Statistical optimization for lipase production from solid waste of vegetable oil industry

Rajesh Kumar Sahoo[1], Mohit Kumar[2], Swati Mohanty[3], Matthew Sawyer[4], Pattanathu K.S.M. Rahman[4], LalaBehari Sukla[5], Enketeswara Subudhi[1*]

1. Centre for Biotechnology, Siksha O Anusandhan University, Bhubaneswar-751003, Odisha, India
2. Shiv Astha Hospital, Haryana-132001, India
3. CSIR-Institute of Minerals and Materials Technology, Bhubaneswar
4. Technology Futures Institute, School of Science and Engineering, Teesside University, Middlesbrough, UK
5. Biofuels and Bioprocessing Research Center, Siksha 'O' Anusandhan University, Bhubaneswar 751030, India

***Corresponding Authors**

Dr. Enketeswara Subudhi

Center of Biotechnology

Siksha O Anusandhan University

Kalinga Nagar, Bhubaneswar

Odisha

Email ID: enketeswarasubudhi@soauniversity.ac.in

Abstract

The production of biofuel using thermostable bacterial lipase from hot spring bacteria out of low cost agricultural residue olive oil cake is reported in the present paper. Using a lipase enzyme from *Bacillus licheniformis*, a 66.5% yield of biodiesel was obtained. Optimum parameters were determined, with maximum production of lipase at a pH of 8.2, temperature 50.8^{o}C, moisture content of 55.7% and bio-surfactant content of 1.693mg. The contour plots and 3D surface responses depict the significant interaction of pH and moisture content with bio-surfactant during lipase production. Chromatographic analysis of the lipase trans-esterification product, biodiesel, from kitchen waste oil under optimized conditions, generated methyl palmitate, methyl stearate, methyl oleate and methyl linoleate.

Keyword: **Response surface methodology; solid state fermentation; lipase; oil-cake industry waste; *Bacillus licheniformis***

1. Introduction

Over the last few decades, residues from various agricultural industries have been investigated for their energy rich wastes and whether value added products can be created from these [1–5]. Oil cakes are byproducts generated by oil mills after oil extraction from seed. They have been used in biotechnological applications for their high nutritional and energy value [3, 6, 7]. India is one of the world's leading producers in oilseeds, oils and oil meals with a current annual target production of 38.6, 16.8 and 17.2 million metric tons (MMT) respectively, with an annual export of oilseeds worth $1.4 billion [8]. Without correct disposal of oil cake wastes or their expensive alternative treatments to make them environmental friendly, they can be an environmental menace. Biotransformation of these agro-industrial residues through fermentation has the potential to overcome these serious environmental problems. Fermentation of oil cakes have been reported to produce bulk-chemicals and value-added products such as amino acids, enzymes including lipase [1, 3], organic acids, single-cell proteins (SCP) etc. [9–13]. Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are one of the enzymes which both hydrolase the lipid and synthesis of esters from glycerol and long-chain fatty acids. The broad-spectrum applicability of thermophilic lipase was investigated using negative value agro-industrial residues from oil producing industries and its potentiality in bioconversion of waste vegetable oil to biodiesel. These enzymes are in great demand in various industries for many reasons such as; they do not require cofactors, they are resistance to organic solvents, and they remain active in broad range of substrates and often exhibit high enantio- and regio-selectivity [14]. Since lipases can be used in a synthetic chemistry such as esterification, trans-esterification, alcoholysis, acidolysis, aminolysis, acylation and resolution of racemic mixtures, they have become potentially important for biotechnological and other industrial applications [9, 15]. Solid substrate fermentation (SSF) is economically and environmentally preferable option over other fermentation methods because of lower water requirements, greater productivity and enhanced enzyme reactivity. Furthermore, the enzymes produced by SSF are retained in the fermented solids bypassing the expensive extraction, purification and immobilization processing.

Solid wastes from the production of vegetable oils (oil cakes) have been widely used for the production of industrial enzymes and other biochemical because they are inexpensive and plentiful in countries like India. Changing one parameter at a time to study the effect of different parameters on the output of any process may not be very accurate, as the interaction effects between multiple factors are not taken in to account [16]. Inherent disadvantage of studying one parameter at a time can be overcome by employing central composite design

(CCD), which is a response surface methodology (RSM) that helps to understand the combined effects of multiple factors in any process [17].

Rhamnolipid bio-surfactants are reported to be enhancer of microbial growth as well as of production and activity of enzyme cellulose, amalyse, xylanase, protease [18] and lipase [19] in several submerged fermentation processes. However, there are very few reports which discuss the influence of bio-surfactants in lipase production in SSF using oilcake waste as substrate. Hasan et al. (2006)[20] reported that the structural properties responsible for conferring enzyme thermostability also contribute towards their stability in harsh environmental conditions including organic solvents. Therefore, highly stable enzymes can be obtained from inoculums of thermophilic or thermotolerant organisms isolated from thermophilic sources such as hot springs for use in solid state fermentation.

In this study, enhanced lipase production was investigated as per CCD under different conditions in solid state fermentation using olive oil cake (OOC) as the substrate and with thermophilic bacteria *Bacillus licheniformis* (JQ991000) isolated from Taptapani hot spring of Odisha, India. Further, lipase was utilized for biofuel production using kitchen waste oil.

2. Materials and Methods

2.1 Materials

All routine chemicals of analytical grade were obtained from Himedia, Mumbai, India. Oil cakes were obtained from the local manufacturing units which were grounded in a pestle and mortar to achieve particle sizes of approximately 3-5mm. p-nitrophenyl laureate and rhamnolipid bio-surfactant were procured from Sigma Chemicals, India. Molecular biology reagents were obtained from Thermo Scientific, India.

2.2 Methods

Screening and identification of lipase producing bacteria from hot spring

Sediment sample was collected from Taptapani Hot spring, Odisha where the temperature is 48±5°C and pH 7.69 ±0.4. Sediment sample was serial diluted and spread on Rhodamine B-olive oil agar plates [21] and incubated for 48 hours at 50 °C. Orange fluorescent halos around colonies under UV rays were recorded as the positive lipase producer. Bacterial colonies exhibiting fluorescence were selected and inoculated into Bushnell Haas Broth containing (BHB) olive oil. After 72 hours, cells were harvested by centrifugation at 10,000X*g* for 10min (HERMLE, Germany) and the supernatant was used as a source of crude lipase.

Genomic DNA was extracted from selected lipase producing bacteria and 16S rRNA gene was amplified by using universal primers 8F (AGAGTTTGATCCTGGCTCAG) and reverse primer 1492R (GGTTACCTTGTTA CGACT T) as described by Sahoo et al.(2014)[22]. The PCR amplified products were sequenced by Xcelris, India. After sequencing, all the nucleotide sequence of 16S rRNA gene of the bacterial strain was deposited in the NCBI-GenBank database.

2.3 SSF Experiments

Solid state fermentation was initiated by inoculating *Bacillus licheniformis* ($2x10^6$ CFU/ml) to 10g of olive oil cake and incubated for 120 hours at 50°C temperature. Oil cake without culture served as control. Oil cake samples were taken for lipase assay every 24 hours.

2.4 Identification of Significant variables using CCD

To identify the best culture conditions for lipase production in solid substrate fermentation, process parameters were optimised by following central composite design under response surface methodology (RSM). RSM involved a mathematical model to appraise the correlation between process parameters and to measure their significant contribution towards lipase production. Different independent variables taken into consideration for studying the lipase production were temperature (°C) (A), pH (B), moisture content (%) (C) and bio-surfactant (mg) (D). Lipase production was taken as dependent response variable (IU/g). The upper and lower limits for the different variables are listed in Table 1.

2.5 Statistical Analysis

Statistical analysis of the employed model was undertaken to determine the analysis of variance using ANOVA, the overall significance of model, its associated probability p(F) through Fisher's test (F-test) and the degree of fit to the regression model by correlation coefficient and determination coefficient (R and R^2). Design Expert 6.0 software (State-Ease Inc, Minneapolis, USA) was used to represent the graphical examination of the experimental results due to effect of independent process variables in the form of contour plots and response surface curves.

2.6 Biodiesel analysis by Gas chromatographic study

Lipase was extracted from the solid substrate and purified through Sephadex G-75 gel permeable column chromatography following method described by Uttatree et al. (2010)[23]. For the biodiesel synthesis, the reaction mixture contained 1:1 kitchen waste oil and methnol, 0.1 M phosphate (pH 7.0) and purified lipase solution. The solution was gently mixed using a vortex and then placed on a shaker at 200 rpm under standard

conditions. 10µl aliquots of the samples were taken after 30 minutes and 24 hours of incubation for analysis by gas chromatography [24]. The constituents of fatty acid mixture were identified by Gas Chromatography analysis using Perkin Elmer Clarus 600 series instrument (Walhton, MA, USA) with a low polarity DB-5 (15 m × 0.25 mm inner diameter and 0.5 µm film thicknesses) capillary column. The initial temperature of column was held at 150°C for 0.5 transactions, which was increased to 300°C at a warming rate of 20°C/min. Nitrogen gas was used as carrier at 1 ml/min flow rate. The temperature of injector was kept at 295°C. The detection was done using flame ionization detector (FID) at 300°C. Calibration curves were prepared for quantification by using standard solutions of four fatty acid methyl esters - methyl palmitate, methyl stearate, methyl oleate and methyl linoleate. Yield of biodiesel after analysis was quantified from the ratio of the total concentration of four methyl esters to those of corresponding fatty acids in the initial reaction mixture.

3. Result and Discussion

3.1 Screening and identification of lipase producing bacteria

After serial dilution of the soil sample, 54 colonies were obtained on BHA plate with olive oil as a carbon source and Rhodamine B as indicator. Among the 54 isolates, only 10 isolates showed halo orange zone on BHA + olive oil + Rhodamine B plate. After 3 days of incubation in BHB + olive oil, 10 bacterial isolates (CBT1-10) could hydrolyse olive oil as sole carbon source and showed lipase activity ranging from 0.03 to 50 IU/ml. Only one of the isolate (CBT1) showed maximum activity of 50 IU/ml and selected for further investigation, which showed 99% similarity with *Bacillus licheniformis*. The nucleotide sequence of the 16S rRNA gene was submitted in NCBI-GenBank and obtained the accession number JQ9911000. Kaur et al. (2016) [25] isolated thermophilic lipase producing *Bacillus licheniformis* from Manikaran Hot spring, Himachal Pradesh, India. Phylogenetic tree of *B. licheniformis,* constructed on the basis of the 16S rRNA gene similarity with already reported lipase producing *Bacillus* sp., showed a distinct phylogenetic line among other lipase producing *Bacillus* sp. (Fig. 1).

3.2 Statistical analysis

A total of 30 experimental runs were carried out as per the plan of CCD which consisted of 16 factorial points, 8 axial points and 6 center points and the corresponding experimental as well as the model predicted lipase activity (IU/g) were presented, the details of which are given in Table S1. Analysis of variance (ANOVA) of the data obtained from experiments was carried out, as shown in Table S2, to identify the significant main effects and also the interaction effects. The f-test showed that the main effects, temperature, moisture and bio-surfactant main effects were significant, but pH had quadratic effect on lipase production. The parameter interaction of pH

with other variables were also significant, hence to maintain hierarchy, pH has been included in the model equation (Equation 1). A quadratic model obtained by regression analysis was found to best fit with the experimental data with R^2 of 0.9236. The F value of 12.95 showed that the model is significant at less than 0.01% (i.e. at 99.99% confidence level). High value of coefficient of variation (CV = 12.97) denotes the reliability of the experiments.

The model equation for estimating lipase activity (Y) for olive oil cake is given by equation (1).

$$Y = - 610.9 + 10.21A + 71.15B + 4.11C + 8.15D - 0.1574\ A^2 - 6.17\ B^2 - 0.0527\ C^2 - 8.31\ D^2 + 0.6262A*B + 0.0416A*C - 0.9933A*D - 0.1668B*C + 4.51B*D + 0.6001C*D$$

……..……equation (1)

Where Y is lipase production (response); A, Temperature; B, pH; C, moisture; D, Biosurfactant.

The lipase activity as calculated using equation (1) is presented in Table S1. The average percentage absolute error in prediction was 9.44%. The lack-of-fitness shows that the error is primarily due to the model rather than the experimental error (Table S2). Since the error in prediction was within the acceptable limit, the model was used for parameter optimisation.

The contour plots and 3D surface responses based on the model equation were obtained using software Design-Expert version 6.0 (State-Ease Inc., Minneapolis, USA). Two parameters were studied at a time, keeping the other two constant at the optimum value. Figure 2 (A and B) show the effect of bio-surfactant and moisture on the lipase activity. Although at low level of moisture content, the effect of bio-surfactant was less significant but at higher level (55%), it is quite significant (61.49 IU/g) and similarly the effect of moisture content was also significant at higher level. Hence, there is a significant interaction between moisture content and bio-surfactant. The maximum lipase activity was 61.5 IU/g at moisture content of around 55% and bio-surfactant of around 1.6 mg. Figure 3 (A and B) show the effect of pH and bio-surfactant at optimized conditions of moisture content and temperature. The pH has a quadratic effect both at low and high level of bio-surfactant, whereas bio-surfactant has little effect at low level of pH and increased effect at higher level. This ensures strong interaction between pH and bio-surfactant. Here, it is observed that the pH is around 8.2 and bio-surfactant concentration is around 1.7 mg. Figure 4 (A and B) show the effect of pH and temperature at optimized conditions of moisture content and bio-surfactant. It is evident from the figure that temperature has significant effect on the lipase activity, which is also reflected in the ANOVA (Table S2). The Figure 4 (A and B) showed the maximum lipase activity at a temperature of around 51^{o}C and pH 8.2, beyond which there was strong decrease in activity.

3.3 Validation of the predicted model

The model was used to optimize the parameters to maximize lipase activity. The optimized conditions were calculated to be at temperature 50.8 ^{o}C, pH 8.2, moisture 55.7% and bio-surfactant 1.693 mg and the maximum lipase activity was calculated to be 61.5 U/g. To validate the predicted model, the lipase fermentation using *B. licheniformis* was carried out again under model predicted optimal conditions as mentioned above. The experimental value was slightly higher (62.3 IU/g) than the predicted (61.5IU/g) lipase synthesis at optimum process parameters which proves the fitness of the used model in predicting the optimum parameter condition for enzyme production.

3.4 Biodiesel production using waste vegetable oil

To understand the potential of lipase to utilise kitchen waste oil, biodiesel yield was determined. Yields of 66.5%±1.7 biodiesel were observed with kitchen waste oil after 24 hours incubation under standard conditions (Fig. 5). Biodiesel production yield in our finding was found to be higher as compared with other studies [26, 27]. Chromatographic analysis of transesterification product at 30 min and 24 hours incubation period is presented in figure 6. After 24 hours, the kitchen waste oil derived biodiesel confirmed to consist of methyl palmitate, methyl stearate and methyl oleate and methyl linoleate.

4. Conclusion

The production of biofuel using thermostable bacterial lipase from low cost agro-industrial residue olive oil cake adopting a low energy incurring approach of solid substrate fermentation is reported. The present investigation deals with bio-surfactant augmented lipase production (62.3 IU/g) under optimized process variables employing the Central Composite Design. The analysis revealed both the influence and interaction of moisture content, pH, temperature and bio-surfactant contributing towards lipase production. The model employed was fit and efficient in predicting suitable parameters for lipase production as the observed value was found close to predicted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was financially supported by the Department of Biotechnology, Government of India (BT/PR7944/BCE/8/1036/2013). The authors are grateful to Prof (Dr.) S.C. Si, Dean, Centre of Biotechnology

and Prof (Dr.) M.R. Nayak, President, Siksha 'O' Anusandhan University, for providing infrastructure and encouragement throughout.

Reference

1. Ramachandran, S.; Singh, S. K.; Larroche, C.; Soccol, C. R.; Pandey, A.. Oil cakes and their biotechnological applications - A review. Biores. Technol. 2007,98, 2000–2009.

2. Gangadharan, D.; Sivaramakrishnan, S.; Nampoothiri, K. M.; Sukumaran, R. K.; Pandey, A. Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. Biores. Technol. 2008, 99, 4597–4602.

3. Salihu, A.; Bala, M.; Alam, M. Z. Lipase production by *Aspergillus niger* using sheanut cake: An optimization study. J. Taibah University Sci.2015, 10.

4. Corre, W. J.; Conijn, J. G. Biogas from agricultural residues as energy source in hybrid concentrated solar power. Procedia Computer Sci. 2016,83, 1126–1133.

5. Gao, J.; Zhang, A.; Lam, S. K.; Zhang, X.; Thomson, A. M.; Lin, E.; Jiang, K.;Clarke, L.; Edmonds, J.; Kyle, G.; Yu, S.; Zhou, Y.; Zhou, S.. An integrated assessment of the potential of agricultural and forestry residues for energy production in China. GCB Bioenergy, 2016,8, 880–893.

6. Vaštag, Ž.; Popović, L.; Popović, S.; Krimer, V.; Peričin, D. Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate. Food Chem. 2011,124, 1316–1321.

7. Cheng, Y. S.; Wu, J. H.; Yeh, L. H. Utilization of *Calophyllum inophyllum* shell and kernel oil cake for reducing sugar production. Biores. Technol. 2016,212, 338–341.

8. Aradhey, A. Oilseeds and Products Annual. Global Agricultural Information Network (GAIN) Report, 2017.

9. Pandey, A.; Selvakumar, P.; Soccol, C. R.; Nigam, P. Solid fermentation for the production of industrial enzymes. Curr. Sci. 1999,77, 149–162.

10. Vandenberghe, L. P. S.; Soccol, C. R.; Pandey, A.; Lebeault, J. M. Solid-state fermentation for the synthesis of citric acid by *Aspergillus niger*. Biores. Technol. 2000,74, 175–178.

11. Nampoothiri, K. M.; Ramachandran, S.; Soccol, C. R.; Pandey, A.. Advances in fermentation technology. Int. Sugar J. 2002, 104, 493–499.

12. Pandey, A. Solid-state fermentation. *Biochemical Eng. J.*2003,*13*, 81–84.

13. Soccol, C. R.; Brand, D.; Mohan, R.; Rodriguez, J. A. L.; Pandey, A.. Coffee husk: a potential alternative material for bioprocesses. Metals Mater. Proc. 2005,17, 195–206.

14. Joseph, B.; Ramteke, P. W.; Thomas, G. Cold active microbial lipases: Some hot issues and recent developments. Biotechnol. Adv. 2008, 26, 457–470.

15. Sharma, S.; Kanwar, S. S. Organic solvent tolerant lipases and applications. The Scientific World J. 2014.

16. Weuster-botz, D.. Experimental Design for Fermentation Media Development : Statistical Design or Global Random Search ?,2000,90, 473–483.

17. Elibol, M.. Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3(2) with response surface methodology. Proc. Biochem. 2004, 39, 1057–1062.

18. Liu, J.; Shi, J.; Li, J.; Yuan, X. Effects of surfactants tween 80 and rhamnolipid on the extracellular enzymes amylase, protease, CMCase and xylanase of one strain. Proceedings - International Conference on Computer Distributed Control and Intelligent Environmental Monitoring, CDCIEM 2011, 2011,1207–1210.

19. Acikel, U.; Erşan, M.; Açikel, Y. The effects of the composition of growth medium and fermentation conditions on the production of lipase by R. delemar. Turkis J. Biol.2011,35, 35–44.

20. Hasan, F.; Shah, A. A.; Hameed, A. Industrial applications of microbial lipases. *Enzyme Microb. Technol.*2006,*39*, 235–251.

21. Olusesan, A. T.; Azura, L. K.; Abubakar, F.; Hamid, N. S. A.; Radu, S.; Saari, N.. Phenotypic and molecular identification of a novel thermophilic *Anoxybacillus* species: A lipase-producing bacterium isolated from a Malaysian hotspring. World J. Microbiol. Biotechnol. 2009,25, 1981–1988.

22. Sahoo, R. K.; Subudhi, E.; Kumar, M. Quantitative approach to track lipase producing *Pseudomonas* sp. S1 in nonsterilized solid state fermentation. Lett. Appl. Microbiol.2014, 58, 610–616.

23. Uttatree, S.; Winayanuwattikun, P.; Charoenpanich, J. Isolation and characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi*. Appl. Biochem. Biotechnol. 2010,162, 1362–1376.

24. Korman, T. P.; Sahachartsiri, B.; Charbonneau, D. M.; Huang, G. L.; Beauregard, M.; Bowie, J. U.

Dieselzymes: development of a stable and methanol tolerant lipase for biodiesel production by directed evolution. Biotechnol. Biofuels, 2013,6, 70.

25. Kaur, G.; Singh, A.; Sharma, R.; Sharma, V.; Verma, S.; Sharma, P.K. Cloning, expression, purification and characterization of lipase from *Bacillus licheniformis*, isolated from hot spring of Himachal Pradesh, India. 3 Biotech, 2016, 6, 49.

26. Sanchez, F.; Vasudevan, P. T. Enzyme catalyzed production of biodiesel from olive oil. Appl. Biochem. Biotechnol.2006,135, 1–14.

27. Dos Santos Corrêa, I. N.; Lorena de Souza, S.; Catran, M.; Bernardes, O. L.; Portilho, M. F.; Langone, M. A. P. Enzymatic biodiesel synthesis using a byproduct obtained from palm oil refining. Enzyme Res. 2011, 814507.

Table S1: Central Composite Design to optimize the significant variables for lipase production by *B. licheniformis* under solid state fermentation

Expt. No.	Temperature (^{o}C)	pH	Moisture (%)	Biosurfactant (mg)	Experimental lipase activity (IU/g)	Model predicted lipase activity (IU/g)
1	42.5	9	60	1.5125	35.25	36.76
2	50	10	50	1.025	40.28	33.41
3	42.5	9	60	0.5375	10.5	11.89
4	50	8	50	1.025	58.23	58.19
5	50	8	50	1.025	57.12	58.19
6	50	6	50	1.025	23	33.58
7	50	8	50	0.05	40.12	42.93
8	50	8	50	1.025	58.65	58.19
9	50	8	50	1.025	59.03	58.19
10	50	8	50	2	56.75	57.65
11	42.5	9	40	0.5375	20.13	23.28
12	50	8	50	1.025	57.72	58.19
13	42.5	7	40	0.5375	40.34	33.83
14	42.5	7	60	1.5125	48.37	45.17
15	57.5	9	40	0.5375	40.12	41.87
16	57.5	9	60	0.5375	45.19	42.96
17	42.5	7	40	1.5125	38.23	38.20
18	57.5	7	40	1.5125	26.3	23.47
19	57.5	7	60	0.5375	39.1	41.39
20	65	8	50	1.025	31.8	30.95
21	57.5	9	40	1.5125	37.38	40.51
22	50	8	50	1.025	58.4	58.19
23	57.5	9	60	1.5125	48.23	53.30
24	50	8	30	1.025	30.52	33.08
25	42.5	9	40	1.5125	40.18	36.45

26	57.5	7	40	0.5375	37.4	33.63
27	42.5	7	60	0.5375	34.5	29.10
28	50	8	70	1.025	40	41.15
29	57.5	7	60	1.5125	48.35	42.93
30	35	8	50	1.025	10.05	14.61

Table S2: ANOVA for Response Surface Quadratic Model [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob> F
Model	4955.389338	14	353.9563813	12.95391	< 0.0001
A	400.7385375	1	400.7385375	14.66602	0.001641
B	0.0459375	1	0.0459375	0.001681	0.9678
C	97.48570417	1	97.48570417	3.567732	0.07840
D	324.6497042	1	324.6497042	11.88136	0.003594
A2	2149.639931	1	2149.639931	78.67142	< 0.0001
B2	1045.551024	1	1045.551024	38.26454	< 0.0001
C2	761.499967	1	761.499967	27.86899	< 0.0001
D2	107.0224313	1	107.0224313	3.916752	0.06647
AB	352.9701563	1	352.9701563	12.91782	0.002657
AC	155.8128063	1	155.8128063	5.702357	0.03053
AD	211.0482563	1	211.0482563	7.723836	0.01404
BC	44.52225625	1	44.52225625	1.629403	0.2212
BD	77.39600625	1	77.39600625	2.832499	0.1131
CD	136.9485063	1	136.9485063	5.011971	0.04076
Residual	409.8642083	15	27.32428056		
Lack of Fit	407.535525	10	40.7535525	87.50342	< 0.0001
Pure Error	2.328683333	5	0.465736667		

Table 1: Experimental range and coded levels of independent variable for lipase production

Process variables	Codes	Ranges and Levels		
		-1 (low)	0 (medium)	1 (high)
Temperature (°C)	A	35	50	65
pH	B	7	8	9
Moisture (%)	C	30	50	70
Biosurfactant (mg)	D	0.5	1	1.5

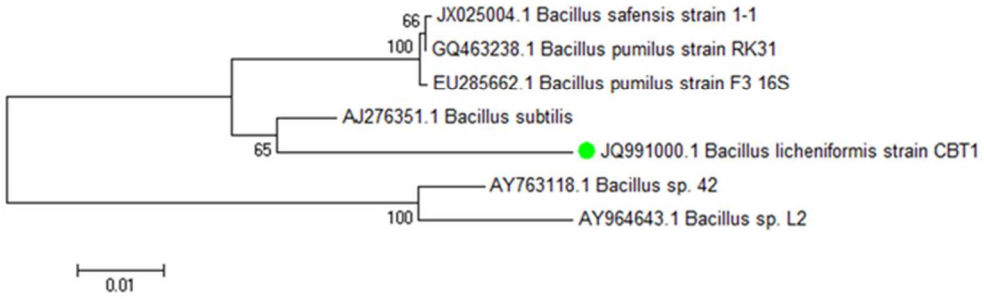

Figure 1: Phylogenetic tree was drawn by using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 7 nucleotide sequences of Bacillus sp. including CBT1 which are reported to be lipase producer.

58x18mm (300 x 300 DPI)

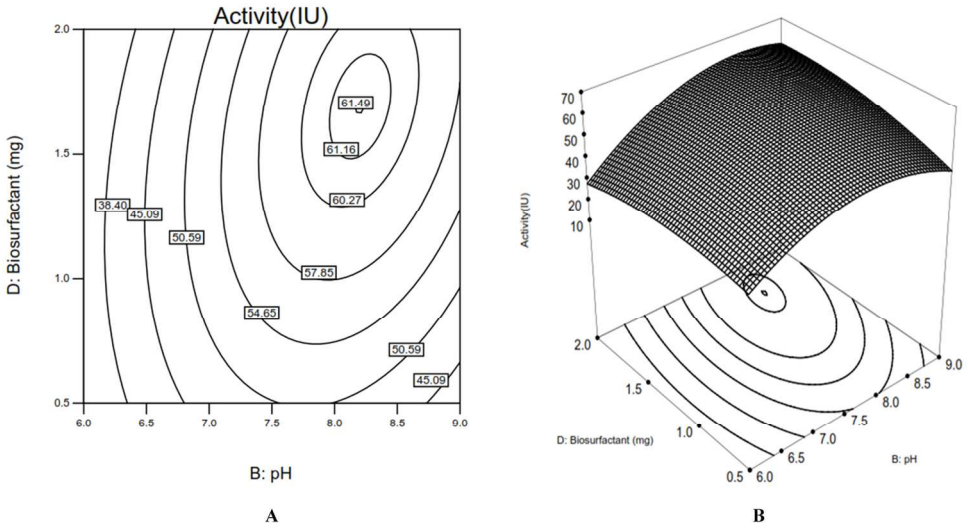

Figure 3: Interactive effect of bio-surfactant and pH on lipase activity as shown in (A) 2-dimensional (B) response surface graph.

161x85mm (300 x 300 DPI)

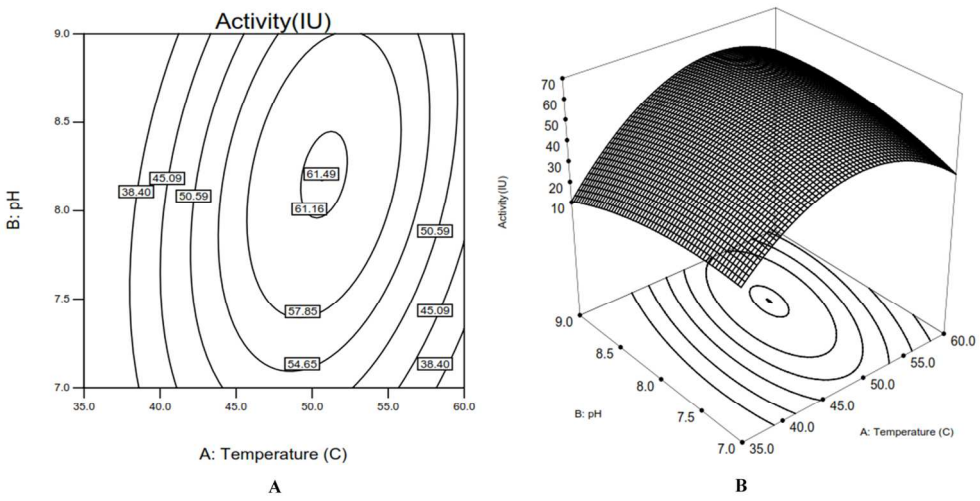

Figure 4: Interactive effect of pH and Temperature on lipase activity as shown in (A) 2-dimensional (B) response surface graph.

150x75mm (300 x 300 DPI)

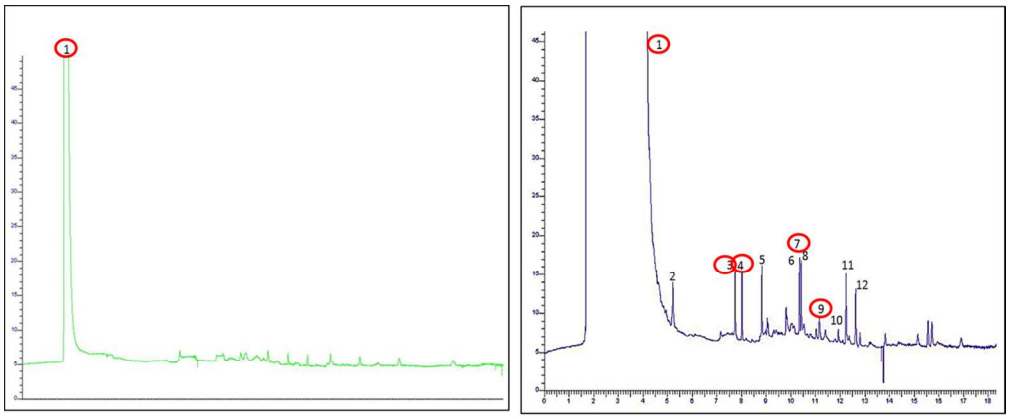

Figure 5: The GC chromatogram of waste kitchen oil derived biodiesel. A) Chromatogram after 30 min, 1: methanol; B) Chromatogram after 24 hours, 1: methanol; 3: methyl palmitate; 4: methyl stearate, 7: methyl oleate; 9: methyl linoleate.

120x53mm (300 x 300 DPI)