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Production and Purification of Lipase from *Bacillus licheniformis* for Biodiesel Production

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ABSTRACT

Lipases enzyme leads to the fat hydrolysis into fatty acid and glycerol at the interface of lipid-water along with the reactions leading to bioconversion in micro and non-aqueous surroundings. In this research, the conditions of culture required for the extracellular production of lipase by the native strain of bacteria isolated from the waste water and sludge samples collected from the sites near district Hisar, India were optimized. The optimization of lipase production was done using FCCCD of RSM. All optimized conditions temperature (55°C), pH (9), inoculum volume [3% (v/v)], agitation rate (200 rpm) and concentration of inducer oil [3% (v/v)] resulted in maximum lipase production of 256.20 µ/ml. The LipBL-WII(c) lipase produced by *Bacillus licheniformis* WII(c) strain was then subjected to sequential method in order to produce complete purification fold of 13.0%, recovery 30.4%, specific activity 760 µ/mg and a m.w. of 41 kDa. The pH and temperature optima for the LipBL-WII(c) were 8 and 55°C, respectively. Moreover, the lipase showed good steadiness amongst various surfactants, metal ions and organic solvents. The values for K_m and V_{max} were observed to be 1 mM and 6.66 mmoles, respectively. The purified lipase was used in the production of biodiesel. LipBL-WII(c) can be utilized for catalyzing the production of biodiesel using olive oil via transesterification reactions due to the higher lipolytic activity in thermophilic conditions and maximum stability in the existence of organic solvents.

Key words: Lipase, *Bacillus* sp., biodiesel, transesterification

INTRODUCTION

Lipase being triacylglycerol-acyl-hydrolases (E.C. 3.1.1.3) catalyzes the esterification the hydrolysis and trans-esterification reaction of carboxylic esters of triacylglycerols, aminolysis, acidolysis and alcoholysis in micro-aqueous milieu and non-aqueous environments (Javed *et al.*, 2018). Various applications of lipase are in the industries: textile, detergent, food, leather, diagnosis, biodiesel, biotransformation, cosmetics and pharmaceutical (Sarmah *et al.*, 2018). The use of lipase is caught up due to its necessity, high cost and poor stability for particular enzyme characteristics in different industries like, ester synthesis requiring lipases stability in organic solvent and detergent industries preferring alkaline stable lipases (Bento *et al.*, 2017).

The extracellular secretion of bacterial lipases is based on the fermentation conditions like, temperature, agitation rate, pH and volume of inoculums, sources of inducer oil and its concentration (Gururaj *et al.*, 2016). For obtaining maximum yields of the enzymes at the minimum cost it is imperative to explore for the optimum conditions for the above discussed parameters. The conventional method of accomplishing these is by upholding OFAT (One factor at a time) method. The conventional methodology has proved expensive, time consuming and laborious in contemplation of many variables simultaneously along with its failure in depiction of cooperative effects among the factors and locating of optimal conditions amongst the variables. For overcoming the above mentioned demerits, the statistical experimental designs by RSM is an effective approach in a number of research areas (Saraswat *et al.*, 2018).

Studies on RSM have been done exhaustibly but fewer studies on production of lipase from *B. licheniformis* have been described which is augmented employing RSM. In this research, the optimizing conditions of the culture

required for the extracellularly lipase production by the native strain of bacteria *B. licheniformis* WII(c) was investigated employing RSM. The influence of various nitrogen, and carbon sources, mineral salts, and inducer oil were studied. Thereafter, for enhancing the production of lipase the optimization of the selected concentration of inducer oil, temperature, agitation rate, inoculum volume and pH was done using face centered CCD (FCCCD). The optimization was followed by purification and the characterization of purified lipase for parameters like, temperature, pH, organic solvent, metal ions, and surfactants. Finally, the application of the enzyme from *Bacillus licheniformis* WII(c) in biodiesel production was accessed.

MATERIALS AND METHODS

Lipase-producing indigenous *B. licheniformisstrain* WII(c) was isolated from Hisar region of Haryana, India. Identification of this strain was performed by sequencing 16S rRNA gene and the sequence was submitted to NCBI to obtain accession no.: OP56979 (Neha *et al.,* 2023). Chemicals and biochemicals were acquired from Sigma and Hi-Media.

In order to induce maximum lipase production in *B. licheniformis* WII(c) various sources of carbon 1.0% (w/v) like, glucose, fructose, sucrose and lactose were screened. The production media containing each kind of carbon source was inoculated with the bacterial culture and kept at 37°C temperature and 120 rpm for 48 h of incubation. After incubation, the fermentation media was then centrifuged at 10000 rpm and 4°C for 10 min. Colorimetric assay was used for determining the lipolytic activity of the obtained culture supernatant.

Various kinds of nitrogen sources (peptone, beef extract, corn steep liquor, yeast extract) were added in the production media at 1.0% (w/v) concentration in order to screen the induction and production of lipase by the bacterial strain. The production media was inoculated with the bacterial culture and kept at 37 °C temperature and 120 rpm for 48 h of incubation. After incubation, the fermentation media was then centrifuged at 10,000 rpm and 4°C for 10 min. Colorimetric assay was used for determining the lipolytic activity of the as obtained culture supernatant.

Different metal salts like $MgSO_4$, $CaCl_2.2H_2O$, $FeSO_4$, $ZnSO_4$ and $MnSO_4$ were screened for maximum lipase production in the bacterial strain by adding salts at 0.02% concentration in the production media. The media was inoculated with the bacterial culture and kept at 37°C temperature and 120 rpm for 48 h of incubation. After incubation, the fermentation media was then centrifuged at 10,000 rpm and 4°C for 10 min. Colorimetric assay was used for determining the lipolytic activity of the as obtained culture supernatant.

Different edible oils (mustard oil, rice bran oil, soybean oil, castor oil and sunflower oil) were tested for inducing lipase production in *B. licheniformis* WII(c) isolate. The fermentation media containing olive oil for the production of lipase was substituted to 1.0% (v/v) of the enlisted oils while maintaining other variables constant. The production media was inoculated with the bacterial culture and kept at 37°C temperature and 120 rpm for 48 h of incubation. After incubation, the fermentation media was then centrifuged at 10,000 rpm and 4°C for 10 min. Colorimetric assay was used for determining the lipolytic activity of the as obtained culture supernatant.

In the process of optimization the selected variables like, temperature, pH, inoculum volume, agitation rate and inducer oil concentration were employed to RSM using FCCCD. Three coded levels (-1, 0 and +1) were used for testing the variables. Table 1 enlists the actual and coded values of the studied variables along with their minimum and maximum ranges. By using Design- Expert® version 13.0 (Stat Ease Inc., Minneapolis, USA) a total of 50 experiments were generated. The average of the triplicates was recorded as (Y) response values in each and every run. The impact assessment of the model developed generated the data which was subjected to ANOVA. The following quadratic polynomial shown in Equation 1 was used for fitting the

Table 1. Independent variables analyzed using FCCCD in terms of coded and actual levels

Variables	Range of levels		
	-1	0	+1
Temperature	35	45	55
pH	7	8	9
Inoculum volume	1	2	3
Agitation rate	100	150	200
Inducer oil concentration	1	2	3

experimental responses using response surface regression.

Lipase activity (Y) = $\beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} DE + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2$ ...(Eq. 1)

Where independent variables referred to as A, B, C, D and E; intercept being β_0; interaction coefficients being β_1, β_2, β_3, β_4, and β_5; quadratic coefficients represented as β_{11}, β_{22}, β_{33}, β_{44} and β_{55}. In ANOVA table, the factor's significance was referred by the value of $P<0.05$.

The supernatant obtained after centrifuging the submerged culture medium for 10 min at 10,000 rpm and 4°C was used as crude enzyme for precipitation using 10 fractions of ammonium sulphate i.e. ranging from 0-100% with constant stirring at 4°C tailed by centrifuging the samples at 10,000 rpm for 20 min. Analysis of lipase activity and protein content was done for each fraction. Ammonium sulphate fractions were then dialyzed using phosphate buffer with pH 7 for 6 h at 4°C using a dialysis bag. The fractions having activity of lipase were pooled, desalted and subjected to column of Sephadex. The enzyme in its bounded form was eluted using pH 8.0 Tris-HCl buffer. The lipase was then eluted from the column at 3 ml/min flow rate. Fractions from Sephadex G-100 column having enzymatic activity were collected, pooled and then subjected to SDS-PAGE for the purification of lipase.

The purity and relative molecular weight of purified lipase were resolved using SDS-PAGE employing 15.0% of resolving gel and 5.0% of stacking gel. Coomaisse Brilliant Blue (R- 250) was used for staining of protein bands.

The stock solution of the substrate pNPP (p-nitrophenylpalmitate, 20 mM) was prepared using isopropanol. The final mixture of reaction was prepared by mixing 75 µl of pNPP of stock solution in 3 ml of Tris buffer (0.05 M, pH-8.0) and then subjected to incubation at 70°C inside a water bath for 10 min. After that, 25 µl of the crude enzyme was supplemented into the reaction mixture followed by the incubation at 35°C in a water bath for 30 min. After 30 min, the addition of 1 ml of stopping reagent (chilled acetone- ethanol (1:1)) halted the enzymatic reaction. In control (blank) set the addition of crude enzyme step was skipped. The liberation of yellow coloured compound (p-nitrophenol) in the reaction mixture was measured at the wavelength of 410 nm alongside a reagent blank in reference to the p- nitrophenol standard curve (2 to 20 µg/ml in TrisHCl buffer (0.05 M, pH-8.0). The very assay was carried out in triplicates leading to presentation of the mean values. One unit (U) of enzyme activity is defined as micromole (µM) of the liberated p-nitrophenol by the p- nitrophenyl ester breakdown by 1.0 ml of the soluble enzyme every minute at 35°C under the standard assay conditions.

The enzyme activity (U/ml/min) was calculated by the following formula:

$$\text{Lipase activity (Units/ml/min)} = \frac{\text{µ moles of p–nitrophenol liberated}}{\text{Volume of enzyme (0.025ml)* Incubation time (30)}} \quad \text{...(Eq. 2)}$$

Specific activity is the enzyme activity per mg of the total protein expressed in µ mol/min/mg.

$$\text{Specific enzyme activity (U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Total protein content (mg/ml)}} \quad \text{...(Eq. 3)}$$

The protein concentration was estimated by using Bradford's method in which BSA (Bovine Serum Albumin) being a standard protein.

The optima of temperature for purified LipBL-WII(c) was determined by incubating purified enzyme aliquots at different temperature ranging from 20-80°C for 24 h at 200 rpm maintaining pH at 8 using Tris-HCl buffer. The maximum activity was considered to be 100% at the beginning of the reaction.

The optima of pH for purified LipBL-WII(c) was estimated after the incubation of purified lipase in various buffers (50 mM): pH- 2 (glycine- HCl), pH 3-5 (acetate-acetic acid), pH 6-7 (phosphate), pH 8- 9 (Tris-HCl), pH 10.0 (glycine- NaOH), pH 11.0 (phosphate-NaOH) and pH 12 (KCl- NaOH). The lipolytic activity of the enzyme was taken to be as 100.0%.

For determining the impact of organic solvents (isopropanol, ethanol, hexane, chloroform and methanol) on the enzyme activity, the purified lipase LipBL-WII(c) was incubated with different organic solvents at a concentration of 50% (v/v) for 6 h at 37°C at 150 rpm. The lipolytic

activity of control (in absence of organic solvent) was considered to be 100.0%.

Purified lipase was incubated with different metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, and Zn^{2+}) of 1 mM concentration at 37°C temperature with 150 rpm for 6 h to assess the impact of metal ions on enzyme activity. Control was devoid of the metal ion solution. The change in the lipase activity as compared to the control's activity was referred to as the relative activity.

Purified LipBL-WII(c) was incubated in presence of various surfactants (Tween-20, 80, SDS and Triton X-100) at a concentration of 1.0% for 6 h at 37°C temperature with 150 rpm in order to assess their effect on the enzyme activity of lipase. No surfactant was used in the sample solution used as a control. The change in the lipase activity as compared to the control's activity was referred to as the relative activity.

The reaction velocity of purified LipBL-WII(c) was studied using p-NPP (p-nitrophenylpalmitate) as a substrate at variable concentration of (0.1–10 mM) in buffer Tris- HCl (50 mM, pH 8.0). The Michaelis constant (Km) and maximum velocity (Vmax) were estimated utilizing Lineweaver-Burk plot.

7.89 ml of olive oil mixed with 0.99 ml of methanol were kept in glass tubes with screw caps, mixed with a 2.6 ml of purified lipase and then incubated at 40°C temperature along with shaking at 220 rpm for 48 h. After the period of incubation, 200 µl of sample was withdrawn from the reaction's mixture and then diluted using 1.0 ml of n-hexane for 2 min. After wards, the centrifugation of samples was done at 10000 rpm for 15 min and 10 µl of the upper layer was subjected to a Thin Layer Chromatography (TLC) plate. Methyloleate was utilized as a reference for biodiesel in TLC. After the development of the plate in 90:10:1 (n-hexane/ethyl acetate/acetic acid), then iodine vapor was used for visualizing the spots for a short period of time. The yield of the biodiesel production was calculated using:

$$\text{Yield} = \frac{\text{Volume of biodiesel produced}}{\text{Volume of oil}} \quad \text{...(Eq. 4)}$$

RESULTS AND DISCUSSION

Carbohydrates, the versatile carbon sources, were used as energy sources for optimal microbial growth. Supplementation of production broth with glucose provided the lipase activity of 59±0.01 µ/ml, while the lowest activity of lipase was detected in the occurrence of fructose (35±0.01 µ/ml) in comparison to the control (48±0.01 µ/ml). In previous study, lactose at 1% (w/v) was found to maximize the production of lipase from *Staphylococcus chromogenes* O1A for optimum lipase production (1.69 µM/min/mg) by Golani *et al.* (2016).

The lipase production by *B. licheniformis* strain WII(c) inside the broth was enhanced in presence of 1.0% (w/v) yeast extract as a nitrogen source (60±0.01 µ/ml). Yeast extract has been generally considered to be good nitrogen source because of its organic nature (molecules of biological origin can be used as a carbon source, also it does not affect osmolarity and there are less chances of inhibition) and complexity (as it contains many growth factors besides proteins lysate). The stimulatory nature of yeast extract on the growth of a *Bacillus* spp. was previously reported by Golani *et al.* (2016).

Metal ions were integral component(s) of many enzyme systems as co-factors as they enhanced the catalytic activity of many enzymes and also confer thermostability to the enzyme(s) by altering the charge arrangement at the catalytic site of the enzyme (Sarac and Ugur, 2015). The effect(s) of addition of selected metal salts (0.02%) in the broth on alkaline lipase production by *B. licheniformis* strain WII(c) was studied. The maximum production of lipase was observed in the occurrence of Mg^{2+} ions (66±0.01 µ/ml) in comparison to the control (54±0.01 µ/ml) without ions. Saraswat *et al.* (2017) discussed that the lipase activity of *Bacillus subtilis* indigenous strain was found to be braced up in the occurrence of metal ions, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+} and K^{+}.

While exploring the effect of various carbon sources (lipidic) including various oils, the olive oil supplementation to the production broth provided an enhanced lipase production extracellularly by *B. licheniformis* strain WII(c) in the broth. The olive oil contains myristic acid glycerides (0.3%), linolic acid (41.7%), palmitic acid (20.0%), oleic acid (35.2%), arachidic acid (0.6%) and stearic acid (2.0%). Thus, it seems that the presence of high percentage of long chain glycerides of palmitic acid, linolinic acid and oleic acid in the olive

oil (1%, v/v) positively affected the lipase production by *B. licheniformis* WII(c). The medium augmented with olive oil showed lipolytic activity of 62±0.01 µ/ml in relation to the control having 55±0.01 µ/ml. On the basis of decreasing order of lipase activity the trend was olive oil> rice bran oil> sunflower oil> mustard oil>soybean oil> castor oil. In the study of Li *et al.* (2019), the lipase was capable in breaking down a number of edible oils like, olive oil, peanut oil, sunflower seed oil, corn oil and sesame oil.

RSM, being an exploratory and successive tool, was used in developing interaction between independent variables such as: temperature, pH, agitation rate, volume of inoculum and concentration of inducer oil for determination of the conditions optimum for highest production of lipase. FCCCD generated a 50 experimental runs set. The response surface quadratic model was created by subjecting the obtained results to ANOVA (analysis of variance). The lipolytic activity (Y) was expressed as a function of (A) temperature, (B) pH, (C) inoculum volume, (D) agitation and (E) inducer oil concentration demonstrated in the second-order polynomial equation as:

Lipase activity (Y) = +121.28 + 21.92 x A +14.15 x B + 10.42 x C +10.72 x D + 11.27 x E + 2.49 x A x B + 4.79 x A x C + 5.89 x A x D + 11.98 x A x E + 3.61 x B x C + 7.72 x B x D +5.46 x B x E + 7.35 x C x D + 3.96 x C x E + 10.1 x D x E + 5.85 x A^2 + 3.10 x B^2 + 3.00 x C^2 + 1.05 x D^2+1.50 x E^2

The model created was considered significant statistically with the value of less than 0.05 indicating that production of lipase could be explained well by this model (Table 2). The F-value of 2768.18 reported the model being suitable and significant for the simulation of production of lipase by any interaction amongst the five independent variables. There is a 0.01% chance that a 'model F-value' this large could occur due to noise. Low probability and high F-value depict a decent prediction model for the lipase production.

The value of R^2 (correlation coefficient) being a variance index falls between 0 and 1. The value of 1 suggests the model efficiency for the navigation of the design accurately, while the value 0 shows a complete inability. The model is stronger and the prediction efficiency of the response is better when the value of R^2 is closer to 1 (Abu *et al.*, 2017). The value of R^2: 0.9995 signified a comparatively high correlation amongst the predicted and experimental values. The response's variability of 99.95% was implied by the above value and can be explained by the model, while the model could

Table 2. ANOVA for the model generated for the optimization of lipase production

Source	Sum of squares	d. f.	Mean square	F-value	p-value	
Model	50665.45	20	2533.27	2768.18	< 0.0001	Significant
A–Temperature	16333.03	1	16333.03	17847.60	< 0.0001	
B–pH	6807.56	1	6807.56	7438.83	< 0.0001	
C–Inoculum concentration	3692.01	1	3692.01	4034.38	< 0.0001	
D–Agitation rate	3909.80	1	3909.80	4272.36	< 0.0001	
E–Inducer oil conc.	4316.64	1	4316.64	4716.92	< 0.0001	
AB	198.00	1	198.00	216.37	< 0.0001	
AC	735.36	1	735.36	803.55	< 0.0001	
AD	1109.21	1	1109.21	1212.06	< 0.0001	
AE	4593.61	1	4593.61	5019.58	< 0.0001	
BC	417.61	1	417.61	456.33	< 0.0001	
BD	1906.53	1	1906.53	2083.32	< 0.0001	
BE	954.85	1	954.85	1043.39	< 0.0001	
CD	1728.72	1	1728.72	1889.02	< 0.0001	
CE	500.86	1	500.86	547.31	< 0.0001	
DE	3280.50	1	3280.50	3584.70	< 0.0001	
A^2	84.74	1	84.74	92.59	< 0.0001	
B^2	23.72	1	23.72	25.92	< 0.0001	
C^2	22.21	1	22.21	24.27	< 0.0001	
D^2	2.71	1	2.71	2.96	0.0959	
E^2	5.54	1	5.54	6.06	0.0201	
Residual	26.54	29	0.9151			
Lack of fit	16.33	22	0.7423	0.5090	0.8930	Not significant
Pure error	10.21	7	1.46			
Cor total	50691.99	49				

not explain only about 0.05% of the entire variation. The values of predicted (0.9986) and adjusted (0.9991) R^2 were in accordance amongst each other. This suggested that the response's trends could be explained by the regression model.

The 0.51 F-value for lack of fit implies the non-significant lack of fit as compared to the pure error. For a model to be fit it is good to be being non-significant. The measurement of signal to noise ratio is known as adequate precision. The model adequacy and adequate signal is confirmed by a ratio of 290.713 greater than 4. The design space could be navigated suitably by using the model. The ratio between the mean value of the observed response and the estimate's standard error is known as the coefficient of variation (CV). The CV value being below 10% can predict the model being practically reproducible. The present study suggests that the reliability and the precision of the conducted experiments are predicted by a relatively lower CV value of 0.78%. The values of mean (123.18), standard deviation (0.96) and predicted residual sum of squares (72.94) were also generated by the model. The significance of the model terms was shown by the probability value of less than 0.05, while the value of 0.1 implied the model terms being non- significant. Every independent variable like A, B, C, D and E were significant terms of model. The effects of interaction on the variables included AB ($P<0.0001$), AC ($P<0.0001$), AD ($P<0.0001$), AE ($P<0.0001$), BC ($P<0.0001$), BD ($P<0.0001$), BE ($P<0.0001$), CD ($P<0.0001$), CE ($P<0.0001$), DE ($P<0.0001$), A^2 ($P<0.0001$), B^2 ($P<0.0001$), C^2 ($P<0.0001$), and E^2 ($P<0.0201$) and the variables were established to be significant ($P<0.05$) (Table 2). In this model, the direct effect of all the independent variables was found to be significant. While the quadratic effect of (A, B, C, and E) was significant, while quadratic effect of agitation rate was non-significant. Likewise, the collaborative consequences of all the independent variables amongst themselves were significant.

The contour plots express the regression equation graphically depicting the interactions amongst the independent variables and their influence on the production of enzyme. The contour plots might be raising ridges, elliptical rounds or saddle points. The generated contour plots were made by varying two factors while keeping the other's constant. The optimum conditions and the interactive effects of the optimized independent variables for enhancing the production of lipase are represented by the contour plots shown in Fig. 1 (a–j). As illustrated by the elliptical contour plot in Fig. 1 (d, f and j), the significant interaction amongst the variables was observed.

As per the suggested model, the predicted optimization result for maximum production of lipase (255.921 µ/ml) was attained when A: temperature, B: pH, C: inoculum volume, D: agitation rate and E: inducer oil concentration were fixed at 55°C, 9, 3.0% (v/v), 200 rpm and 3.0% (v/v), respectively. According to the variables predicted by the model it was further deep-rooted by carrying out lab experiments in conical flasks. The obtained results exhibited a highest mean lipase production of 256.20 µ/ml (72 h) through the late log phase, was in pact with the predicted response. This inferred an approx. 99.8% of validity accomplished a suggestion that the model presented a satisfactory prediction of the lipase activity. Culture conditions of *B. licheniformis* WII(c) were optimized and led to an approx. 5.124 fold rise in lipase production in reference to the non-optimized medium. A great similarity was observed amongst the experimental and predicted results assuring the applicability and accuracy for the optimization of process studies of the model. The statistical analysis and experimental design were depicted to be effective in inferring the significant variables and various optimum parameters for highest lipolytic activity. Improvement of the production of lipase by *B. licheniformis* WII(c) using FCCCD was significantly in reference with that of *Pichia guilliermondii* (Abu *et al.*, 2017), *Bacillus cereus* (Awad *et al.*, 2015) *Bacillus aryabhattai* (Adetunji and Olaniran, 2018), *Aspergillus niger* (Jia *et al.*, 2015) and *Thalasso spirapermensis* (Kai and Peisheng, 2016).

The lipase produced extracellularly from WII(c) was initially purified engaging ammonium sulfate precipitation method tailed employing dialysis and then ion exchange chromatography. The production of enzyme after culture conditions optimization was exposed to ammonium sulfate precipitation in order to salt out the proteins. Table 3 depicts that ammonium sulphate (80%) saturation of the supernatant precipitated the maximum amount of lipase i.e 55.9 µ/ml by *B.*

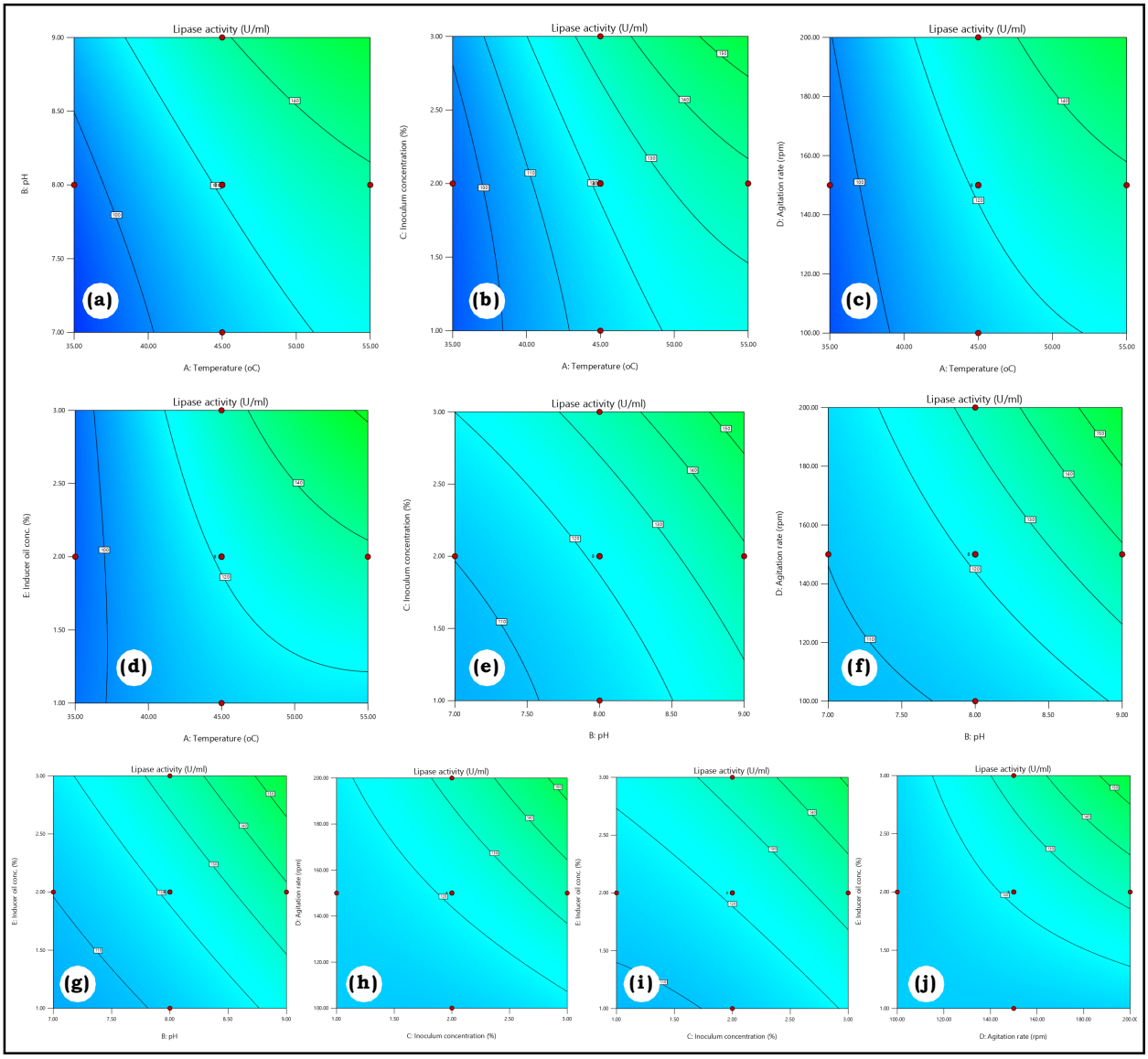

Fig. 1. Contour plots of production of lipase by *B. licheniformis* WII(c) showing interactive effect amongst (a) temperature and pH, (b) temperature and inoculum volume, (c) temperature and agitation rate, (d) temperature and inducer oil concentration, (e) pH and inoculum volume, (f) pH and agitation rate, (g) pH and inducer oil concentration, (h) agitation and inoculum volume, (i) inoculum volume and inducer oil concentration and (j) agitation rate and inducer oil concentration.

Table 3. Purification of lipase LipBL- WII(c) produced by *Bacillus licheniformis* WII(c)

S. No.	Purification step	Volume (ml)	Total activity (µ)	Total protein (mg)	Specific activity (µ/mg)	Purification fold	Yield (%)
1.	Crude enzyme	100	5000	85.2	58.68	1.00	100.00
2.	Ammonium sulphate precipitation	40	2236	8.2	272.68	4.64	44.72
3.	DEAE-cellulose chromatography	10	1520	2.0	760.00	13.00	30.40

licheniformis. It discovered 44.72% yield with 4.64 purification fold. Ammonium sulfate precipitation was tailed by ion-exchange chromatography. The enzyme after desalting was exposed to Sephadex G-100 column for ion exchange chromatography. The purification was attained by ion exchange chromatography while the comparative negative charge on lipase assistances in binding to the positively charged Sephadex G-100. Out of the total

fractions taken throughout elution procedure; fraction no. 11 contained the highest lipase activity. The purified lipase of WII(c) unveiled a final specific activity of 760 µ/mg with 13 fold of purification and 30.4% yield (Table 3). Low outcome of purified enzyme was due to loss during the ammonium sulfate precipitation. After the purification of extracellular lipase the LipBL-WII(c) from *B. licheniformis* its molecular weight was determined by SDS PAGE which was 41 kDa (Fig. 2). The lipase purity was confirmed using SDS-PAGE which discovered the presence of a solo band of protein. The m.w. of lipase (BSK-L) produced intracellularly was determined to be 45 kDa, which was comparable to lipase from *Acinetobacter* sp. AU07 strain (Gururaj *et al.*, 2016). Lipase produced by *Candida viswanathii* was purified by hydrophobic interaction chromatography, showing m.w. of 18.5 kDa as determined by SDS-PAGE (De Almeida *et al.*, 2016). Li *et al.* (2019) reported a lipase produced extracellularly from *A. pullulans* and was purified with a 17.7 µ/mg specific activity of protein employing ultrafiltration using a DEAE-Sepharose column. The molecular weight of the lipase was 39.5 kDa, determined by SDS-PAGE. Lipase from strain *Streptococcus* sp. N1 was partly purified by 60% ammonium sulphate precipitation with an activity of 654 µ/ml. The m.w. of enzyme was determined to be 40 kDa employing SDS-PAGE technique (Sharma and Goswami, 2020).

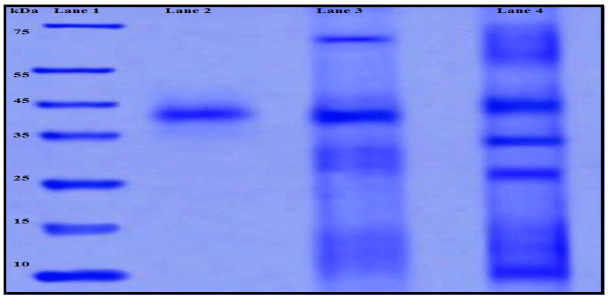

Fig. 2. SDS PAGE of LipBL-WII(c) lipase obtained from *B. licheniformis:* Lane 1: molecular marker, Lane 2: purified lipase from ion exchange chromatography, Lane 3: ammonium sulphate precipitated lipase and Lane 4: crude enzyme.

The optimal temperature for purified LipBL-WII(c) from *B. licheniformis* was 55°C, with 105% relative lipase activity. LipBL-WII(c) showed activity from temperature 20 to 80°C. The retaining of relative activity was witnessed at 40°C (85%), 45°C (92%), 50°C (105%), 55°C (98%), 60°C (84%), 65°C (56%), 70°C (42%), 75°C (33%) and 80°C (22%) showing that the lipase was thermo-tolerant. On increasing the temperature beyond 50 °C, the activity of lipase decreased indicating a thermal destruction of the tertiary enzyme structure. The optimal activity of the lipase from *Anoxybacillus flavithermus* was at temperature 50°C and was detected to be firm from 25 to 50°C for incubation of 24 h (Bakir and Metin, 2016). The purified enzyme produced by *Bacillus subtilis* was moderately active at various temperature ranges, while it presented highest activity at 70°C (124.00 µ/ml) as reported by Mazhar *et al.* (2016). Optimal temperature for the activity of enzyme was found to be 40°C with decent stability showing at temperature range of 10-30°C for the lipase produced by the bacteria identified as *Acinetobacter haemolyticus* (Sarac and Ugur, 2015). Saraswat *et al.* (2017) reported the alkaline and thermo-tolerant lipase (BSK-L) production from the strain *B. subtilis.* Lipase had an optimum activity at temperature 37°C and exhibited active activity of lipase at temperature range 30 to 60°C.

The optimal pH of purified LipBL-WII(c) was estimated to be 8 and exhibited stability in pH from 4 to 10 retaining about 50% of the relative activity on incubating at pH 10.0 for 24 h, while only 10 and 25% of the relative activities were reserved at pH 4.0 and pH 5.0, respectively. Lipase from *Cohnella* sp. A01 showed optimum activity at pH 8.5 and was ideally stable at the pH from 8.5-10 for the incubation of 180 min (Golaki *et al.*, 2015). The pH optima for the purified lipase produced by *B. subtilis* was pH 8 and the enzyme activity possessed by the enzyme at the pH was 174.06 µ/ml (Mazhar *et al.*, 2016). Rmili *et al.* (2019) reported that lipase SCL produced by *Staphylococcus capitis* retained greater than 60% of its initial activity over an extensive pH values ranging from 5 to 11 with a maximum stability between pH 9 and 11 after 1 h of incubation at room temperature. Sarac and Ugur (2015) reported that the lipase produced by *Acinetobacter haemolyticus* showed the pH optima at 9 with a good stability exhibiting at pH ranging between 5.0-11.0. The lipase (BSK-L) produced from the strain *B. subtilis* articulated thermo-tolerant and alkaline characters. Lipase showed an optimum activity at pH 8.0, while it was found to be stable at pH range 6.0 to 9.0 (Saraswat *et al.*, 2017).

Purified LipBL-WII(c) from *B. licheniformis* WII(c) was incubated in presence of 50% (v/v) organic solvents for 6 h and observed notable retaining of relative activity with hexane (103%), ethanol (87.6%), methanol (101%) and isopropanol (91.6%), while moderately tolerant to chloroform (41.4%) stating that this lipase unveiled decent stability with most of the organic solvents. Similarly, *Bacillus* sp. was found to be tolerant to methanol, ethanol, and acetonitrile making the strain produce a solvent tolerant lipase enzyme rendering it to be a likely nominee of the solvent tolerant lipase for a variety of industrial applications (Jaiganesh and Janganathan, 2018). Lipases tolerant to organic solvent are crucial for the production of biopolymeric, fine chemicals and biodiesel material production (Javed *et al.*, 2018). The lipase produced by *Aureobasidium pullulans* exhibited a noteworthy stability in some 30% (v/v) organic solutions including hexane, DMSO (dimethyl sulfoxide), n-propanol and isopropanol (Li *et al.*, 2019). Malekabadi *et al.* (2018) described that the KM12 lipase produced by *B. licheniformis* showed substantial stability in various organic solvents after 7 and 21 days of incubation, particularly in polar organic solvents. The purified lipase from *B. licheniformis* reserved a decent activity in the occurrence of both hydrophilic (DMSO) and n-hexane being hydrophobic alongwith solvents that specified its worth in stimulating both hydrolytic alongwith synthetic reactions (Sharma and Kanwar, 2017).

Purified LipBL-WII(c) was incubated with 1 mM molarity of metal ions for 6 h and perceived for a change in the relative activity of lipase on the treatment by Fe^{2+} (22%) and Mn^{2+} (42%), while a decent tolerance was perceived in the occurrence of Ca^{+1} (115%), Mg^{2+} (85.2%) and Zn^{2+} (65%). A decrease in the lipase activity on treatment with EDTA (a metal ion chelator) along with Ca^{2+} (71.5%) and Mn^{2+} (62.8%), as metal ions assisted in the structural and functional maintenance of the enzyme as described by Golaki *et al.* (2015). It is significant that the enzyme lipase stable in the presence of metal ions could be valuable in formulation of detergents. The lipolytic activity of strain RSJ-1 of *Bacillus* sp. was retained in the presence of Ba^{2+} , Ca^{2+}, Mg^{2+} and Na^{+}, while it was greatly repressed by Zn^{2+}, Cs^{+}, K^{+} and Co^{2+}, whereas EDTA had nil impact on the activity enzyme (Sharma *et al.*, 2017). While lipase activity of the extracellular lipase from *Azospirillum* sp. was decreased by the addition of Mg^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} metal ions (Lestari *et al.*, 2016). Li *et al.* (2019) showed that the catalytic activity of the lipase produced from *Aureobasidium pullulans* was heightened by Ca^{2+} and was marginally inhibited by Zn^{2+} and Mn^{2+} at a concentration of 10 mmol/l. Golani *et al.* (2016) reported that amongst the metal ions tested Ca^{2+} heightened the lipase activity up to (1.67 µm/min/mg) and Zn^{2+} depicted a lower enzyme activity (0.28 µm/ml/mg).

The effect of different surfactants on the LipBL-WII(c) was examined by incubating the lipase with various surfactants for 6 h. The relative activity was upgraded by the adding Triton X-100 (102%), Tween 20 (92%), Tween 80 (95%) and SDS (87%). On the other hand, Golaki *et al.* (2015) stated that the bio-catalytic activity of the enzyme was considerably reduced to 16.2% by the surfactant SDS. Kaur *et al.* (2016) reported that the enzyme produced by *B. licheniformis* could preserve its activity in occurrence of different detergents (Tween-20, 40, SDS and Triton X-100). Li *et al.* (2019) described that the lipase from *Aureobasidium pullulans* was stimulated by the non-ionic surfactants (Tween-80, Triton X-100 and Tween-20) and the anionic surfactant SDS, but it was severely repressed by the cationic surfactant CTAB. Sharma and Kanwar (2017) reported that all surfactants (SDS, Tween-20, 40, 60, 80 and Triton X-100) had an inhibitory outcome on the production of lipase by *B. licheniformis.*

Lineweaver-Burk plot was used to determine the kinetic parameters (Km and Vmax) of enzyme lipase. The values of Km and Vmax for lipase by utilizing pNPP (p-nitrophenylpalmitate) as a substrate were determined to be 1.0 mM and 6.66 mmoles/min/mg, respectively (Fig. 3). A low value of Km implied a greater affinity of enzyme towards its substrate, while a high Km implied a less affinity. Kumar *et al.* (2020) stated the values of Km and Vmax being 0.104 mM and 3.58 µ/mg, respectively, for lipase from *Chryseobacterium polytrichastri*, which depicted the interaction of enzymes toward its substrates. Sarac and Ugur (2015) described the lipase production from *Acinetobacter haemolyticus* depicting lipase's Km and Vmax as 0.8 mM and 3.833 mmoles/min/mg, respectively. Km and Vmax values of the lipase

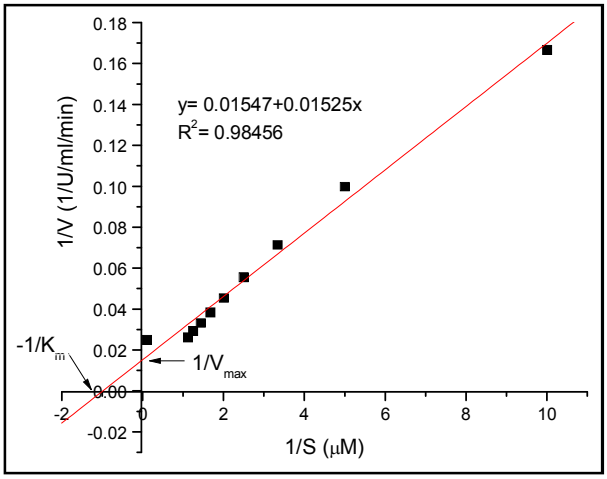

Fig. 3. Line weaver- Burk plot of purified LipBL-WII© from *Bacillus licheniformis*.

production by the native *B. subtilis* strain Kakrayal_1 (BSK-L) were perceived to be 2.2 mM and 6.67 mmoles/min/mg, respectively (Saraswat *et al.*, 2018). Sharma and Kanwar (2017) verified that the Vmax and Km of the purified lipase of *B. licheniformis* SCD11501 were 2.27 and 0.43 mmoles/ml/min, respectively for the breakdown of p-NPP. The values of Vmax and Km of purified lipase for isolates G14, B10 and OI were 17.6, 24.4 and 135 mmoles/min/mg and 1.3, 1.6 and 0.681 mM, respectively (Shart and Elkhalil, 2020).

Purified LipBL-WII(c) catalyzed the biodiesel production in the occurrence of olive oil and methanol (Fig. 4). The yield of the biodiesel produced using olive oil was 88%. Purified LipBL-WII(c)'s characteristics properties and stability amongst organic solvents made them useful for the trans-esterification process for the production of biodiesel as a bio-catalyst. For the mentioned purpose, the solidity of enzyme in the existence of various organic solvents like methanol and ethanol were examined as the stability of the enzyme in these solvents was extremely necessary for the enzymatically biodiesel formation.

CONCLUSION

In this research, culture conditions were optimized for enhancing the production of lipase by *B. licheniformis* manually and by FCCCD. The production of lipase was affected by the parameters like, temperature, pH, agitation rate, volume of inoculum, inducer oil concentration, carbon source, nitrogen sources, inducer oil and mineral salts. The results of

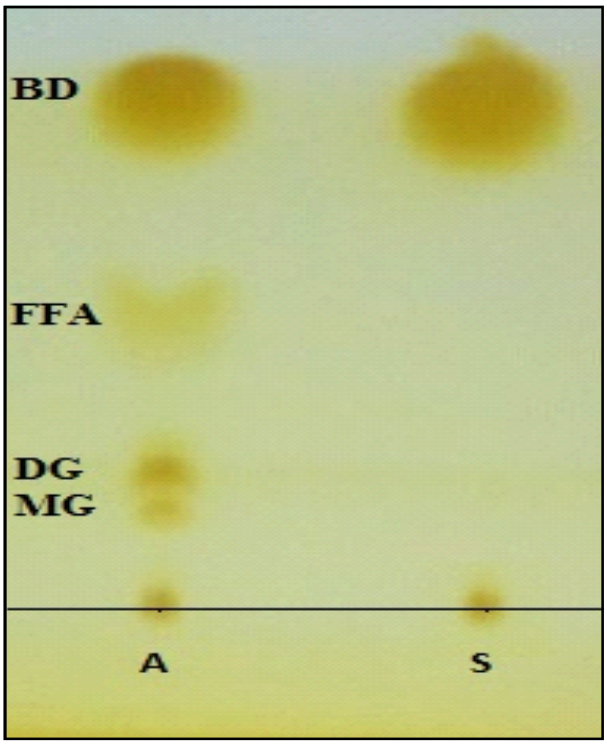

Fig. 4. Purified LipBL-WII(c) catalyzed production of biodiesel, TLC plate. S: standard (methyl oleate), A: sample. BD: biodiesel, DG: diglyceride, FFA: free fatty acid and MG: monoglyceride.

the observed and predicted yield of lipase confirmed the accuracy and validity of the generated model. After the optimization of the culture parameters by RSM there was an overall 5.4 fold increase in the production of lipase. The lipase was consecutively purified utilizing precipitation method employing ammonium sulphate and ion-exchange chromatography, attaining the m.w. of 41 kDa with 13-fold purification value and specific activity of 760 μ/mg. Characterization of purified lipase LipBL-WII(c) was done in order to exhibit the maximum efficiency of conversion. Furthermore, because of its evident characteristics like, tolerance to surfactants, organic solvents and metal ions, lipase could add to sewage treatment, organo-synthesis and detergent formulation. The proficiency of organic solvent tolerance was oppressed in order to seek out application in the biodiesel production using enzymatic catalyst. The lipase was in order to have the ability to catalyze the synthesis of biodiesel using olive oil. This is the reporting of the purification, characterization and potential application of *B. licheniformis* lipase in biodiesel production.

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