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Yeast and bacteria co-culture-based lipid production through bioremediation of palm oil mill effluent: a statistical optimization

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Abstract

In the present study, a co-culture of yeast (*Lipomyces starkeyi*) and bacterium (*Bacillus cereus*) was used to optimize lipid accumulation capability and simultaneous treatment of wastewater using palm oil mill effluent (POME) as a carbon source. The influence of process parameters (i.e., inoculum composition, pH, temperature, and time) on the lipid accumulation and the chemical oxygen demand (COD) removal were optimized using design of experiments (DoE) as a statistical tool. The DoE results suggested that the maximum lipid accumulation of 2.95 g/L and COD removal efficiency of 86.54% could be obtained while the inoculum composition, pH, temperature, and incubation time were 50:50, 6.50, 32.5 °C, and 90 h, respectively. The predicted results were very close to the experimental results (< 5% deviation); hence, the proposed model could be useful to predict the lipid accumulation and COD removal performance of a yeast and bacteria co-culture.

Keywords Palm oil mill effluent · Bioremediation · Lipid accumulation · Yeast and bacteria co-culture · Optimization

1 Introduction

The use of conventional fossil fuels and mineral resources as energy sources is untenable due to their limited reserves and accumulation of greenhouse gases in the environment [1, 2]. Therefore, sustainable and cost-effective renewable energy sources are required to meet energy demand as well as support an ecofriendly environment. Besides, the amount of industrial wastewater generation is increasing day by day that cannot be retained due to population growth and industrialization [3, 4]. Among the different industrial wastewaters, palm oil mill effluent (POME) is a predominant wastewater which is considered mostly threatening to the environment [5, 6]. Direct discharge of POME on arable land causes serious pollution to the environment as it contains high amount of ammonia, phenolic compounds, low pH (~4.5), and high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in the range of 50,000–100,000 mg/L and 25,000–54,000 mg/L, respectively [3]. Nonetheless, several researchers have reported that POME contains a considerable amount of nutrients such as carbohydrates, vitamins, proteins, and minerals (e.g., K, N, Mg, Ca, P, Fe, B, Zn, Mn, and Cu) which can stimulate the growth of heterotrophic microbes [7, 8]. Therefore, the use of this industrial wastewater as a feedstock for producing non-fossil biofuels could be a promising solution, as it simultaneously addresses the demand for renewable carbon fuels and the diminution of the environmental burden posed by palm oil milling.

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Microbial lipids are regarded as potential substitutes of vegetable oils and animal fats for producing non-fossil biofuels (i.e., biodiesel) and oleochemicals as the oil properties are similar in types, composition, and structure of fatty acids. Oleaginous microorganisms such as bacteria [9], yeasts [10, 11], molds [12], and algae [13, 14] are capable of accumulating more than 20% of lipids [3, 7]. However, the yeast and algae are the most explored oleaginous microbes due to their high lipid contents. Recently, bacteria have gained attention as potential candidates due to their simpler genome constructions, higher metabolic rate, and the capability to produce different kinds of lipids compared to multicellular eukaryotes [15]. To date, several oleaginous yeasts including *Lipomyces*, *Candida*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Trichosporon*, and *Yarrowia* have been extensively studied for microbial lipid accumulation. Among them, *Lipomyces starkeyi* has been widely reported as potential microbe because they are capable of utilizing a wide range of carbon source and produce fatty acids that are similar to vegetable oils [16]. Besides, few studies have explored that several species of bacteria such as *Bacillus cereus*, *Bacillus subtilis*, *Rhodococcus opacus*, and *Pseudomonas* spp. could produce a considerable amount of lipids as triacylglycerols (TAGs) [7, 17, 18]. Moreover, the strains of *Bacillus* including *B. subtilis* and *B. cereus* produce varying degrees of enzymes such as cellulase and lipase which would promote wastewater treatment [5].

Recently, it has been revealed that the lipid accumulation and COD removal could be enhanced by using the mixed cultures compared to the individual cultures [5, 19, 20]. Cai et al. [21] demonstrated that the biomass growth was considerably higher ($p < 0.05$) while inoculated with a mixed culture of *Isochrysis galbana* (algae) and *Ambrosiozyma cicatricosa* (yeast) than the monocultures. In a recent study, Cheah et al. [22] obtained a satisfactory biomass growth of 2.04 g/L (with a productivity of 185.71 mg/L/day) and lipid content (16.04%) by co-culturing bacteria (i.e., *Pseudomonas* sp.) on microalgae (i.e., *Chlorella sorokiniana* CY-1) through an effective POME bioremediation (COD removal). In addition, Bala et al. [5] found that the COD removal efficiency (90.64%) was significantly higher for bacterial co-culture (*B. subtilis* 106 PB and *B. cereus* 103 PB) than for the monocultures. Nevertheless, a co-culture culture of microalgae (*Chlorella vulgaris*) and yeast (*Rhodotorula glutinis*) produced a higher biomass (4.63 g/L) and lipid accumulation (2.88 g/L) compared to the pure cultures [19]. This is because, in the mixed cultures, two or more preselected microbes could be synchronously grown within the same substrate to mutually utilize complementary metabolic activities to survive, grow, and reproduce [23]. Therefore, the symbiotic association of yeast with bacterium could be advantageous for enhancing lipid accumulation and bioremediation of POME which has not been studied yet.

In co-culture system, the composition of co-culture inoculum could have significant effect on the performance of growth and lipid accumulation. Besides, various experimental conditions such as pH, time, and temperature strongly influence the performance of microbial lipid production [24, 25]. In some recent studies, it has been observed that lipid content and fatty acid compositions fluctuate based on the nature of microorganisms and the environmental conditions including substrate type and concentration, incubation temperature and period, medium pH, static and shaking condition, and nutrients [26, 27]. Therefore, optimization of operational parameters is emergent to enhance the performance of microbial lipid production through facilitating process efficiency [24]. Recently, the multivariate statistical optimization techniques like response surface methodology (RSM) have attracted considerable attention to optimize process parameters since a significant number of parameters could be optimized using this tool [25]. A wide experimental area with minimum runs can be explored using this technique. The contribution of individual factors on the different responses and their interactions can also be obtained [28]. Furthermore, the impact of a particular factor at various levels of other factors is evaluated; thus, more precise assumptions could be achieved over the whole experimental area [29]. Thus, the constraints of the conventional optimization method called "one variable at a time" can be overwhelmed. Among the RSM-based models, the most popular class of second-order regression metamodel designs is central composite design (CCD) because this is a factorial or fractional factorial design with center points, expanded with a group of axial points to model a response variable with curvature, and moreover efficiently estimate first- and second-order terms [30]. The CCD models are optimized for fitting quadratic models and include equal predictability in all directions from the center. Specially, the CCD is a more useful methodology for modeling various technological processes in the case of small number of preformed experiments compared with the "one variable at a time" approach [31].

Abdelhamid et al. [24] optimized some variables such as pH, time, and temperature for lipid production from *Penicillium commune* NRC2016 using traditional method, where they achieved the maximum performance after 5 days of incubation using an initial pH and temperature of 7.0 and 20 °C, respectively. However, the method did not depict complete effects of parameters on response and interactive influences of different variables could not be explained. In another study, Shoaib et al. [25] optimized cultural conditions such as pH, temperature, and time to 6.0, 28 °C, and 7 days, respectively, for lipid accumulation using *Aspergillus wentii* Ras101 by following RSM. However, the optimization of inoculum compositions along with the aforementioned parameters (i.e., pH, temperature, time) which determine the performance of lipid accumulation in a yeast-bacteria co-culture has not been studied. In this study, several operational conditions such as pH, temperature, and time with co-culture inoculum compositions were optimized using CCD to maximize the lipid

production and COD removal efficiency. Moreover, the effects of different variables and their interactive influences on the responses were explained. In designing co-culture inoculum, the bacteria spp. *B. cereus* was selected due to its capability of utilizing a broader range of substrates including real wastewater, especially POME, while the yeast spp. *L. starkeyi* was preferred as a robust lipid producer.

2 Materials and methods

2.1 Sample collection and culture medium preparation

Raw POME was acquired from a local palm oil mill (LKPP Corporation Sdn. Bhd.) located in Gambang, Pahang, Malaysia, and stored at 4 °C to avoid further deterioration of ingredients. The solid and debris present in the POME were removed using a Whatman No. 1 filter paper. The composition of POME was determined by following APHA methods [32] and presented in Table S1. The raw POME (after filtration) was considered the 100% POME sample, and 50% POME sample was prepared by adding an equivalent amount of deionized water, as it was found to be a suitable medium for microbes in our earlier study [3, 7]. The pH was set to 7.0 ± 0.10 using 1 N NaOH.

2.2 Inoculum preparation

In this study, a wild-type pure culture of *B. cereus* and ATCC culture of *L. starkeyi* were used as inoculum. The strain of *B. cereus* (accession no. MF 661883) [33] was attained from the Laboratory of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Malaysia. The pure culture of *L. starkeyi* ATCC 56304 was obtained from the University of Naples Federico II (Laboratory of Biochemical Engineering), Italy. The 10 mL agar slants (agar, 2% w/v; yeast extract, 10 g/L; peptone, 10 g/L; and glucose, 10 g/L) were prepared to grow and maintain the stock culture. The fresh cells were prepared by sub-culturing 1 mL of broth on the solid agar Petri plates and the inoculated plates were incubated at 30 °C for 24 h. A working cell bank culture was then prepared by dissolving 10 loops of microbes from the subcultured cells in 10 mL of sterile water. Then, the liquid cultures of yeast and bacteria were prepared in Luria-Bertani broth (10% v/v) as growth medium inoculating with 1 mL of working cell bank cultures at 30 °C and 150 rpm overnight. Finally, the POME medium was inoculated with 5% (v/v) of this primary inoculum. The cell concentrations of primary inoculums were measured at OD_{600} = 1.5 using a UV spectrophotometer (Shimadzu, model UV-180 240 V).

2.3 Wastewater treatment analysis

The co-culture (*B. cereus*:*L. starkeyi*) was inoculated and enriched in POME for 5 days (120 h). Briefly, the 200 mL of POME samples (50% POME) were taken in the 500 mL of Erlenmeyer flasks and autoclaved (at 121 °C for 20 min). The POME samples were then inoculated by the 5% (v/v) of primary inoculum and incubated for 120 h at 150 rpm. The COD of POME samples (before and after fermentation) were analyzed by using the APHA method [32], and the COD removal efficiency was calculated by following Eq. 1.

$$\text{Removal efficiency}\ (\%) = \frac{(C_\mathrm{i} - C_\mathrm{f})}{C_\mathrm{i}} \times 100 \quad (1)$$

where the initial COD in POME before treatment is expressed as C_i and the COD after treatment is termed as C_f.

2.4 Biomass harvesting and lipid extraction

The microbial biomass was harvested for different duration of enrichment. Briefly, the biomass was separated from liquid cultures (200 mL) by centrifuging (8000×*g*; 10 min) and weighted by an analytical balance. To obtain dry biomass, the wet biomass was dried in an oven at 60 °C until a constant weight was observed. Thereafter, the microbial lipids were extracted by using electroporation (EP) technique, according to the method described by Karim et al. [34]. Briefly, the dry biomass (50 mg) was taken in the EP reactor, and thereafter, electrical pulses (4 kV) were applied for 10 min with a frequency of 100 Hz. The treated mixture was centrifuged for 10 min (2000×*g*) to separate the solvent phase. Finally, a Rotavapor (Buchi, R-100) was used to obtain dry lipids. All experiments were performed in triplicate to confirm each observation.

2.5 Experimental design and data analysis for optimization

The COD removal efficiency and lipid accumulation capacity were considered dependent variables, whereas the inoculum composition, medium pH, incubation temperature, and cultivation time were regarded as predictor variables to establish a mathematical model. The approach of sequential analysis using design of experiments (DoE) was proposed where each regressor was coded as shown in Eq. 2. Design-Expert version 7.1.6 software (Stat-Ease Inc., USA) was used for generation and evaluation of experimental design. The polynomial mathematical model generated by circumscribed central composite design is as follows:

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \left(\sum_{i=1}^{n} b_{ii} x_i\right)^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad (2)$$

In the equation, Y = predicted response; b_0 = constant coefficient; b_i = linear coefficient; b_{ii} = quadratic coefficient; b_{ij} = the interactions of coefficient; and x_i and x_j are coded values of a reactor.

2.6 Statistical analysis

The results obtained from experimental run were analyzed by Design-Expert (version 7.1.6). The model adequacy and error independency were used for each of the variables to diagnose the fitted models. The significance of the fitted model was evaluated by using the analysis of variance (ANOVA). One-way ANOVA was applied to estimate the significance of the model ($p < 0.05$). The surface response plots and contour plots were studied to reveal the effect of independent factors (inoculum composition, medium pH, incubation temperature, and cultivation time) on the measured responses (COD removal efficiency and lipid accumulation capacity). Furthermore, different statistics were used to analyze the adequacy of the model and the coefficient of determination, R^2, was used to assess the integrity of the fitted model. In addition, the ratio of the signal to noise was evaluated by adequate precision statistics [29, 35]. How well the model fitted with the ith point and how far that point was from the rest of the data were evaluated by using Cook's distance statistics. The large distance data (greater than unity) requires to be assessed with more attention since the point is more influential than the other [29]. Normal probability plot of the residuals was used to check the normality of the errors. The consistency of variances was measured by plotting the residuals versus the time sequence of the runs, predicted values, and each independent variable. The data transformation on the response was used to ease the issues, while the discrepancies were observed. The replicates of center points were inserted to the factorial designs to analyze the adequacy of the model to capture the curvature expressed in the response. The set of equations derived from the differentiation of the fitted model was solved to calculate the predicted response for optimum value and their levels of independent variable. The independent variables used for the lipid accumulation and COD removal are shown in Table 1.

3 Results

3.1 Regression model analysis

Under the experimental conditions of CCD, several experiments were conducted with a different combination of parameters to estimate the interactive effects of variables (Fig. 1; Tables S2 and S3). The lipid production and COD removal efficiency data were fitted using a quadratic model. The predicted data and experimental data were very close to each other and distributed symmetrically, indicating that the good agreement within predicted and actual data for both COD removal efficiency and lipid accumulation (Fig. S1 and Fig. S2).

The interactive effect of inoculum composition, medium pH, incubation temperature, and cultivation time on the COD removal and lipid accumulation performance over operation period is illustrated in Fig. 1. The effect of responses can be observed by changing the variables from a single reference point. The perturbation plot in Fig. 1b showed that inoculum composition, medium pH, and incubation temperature have significant impact on lipid production, where the effect of variables could be compared with the center point of design space. Similar profile was observed in the case of COD removal (Fig. 1a). A steep curvature in inoculum composition, "(A)" curve, suggests that COD removal is sensitive to this factor. The comparatively flat "(B)" and "(C)" curve shows slight lower sensitivity than "(A)" to COD removal. It is clear from the perturbation plot that inoculum composition has comparatively more significant factor to influence lipid yield as well as COD removal.

The empirical relationship between responses (lipid accumulation and COD removal efficiency) and the variables are analyzed using the equations given below (Eq. 3 and Eq. 4).

$$\begin{aligned}\text{COD removal efficiency (Coded)} &= 83.67 + 0.083 \times x_1 + 0.67 \times x_2 + 2.08 \times x_3 \\ &\quad + 2.58 \times x_4 - 0.37 \times x_1 \times x_2 + 0.13 \times x_1 \times x_3 \\ &\quad + 0.000 \times x_1 \times x_4 + 0.13 \times x_2 \times x_3 + 0.000 \times x_2 \\ &\quad \times x_4 - 0.50 \times x_3 \times x_4 - 2.27 \times x_1^2 - 1.15 \times x_2^2 - 2.02 \\ &\quad \times x_3^2 - 0.65 \times x_4^2 \end{aligned} \quad (3)$$

$$\begin{aligned}\text{Lipid accumulation (Coded)} &= 2.78 - 0.14 \times x_1 - 0.093 \\ &\quad \times x_2 + 0.012 \times x_3 \\ &\quad + 0.19 \times x_4 - 0.13 \times x_1 \\ &\quad \times x_2 + 0.17 \times x_1 \times x_3 \\ &\quad + 0.013 \times x_1 \times x_4 - 0.11 \\ &\quad \times x_2 \times x_3 \\ &\quad + 9.375E{-}003 \times x_2 \\ &\quad \times x_4 - 0.022 \times x_3 \\ &\quad \times x_4 - 0.41 \times x_1^2 - 0.29 \\ &\quad \times x_2^2 - 0.29 \times x_3^2 - 0.15 \\ &\quad \times x_4^2 \end{aligned} \quad (4)$$

Table 1 Variables used for lipid accumulation and COD removal

Variables	Levels				
	−2	−1	0	+1	+2
Concentration of inoculum A (*B. cereus*) (%), x_1	10	30	50	70	90
pH, x_2	5.5	6.0	6.5	7.0	7.5
Temperature (°C), x_3	27.5	30.0	32.5	35.0	37.5
Incubation time (h), x_4	70	80	90	100	110

3.2 Statistical analysis

The ANOVA tables obtained from the model for the COD removal efficiency and lipid accumulation are presented in Tables S4 and S5, respectively. The *F* values corresponding to the x and y axes were determined from the obtained model in the case of COD removal efficiency (7.85) and lipid accumulation (10.28), which ascribes the accuracy and precision of the model. The adj-R^2 value was obtained as 0.7678 for COD removal efficiency while the value of 0.8175 was observed for lipid accumulation. The higher values of adj-R^2 indicate that the model was developed using adequate data; hence, the prediction of model was reliable to obtain accurate performance. It is worth noting that the pred-R^2 values for COD removal efficiency and lipid accumulation were observed as 0.3622 and 0.4591, respectively. In addition, the higher adequate precision values of 9.693 and 10.604 for COD removal efficiency and lipid accumulation, respectively, indicate an adequate signal, and therefore, this model could be useful to determine the design of space. Nevertheless, the high R^2 values (close to 1) for the model suggesting the experimental data were adjusted with the model [36]. The higher R^2 values for COD removal efficiency (R^2, 0.8799) and lipid accumulation (R^2, 0.9056) confirming the model successfully explained the relationship between variable parameters and operational conditions accurately. The R^2 values for COD removal efficiency and lipid accumulation indicate that 87.99% and 90.56% of the variability in response could be explained by these fitted models, respectively. These results suggest that the employed models were acceptable to estimate the responses for both lipid accumulation and COD removal efficiency.

The statistical significance of equation terms was estimated based on *p* values. Generally, the model terms are significant when this value is less than 0.0500, while the values greater than 0.1000 indicate that the model terms are not significant. ANOVA analysis for the quadric model of COD removal efficiency is presented in Table S4. The *p* value of the model (0.0001) showed that the model was very significant. The linear term temperature (x_3: 0.0003) and time (x_4: < 0.0001) were also significant, whereas the quadratic effect of inoculum composition (x_1^2: < 0.0001), pH (x_2^2: 0.0142), and temperature (x_3^2: 0.0002) was found to be very significant. However, the interactions of the manipulated variables were not significant for COD removal efficiency. For lipid accumulation, the *p* value of the model (< 0.0001) indicated that the model was very significant (Table S5). The linear term inoculum composition (x_1: 0.0146), pH (x_2: 0.0423), and time (x_4: 0.0027) were also significant, whereas the quadratic effect of all four variables such as inoculum composition (x_1^2: < 0.0001), pH (x_2^2: < 0.0001), temperature (x_3^2: < 0.0001), and time (x_4^2: 0.0079) was found to be very significant. Moreover, on assessing interactions between the manipulated experimental variables, the *p* values of cross-terms provide some insights, as they do

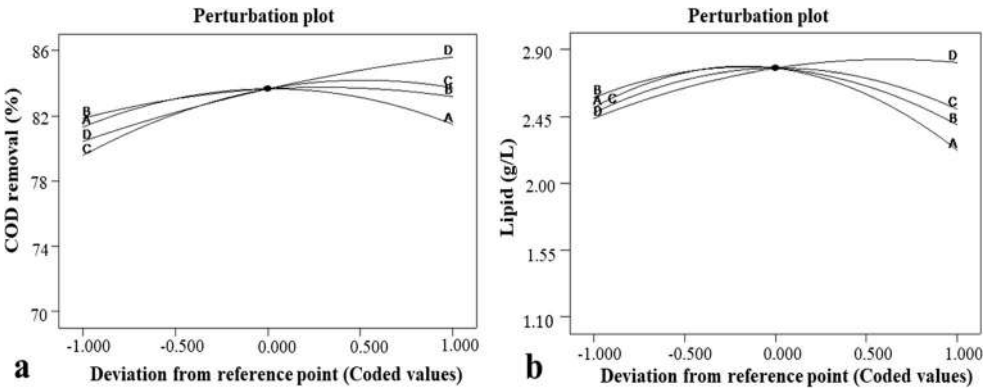

Fig. 1 Perturbation plot illustrating the interactions of the independent variables using different combinations of parameters for **a** COD removal efficiency and **b** lipid accumulation, where (A), (B), (C), and (D) represent the inoculum compositions, medium pH, incubation temperature, and time, respectively

regarding effects of each single variable by itself. The interaction of the inoculum composition and temperature (x_1x_3: 0.0208) was significant to lipid accumulation performance.

3.3 COD removal efficiency

The effect of targeted operational parameters (i.e., inoculum composition, temperature, pH, and time on the COD removal efficiency) is presented in Figs. 2a–c and 3a–c. As can be seen from the figures, the COD removal efficiency increased from pH of 6.0 to 6.5 and the maximum COD removal was availed at pH ~ 6.5. Likewise, the COD removal efficiency was improved by increasing the temperature from 30.0 to 32.5 °C, and the highest COD removal was obtained at 32.5 °C. Nevertheless, further increasing in the pH and temperature substantially reduced the COD removal efficiency. Besides, the inoculum compositions also significantly influenced the COD removal efficiency. The COD removal efficiency enhanced with the increasing concentration of inoculum A (*B. cereus*) from 30 to 50%, then started to decrease while further increased to 70%. Interestingly, the equal ratio of both microbes obtained the highest COD removal efficiency. Then again, the COD removal efficiencies were enhanced with the function of incubation time and reached the plateau after 90 h of operation.

3.4 Lipid accumulation capacity

The interaction between dependent and independent parameters was analyzed with the 3D response curves. The lipid accumulation was varied by modifying operational parameters (i.e., the inoculum composition, temperature, pH, and time) as presented in Fig. 4a–c and Fig. 5a–c. It is apparently observed that the inoculum composition, pH, and incubation time significantly influenced lipid accumulation. The equivalent concentration (50:50) of yeast and bacteria seems to be an optimum ratio for obtaining maximum lipid production. Besides, the lipid accumulation increased with the augmenting temperature, but the temperature above 32.5 °C showed the descending trend. The lipid accumulation increased when raising the pH from 6.0 to 6.5, and the maximum lipid was obtained at pH ~ 6.5. However, further increment in pH negatively affected on the upward trend of lipid accumulation. Likewise, the incubation time maintained a positive correlation on lipid accumulation while the maximum lipid accumulation was reached after

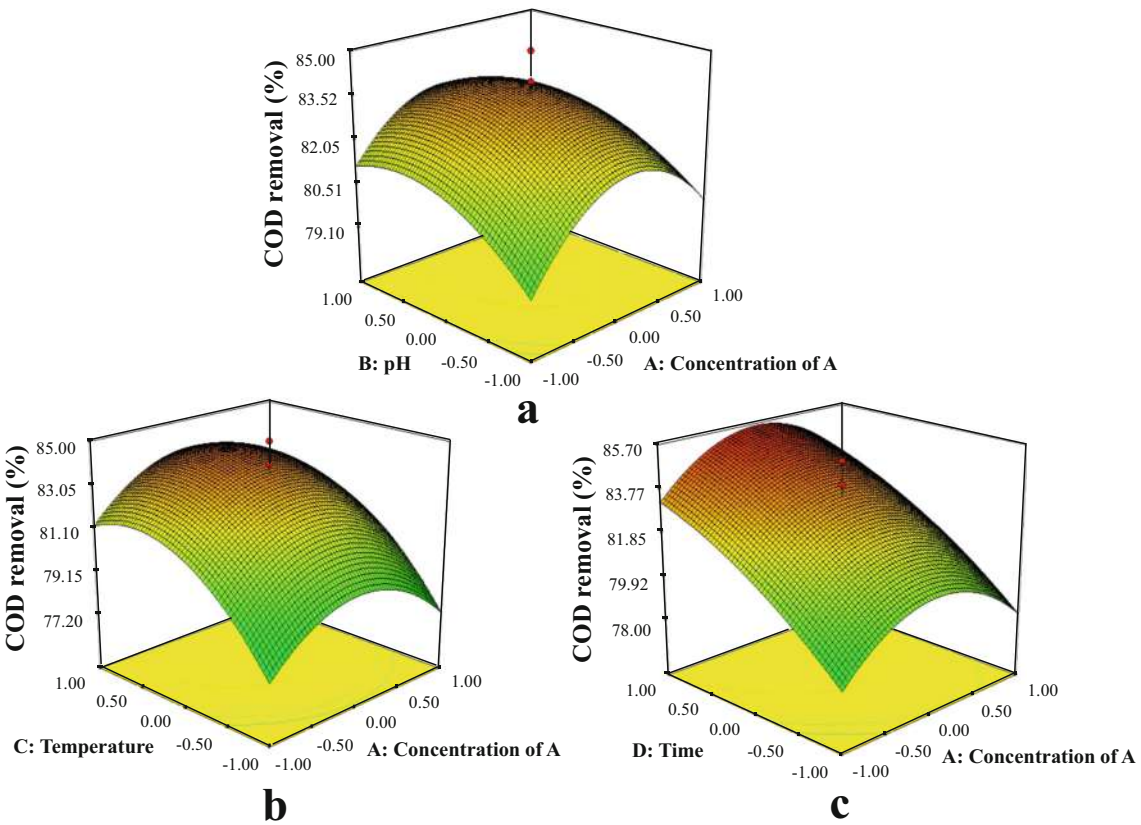

Fig. 2 Three-dimensional response surface plots showing the interaction between **a** inoculum composition and pH, **b** inoculum composition and temperature, and **c** cultivation time and inoculum composition

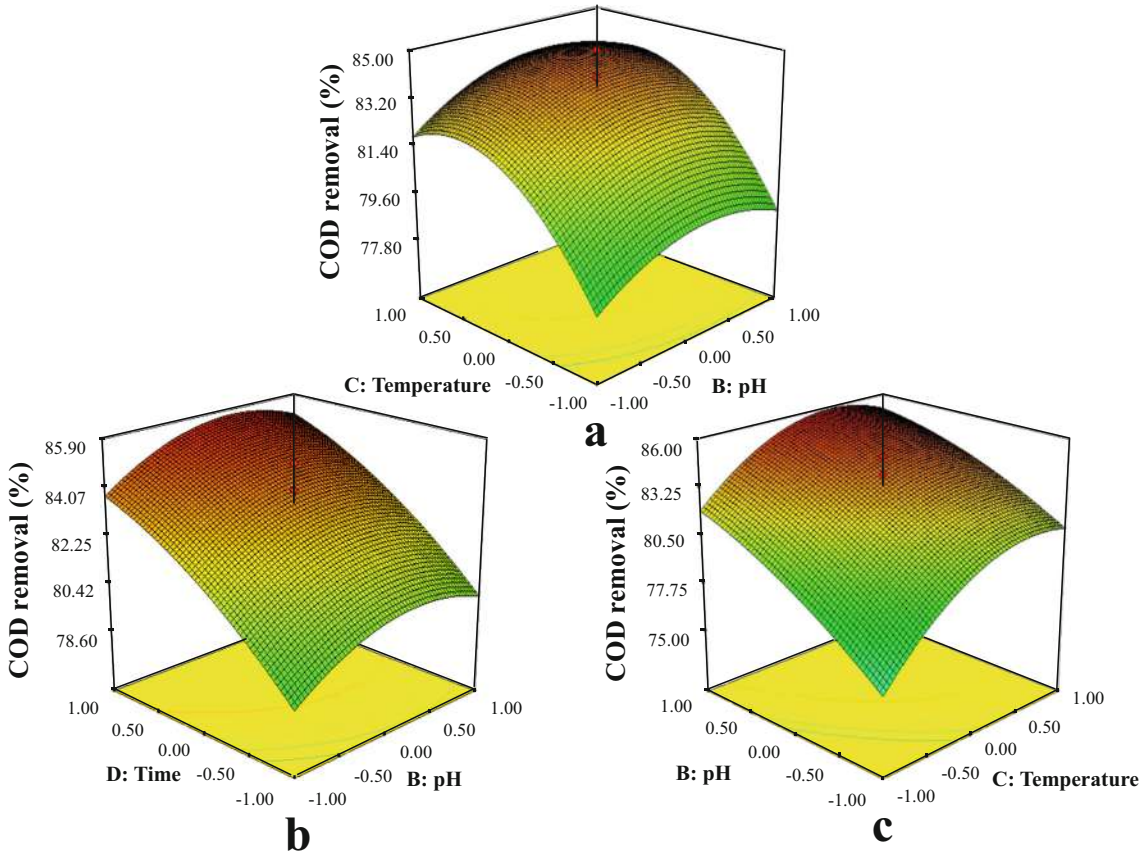

Fig. 3 Three-dimensional response surface plots showing the interaction between **a** temperature and pH, **b** cultivation time and pH, and **c** cultivation time and temperature

90 h of operation, but subsequently, the lipid accumulation showed a quasi-steady fashion.

Based on mathematical equations, individual parameters were optimized to get optimum performance of lipid accumulation and COD removal efficiency. The optimum parameters are shown in Table 2. According to Table 2, the maximum performance can be achieved while the inoculum composition, pH, temperature, and incubation time would be 50:50, 6.50, 32.5 °C, and 90 h, respectively. Under these conditions, the model estimated that the maximum lipid accumulation efficiency would be 2.95 g/L while the maximum COD removal efficiency would be 86.54%. To justify the model prediction, an experiment was conducted in three replicates by following model given operational parameters. At optimized process parameters, we observed that the COD removal efficiency and lipid accumulation were 84.57% and 2.81 g/L, respectively, with error values (2.28 and 4.75, respectively) between predicted and experimental results. The validation confirmed a good agreement between predicted responses and experimental results. Hence, this model could be applied to predict the performance of co-culture inoculated reactor with varying operational conditions.

4 Discussion

In this study, the effect of operational parameters (i.e., pH, inoculum composition, time, and temperature) on the performance of lipid accumulation and wastewater treatment (COD removal) by a co-culture of *B. cereus* and *L. starkeyi* was optimized using RSM. Recently, the co-culture inoculums have gained attention from the researchers for enhancing lipid production because the collective output of co-culture is generally higher than their pure cultures [37–39]. In the present study, several ratios of both microbes were investigated to achieve highest performance in the lipid accumulation and COD removal efficiency. However, among them, the equivalent concentration (50:50) of both microbes attained the highest performance compared to other ratios. This might be due to the synergistic interaction between two microbes. Indeed, the ratio of inoculum greatly influences the microbial synergistic interaction and lipid accumulation. For instance, the higher biomass (2.04 g/L) was obtained by the co-culture inoculum of *Pseudomonas* sp. and *C. sorokiniana* with a ratio of 1:1 in the POME [40]. At this inoculum ratio, lipid content (16.04%) was about twofold higher than other ratios of 2:1 or 1:2. Similarly, Cheirsilp et al. [19] obtained higher lipid

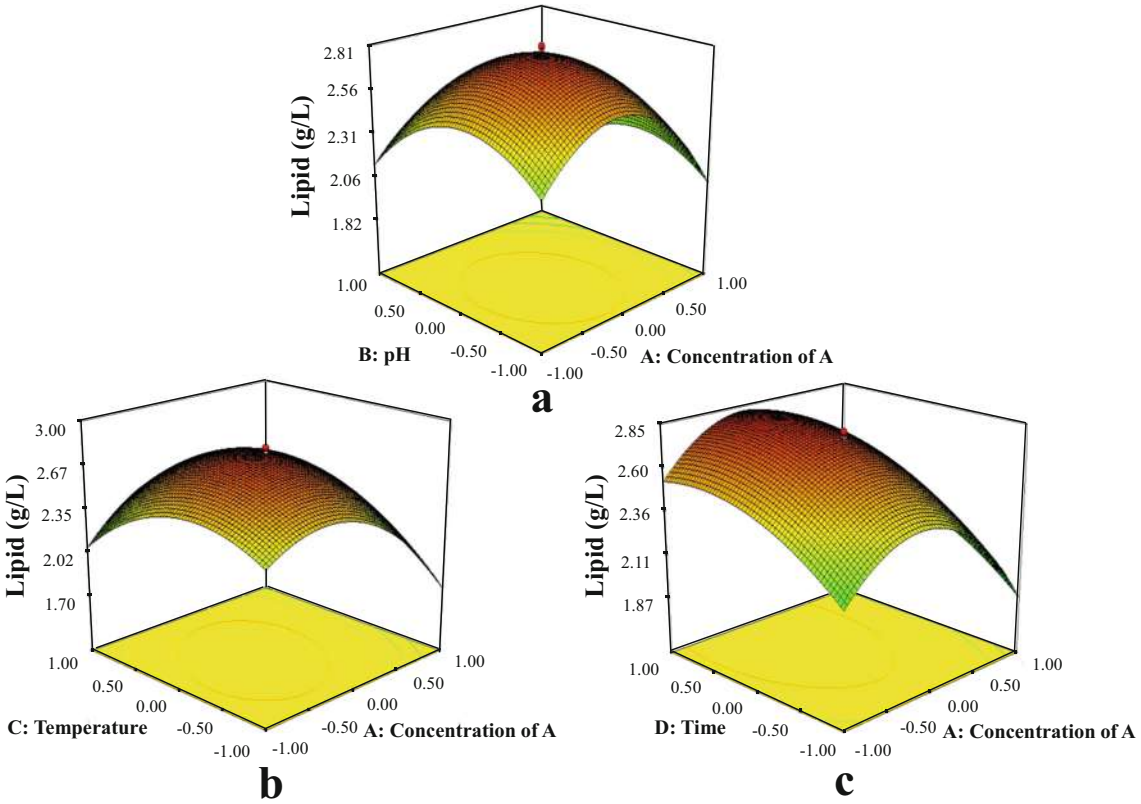

Fig. 4 Three-dimensional response surface plots showing the interaction between **a** inoculum composition and pH, **b** temperature and inoculum compositions, and **c** cultivation time and inoculum composition

production (2.88 ± 0.16 g/L) and COD removal (79.0 ± 1.1%) using a co-culture of *R. glutinis* (yeast) and *C. vulgaris* (microalga) by maintaining the ratio of 1:1 (Table 3). In another study, the ratio of 0.20–0.25 for bacteria and microalgae was observed as optimum culture conditions to obtain maximum lipid accumulation [27]. Therefore, it can be speculated that the inoculum composition significantly influences the growth of microbes as well as lipid accumulation.

The initial pH of the substrate significantly influences the microbial biomass growth as well as lipid production because the H^+ concentration severely influences the growth and sporulation process of microbes [43]. The influence of substrate pH on lipid accumulation performance has already been studied by several researchers [19, 24, 25]. In general, the optimal growth of microbes could be obtained at neutral pH. In this study, it was observed that lipid accumulation augmented with the increase in initial pH from 5.5 to 6.5, and then slowly decreased for further enhancement to 7.5. This could be due to the smaller biomass growth in higher medium pH because the *L. starkeyi* could assimilate phenolic compounds present in the POME leading to the formation of hydroxide ions [3]; hence, the pH was increased with the incubation time. Consequently, the pH of substrate would have reached a basic condition faster and inhibited the microbial growth. Some studies have reported that the acidity of medium could inhibit the microbial lipid accumulation due to the interruption in metabolic cycle of microbes [44]. Zhao et al. [45] showed that the maximum growth of *Lactobacillus* bacteria was at an initial pH of 6.5; however, pH below 5.0 was not favorable for the growth of bacteria. Therefore, it can be concluded that the neutral pH is imperative to achieve optimal microbial growth as well higher COD removal and lipid accumulation.

Nevertheless, the incubation temperature significantly influenced the biomass and lipid accumulation. Several previous studies indicated that the biomass growth and lipid accumulation were increased with an increasing temperature ranging from 25 to 35 °C [25, 46]. This might be due to the higher metabolic/enzymatic activity of microorganisms at this temperature range [24]. This study found that the COD removal efficiency and lipid production increase with rising the incubation temperature from 27.5 to 32.5 °C, and gradually decrease at incubation temperatures higher than 32.5 °C. Our results were in accordance with Zhao et al. [45] that the optimum temperatures for both bacteria and yeast were observed at 28–32 °C, and they obtained the highest growth rate at 31 °C using a co-culture of yeast and bacteria. A recent study by Subhash and Mohan [26] observed that the temperature of 30 °C was suitable to have maximum growth as well as lipid accumulation by oleaginous fungus *Aspergillus awamori*. The maximum lipid production (39.0 ± 1.43% lipid/dry biomass)

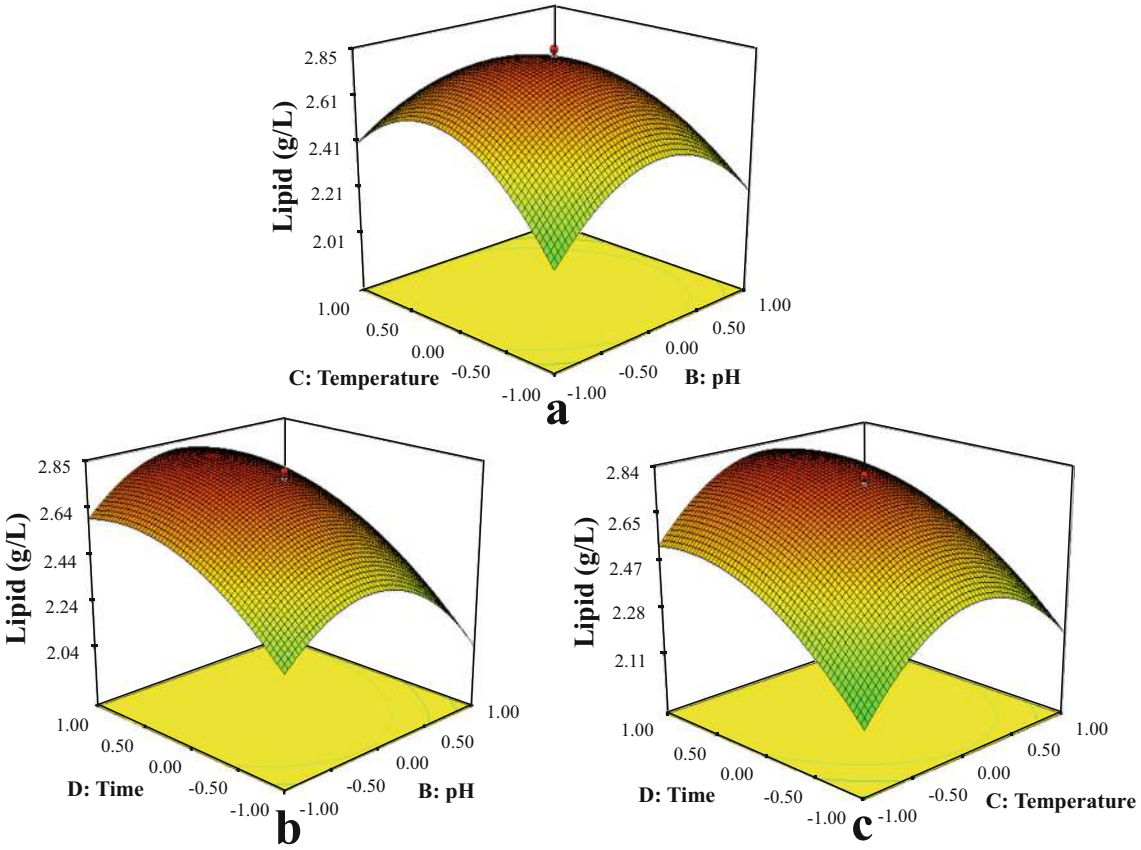

Fig. 5 Three-dimensional response surface plots showing the interaction between **a** temperature and pH, **b** cultivation time and pH, and **c** cultivation time and temperature

was observed at 30 °C for *Penicillium brevicompactum* NRC 829, while a lower biomass and lipid production was observed at increasing or decreasing incubation temperature [46].

The incubation time had a great impact on the performance of lipid accumulation and COD removal. At initial stage, the COD removal efficiency significant increase could be due to the quicker growth of microorganisms. However, afterwards (after 3 to 4 days), the COD removal efficiency reached to the stationary phase due to the sluggish microbial growth rate. The COD removal efficiency slightly fluctuated after 4 days, attributed to the fact that the growth of microorganisms was hindered as the nutrients of POME medium depleted over time. Lipid production also raises with increases of incubation time in the initial period of operation, but it started decreasing after a few days of operation due to the degradation of stored lipid by microorganisms. This fact is well established that the oleaginous microorganisms are usually seen to store lipids at initial stage, especially during the lag/log phase, and started breaking down under carbon deficiency conditions [7, 19, 47]. In the present study, we observed that the lipid accumulation and COD removal efficiency were improved with an increasing of incubation time from 80 to 100 h and obtained a maximum COD removal efficiency (~ 85%) and lipid accumulation (2.81 g/L) at 90 h of incubation time. These results are compatible with Ali et al. [46], where they reported that *Aspergillus* spp. obtained maximum lipid accumulation after 5 days (120 h) of incubation. Likewise, a mixed culture of *R. glutinis* (oleaginous yeast) and *C. vulgaris* (microalgae)

Table 2 The best operational conditions for the process and experimental results to confirm optimization capability

Factors				Desirability	Response[a]					
					COD removal efficiency (%)			Lipid accumulation (g/L)		
x_1 (%)	x_2	x_3 (°C)	x_4 (h)		Prediction	Actual	Error (%) [b]	Prediction	Actual	Error (%) [b]
50	6.50	32.50	90	0.928	86.54	84.57±2.35	2.28	2.95	2.81±0.32	4.75

[a] Observed response value: mean ± S.D. ($n = 3$)

[b] [Difference between predicted value and actual value/Predicted value] × 100

Table 3 Performance of co-culture inoculums to produce microbial lipids through COD removal from different wastewaters

Strains	Co-culture consortia	Conditions	Biomass (g/L)	Lipid (g/L)	COD removal (%)	References
Pseudomonas sp. on *Chlorella sorokiniana* CY-1	Bacteria-microalgae, 1:1	30% (v/v) POME, 5 days	2.04	0.33	53.70	[22]
Klebsiella variicola and *Pseudomonas aeruginosa*	Bacteria-bacteria, 1:1	50% (v/v) POME, 11 days	–	–	69.28	[41]
Bacillus cereus 103 PB and *Bacillus subtilis* 106 PB	Bacteria-bacteria, 1:1	POME, 5 days	–	–	90.64	[5]
Rhodotorula glutinis and *Chlorella vulgaris*	Yeast-microalgae, 1:1	Sugar cane plant wastewater (molasses), 7 days	4.63	2.88	79.00	[19]
Scenedesmus obliquus with *Pseudomonas* sp.	Microalgae-bacteria, 2:1	BG11 medium, 10 days	2.96	0.68	–	[42]
Bacillus cereus and *Lipomyces starkeyi*	Bacteria: yeast, 1:1	50% (v/v) POME, 90 h		2.81	84.57	This study

obtained highest biomass as well as lipid accumulation after 5 days of cultivation in industrial waste, then slightly decreased in lipid production on day 7 [19]. Ali and El-Ghonemy [48] noticed that the *Aspergillus* sp. and *Trichoderma viride* achieved maximum lipid accumulation after 5 days of incubation. Therefore, the COD removal efficiency and lipid accumulation were significantly dependent on the incubation time.

5 Conclusions

In this study, the influence of several operational parameters (i.e., inoculum composition, pH, temperature, and time) of co-culture (*B. cereus* and *L. starkeyi*) inoculum was optimized using RSM for microbial lipid accumulation and concurrent bioremediation of POME. The statistical model suggests that the maximum lipid accumulation of 2.95 g/L and COD removal of 86.54% could be obtained under the conditions of inoculum composition, 50:50; pH 6.5; temperature, 32.5 °C; and incubation time, 90 h. An experiment was conducted by following optimum parameters as suggested by the model to justify the accuracy of model predictions. We obtained less than 5% deviation between model predictions and real experimental results which apparently justify the use of the proposed model. The results of the study suggest that the performance of microbial lipid production and bioremediation could be improved using yeast-bacteria co-culture inoculum in certain optimum conditions. However, further studies are required to develop a mechanistic model to know the insight of co-culture inoculum influences on lipid production and POME bioremediation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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