Applied Biochemistry and Biotechnology (2022) 194:4066–4080
https://doi.org/10.1007/s12010-022-03974-w

ORIGINAL ARTICLE

Biodiesel Production from *Brassica juncea* Using Oleaginous Yeast

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Received: 20 February 2022 / Accepted: 10 May 2022 / Published online: 20 May 2022

Abstract

The present study explores the potential of *Brassica juncea* as a low-cost substrate for biodiesel production through the growth of oleaginous yeast. Firstly, the selected lignocellulosic biomass, i.e., *Brassica juncea*, was thermochemically pretreated using dilute sodium hydroxide. Optimization of thermochemical pretreatment resulted in significant removal of lignin and hemicellulose with 8.4% increase in cellulose content. Further, the sugar hydrolysate of pretreated biomass was used as a substrate for the growth of selected oleaginous yeast (*Cryptococcus* sp. MTCC 5455). Lipid and biomass production was optimized using central composite design (CCD) based on response surface methodology (RSM). Maximum biomass and lipid content of 32.50 g/L and 11.05 g/L, respectively, was obtained at 30 °C temperature, pH 6.0, and after 5 days of incubation. The oleaginous yeast lipid was further transesterified using immobilized lipase. The highest fatty acid methyl ester 15% FAME yield was obtained after 10 h of enzymatic reaction. Next, the results of specific gravity, viscosity, flash points, and cloud point of obtained biodiesels were conformed to the ASTM D975 standard. Overall, the present study put forth the cost-effective approach for lignocellulosic biomass-based oleaginous lipid production toward the green synthesis of biodiesel.

Keywords Oleaginous yeast · *Brassica juncea* · Statistical optimization · Transesterification · Fatty acid methyl ester

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Introduction

Biodiesel is a potentially sustainable fuel derived from oleaginous lipids in plants, animals, and microbes. Interest in 3rd generation of biodiesel from oleaginous microorganisms (OM) has increased dramatically to meet the rising demand for fuel [1]. Biodiesel is usually produced through the transesterification of plant-based oils, which has many drawbacks in crop availability and arable land. It is unreasonable to rely on edible feedstock (as raw materials) for biodiesel production because a large portion of the world's population depends on edible vegetable oil produced about 30 billion tons per year. Vegetable oils for biodiesel production could lead to a shortage of edible oils, causing food prices to rise and sparking food-fuel priority. To meet the energy demand, unconventional resources should be investigated in the future. Biodiesel manufacturing should be less expensive and higher quality, utilizing non-edible oils and other sources. To address this problem, scientists are studying the synthesis of microbial oil from various oleaginous organisms and converting it into biodiesel [2, 3]. Single-cell oil (SCO), also known as microbial oil, it is a promising sustainable oil with a fatty acid composition similar to vegetable oils. Oleaginous microorganisms can store lipids over 20–60% (w/w) of their total dry biomass weight. During nitrogen-limited conditions, excess carbon sources (such as glucose and xylose) can be used not only for biomass synthesis but also for lipid synthesis, which is used mainly for biodiesel synthesis (FAME) [4, 5]. Yeasts have been identified as promising organisms because of relatively shorter duplication time, unicellular, ease of growing in a controlled environment, and ability to grow on multiple substrates (i.e., municipal solid waste, industrial waste, crude glycerol, lignocellulosic biomass) at high cell densities [6–8]. The most commonly known oleaginous yeast species are *Trichosporon*, *Cryptococcus*, *Lipomyces*, *Yarrowia*, *Candida*, and *Rhodotorula* [9, 10]. Lignocellulose hydrolysates have been converted to microbial lipids in several investigations. Lignocellulosic materials are renewable, readily available, inexpensive raw materials converted to fuels and other valuable products. Lignocellulosic products are made up of cellulose (40–50%), hemicellulose (20–30%), and lignin (10–20%). Lignin is the only non-carbohydrate constituent comprising aromatic compounds. It is associated with cellulose and hemicelluloses via ester and ether linkages, making them inaccessible for enzymatic degradation. Therefore, pretreatment is required to remove the lignin rendering enzymatic digestion of cellulose and hemicellulose [11, 12]. Hydrolyzed cellulose and hemicellulose produce hexose and pentose sugars that oleaginous yeasts can utilize for their growth and lipid synthesis. The *Brassica juncea* is one of these LCBs. India is the world's third-largest mustard producer. After the seeds have been harvested, the plant biomass is mostly cellulose, hemicellulose, and lignin. After pretreatment and saccharification, its carbohydrate components (cellulose and hemicellulose) can be used to make biodiesel [13]. The present study focused on microwave-assisted dilute alkaline pretreatment of *Brassica juncea* followed by maximization of microbial oil production from pretreated biomass hydrolysate using oleaginous yeast. Further microbial oil was transesterified for biodiesel production. The properties of produced biodiesel was compared with the biodiesel standards.

Materials and Methods

Pretreatment of *Brassica juncea* Biomass

Five grams of biomass was thermochemically treated in a microwave oven (Thomas Scientific EMS-820 microwave oven, 220 V) at different concentrations of NaOH (0.1–2 M), different

temperatures (80–200 °C) for different incubation times (1–120 min), and at various substrate concentrations (1–25%). Treated biomass was washed thoroughly with distilled water to reach a neutral pH and dried in a hot air oven at 60 °C to a constant weight. Each pretreated biomass type was enzymatically hydrolyzed using cellulase produced from *Trichoderma viride* MTCC 800 using a previously optimized method [14]. After enzymatic hydrolysis, the DNS method measured the total reducing sugar [15]. Compositional analysis of untreated and treated biomass followed the NREL protocol [16, 17]. Sugar hydrolysate obtained from pretreated biomass was further used for oleaginous yeast growth.

Oleaginous Yeast

In the present study, *Cryptococcus* sp. MTCC 5455 was used for lipid production. It was procured from Microbial Type Culture Collection, Chandigarh, India. The oleaginous yeast colony was maintained on a YEPD slant containing (g/100 mL) glucose 2.0, yeast extract 1.0, peptone 2.0, and agar 4.0, incubated at 25 °C for 2 days and transferred once every 2 weeks and stored in a refrigerator at 4 °C.

Selection of Oleaginous Yeast Growth Parameters

The oleaginous yeast growth parameters were selected by studying the yeast growth at varying temperatures (20–40 °C), pH (4.0–8.0), and incubation time (1–8 days) at 150 rpm. The growth of yeast was optimized in a minimal medium containing (5% sugar hydrolysate, 0.25% sodium nitrate, 0.1% potassium dihydrogen orthophosphate, 0.05% magnesium sulfate, and 0.05% potassium chloride) in an Erlenmeyer flask (250 mL) covered with cotton plug and autoclaved at 121℃ temperature, 15 psi pressure for 15 min. Yeast inoculum was prepared in the YPD liquid medium (yeast extract 1%, peptone 2%, glucose 2%, pH 6.0). The yeast growth was determined after varied temperature, pH, and incubation time by measuring the dry cell weight [7, 18].

Modeling and Optimization of Lipid and Biomass Production Through Response Surface Methodology (RSM)

The total number of experimental combinations in the central composite design (CCD) was 2 k + 2 K + n0, where K is the number of independent variables and n0 is the number of repeated trials at the central point, indicating that this approach needed 20 experiments [19]. A total of 20 experiments were included in CCD, each having a three-level complete factorial design with three replications of the central and axial points. Lipid (g/L) and biomass production (g/L) were chosen as the dependent variables for this study. Temperature (25–35 °C), pH (5–7), and incubation period (4–6 days) were selected as independent variables.

Extraction of Lipid from Oleaginous Yeast

The Bligh and Dyer method was utilized for lipid extraction from the yeast cell biomass at optimized conditions [20]. Cells were disrupted using a benchtop homogenizer, 1 g

yeast cell biomass was transferred into a glass vial, and a 3.75 mL chloroform/methanol (1/2) mixture was added and vortexed for 10–15 min. Then 1.25 mL chloroform was added and vortexed for 1 min. After adding 1.25 mL of water and mixing for 1 min, the contents were centrifuged. The upper phase was discarded, and the lipid extract from the lower phase was redissolved in a small volume of chloroform/methanol (2/1) mixture [20]. The yeast microbial oil was stored at 4 ℃ for the further transesterification process.

Transesterification Reaction

Immobilized lipase obtained from previous studies with 10 U/ml activities was used for transesterification reactions [21]. For biodiesel production, transesterification reactions were carried out in a 250-mL Erlenmeyer flask containing 2.5 mL oleaginous yeast oil, 25 mL methanol and water (4 ≈ 35 wt %, based on the total mass of oil and methanol) were preheated to 20–60 °C in a water bath with a thermostatic magnetic stirrer. Then 50 mL n-hexane and immobilized lipase (10 U/ml) were added to the reaction mixture [22]. The flask was covered with a lid and incubated in an orbital shaker at 35℃ and 150 rpm for 10 h. The mixture was then transferred to a separating funnel and allowed to settle for 14 h for the formation of 2 layers. The biodiesel layer was separated from the sediment layer and washed with water a couple of times to remove any remaining impurities. The obtained biodiesel was stored and then analyzed using gas chromatography. Further, the lipase-catalyzed transesterification reaction was optimized by varying the reaction temperature (25–40℃), pH (4.0–9.0), and reaction time (1–15 h). For pH optimization, immobilized lipase was stored in phosphate buffer with different pH, and it was used for transesterification reaction. Samples were taken at specific time intervals and centrifuged at 5000 rpm for 10 min. After centrifugation, the clear supernatant was taken and diluted adequately with n-hexane for gas chromatographic analysis.

Biodiesel Characterization

The produced biodiesel was washed and dried following the standard method [23]. The dried biodiesel was used to characterize different parameters such as viscosity, flash point, and cloud point properties to ensure that the obtained biodiesel meets the specific standard values [24]. The viscosity was measured with the Redwood viscometer**.** The specific gravity of biodiesel produced was confirmed with ASTM D6751 standards for biodiesel. Flashpoint was determined according to ASTM D7094, and cloud points were determined according to ASTM D7683 [22].

Analytical Methods

2 mL of yeast culture was transferred to pre-weighed 2 mL Eppendorf tubes for dry biomass preparation. The tubes were weighed the following centrifugation again at 10,000 rpm for 5 min, washing and drying at 50 °C overnight. Measurements were done by subtracting the weight of the sample tubes from their respective pre-weights. The FAME obtained by transesterification was further analyzed on a gas chromatography system

(Shimadzu, Tokyo, Japan) equipped with a flame ionization detector and capillary column (30 m × 0.25 mm with 0.25-µm film thickness). Helium was used as carrier gas with a 1.0 mL/min flow rate. One microliter of the sample was injected in splitless mode. The column temperature was initially raised from 50 to 180 °C at a 25 °C/min gradient. Subsequently, it was further raised to 220 °C at a gradient of 10 °C/min and held for 1 min, then further increased to 250 °C at a gradient of 15 °C/min. The retention times of the obtained FAMEs were identified by comparing them with standard FAME (F.A.M.E. Mix C8-C24, Sigma-Aldrich) [25].

Results and Discussion

Microwave-Assisted Dilute Alkali Pretreatment of Brassica juncea

The distribution of major components in *Brassica juncea* is tabulated in Table 1. Cellulose, hemicellulose, and lignin content were 35.5%, 24.3%, and 21.5%, respectively. Alkali pretreatment resulted in 60.25% of total solid recovery with a substantial decrease in lignin (21.5 to 8.20%) and hemicellulose (24.3 to 12.35%) content. Cellulose content was increased from 35.5 to 38.75%, with an increment value of nearly 8.4%. This alkaline treatment efficiently enriched the cellulose content while removing lignin and hemicellulose. The presence of lignin and hemicellulose in the cell wall of ligno-cellulosic biomass forms a protective barrier that prevents any enzymatic or chemical degradation. Therefore, to enhance the biomass digestability, various pretreatments are carried out [23]. In the current study, *Brassica juncea* was pretreated with dilute alkali. Alkali treatment boosts the disruption of lignin, while microwave irradiation aids in improving this disruption by selectively heating the more polar parts and thereby creating "hot spots" within the heterogeneous material [16]. Figure 1a–d showed reducing sugar yields obtained from pretreated biomass for different treatment conditions concerning residence time, substrate concentration, temperature and alkali concentration. Reducing sugar content in treated biomass increased gradually with a rise in reaction time until 30 min; however, prolonged exposure beyond 30 min showed a negligible effect. The longer residence time can result in insignificant loss of cellulose and hemicellulose content, affect the enzymatic digestibility, and result in certain inhibitory compounds [26]. The reducing sugar yield was not enhanced even with the pretreatment conditions beyond 10% substrate loading, 30 min, and reaction time at 150 °C. CH2OH groups present in cellulose are involved in hydrogen bonding and require specific threshold energy for localized rotations around the bonds. Temperature also plays a significant role in microwave-treated pretreatment, where profound results were obtained until 180 °C; after that, lower reducing sugars were attained due to the formation of inhibitory products [27]. Nuchdang et al. [28] also reported a lower

Table 1 Biochemical composition of *Brassica juncea*. All values are represented as ± s.d of three replicates

Chemical constituent (%, dw/v)	Control	Pretreated
Cellulose	35.5 ± 1.5	38.75 ± 1.1
Hemi cellulose	24.3 ± 0.9	12.35 ± 0.8
Lignin	21.5 ± 1.0	08.20 ± 0.6

reducing sugar yield beyond the 120 °C and 30 min with the microwave-based pretreatment of *Brachiaria mutica.* Sombatpraiwan et al. [29] also reported the lower reducing sugar yields beyond the threshold limits of microwave-based pretreatment conditions with Cassava (i.e., 840-W microwave capacity, 9-min irradiation time, and 3% w/v NaOH) [29]. The optimum requirements of microwave-based pretreatment vary with the substrate source, and microwave capacity, irradiation time, and alkali concentration seem to be the influential parameters in microwave-based pretreatment studies.

Selection of Oleaginous Yeast Growth Parameters

The present study aimed at microbial lipid production using oleaginous to assimilate the complex sugars in biomass hydrolysate and select the best growth conditions (temperature, pH, and incubation time) for better lipid production. The accumulation of lipids by the yeast mainly depends on microbial physiology, nutrient limitation, and other physiological factors, i.e., pH and temperature of the medium [8]. The highest biomass and lipid content, 30.15 g/L and 9.09 g/L, respectively, were obtained after 5 days of incubation (Fig. 2a–c). Cell biomass gradually increased up to 5 days. Beyond that fifth day, no significant increase in biomass concentration was observed because cells began to enter the death phase due to nutrient depletion. Lipid accumulation was increasing with an increase in biomass concentration. The maximum biomass (32.48 g/L) and lipid

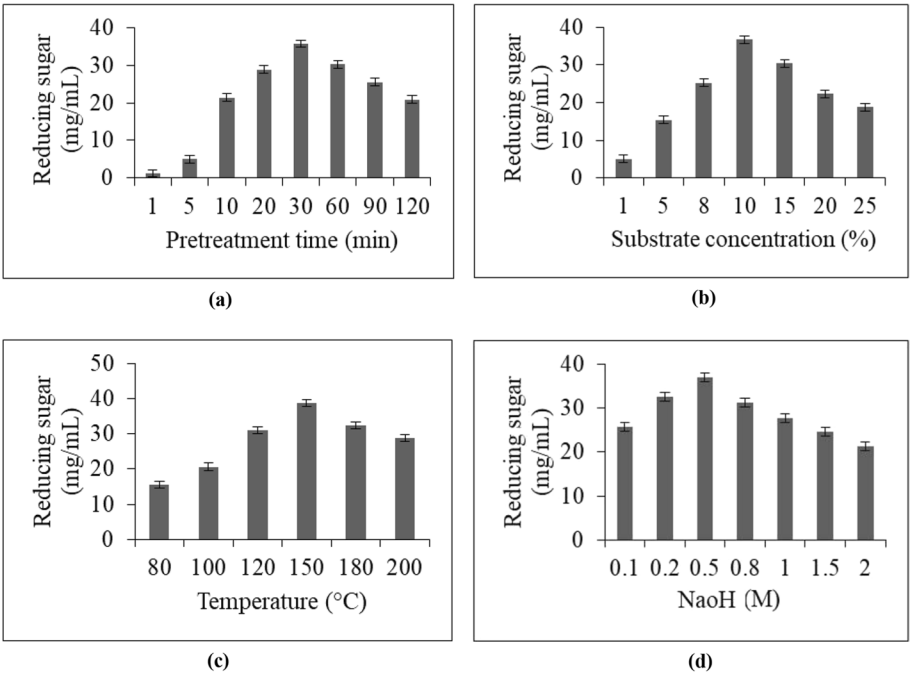


Fig. 1 Effect of **a** incubation time, **b** substrate concentration, **c** temperature, and **d** NaOH concentration on reducing sugar production from pretreated *Brassica juncea.* All values are represented as + s.d of three replicates

content (10.35 g/L) were at pH 6.0. The lowest was at pH 5.0, with 15.19 g/L biomass and 5.37 g/L lipid content.

The temperature affects yeast metabolism and its biochemical pathways, growth rate, and lipid synthesis and alters cellular fatty acid composition. In the present study, maximum biomass (26.34 g/L) and lipid content (9.43 g/L) was observed at 30 °C, and the lowest results were obtained at 25 °C. Osman et al. [30] also reported 30 °C as a suitable temperature for *Rhodotorula diobovata* with reported biomass, lipid yield, and lipid contents of 11.77 g/L, 5.12 g/L, and lipid content (43.51%), respectively. In another study, Jiru et al. [10] also reported that 25–30 °C were the favorable temperature conditions (along with the pH 5.5 and 5-day incubation time) for *Rhodotorula kratochvilovae* for attaining a maximal biomass yield (15.34 ± 1.47 g/L) and lipid yield (8.60 ± 0.81 g/L). Laker et al. [26] also reported that 25 °C is the suitable growth condition for oleaginous yeast for better biomass production (10 g/L) and lipid yields (1.25 g/L) using solid waste as a substrate. Overall, the results show that 25–30 °C were suitable temperature conditions for oleaginous yeasts for maximum biomass and lipid yields.

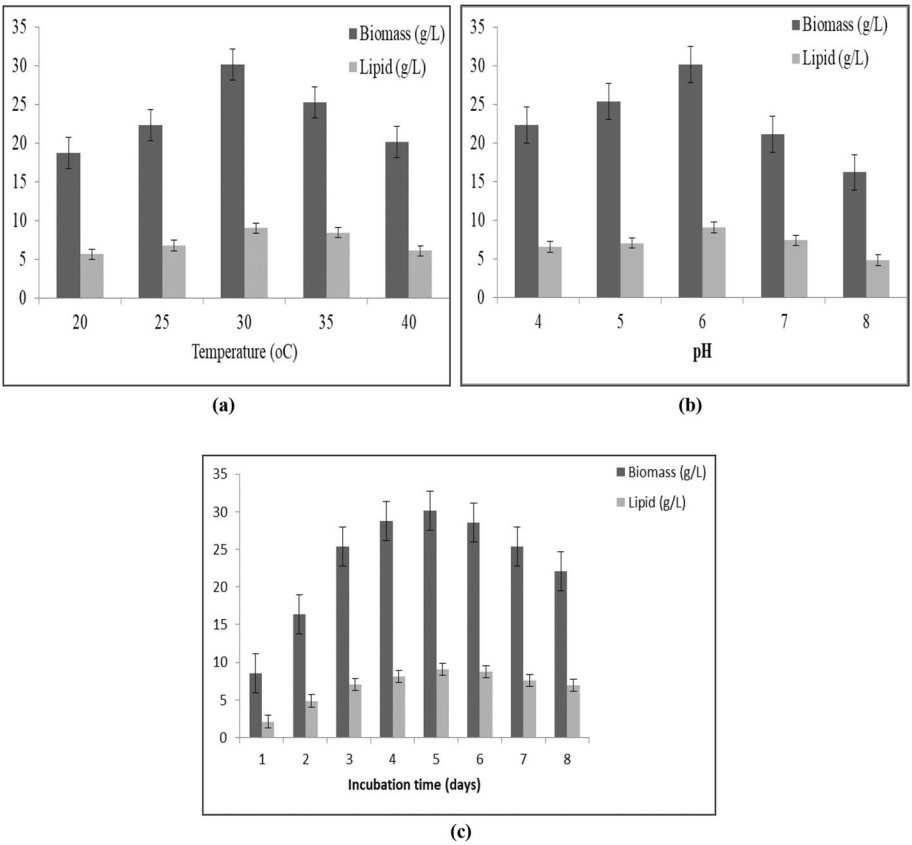


Fig. 2 Effect of **a** temperature, **b** pH, and **c** incubation time on biomass and lipid production by oleaginous yeast. All values are represented as + s.d of three replicates

RSM-Based Modeling and Optimization of Oleaginous Lipid and Biomass Production

The experimental responses of the dependent variable (lipid and biomass production) on a three-level central composite design matrix are summarized in Table 2. The regression equation coefficients were determined, and the data were fitted to a second-order polynomial equation. The following regression Eq. (1) and Eq. (2) can be used to represent the response, lipid, and biomass production from oleaginous yeast:

$$\text{Lipid(g/L)} = +10.85 - 0.004 \times \text{Temperature} + 0.52 \times \text{pH} + 0.35 \times \text{Incubationtime} - 1.35 \times \text{Temperature} \times \text{Temperature} - 1.64 \times \text{pH} \times \text{pH} - 0.45 \times \text{IncubationtimeXIncubationtime} + 0.90 \times \text{Temperature} \times \text{pH} - 0.33 \times \text{Temperature} \times \text{Incubationtime} - 0.66 \times \text{pH} \times \text{Incubationtime} \quad (1)$$

$$\text{Biomass(g/L)} = +32.53 + 0.02 \times \text{Temperature} + 2.47 \times \text{pH} - 0.49 \times \text{Incubationtime} - 6.52 \times \text{Temperature} \times \text{Temperature} - 8.33 \times \text{pH} \times \text{pH} - 0.77 \times \text{IncubationtimeXIncubationtime} - 1.56 \times \text{Temperature} \times \text{pH} + 2.01 \times \text{Temperature} \times \text{Incubationtime} + 0.91 \times \text{pH} \times \text{Incubationtime} \quad (2)$$

Furthermore, the RSM analysis provides information on quadratic factors and combined impacts on the linear effect of lipid and biomass production (g/L). The ANOVA (Table 3) results suggested the significant effects ($P < 0.05$) of linear, square, and interaction effects of production variables on lipid and biomass production. The relative close

Table 2 Experimental design for lipid and biomass productivity from oleaginous yeast using mustard biomass as substrate

Exp	Temp	pH	IT	Lipid (g/L)		Biomass (g/L)	
				Experimental	Predicted	Experimental	Predicted
1	25	5	4	06.48	06.36	16.01	16.26
2	35	5	4	05.37	05.31	15.19	15.40
3	25	7	4	07.27	07.10	22.67	22.51
4	35	7	4	09.50	09.89	15.46	15.42
5	25	5	6	09.16	09.18	09.24	9.46
6	35	5	6	06.46	06.63	16.30	16.63
7	25	7	6	07.05	07.07	19.36	19.32
8	35	7	6	08.24	08.36	20.35	20.27
9	25	6	5	09.22	09.13	26.25	25.99
10	35	6	5	09.57	09.26	26.45	26.03
11	30	5	5	08.79	08.49	22.74	21.73
12	30	7	5	09.43	09.72	26.34	26.67
13	30	6	4	09.70	09.97	32.50	32.25
14	30	6	6	10.90	10.62	31.70	31.27
15	30	6	5	10.35	10.55	32.67	32.53
16	30	6	5	11.20	10.55	32.48	32.53
17	30	6	5	11.09	10.55	31.80	32.53
18	30	6	5	11.05	10.55	32.50	32.53
19	30	6	5	11.01	10.55	32.81	32.53
20	30	6	5	10.80	10.55	31.56	32.53

values of "adjusted R^2" to 1.0 (0.9250 in case of lipid and 0.9858 with biomass) show the accuracy and fitness of the developed RSM model for lipid and biomass production. Due to a very high *F*-value (69.93 and 342.55 for lipid and biomass production, respectively) and a meager probability value (< 0.001), ANOVA of the regression model for lipid and biomass production revealed that models were significant [29]. The graphical depiction of the regression equation is the 3D response surface plots. Examining three-dimensional response surface plots as a function of two factors at a time while maintaining all other factors at fixed levels might help to understand the relationships between the factors and response. The 3D response surface plot for the optimal lipid and biomass production conditions from oleaginous yeast is shown in Fig. 3a–d. Each graph depicted the impact of two factors on oleaginous yeast lipid and biomass production. The optimums of the three variables were as follows: temperature of 30 °C, pH 6, and the incubation period of 5 days. Usually, oleaginous yeast cultures are held for at least 5–7 days before the maximum lipid content is achieved. The present studies obtained maximum biomass and lipid content within 5 days of incubation. In most cases, if yeast culture is held for a more extended period, it can use storage lipids as an energy source after depleting fermentable sugars in the medium [31]. The maximum RSM-predicted optimization results were 10.55 g/L lipid yield and 32.53 g/L biomass production was, aligned with the triplicate experimental validation results, i.e., 11.05 g/L lipid yield and 32.50 g/L biomass production. The enhancement of lipid yield and biomass yields was impeded due to the inducible stress response of xylose presence in hydrolysate and the favorable glucose hydrolysate [31]. Brandenburg et al. [32] reported the presence of xylose stress response, limiting the lipid yield to 7.68 g/L using wheat straw hydrolysate by *Lipomyces starkeyi* CBS 1807 and a growth pH of 6.0. In another study, Thangavelu et al. [25] reported a 5.99 g/L biomass yield and 2.68 g/L lipid yield at a pH of 6.0 and 30 °C using *Candida tropicalis* ASY2. The RSM-based modeling of oleaginous lipid production showcased the primary importance of growth parameters (pH and temperature) and the xylose stress response on the total lipid yields [25, 31, 32]. The developed RSM model for oleaginous lipid production, caring for the interaction effects of oleaginous yeast growth parameters, helps in subsequent lipid production capabilities on a pilot scale.

Lipase-Catalyzed Transesterification Reaction

The yield of fatty acid methyl esters is affected by temperature, pH, and reaction time, among other things. The effect of incubation time on the yield of fatty acid methyl ester (FAME) was investigated for 1–15 h. The maximum FAME yield was obtained after 10 h of incubation time, as shown in Fig. 4a; further, the FAME's yield started to decline due to the loss of enzyme activity by the methanol and phospholipids. The inhibitory effects of methanol on FAME yield were also reported in the immobilized lipase-mediated transesterification studies [8, 33]. Shah et al. [33] obtained 89% FAME yield in 10 h using the immobilized lipase of *Chromobacterium viscosum* as a catalyst for ethanolysis of Jatropha oil. The effect of temperature on the FAME's yield was studied from 25 to 40 °C. From Fig. 4b, the maximum FAME yield was attained at 35 °C, and lower FAME yield was observed below and above 35 °C. The reaction rate increases typically as temperature rises due to less mass transfer limitations and an improvement in rate constants which aids in attaining higher FAME yield up to a specific temperature. The lower FAME yields at higher temperatures were attributed to the inhibition of lipase activity due to the denaturation [34]. Andrade et al. [35] and Shah et al. [33] also reported that temperatures of 35–45 °C are the better conditions for

Table 3 ANOVA analysis of response surface quadratic model for lipid and biomass productivity from oleaginous yeast using mustard biomass as substrate

Source	Seq SS		Adj SS		Adj MS		F		P	
	Lipid	Biomass	Lipid	Biomass	Lipid	Biomass	Lipid	Biomass	Lipid	Biomass
Regression	9	9	59.55	1078.92	6.62	119.88	69.93	342.55	< 0.001	< 0.001
Linear	3	3	3.95	63.40	1.32	21.13	13.93	60.38	0.001	< 0.001
Square	3	3	44.75	957.25	14.92	319.08	157.65	911.76	< 0.001	< 0.001
Interaction	3	3	10.84	58.28	3.61	19.43	38.20	55.51	< 0.001	< 0.001
Residual error	10	10	0.95	3.50	0.09	0.35				
Lack-of-fit	5	5	0.47	2.23	0.09	0.45	1.01	1.76	0.497	0.275
Pure error	5	5	0.47	1.27	0.09	0.25				
Total	20	20	60.49	1082.42						

$R^2 = 98.44\%$ (lipids g/L) $R^2 = 92.50\%$ (lipids g/L)

$R^2 = 99.68\%$ (biomass, g/L) $R^2 = 98.58\%$ (biomass, g/L)

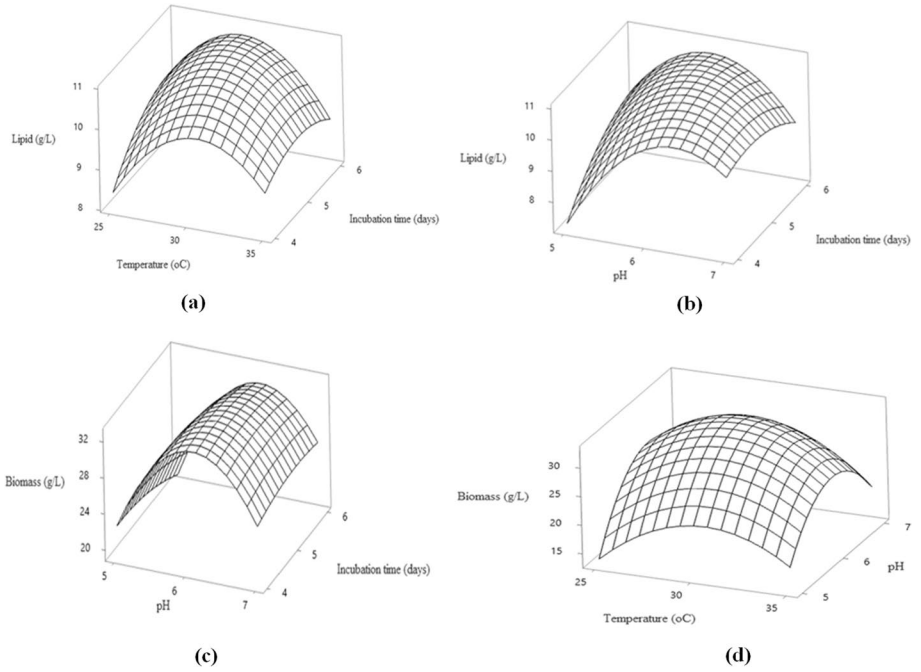


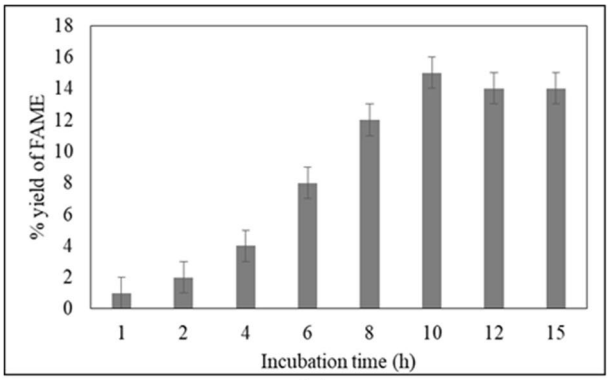
Fig. 3 RSM plots showing the effect of **a** temperature and incubation time and **b** pH and incubation time on lipid production and the effect of **c** pH and incubation time and **d** temperature and pH

immobilized lipase-mediated transesterification of oils. The small addition of water aids in the immobilized lipase-mediated transesterification of oils which aids in the catalytic activity of immobilized lipase towards enhanced FAME yield [8, 33, 35].

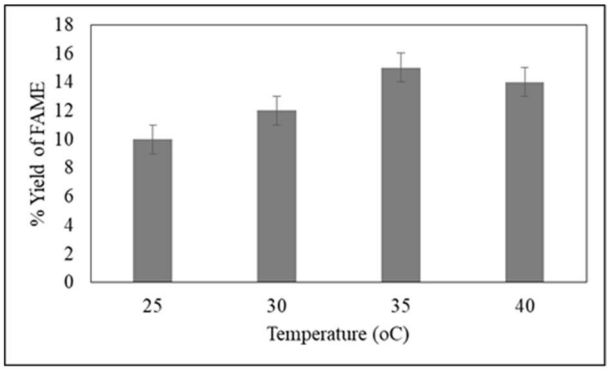
.The enzyme is susceptible to pH changes because changes in pH can affect the ionization states of the enzyme and thus affect its selectivity and activity. Therefore, the effect of pH on FAME yield of immobilized lipase-mediated transesterification of oleaginous lipid was studied from pH 4.0–9.0 (Fig. 4c). The results showed a higher FAME yield at pH 6.0; beyond that, the lower FAME's yield was observed due to the enzyme denaturation associated with the higher pH [34]. Hasan et al. [36] reported that a pH of 7.0 is the best for favorable immobilized *Candida rugosa lipase*-mediated transesterification of waste cooking oil.

Biodiesel Characterization

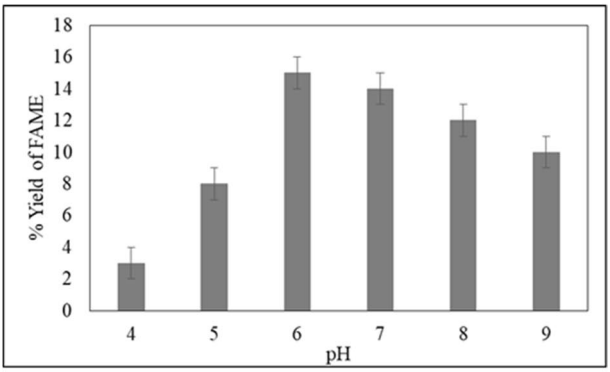
To confirm its acceptability as a fuel in diesel engines, the biodiesel made from oleaginous yeast lipid was characterized and compared to the ASTM D975 fossil diesel standard. The fuel property of the produced biodiesel is shown in Table 4. The specific gravity of the produced FAME was 0.85 ± 0.25, which compares well with the biodiesel standards. Viscosity is a measurement of a liquid's resistance to flow caused by internal friction between two parts of the fluid flowing over each other [37]. The viscosity of produced FAME was 4.8 ± 0.5, equivalent to that of standard gasoline fuel. The flashpoint is the lowest temperature when the vapor above the oil ignites when a test flame is applied. The flashpoint of the



(a)



(b)



(c)

Fig. 4 Effect of **a** incubation time (h), **b** temperature (°C), and **c** pH on enzymatic transesterification of microbial lipid. All values are represented as + s.d of three replicates

presently produced FAMEs was 135 °F, which is within the standard limits (140 °F). The flashpoint of biodiesel corresponds to the percentage of saturated hydrocarbon compounds and the residual alcohol. The low-temperature property of FAMEs is the cloud point, the temperature at which oil becomes cloudy or hazed. The cloud point of produced FAMEs was 50 °F, the most common indicator of a fuel's tendency to crystallize [38]. The superior

Table 4 Characterization of biodiesel. All results were represented as ± s.d of three replicates

Property	Oleaginous FAMEs	Standard petrol diesel
Viscosity at 40 °C (cp)	4.8 ± 0.5	5.5
Specific gravity	0.85 ± 0.25	0.98
Flash point (°F)	135 ± 0.12	140
Cloud point (°F)	50 ± 1.2	40

fuel properties associated with the immobilized lipase-mediated transesterification of oils were also reported with the Jatropha [21] and Simarouba [22] oils.

Conclusion

The present study put forth biodiesel production from oleaginous lipids of *Cryptococcus* sp. MTCC 5455 was produced using pretreated less-cost *Brassica juncea* as a carbon source. Using pretreated (dilute alkali coupled microwave irradiation) *B. juncea by Cryptococcus* sp. as a carbon source results in a 32.50 g/L biomass and 11.05 g/L lipid content. The immobilized lipase-mediated oleaginous lipid transesterification results in a 15% FAME yield with the transesterification variables of 10-h incubation time, pH 6.0, and 35 °C. The produced FAME's fuel properties (specific gravity, viscosity, flash point, and cloud point) conform to the ASTM D975 biodiesel standards. Further optimization and blending studies of produced FAMEs are needed for foreseeable transportation fuel.

Acknowledgements The authors sincerely acknowledge Prof. Ina Shastri, Vice Chancellor of Banasthali Vidyapith, for providing infrastructure to carry out this work.

Author Contribution Ashok Kumar Yadav performed all the experiments. Arindam Kuila planned the work and wrote the manuscript along with Ashok Kumar Yadav. Vijay Kumar Garlapati supervised the work and approved the final draft for submission.

Declarations

Ethical Statement Not applicable.

Consent to Participate and Publication All the authors agreed to submit the work to this journal.

Conflict of Interest The authors declare no competing interests.

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