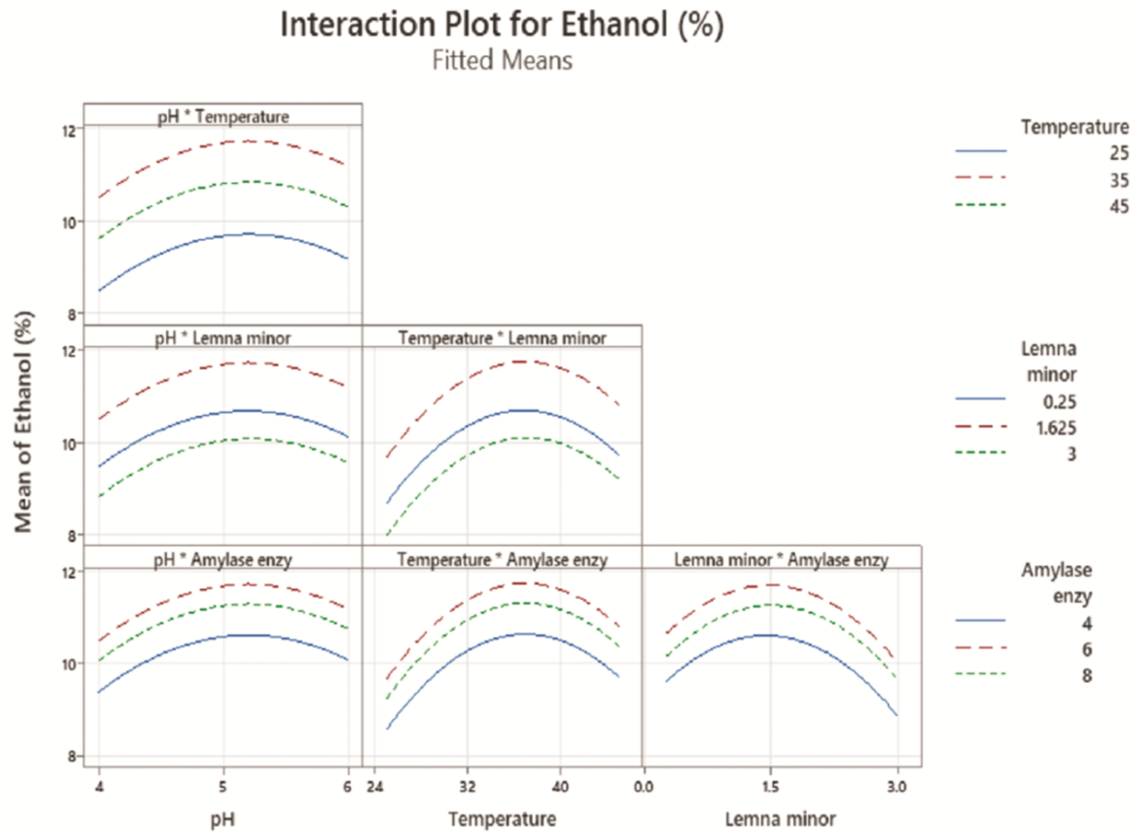


Fig. 4 — Interaction plot between the factors

experimental value of 1072.4 U/mL indicating the applicability and accuracy of RSM in optimizing the enzyme optimization process.

RSM by Central composite design 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 S4. A Linear square model relating production in % (response) was chosen by CCD (Supplementary Information, Table S5) and was represented as coded parameters by the linear equation as given in Eq. (4).

Regression Equation in Uncoded Units

$$\begin{aligned}\text{Ethanol (\%)} = {} & -40.40 + 8.87\text{pH} + 1.072\ \text{Temperature} + 2.097\ \textit{Lemna minor}\ \text{biomass (\%)} \\ & + 2.452\ \text{Amylase enzyme extract (mL)} - 0.853\ \text{pH} * \text{pH} \\ & - 0.01453\ \text{Temperature} * \text{Temperature} \\ & - 0.713\ \textit{Lemna minor}\ \text{biomass(\%)} * \textit{Lemna minor}\ \text{biomass(\%)} \\ & - 0.1913\text{Amylase enzyme extract (mL)} * \text{Amylase enzyme extract (mL)} \qquad \ldots(4)\end{aligned}$$

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 (Fig. 4). ANOVA demonstrated the model's adequacy, with an F-value of 7.75 and a P-value of < 0.05 (Supplementary Information, Table S6), 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 S5).

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 occured 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 have an immediate impact on subsequent pH in continuous processes like simultaneous fermentation and

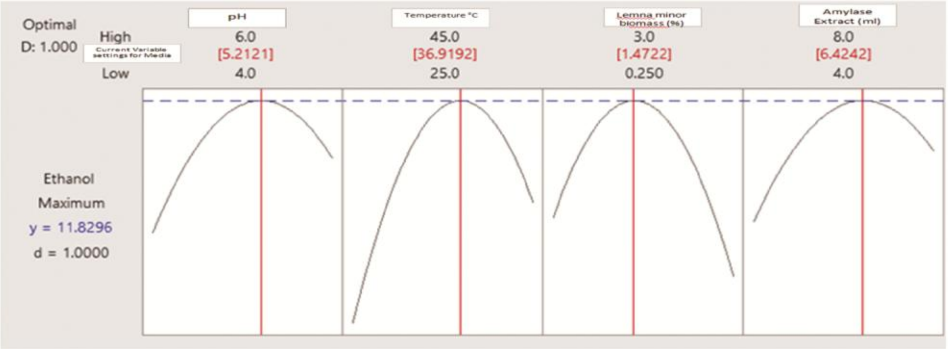


Fig. 5 — Optimized media for maximum ethanol production produced by response optimizer

Table 2 — Ethanol production by mono and cocultured bacterial strains estimated by GCMS data

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

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. Additionaly F-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.

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, *Lemna minor* biomass, and crude enzyme volume. These plots given in depicted Fig. S2 (Supplementary information) shows the influence of several factors and the interaction effect of each parameter on the response

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 % (Fig. 5). The highest ethanol production predicted by the software was closer to the experimental value of 12.1% indicating the applicability and accuracy of RSM in optimizing enzyme optimization process.

Ethanol estimation in monoculture and coculturing of *Candida shehatae* and *Saccharomy cescerevisiae*

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 2, Fig. 6). Hawaz *et al*. used cocultured

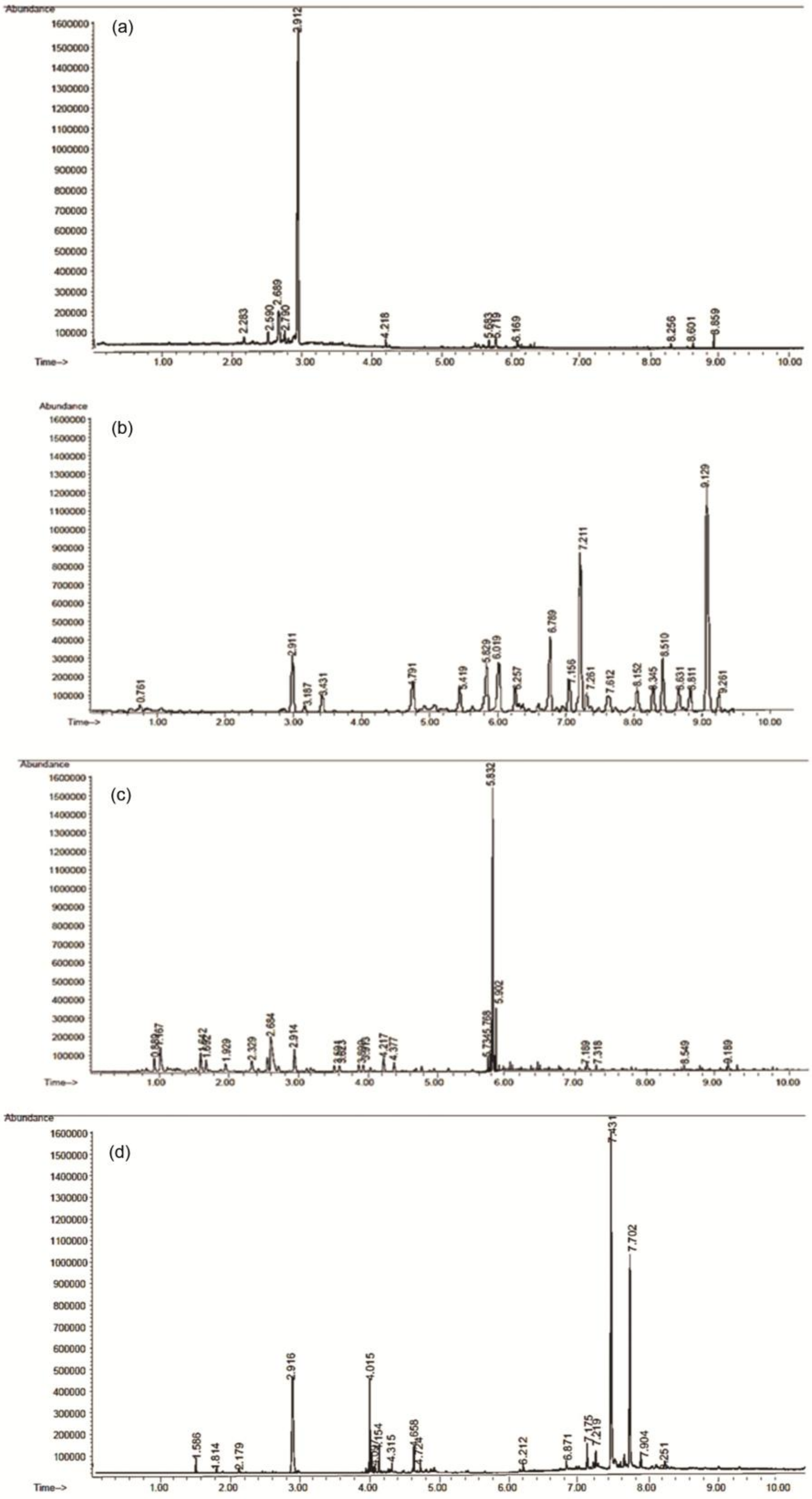


Fig. 6 — GCMS of (a) standard ethanol 95% (749.778 g/L ethanol), (b) *Candida shehatae* cultured in optimized ethanol production media, (c) *Saccharomyces cerevisiae* cultured in optimized ethanol production media and (d) co-cultured media

Saccharomyces cerevisiae and *Wickerhamomyces anomalous* in RSM-optimized fermentation conditions using sugarcane molasses as an initial substrate were used 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*. found that coculturing of *Candida shehatae* HM 52.2 with *S. cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate effectively utilized glucose and xylose simultaneously and increased ethanol yield[45]. Similar results were also observed with the present research work with the advantage of that there is no use of synthetic medium. Density of ethanol is 0.78924 g/mL, which is used to convert ethanol percentage during ethanol production as given in Eq. (5), where x is the ethanol %.

$$\text{Concentration (g/L)}=10x\times0.78924 \qquad \ldots(5)$$

Conclusion

Enhancement of ethanol production by starch using *Lemna minor* was successfully done in this study. 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. With 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. Amylolytic cocktail activity was significantly increased by manipulating variables like pH, temperature, substrate composition, and nitrogen sources, as per the study. CMC stimulates the formation of cellulose lowers the chances of contamination and provides an ongoing carbon supply. Ethanol and enzyme production were increased under RSM-optimized conditions, which increased productivity and cost-effectiveness. Co-cultivation of *Candida shehatae* and *Saccharomyces cerevisiae was* better than monoculturing during 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.

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Declaration of Competing Interest

The authors have declared no conflict of interest.

Supplementary Information

Supplementary information is available on the website http://nopr.niscpr.res.in/handle/123456789.

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