Process Biochemistry 89 (2020) 46–54

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Sequential optimization and large scale production of lipase using tri-substrate mixture from *Aspergillus niger* MTCC 872 by solid state fermentation

Venkatesh Mandari, Ashutosh Nema, Santhosh Kumar Devarai*

Industrial Bioprocess and Bioprospecting Laboratory, Department of Chemical Engineering, Indian Institute of Technology Hyderabad, Laboratory No: A-224, Kandi, Sangareddy-Dist, Telangana, 502285, India

ARTICLE INFO

Keywords:
Lipase
Prosopis Juliflora (PJ)
Solid state fermentation (SSF)
Specific activity
Simplex Centroid Mixture Design (SCMD)
Central Composite Face-Centered Design (CCFD)

ABSTRACT

Tri-substrate mixture of *Prosopis juliflora* (PJ), red gram husk (RGH) and cotton seed cake (CSC) has been studied for the production of lipase (E.C. 3.1.1.3) using *Aspergillus niger* MTCC 872 in solid state fermentation. Simplex centroid mixture design (SCMD) was implemented to optimize the tri-substrate mixture composition consisting of PJ, RGH and CSC. Mixture taken in the ratio of 6.66:1.66:1.66 for PJ:RGH:CSC has shown highest lipase activity of 212.20 ± 6.36 U/gds at 30 °C, 7 pH and 70 % initial moisture content (v/w). Sequential optimization of physical parameters was done using the central composite face-centered design (CCFD). The optimum mixture composition has shown the highest lipase activity of 269.87 ± 8.09 U/gds at 35 °C, 7 pH and 70 % initial moisture content (v/w). ANOVA analysis for SCMD and CCFD confirms the model's significance with R^2 values of 0.9989 and 0.968. A 1.27 fold increased lipase activity was obtained after physical parameters optimization. Large scale production using 1 kg substrate was carried out in tray bioreactor with different bed heights and the highest lipase activity of 208.79 ± 6.26 U/gds was obtained. This study signifies the enhancement of lipase production using substrate PJ for lipase production along with the other agricultural residues.

1. Introduction

In the past 200 years, fossil fuel consumption has increased rapidly, leaving the climate seriously impacted by global warming, and fossil fuel reserves depleted. These reserves may last only for the next 40 years as per the present fuel consumption statistics [1]. An increase in the consumption of fossil fuels increases demand. If oil depletes without alternate fuels, its price will rise [2]. So the scientists are working on liquid biofuels. Biodiesel emerged as the alternate source of the petroleum-based diesel fuel. Biodiesel manufactured by the transesterification of vegetable oils and short-chain alcohols in the presence of catalyst [3]. In previous studies, chemical (homogeneous and/or heterogeneous) catalysts such as H_2SO_4, KOH, SO_4/Fe-Al-TiO_2, Cu/Zn/ɣ-Al_2O_3 and Ti(SO_4)O, etc. are used in transesterification reaction although there exist several flaws with these methods [4–7]. Enzyme-catalyzed biodiesel production has several advantages over chemical-catalyzed transesterification, for example, the requirement of low energy, environmental friendly, relatively easy removal of glycerol and the complete conversion of free fatty acids (FFA) [8].

Lipases (E.C. 3.1.1.3) are a group of enzymes that catalyze the hydrolysis of triacylglycerols into di-acylglycerol, mono-acylglycerol then glycerol and FFA at the water-lipid interface. Lipases are ubiquitous and microbial lipases are advantageous over derived from plants and animals as they can be modified at the gene level and ease of cultivation [9]. Lipases exhibit a high degree of activity and stability. They do not require co-factors and can be easily immobilized on different matrices. As surface-active enzymes, lipase activity highly depends on the interfacial area of the organic-aqueous phase. Lipases can use relatively broad spectrum substrates, stability towards high temperature, pH, and they are enantioselective and regioselective [10]. Lipases can synthesize FFA and organic alcohols on the reverse reaction of carboxylate esters in the presence of excess water in a non-aqueous environment [11,12].

Lipases belong to the α/β hydrolases family. In general, microbial lipases molecular weight ranges between 19–60 kDa. The active site of lipases consists of three amino acid residues: a nucleophile is most commonly serine or cysteine, aspartate as acid and histidine as base residues [13]. The core consists of eight different beta strands (β1- β8) connected to six alpha helices (αA- αF). The nucleophile serine locates on C-terminal at the end of the β5 strand. A mobile lid covers the active

* Corresponding author.
E-mail addresses: ch17resch11004@iith.ac.in (V. Mandari), ch17mtech11008@iith.ac.in (A. Nema), devarai@iith.ac.in (S.K. Devarai).

https://doi.org/10.1016/j.procbio.2019.10.026
Received 24 June 2019; Received in revised form 14 October 2019; Accepted 22 October 2019
Available online 29 October 2019
1359-5113/

Nomenclature

ANOVA	analysis of variance
CSC	cottonseed cake
CCFD	Central Composite Face-Centered Design
FFA	free fatty acid
PJ	*Prosopis juliflora*
p-NPP	*p*-nitrophenyl palmitate
RGH	red gram husk
SCMD	Simplex Centroid Mixture Design
SmF	submerged fermentation
SSF	solid state fermentation

site. Whether this lid is open or closed will determine the active or inactive state of the enzyme. At the oil-water interface, the lid will open to give access to the active site to the substrate [14,15].

In an organic medium, lipase can also catalyze esterification and interesterification reactions [11]. Lipases have wide industrial applications due to their high substrate specificity, mild operating conditions, and high purity of the end product. So, there is a huge demand for lipase due to its widespread industrial applications, including detergent formulation, biodiesel production, pharmaceutical, food, cosmetic, and fine chemicals. The global market value for lipase predicted to reach $590.5 million by 2020, with an annual growth of around 6.5 % between 2015 and 2020 [16–18].

The use of cheap agro-industrial wastes as the substrate in solid state fermentation (SSF) gaining more attention due to higher activity compared to submerged fermentation (SmF). Production of the enzyme in the fermentation process requires the use of carbon and nitrogen sources for the growth of microorganisms. In SmF, microorganism grows on freely flowing liquid media. Therefore, SmF has notable advantages, such as maintenance and monitoring pH, dissolved oxygen, Temperature, etc. Contrarily, SSF provides a natural habitat for the most microorganisms, especially for fungi and mold. SSF requires less energy demand, cheap raw materials, and it is less susceptible to bacterial contamination [19]. The use of mixed substrates is viable for the growth of microorganisms in SSF. Mixture substrate can provide better surface area and accessibility of the nutrition to the microorganism [20].

Statistical design of experiments is a process that is used to plan an experiment to generate and analyze the data using different available methods which helps in determining objective conclusions from the data. Any statistical method runs on three basic principles which are randomization, replication and blocking. Randomization is the technique in which the allocation of the experimental data and the order in which the experiments conducted are completely random that makes the process effective. Replication is the process in which different sets of factor combinations are run repeatedly to process the input data. Replication brings out the variability between different runs of the experiment. Finally, blocking is the technique that is used to improve the accuracy of the data by comparing it with different factors used in the experiments. It helps to reduce or eliminate unnecessary factors that may be present in the experimental data. There are different methodologies to design a different set of experiments which include factorial design, mixture design, response surface methodology, etc. [21].

In the current study, lipase was produced by SSF using fungal strain *Aspergillus niger* MTCC 872 with highly lipolytic solid substrate *Prosopis Juliflora* (PJ) pods. Other agro-industrial wastes Red Gram Husk (RGH) and Cottonseed Cake (CSC) also used as a tri-substrate mixture for the better lipase production. SCMD was used to optimize the mixture composition of the tri-substrates. Then using the SCMD results, CCFD was used to optimize the physical parameters to enhance the lipase activity. With all the optimized conditions, large scale production of lipase was done in a tray bioreactor with varying bed heights.

2. Materials and methods

2.1. Microorganism and growth conditions

The fungal strain *Aspergillus niger* MTCC 872 was procured from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India. The fungi were revived from glycerol stock stored at −20 °C by streaking on Potato Dextrose Agar (PDA) slants. Later, PDA slants incubated at 30 °C for 96 h. After the incubation, the slants stored at 4 °C for the sub-culturing. The sub-cultured slants were used as the inoculum for the production of lipase.

2.2. Solid substrates and other chemicals

Solid substrates, cottonseed cake and red gram husk, were purchased from the local market, Hyderabad. *Prosopis juliflora* pods collected from the surroundings of the Indian Institute of Technology Hyderabad. K_2HPO_4, NaOH and $CuSO_4$ from SDFCL, KH_2PO_4 and Folin-Ciocalteu reagent from SRL. Na_2CO_3 from FINAR and p-NPP from Sigma Aldrich. All the chemicals used are analytical grade.

Prosopis Juliflora, mesquite, is a well-known invasive plant species widely spread in arid zones of the world, mainly Africa, India, Pakistan, Brazil, and Australia. It belongs to the *Fabaceae* family, and it is native to South America and Central America. PJ trees flower twice a year (February-March and August-September) causes rapid spread and distribution, especially in invaded habitats [22]. It extensively used in the diet of lambs, cattle, goats with good nutritional values. PJ pods are rich in protein, iron, carbohydrates (30–75%), fructose (3.2–12%), glucose (7.5–75%), maltose (< 0.4 %), lactose (0.7 %), inositol (5 %), raffinose (1 %) and reducing sugar (2–20%) [23].

Elemental composition (% weight basis) of the substrates PJ, RGH and CSC were analyzed using the CHNS-O organic elemental analyzer. Carbon, Nitrogen, Hydrogen and Sulfur compositions tabulated in Table 1.

2.3. Lipase production by SSF

Solid substrates with individual and mixture composition making a total quantity of 10 g taken in an Erlenmeyer flask. Potassium phosphate buffer with 0.05 M added to maintain the pH and moisture content required for the growth of the microorganism. The solid substrate was sterilized by autoclaving at 121 °C and 15 psi for 15 min. The inoculum with a spore concentration (counted using haematocytometer) of 5.4 million cells per mL added to the solid media. Then, the flask kept in the incubator with the required temperature to allow the microorganism for growth.

Samples collected at the regular intervals for analyzing the lipase activity. For every 0.5 g of sample, 10 mL of potassium phosphate buffer with 0.05 M, and 7.0 pH added to extract the lipase in a shaker incubator at 30 °C, 150 rpm for 60 min. The extracted samples filtered using Whatman No.1 filter paper. This filtered sample was used to

Table 1
Elemental composition (% weight basis) of solid substrates analyzed by CHNS-O organic elemental analyzer.

Element	*Prosopis Juliflora*	Red Gram Husk	Cottonseed Cake
Carbon	43.5796	38.6644	44.7224
Nitrogen	5.1363	6.1397	8.2050
Hydrogen	6.111	6.0517	6.4453
Sulfur	0	8.7196	0
Other	45.173	40.4606	40.6273

determine the lipase and protein assay.

2.4. P-Nitrophenyl palmitate (p-NPP) lipase assay

The lipase activity determined spectrophotometrically by the modified para-nitrophenyl palmitate (p-NPP) method [24]. The reaction mixture consists of 0.65 mL of 0.05 M potassium phosphate buffer, 0.1 mL of 0.025 M p-NPP in absolute ethanol, and 0.25 mL filtered crude enzyme. The reaction conducted in a test tube placed in a shaker incubator at 37 °C and 130 rpm for 30 min. After the reaction, the samples were taken out, and 0.25 mL of 0.1 M Na_2CO_3 added to the reaction mixture to quench the reaction. This mixture then centrifuged at 4 °C and 10,000 rpm for 15 min. 1 mL supernatant taken out to another test tube and diluted with 2.5 mL double distilled water. The resulted solution assayed using spectrophotometer at OD 410 nm. The samples assayed in triplicates. One unit of lipase defined as 1.0 µmol of p-nitrophenol liberated per minute under assay conditions.

2.5. Protein assay

Protein assay determined by Lowry protocol to determine the specific activity of the sample [25]. Initially, a mixture of A (2 % of Na_2CO_3 (w/v) in 0.1 N NaOH), B (1 % of $CuSO_4$ (w/v)) and C (2 % of Sodium Potassium Tartrate, C_4H_4KNa (w/v)) prepared. 1 mL of A + B + C mixture of 100:1:1 vol ratio of A, B and C respectively, was taken in a test tube. 0.2 mL of the filtered crude enzyme and 0.1 mL Folin reagent (diluted with double distilled water 1:1 vol) added to it and allowed to react in a dark place at room temperature for 30 min. After the reaction, the mixture diluted with 2.6 mL of double distilled water and protein analysis was done spectrophotometrically at OD 660 nm. All the samples assayed in triplicates. Though the individual solutions for the Lowry solution can be prepared in advance, it should mix on the day of protein measurement. Specific activity (µmol/min.mg = U/mg) defined as the moles of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins.

3. Optimization of Lipase for enhanced activity

Two statistical design methods used for the optimization of lipase activity. (1) Simplex Centroid Mixture Design and (2) Central Composite Face-Centered Design.

3.1. Simplex centroid mixture design

Design Expert v.9.0.5.1 software was used to determine the optimum mixture composition of the solid substrates PJ, RGH, and CSC. The SCMD is an unconstrained design because the proportions of all the components range from 0 to 1. The SCMD is distributed uniformly in the interior of the triangle that helps to detect the curvature of the response surface. The experimental design comprised of ten combinations: three of them are pure mixtures, each of the components are duplicate that located at the vertex of the triangle. Three binary blends, each mixture contains two possible components blend located at the center edge (one duplicate out of three). Combination of three components blend but not in equal proportions located at axial check blend and one center point or centroid where all the three components are in equal proportions included in the blend.

3.2. Central Composite Face-Centered Design

Response surface methodology was used to optimize the screened variables and studying their interaction effects for enhanced lipase production by SSF. This method involves performing the statistically designed experiments, estimating the mathematical coefficients, predicting the response and checking the adequacy of the model [26]. Design Expert v9.0.5.1 is used to design the experiments of Central Composite Face-Centered Design. The design consists of 20 experimental runs with six replicated center points, eight star points and six axial points.

The relation between the dependent and independent variables expressed in terms of a second-order polynomial Eq. (1).

$$Y = \beta_0 + \beta_1 A_1 + \beta_2 A_2 + \beta_3 A_3 + \beta_{12} A_1 A_2 + \beta_{13} A_1 A_3 + \beta_{23} A_2 A_3 + \beta_{11} A_1^2 + \beta_{22} A_2^2 + \beta_{33} A_3^2 \quad (1)$$

Where A_1, A_2 and A_3 are the levels of the factors, β_0 is a constant, β_1, β_2 and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the interactive coefficient estimates and β_{11}, β_{22}, and β_{33} are the quadratic coefficients.

Statistical analysis of the model is performed to evaluate ANOVA, which consists of F-test and coefficient of determination (R^2). F-test measures the overall model significance, and the R^2 measures the goodness of fit of the regression model.

4. Results and discussions

4.1. Optimization of substrate mixture for enhanced production of lipase using SCMD

The mixture components PJ, RGH and CSC have been taken on a weight basis, fixing the total weight to 10 g. Three substrates were used to study the effect of lipase activity and specific activity fixing initial physical parameters at 30 °C, 7 pH and 70 % initial moisture content (v/w). Design Expert software generated 14 experimental runs. Lipase activity and its corresponding specific activity obtained with different solid substrate mixture composition in SSF tabulated in Table 2. All the experiments conducted in duplicates for better understanding and consistency of data.

The highest lipase activity of 204.92 ± 6.14 U/gds was obtained for RGH at 48 h, whereas for PJ and CSC are 205.87 ± 6.17 and 205.92 ± 6.23 U/gds obtained respectively at 72 h. A binary mixture of PJ:RGH has shown the highest activity of 199.78 ± 5.99 U/gds and a mixture of PJ:CSC and RGH:CSC has shown comparable lipase activities of 180 ± 5.40 and 171.68 ± 5.15 U/gds respectively at 72 h. For ternary mixtures in different proportions, highest lipase activity of 212.20 ± 6.36 U/gds for mixture composition of 6.66:1.66:1.66 for PJ:RGH:CSC and its corresponding specific activity found to be 62.21 ± 1.86 U/mg. Lipase activity for similar mixture composition of CSC:PJ:RGH and RGH:PJ:CSC has shown the highest activity of 183.48 ± 5.50 and 139.56 ± 4.18 U/gds respectively. The ternary mixture of all the components taken in equal ratios has shown the highest activity of 162.06 ± 4.86 U/gds. All the ternary mixture have shown the maximum activity at 48 h, as shown in the Fig.1. In another

Table 2
Lipase activity and its specific activity obtained with different solid substrates mixture composition.

Run	Mixture Composition (g)			Response	
	PJ (A)	RGH (B)	CSC (C)	Lipase Activity (U/gds)	Specific Activity (U/mg)
1	0	10	0	205.02 ± 6.15	42.96 ± 1.28
2	1.66	6.66	1.66	139.56 ± 4.18	27.29 ± 0.18
3	10	0	0	206.80 ± 6.20	45.78 ± 1.37
4	5	0	5	180.09 ± 5.40	38.85 ± 1.16
5	5	5	0	199.78 ± 5.99	54.56 ± 1.63
6	0	5	5	171.68 ± 5.15	26.72 ± 0.80
7	0	0	10	207.67 ± 6.23	43.66 ± 1.30
8	5	5	0	200.24 ± 6.00	54.36 ± 1.63
9	10	0	0	204.95 ± 6.14	52.96 ± 1.58
10	1.66	1.66	6.66	183.48 ± 5.50	35.32 ± 1.05
11	3.33	3.33	3.33	162.06 ± 4.86	29.44 ± 0.88
12	0	10	0	204.82 ± 6.14	42.96 ± 1.28
13	6.66	1.66	1.66	212.20 ± 6.36	62.21 ± 1.86
14	0	0	10	208.23 ± 6.24	42.95 ± 1.28

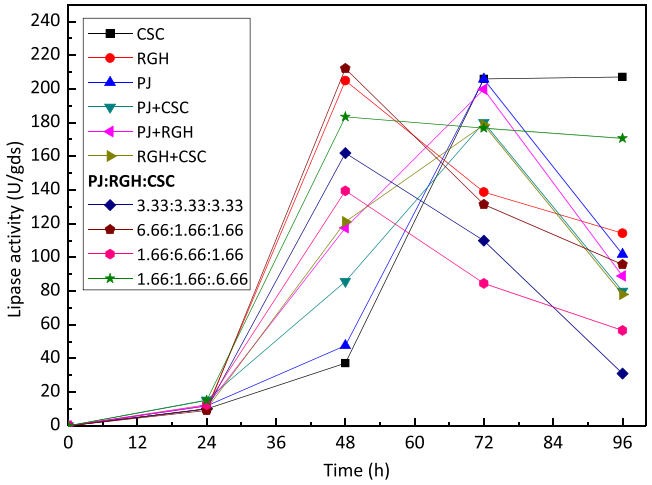

Fig. 1. Lipase activity for different compositions of solid substrates (PJ, RGH and CSC) with *Aspergillus niger* MTCC 872 in SSF.

study taking the ternary mixture wheat bran, coconut oil cake and wheat rawa with different compositions have produced enhanced lipase activity compared to the single substrate [20].

It was observed that the lipase activity for RGH has increased after 24 h till 48 h then the activity declined. In the case of PJ and CSC, appreciable growth observed after 48 h. CSC has maintained its activity until 96 h, whereas a decline in the activity after 72 h observed for PJ. Comparable highest lipase activity obtained for all three individual substrates. In another study conducted by Aguieiras et al. [16] on cottonseed meal using *Rhizomucor miehei* produced an activity of 93 U/g after 96 h. The maximum activity for RGH within 48 h maybe because of the high surface area provided by RGH. If the surface area is high, then the accessibility of the nutrition to the microorganism for its growth is easy. The highest lipase activity for different compositions of solid substrates represented in 3D surface plot and its corresponding contour plots were shown in Fig. 2(a and b). From the contour plot, it can be seen that the binary mixture of PJ and RGH composition ranging from 1.66 to 3.33 g of PJ and 3.33 to 5 g of RGH shows a reduction, whereas increasing PJ composition from 3.33 and above by decreasing the RGH composition increased the lipase activity. Keeping the higher composition of PJ or CSC in the binary mixture increased the lipase activity, but the maximum was obtained when PJ composition is higher. In the ternary mixture, PJ showed the maximum impact for producing the highest lipase activity.

The standard deviation of 1.15 and R^2 value of 0.9989 was observed for the special quartic model and also the predicted and adjusted R^2 values give a reasonable agreement for the model which provides the best fit for the data. SCMD model summary was shown in Table 3. The analysis of variance (ANOVA) data for lipase activity that shows the sum of squares, the mean sum of squares, degree of freedom and F-value for the model and different parameters tabulated in Table 4. The F-value of 569.10 obtained from the model implies that the model is significant. The higher F-value for all the model parameters suggests that the model equation can explain the variation in the lipase activity. The validity of F-value further confirmed by evaluating the corresponding p-value to identify the statistical significance of the model and parameters. A p-value < 0.05 is considered to be statistically significant with a high confidence level (> 95 %) whereas p-values > 0.1 are insignificant. The p-value (< 0.0001) of the overall regression model for

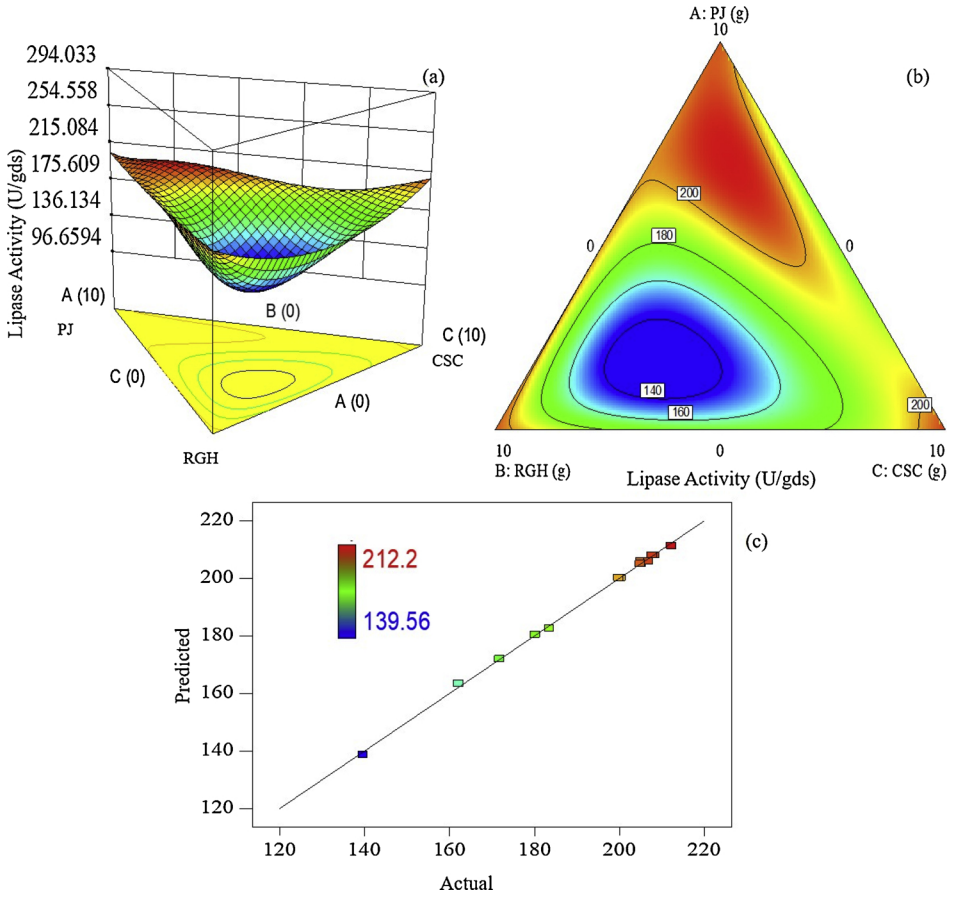

Fig. 2. Optimum lipase activity plots from simplex centroid mixture design for tri-substrate mixture (a) 3-D surface plot (b) 2-D contour plot and (c) The comparison between predicted and actual lipase activity.

Table 3
Simplex centroid mixture design (SCMD) model summary.

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	22.70	0.0618	−0.1088	−0.4579	8805.81	
Quadratic	17.19	0.6088	0.3643	−0.0612	6409.68	
Special Cubic	17.75	0.6348	0.3218	−4.4556	32,951.73	
Cubic	1.15	0.9989	0.9971	0.9070	561.47	Aliased
Special Quartic	1.15	0.9989	0.9971	0.9070	561.47	Suggested
Quartic	0.71	0.9997	0.9989			Aliased

Table 4
Analysis of variance (ANOVA) of special quadratic model using simplex centroid mixture design (SCMD).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6033.31	8	754.16	569.10	< 0.0001	significant
Linear Mixture	373.25	2	186.63	140.83	< 0.0001	
A·B	37.68	1	37.68	28.43	0.0031	
A·C	570.94	1	570.94	430.84	< 0.0001	
B·C	962.33	1	962.33	726.19	< 0.0001	
A^2BC	839.87	1	839.87	633.78	< 0.0001	
AB^2C	2210.59	1	2210.59	1668.14	< 0.0001	
ABC^2	48.64	1	48.64	36.70	0.0018	
Residual	6.63	5	1.33			
Lack of Fit	4.63	1	4.63	9.29	0.0381	significant
Pure Error	1.99	4	0.50			
Cor Total	6039.93	13				

Table 5
Range of physical parameters and their coded values for central composite face-centered design (CCFD).

Physical parameter	Low (-1)	Medium (0)	High (+1)
Temperature	25	30	35
pH	6	7	8
Moisture Content	60%	70%	80%

Table 6
Lipase activity and its specific activity obtained for physical parameter optimization using central composite face-centered design (CCFD).

Run	Temperature (T) (°C)	pH	Moisture Content (MC) (v/w)	Lipase activity (U/gds)	Specific activity (U/mg)
1	0	0	+1	195.44 ± 4.82	58.12 ± 1.74
2	−1	−1	−1	206.22 ± 4.57	48.33 ± 1.44
3	0	0	0	213.30 ± 6.35	60.05 ± 1.80
4	+1	+1	+1	178.54 ± 6.84	65.92 ± 1.98
5	0	0	0	212.38 ± 6.37	60.39 ± 1.81
6	+1	−1	−1	220.55 ± 6.61	61.97 ± 1.85
7	0	0	−1	155.75 ± 4.67	49.33 ± 1.48
8	+1	−1	+1	250.77 ± 7.52	66.03 ± 1.98
9	−1	−1	+1	192.49 ± 5.77	61.69 ± 1.85
10	0	0	0	212.10 ± 6.36	60.50 ± 1.81
11	0	0	0	212.30 ± 6.36	60.80 ± 1.82
12	0	+1	0	198.19 ± 5.94	59.98 ± 1.80
13	−1	+1	+1	168.75 ± 5.06	52.18 ± 1.56
14	+1	0	0	269.87 ± 8.09	72.67 ± 2.18
15	0	0	0	212.10 ± 6.36	60.1 ± 1.80
16	+1	+1	−1	241.66 ± 7.24	67.61 ± 2.02
17	0	−1	0	195.44 ± 5.86	60.78 ± 1.82
18	−1	0	0	206.22 ± 6.18	62.79 ± 1.88
19	0	0	0	213.30 ± 6.39	61.64 ± 1.84
20	−1	+1	−1	178.54 ± 5.35	49.00 ± 1.47

the lipase activity was found to be highly significant, and the special quartic model equation efficiently represented by the actual relationship between the lipase activity and the varying mixture composition. The high regression coefficient ($R^2 = 0.9989$) of the model equation also confirmed the same. The F-value of 9.29 for "Lack of fit" implies that it is significant and also the p-value suggests that there is only 3.81 % chance that it could occur due to noise.

Lipase activity (U/gds) =

$$205.95\ *A + 205\ *B + 208.03*C - 21.25*A*B - 106.38*A*C - 138.11\ *B*C + 2863.06*A^2*B*C - 4644.92*A*B^2\ *C + 698.85*A*B*C^2 \quad (2)$$

Where A is *Prosopis Juliflora* (PJ), B is Reg Gram Husk (RGH) and C is Cotton Seed Cake (CSC).

From Eq. (2), obtained from the special quartic model, it is clear that the individual components have a positive effect, whereas binary component mixtures have shown the antagonistic effect on the lipase activity. In case of the binary mixture, RGH and CSC have shown the highest antagonistic effect compared with the other two mixtures. For ternary mixture comprising PJ in more quantity has shown synergistic effect whereas mixture comprising more RGH has an antagonistic effect on lipase activity. From the above results, it is evident that RGH is providing sufficient surface area to support the fungal growth, but its nutrients are getting readily depleted. CSC helps in sustaining the growth for a longer period providing nutrients for the fungal growth. PJ is the key component that provides nutrients to uphold the lipase activity. From the predicted equation using special quartic model, it is clear that the experimental values are nearer to the predicted values of lipase activity as shown in Fig. 2(c).

Point prediction of the model was confirmed with the experimental run using the composition of 7.72:1.82:0.46, PJ:RGH:CSC at 30 °C, 7.0 pH and 70 % initial moisture content (v/w). The predicted and experimental activities at this condition was 207.02 U/gds and 201.74 U/gds at 48 h. The specific activity observed was 48.72 U/mg.

The lipase activity for the tri-substrate mixture composition of 6.66:1.66:1.66 g of PJ:RGH:CSC obtained was similar to those exhibited by individual substrate components. But the specific activity for this mixture composition is much higher than the individual components. As specific activity signifies the purity of the enzyme in the mixture, enzymes with higher specific activity will have higher stability, which helps to use the enzyme in hydrolysis for an elongated time [27,28]. Hence this mixture composition was chosen for the next optimization process.

4.2. Optimization of physical parameters using the central composite face-centered design (CCFD)

Based on the SCMD, the optimized mixture composition of 6.66:1.66:1.66 g of PJ:RGH:CSC respectively, was chosen for CCFD under RSM to determine the optimum physical parameters, temperature (25 °C, 30 °C and 35 °C), pH (6, 7 and 8) and initial moisture content (v/w, 60 %, 70 % and 80 %) to enhance the lipase activity. Independent variables, temperature, pH and initial moisture contents were studied at three different levels, as shown in Table 5. And their corresponding results (lipase and specific activities) were shown in Table 6. All the experiments conducted in duplicates and average values were reported.

The highest lipase activity of 269.87 ± 8.09 U/gds and its corresponding specific activity 72.67 ± 2.18 was obtained at 30 °C, 7 pH

Table 7
Central composite face-centered design (CCFD) model summary.

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	22.55	0.5526	0.4687	0.3254	12,262.05	
2FI	23.24	0.6137	0.4353	0.4636	9751.00	
Quadratic	7.63	0.9680	0.9392	0.8575	2589.95	Suggested
Cubic	9.63	0.9694	0.9030	−36.5579	6.827E+005	Aliased

Table 8
Analysis of variance (ANOVA) of the quadratic model using central composite face-centered design (CCFD).

Source		Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model		17,595.63	9	1955.07	33.59	< 0.0001	significant
Linear	A-Temperature	9770.63	1	9770.63	167.87	< 0.0001	
	B-pH	1.31	1	1.31	0.023	0.8837	
	C-Moisture Content	272.48	1	272.48	4.68	0.0558	
Two way interaction	AB	1.58	1	1.58	0.027	0.8726	
	AC	21.88	1	21.88	0.38	0.5535	
	BC	1086.95	1	1086.95	18.67	0.0015	
Square	A^2	4113.52	1	4113.52	70.67	< 0.0001	
	B^2	17.94	1	17.94	0.31	0.5910	
	C^2	4625.93	1	4625.93	79.48	< 0.0001	
Residual		582.04	10	58.20			
	Lack of Fit	580.80	5	116.16	467.50	< 0.0001	significant
	Pure Error	1.24	5	0.25			
Total		18,177.67	19				

and 70 % initial moisture content (v/w) at 72 h. The quadratic model suggested from the experimental results were shown in Table 7. The coefficient of determination, R^2 value is 0.968, and the difference between the adjusted and predicted R^2 value < 0.2. The value of adequate precision which measures the signal to noise ratio of 23.249 was obtained. A ratio greater than 4 is desirable, which signifies the acceptability of the model.

The ANOVA analysis for the quadratic model shown in Table 8. F-values 33.59 and lack of fit values of 467.5 imply the significance of the suggested model. There are only 0.01 % chances that the values this large could occur due to noise. Further, F-values were validated by evaluating the corresponding p-values, which will identify the statistical significance of the model and parameters. In this case, the significant terms are A, BC, A^2 and C^2.

Equation (3) in terms of coded factors represents empirical relation between the lipase activity and the screened variable expressed as a second-order polynomial equation.

Lipase activity (U/gds) =

$$207.15 + 31.26\,A + 0.36\,B + 5.22\,C - 0.44\,AB - 1.65\,AC - 11.66\,BC + 38.68A^2 - 2.55B^2 - 41.01C^2 \quad (3)$$

Where A is the temperature in °C, B is pH and C is percentage of initial moisture content (v/w).

Individual parameters, temperature, pH and initial moisture content are efficacious for lipase activity. Interaction terms have produced an antagonistic effect out of which interaction between pH and initial moisture contents contributed the maximum. Square term of temperature shows the positive effect, and the other two has an obstructive effect on lipase activity.

4.2.1. Optimization of the combined effect of physical parameters on lipase activity

Many reports suggest that physical parameters like temperature, pH and initial moisture content greatly influence the production of lipase [9,29]. In the present study, an attempt has been made to enhance the lipase activity at flask level by optimizing physical parameters. Three-dimensional response surface plot was constructed to obtain optimum value for each variable. The 3D response surface for lipase activity was plotted on Z-axis against the two independent variables on X-axis and Y-axis while maintaining the third variable at the central level shown in Fig. 3(a, c &e). This optimum physical parameter zone can be seen in the two-dimensional response contour plots, as shown in Fig. 3(b, d &f).

The 3D response surface and contour plots for the interaction effect of temperature and pH, keeping the initial moisture content of 70 % (v/w) as a constant as shown in Fig. 3(a & b). Optimized substrate composition was incubated at different temperatures ranging from 25 °C to 35 °C with an increment of 5 °C and pH ranging from 6 to 8. Maximum lipase activity of 269.87 ± 8.09 U/gds was observed at 35 °C. Further increase in the temperature might result in the denaturation of the protein structure of the enzyme. It is clear from the graph, an increase in the temperature favors the lipase activity at all pH values. Interaction between temperature and moisture content at pH 7 shown in Fig. 3(c & d). An increase in moisture content and temperature favors lipase activity. A saddle shape obtained for interaction of initial moisture content and temperature. Increase in initial moisture content above 70 % results in a decline of lipase activity. Because at higher moisture content, there is a chance of agglomeration of the solid substrate. This leads to a decrease in the porosity of the substrate, thereby affecting fungal growth. From the plot, it confirmed that an increase in temperature supports lipase activity at all levels of initial moisture content. Low moisture levels would result in minimal growth due to a reduction in nutrient diffusion and low substrate swelling [30]. Thus optimum temperature and initial moisture content (v/w) for lipase activity were observed at 35 °C and 70 % respectively. Effect of interaction between pH and moisture content at a constant temperature of 30 °C shown in Fig. 3(e & f). An increase in initial moisture content favors lipase activity at all pH. The pH affects the stability of enzymes by changing the electrostatic interactions of their protein structure, causing changes in the amino acid ionization status, which defines the secondary and tertiary structures of protein and therefore its activity and stability [31]. It is clear from the plot that interaction of pH and initial moisture content has no significant effect on the lipase activity. Highest lipase activity of 269.87 ± 8.09 U/gds was obtained for a substrate mixture containing 6.66:1.66:1.66 of PJ:RGH:CSC at 35 °C, pH 7 and 70 % of

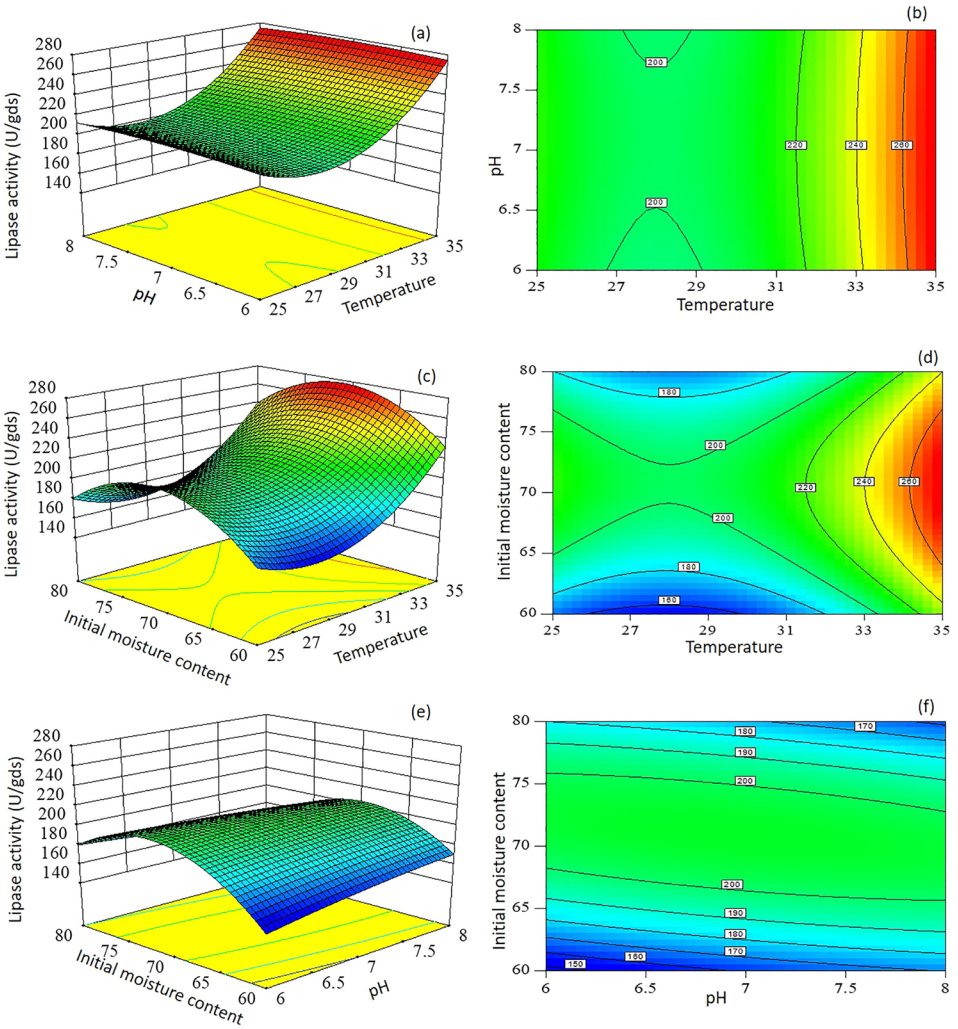

Fig. 3. Interaction effect of physical parameters by response surface plots. (a & b) Temperature and pH at 70 % initial moisture content (c & d) Temperature and initial moisture content at pH 7.0 and (e & f) pH and initial moisture content at 35 °C.

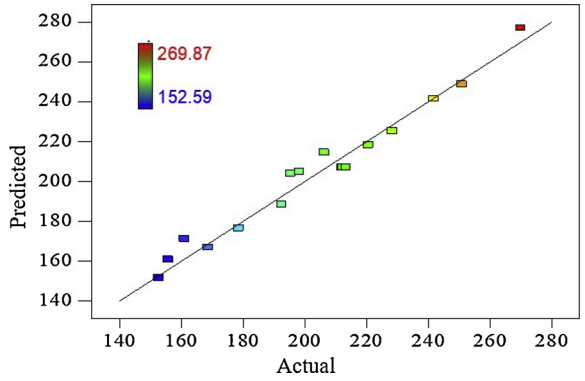

Fig. 4. The comparison between predicted and actual lipase activity from central composite face-centered design (CCFD).

temperature, pH and initial moisture content (v/w) respectively. The predicted and actual lipase activity with the R^2 value of 0.968, which gives a comparable activity, as shown in Fig. 4. An increase of 1.27 folds in lipase activity at flask level was observed after optimizing the physical parameters.

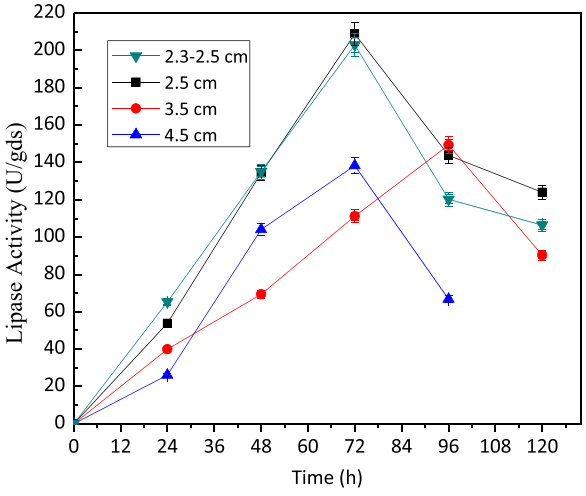

Fig. 5. Large scale production of lipase using different bed heights in tray bioreactor with optimized mixture composition and physical parameters.

5. Large scale production of lipase in tray bioreactor

Optimized process conditions were scaled up from the flask level to the semi-pilot production level. Lipase production was scaled up using

Table 9
Validation of the experimental model for central composite face-centered design (CCFD) using point prediction tool.

Temperature (°C)	pH	Initial Moisture Content % (v/w)	Lipase Activity (U/gds)	
			Predicted	Experimental
34.91	6.65	68.1	271.94	263.4 ± 7.90
34.99	6.33	71.86	276.5	265.8 ± 7.97

1 kg solid substrate in tray bioreactor with dimensions of 45.2*42.7*2.5 (l*b*h) cm with intermittent mixing at different bed heights. The lipase activity with different bed heights shown in Fig. 5. The highest lipase activity of 208.79 ± 6.26 U/gds was found for the bed height of 2.5 cm. The large scale production yielded 77.32 % of the optimum activity obtained at 10 g flask level. Maintaining the moisture content in the tray bioreactor was difficult because, at an optimized temperature of 35 °C, the moisture gets evaporated. To maintain the moisture content in the tray bioreactor, at every 6 h of time interval moisture content was analyzed, and the required amount of buffer was added. The observation of a reduction in yield could be moisture loss due to an increase in temperature (35–38 °C) during the growth of the culture. Comparable results are obtained when the substrates unevenly distributed for bed height ranges from 2.3 to 2.5 cm and 2.5 cm. Additional supports were used to maintain the bed height of 3.5 and 4.5 cm. Increasing bed height resulted in a decrease in lipase activity. The reason for the reduction of activity could be difficult for the spore penetration into the substrates to access the nutrients and due to the moisture loss.

6. Validation of model

To validate the suggested model for maximizing the activity, two sets of experiments performed concerning all three parameters using point prediction. The results for the predicted and experimental values of lipase activity obtained are comparable as shown in Table 9.

7. Conclusion

This study provides the evidence for utilizing the *Prosopis Juliflora* as a solid substrate for production of lipase using *Aspergillus niger* MTCC 872 by solid state fermentation. This study also involves the use of two optimization techniques SCMD and CCFD for enhanced lipase production. Mixture design technique, SCMD, was used to optimize the tri-substrate mixture consisting of PJ, RGH and CSC in the ratio of 6.66:1.66:1.66 which has provided the maximum activity of 212.20 ± 6.36 U/gds. And then CCFD under RSM was used to optimize the physical parameters. The highest lipase activity of 269.87 ± 8.09 U/gds was obtained for the tri-substrate mixture at 35 °C, 7 pH and 75 % initial moisture content (v/w). The developed models have shown a good correlation between experimental and predicted value with the R^2 values of 0.9989 and 0.968 for SCMD and CCFD, respectively. It was observed that there is an increment of 1.27 fold in lipase activity after the optimization of physical parameters. Large scale production of lipase in tray bioreactor with the bed height of 2.5 cm has shown the highest activity of 208.79 ± 6.26 U/gds. Large scale production yielded 77.32 % of the maximum lipase activity of the flask level study. This study has shown that the use of PJ as a substrate can be an effective solution to meet the increasing demand for lipase production.

Declaration of Competing Interest

The authors declare that they have no conflict of interest

Acknowledgements

The authors are indebted to Dr. Kruthi Doriya and Mr. Anup Ashok for helping to conduct the experiments at the initial stages and their support whenever required in the laboratory. Special thanks to Ms. Swarnalatha for facilitating analysis of the elemental composition of the solid substrates. The authors are grateful to Science and Engineering Research Board-Department of Science and Technology, Government of India for supporting the research with grant number SERB-DST- ECR/2017/000241.

References

[1] A. Marwaha, P. Rosha, S.K. Mohapatra, S.K. Mahla, A. Dhir, Waste materials as potential catalysts for biodiesel production: current state and future scope, Fuel Process. Technol. 181 (2018) 175–186, https://doi.org/10.1016/j.fuproc.2018.09.011.
[2] R.A. Barreto, Fossil fuels, alternative energy and economic growth, Econ. Model. 75 (2018) 196–220, https://doi.org/10.1016/j.econmod.2018.06.019.
[3] P. Selvakumar, P. Sivashanmugam, Optimization of lipase production from organic solid waste by anaerobic digestion and its application in biodiesel production, Fuel Process. Technol. 165 (2017) 1–8, https://doi.org/10.1016/j.fuproc.2017.04.020.
[4] A. Arumugam, D. Thulasidharan, G.B. Jegadeesan, Process optimization of biodiesel production from *Hevea brasiliensis* oil using lipase immobilized on spherical silica aerogel, Renew. Energy 116 (2018) 755–761, https://doi.org/10.1016/j.renene.2017.10.021.
[5] N.F. Sulaiman, W.A.W.A. Bakar, S. Toemen, N.M. Kamal, R. Nadarajan, In depth investigation of bi-functional, Cu/Zn/γ-Al_2O_3 catalyst in biodiesel production from low-grade cooking oil : optimization using response surface methodology, Renew. Energy 135 (2019) 408–416, https://doi.org/10.1016/j.renene.2018.11.111.
[6] J. Gardy, A. Osatiashtiani, O. Céspedes, A. Hassanpour, X. Lai, A.F. Lee, K. Wilson, M. Rehan, A magnetically separable SO_4/Fe-Al-TiO_2 solid acid catalyst for biodiesel production from waste cooking oil, Appl. Catal. B Environ. 234 (2018) 268–278, https://doi.org/10.1016/j.apcatb.2018.04.046.
[7] J. Gardy, A. Hassanpour, X. Lai, M.H. Ahmed, Synthesis of Ti(SO_4)O solid acid nano-catalyst and its application for biodiesel production from used cooking oil, Appl. Catal. A Gen. 527 (2016) 81–95, https://doi.org/10.1016/j.apcata.2016.08.031.
[8] M. Ilmi, C. Hidayat, P. Hastuti, H.J. Heeres, M.J.E.C. Van Der Maarel, Biocatalysis and agricultural biotechnology utilisation of Jatropha press cake as substrate in biomass and lipase production from *Aspergillus niger* 65I6 and *Rhizomucor miehei* CBS 360.62, Biocatal. Agric. Biotechnol. 9 (2017) 103–107, https://doi.org/10.1016/j.bcab.2016.12.004.
[9] A.R. Behera, A. Veluppal, K. Dutta, Optimization of physical parameters for enhanced production of lipase from *Staphylococcus hominis* using response surface methodology, Environ. Sci. Pollut. Res. (2019).
[10] B. Andualema, A. Gessesse, Microbial lipases and their industrial applications: review, Biotechnology 11 (2012) 100–118, https://doi.org/10.3923/biotech.2012.100.118.
[11] N.B. Melani, E.B. Tambourgi, E. Silveira, Lipases: from production to applications, Sep. Purif. Rev. 0 (2019) 1–16, https://doi.org/10.1080/15422119.2018.1564328.
[12] C. Papagora, T. Roukas, P. Kotzekidou, Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology, Food Bioprod. Process. 91 (2013) 413–420, https://doi.org/10.1016/j.fbp.2013.02.008.
[13] J.K. Poppe, R. Fernandez-Lafuente, R.C. Rodrigues, M.A.Z. Ayub, Enzymatic reactors for biodiesel synthesis: present status and future prospects, Biotechnol. Adv. 33 (2015) 511–525, https://doi.org/10.1016/j.biotechadv.2015.01.011.
[14] P. Kanmani, J. Aravind, K. Kumaresan, An insight into microbial lipases and their environmental facet, Int. J. Environ. Sci. Technol. 12 (2015) 1147–1162, https://doi.org/10.1007/s13762-014-0605-0.
[15] M. Aarthy, P. Saravanan, M.K. Gowthaman, C. Rose, N.R. Kamini, Enzymatic transesterification for production of biodiesel using yeast lipases: an overview, Chem. Eng. Res. Des. 92 (2014) 1591–1601, https://doi.org/10.1016/j.cherd.2014.04.008.
[16] E.C.G. Aguieiras, D.S.N. de Barros, R. Fernandez-Lafuente, D.M.G. Freire, Production of lipases in cottonseed meal and application of the fermented solid as biocatalyst in esterification and transesterification reactions, Renew. Energy 130 (2019) 574–581, https://doi.org/10.1016/j.renene.2018.06.095.
[17] A. Salihu, M. Bala, S.M. Bala, Application of plackett-burman experimental design for lipase production by *Aspergillus niger* using shea butter cake, ISRN Biotechnol. 2013 (2013) 1–5, https://doi.org/10.5402/2013/718352.
[18] S.N.S. Ávila, M.L.E. Gutarra, R. Fernandez-lafuente, E.D.C. Cavalcanti, D.M.G. Freire, Multipurpose fixed-bed bioreactor to simplify lipase production by solid-state fermentation and application in biocatalysis, Biochem. Eng. J. 144 (2020) 1–7, https://doi.org/10.1016/j.bej.2018.12.024.
[19] C.R. Soccol, E.S.F. da Costa, L.A.J. Letti, S.G. Karp, A.L. Woiciechowski, L.P. de S. Vandenberghe, Recent developments and innovations in solid state fermentation, Biotechnol. Res. Innov. 1 (2017) 52–71, https://doi.org/10.1016/j.biori.2017.01.002.doi:10.1016/j.biori.2017.01.002.
[20] N.G. Edwinoliver, K. Thirunavukarasu, R.B. Naidu, M.K. Gowthaman, T.N. Kambe, N.R. Kamini, Scale up of a novel tri-substrate fermentation for enhanced production

of *Aspergillus niger* lipase for tallow hydrolysis, Bioresour. Technol. 101 (2010) 6791–6796, https://doi.org/10.1016/j.biortech.2010.03.091.
[21] D.C. Montgomery, Design and Analysis of Experiments, eighth ed., John Wiley & Sons, New Jersey, 2012.
[22] S.S. Al-Abdali, A.S. Al-Dhuhli, H.A. Al-Reasi, Preliminary investigations of allelopathic effects and herbicide-based eradication of mesquite (Prosopis juliflora), Sultan Qaboos Univ. J. Sci. 24 (2019) 11–17, https://doi.org/10.24200/squjs.vol24iss1pp11-17.
[23] S. Chovatiya, S. Bhatt, A. Shah, P. Dube, An investigation on the use of *Prosopis juliflora* pods as a carbohydrate source supplemented with probiotics in the diet of *Labeo rohita* fingerlings, Iran. J. Fish. Sci. 17 (2018) 327–345, https://doi.org/10.22092/IJFS.2018.115827.
[24] S. Sigurgísladóttir, M. Konráòsdóttir, Á. Jónsson, J.K. Kristjánsson, E. Matthiasson, Lipase activity of thermophilic bacteria from icelandic hot springs, Biotechnol. Lett. 15 (1993) 361–366, https://doi.org/10.1007/BF00128277.
[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[26] A.R. Ismail, S.B. El-Henawy, S.A. Younis, M.A. Betiha, N.S. El-Gendy, M.S. Azab, N.M. Sedky, Statistical enhancement of lipase extracellular production by *Bacillus stratosphericus* PSP8 in a batch submerged fermentation process, J. Appl. Microbiol. 125 (2018) 1076–1093, https://doi.org/10.1111/jam.14023.
[27] J. Pei, Q. Pang, L. Zhao, S. Fan, H. Shi, *Thermoanaerobacterium thermosaccharolyticum* β-glucosidase: a glucose-tolerant enzyme with high specific activity for cellobiose, Biotechnol. Biofuels 5 (2012) 1–10, https://doi.org/10.1186/1754-6834-5-31/10.1186/1754-6834-5-31.
[28] Y. Yücel, Biodiesel production from pomace oil by using lipase immobilized onto olive pomace, Bioresour. Technol. 102 (2011) 3977–3980, https://doi.org/10.1016/j.biortech.2010.12.001.
[29] A. Sharma, D. Bardhan, R. Patel, Optimization of physical parameters for lipase production from *Arthrobacter* sp. BGCC#490, Indian J. Biochem. Biophys. 46 (2009) 178–183.
[30] Z. Baysal, F. Uyar, Ç, Aytekin, Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water, Process Biochem. 38 (2003) 1665–1668, https://doi.org/10.1016/S0032-9592(02)00150-4.
[31] E. Rajakumara, P. Acharya, S. Ahmad, R. Sankaranaryanan, N.M. Rao, Structural basis for the remarkable stability of *Bacillus subtilis* lipase (Lip A) at low pH, Biochim. Biophys. Acta 1784 (2008) 302–311, https://doi.org/10.1016/j.bbapap.2007.10.012.