

Statistical Optimization of Parameters for the Enhancement of Lipase Activity

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Research

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Abstract

In this present study, lipase producing bacteria were isolated and screened from an indigenous soil sample and was used for lipase production with high enzyme activity. Different production media were screened and lipase production was found to be induced by olive oil, 14 mL/L, in the production medium. It was observed from Luedeking and Piret model that the lipase production was mixed growth associated with its maximum activity at 37 °C and pH 7. To understand the interaction of different parameters, statistical optimization using Response Surface Methodology was performed and the standardized conditions obtained were as follows: Peptone 10 g/L, yeast extract 7.5 g/L and olive oil 14 mL/L. The predicted data were validated and the model predicted was significant with maximum specific activity of 1.1 $\mu\text{mole}/\text{min}/\text{mg}$ proteins. The lipase specific activity was found to be enhanced by 10% and 23% after a single parameter and statistical optimization respectively.

1. Introduction

Enzymes are used as biological catalysts due to their high specificity and economic advantages without any adverse environmental impact. Lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) are important enzymes due to their various industrial applications (Salihu et al., 2012). Lipase produced from bacteria is called glycoproteins (Bajpai, 1999; Eugene, 1974; Nakamura and Nasu, 1990; Nouredini et al., 2005) and it hydrolyze triacylglycerol ester linkage that in turn used in industries for the processing of fats and oils, cosmetics, paper manufacturing degreasing of leather and pharmaceutical industries (Hasan-Beikdashti et al., 2012). Transesterification of vegetable oils into biodiesel (Chattopadhyay et al., 2011) is another significant application of lipase in the agriculture industry.

Lipase can be extracted from different sources like bacteria, fungi, plant or animal. Microbial lipase production is preferred due to its higher yield over plant or animal. Different substrates and different sources viz. agricultural soil, oil spilled soil, coconut industry soil, natural hot spring, salt pan, residual grease, mucus layer and gill of fish, rock lobster, wastewater, oil mill waste, marine fungus etc. were reported for isolation of lipase-producing bacteria (Habibollahi and Salehzadeh, 2018; Kumar et al., 2017; Golani et al., 2016; Alhamdani and Alkabbi, 2016; Papagora et al., 2013).

Optimization of various physico-chemical conditions for lipase production was classically carried out by single parameter optimization method. In this process, values for one parameter were changed keeping all other parameters constant. Though this method is simple, interaction or influence of one parameter to another cannot be analyzed (Liu et al., 2012). To overcome this problem, statistical optimization such as Response Surface Methodology (RSM) is widely used by researchers to check the interaction of parameters and optimization Sarve, 2015; Papagora et al., 2013; Su et al., 2011; Acikel et al., 2010).

The major challenge is to produce lipase with a high specific activity and single parameter optimization alone cannot able to increase activity significantly. Thus, the present study aims to optimize different

physicochemical parameters statistically to enhance lipase activity produced by bacteria isolated from the native soil sample.

2. Materials And Method

2.1. Materials

The Bradford protein estimation kit was purchased from Bangalore Genei Pvt. Ltd. (Bangalore, India). Tributyrin was obtained from Himedia (Mumbai, India). All other chemicals were procured from SRL (Mumbai, India) and were analytical grade.

2.2. Isolation and screening of lipolytic bacteria

Lipase producing microbial cultures was isolated from indigenous soil and screened on an LB agar plate supplemented with Rhodamine B (0.9% w/v) and olive oil (1% v/v). The colonies were visualized under UV-transilluminator at 310 nm and positive colonies with shiny orange halos were used for further studies.

2.3. Screening of different production medium

In this study, different media were used for the production of lipase. The media compositions per liter are: [A] LB media; [B] LB media, olive oil (1 mL); [C] Peptone (10 g), yeast extract (5 g), NaCl (1 g), Na₂HPO₄ (8.63 g), NaH₂PO₄ (6.08 g), MgSO₄·7H₂O (0.5 g), olive oil (10 mL) (Kulkarni et al., 2002); [D] Peptone (10 g), yeast extract (5 g), NaCl (1 g), Na₂HPO₄ (8.63 g), NaH₂PO₄ (6.08 g), MgSO₄·7H₂O (0.5 g) (Ramyasree and Dutta, 2013); [E] Peptone (10 g), yeast extract (5 g), NaCl (1 g), Na₂HPO₄ (8.63 g), NaH₂PO₄ (6.08 g), MgSO₄·7H₂O (0.5 g), olive oil (10 mL), FeSO₄ (0.6 g) (Hasan-Beikdashti et al., 2012). The media that provide lipase with maximum activity was selected and all subsequent studies were performed using that production medium.

2.4. Study of growth association using Luedeking and Piret Model

Specific growth rate and product formation rate was measured in selected production media and Luedeking-Piret Model was fitted to determine the model constants to predict the association between specific growth rate and product formation rate. The model equation is:

$$\frac{1}{x} \frac{dP}{dt} = \alpha x + \beta \quad (\text{Eq. 1})$$

Where, P = product concentration, x = cell concentration, μ = specific growth rate, t = time, α and β are coefficients.

2.5. Enzyme Assay

Lipase activity was measured by the method described in earlier reports (Chattopadhyay and Sen, 2012). In brief, One mL of tributyrin was added to 9 mL of 20 g/L polyvinyl alcohol (PVA) solution. The reaction mixture composed of 500 µl of the emulsion, 400 µl of phosphate buffer (0.1 M, pH 7.0) and 100 µl of the enzyme solution was incubated at 37 °C for 1 h in an incubator shaker at 120 rpm. The reaction was terminated by addition of 2 mL of chilled acetone – ethanol mixture (1:1) and the liberated fatty acids were titrated with 0.01 N NaOH using phenolphthalein as indicator. The activity was expressed in terms of micromoles of free fatty acids liberated by enzyme per min under assay condition. Total protein was estimated using Bradford reagent where BSA (Bovine Serum Albumin) was used as a standard. The specific activity was calculated by dividing enzyme activity with total protein and expressed in µmole/min/mg proteins unit.

2.6. Optimization of physicochemical parameters for lipase production

Different physicochemical parameters were standardized for lipase production by a single variable change method keeping all other conditions constant. Temperature was varied by 30, 35, 45 and 50 °C; pH by 6.5, 7.0, 7.5 and 8.5; peptone concentration was varied by 2, 6, 10, 14 and 18 g/L; and yeast extracts concentration by 1, 3, 5, 7, 9 and 11 g/L. Different oil source such as olive oil, rice bran oil, sunflower oil, mustard oil and coconut oil was used. To check the effect of oil concentration on lipase production, a varying concentration of 0, 5, 10, 15 and 20 mL/L oil was used.

2.7. Experimental design

Response Surface Methodology (RSM) was performed using Design Expert Software version 11.0; 2018, and a five-level Central Composite Design (CCD) with a quadratic model was used to optimize the most significant variables like (A) peptone concentration, (B) yeast extract concentration and (C) olive oil amount (% v/v). The response was considered as a specific activity of lipase. The experimental values for different levels are shown in Table 1. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The experimental data obtained were fitted into the model equation and analysed. The data obtained for specific activity after optimization of media was compared with the predicted value of Response Surface Methodology (RSM).

Table 1
Levels of variables used for CCD

Variables	Symbols	Levels				
		- α	-1	0	+1	+ α
Peptone (g/l)	A	6.63	9	10	11	13.3
Yeast extract (g/l)	B	3.3	6.25	7.5	8.75	11.7
Olive oil (% v/v)	C	7.27	12	14	16	20.7

3. Results And Discussions:

3.1. Isolation and screening of Bacteria

The lipase producing bacteria flourished under UV transilluminator in presence of Rhodamine B dye at 310 nm will be selected, subculture and identified as *Bacillus* sp. Lipase production in the medium leads to hydrolysis of olive oil resulting in the formation of free fatty acids. Free fatty acids form a fluorescent complex with Rhodamine B that is incorporated in the medium. Thus the lipase-producing colonies give a fluorescent halo that is visible under UV light (Dhiman and Chapadgaonkar, 2013)

3.2. Lipase activity in different production media

The lipase activity in different production media is presented in Fig. 1. From the results, it is clear that media C (Kulkarni et al., 2002) is found to be the best in terms of lipase activity and it was used in the further experiment for production of lipase and optimization studies.

3.3. Association of specific growth rate and product formation rate

Lipase production was started after 90 h of growth as shown in Fig. 2a, which indicate that the product formation is not fully associated with growth. The relation between specific growth rate and product formation rate was shown in Fig. 2b. From the model equation it can be observed that the product formation is mixed growth associated with the values of model constants are $\alpha = 3.9198$ and $\beta = 0.0106$.

3.4. Optimization of parameters

3.4.1. Effect of pH

It was observed from the results that the bacterium is capable of producing lipase from initial pH 6.5 to pH 8.5. The enzyme production varied considerably from 1000 to 3300 $\mu\text{mole}/\text{min}$. The bacteria showed optimum lipase production in between the pH 7.0 to 7.5 (3300 $\mu\text{mole}/\text{min}$) as shown in Fig. 3(I). When the pH is optimized the active site is fully ionized while in low or high in pH some charge is neutralized that is reflected by the decreasing of enzyme activity. Golani et al. (2016) also found that optimum lipase production at pH 7.0. However, it was noted that the lipase production was declined with an increase in pH from 7.0 to 10.0 but was able to produce lipase towards alkaline pH which shows its alkalitolerant nature. Chaturvedi and Khare (2016) optimized pH for lipase production and the optimum pH for lipase production was found to be 6.0.

3.4.2. Effect of temperature

Lipase activity was determined at a range of 30 to 50 °C which is showed in Fig. 3(II). The maximum activity was observed at 37 °C temperature (2500 $\mu\text{mole}/\text{min}$) at pH 7. With the increasing temperature, kinetic energy increases that in turn increase enzyme activity, whereas after optimum temperature, activity decreases due to denaturation of the protein. The Study of the effect for the optimization of

temperature on lipase production by Golani et al. (2016) also showed that the bacteria produce lipase in wide range of temperature from 22 to 42 °C. They found optimum temperature 37 °C for lipase enzyme production. According to Chaturvedi and Khare (2016) lipase activity was determined at a range of 30 to 50 °C and the maximum activity was observed at 37 °C temperature and pH 7.

3.4.3. Effect of peptone concentration

Among the five different concentration of peptone, 10 g/L peptone in media showed maximum lipase production (3300 µmole/min) and enzyme activity found decreased with the increase of peptone dose which is showed in Fig. 3(III). Similar results were obtained by other researchers where optimum peptone concentration was found to be 10 g/L (Paul et al., 2015).

3.4.4. Effect of yeast extract concentration

From Fig. 3(IV), it can be observed that enzyme activity enhanced with yeast extract concentration and finally remain same after 10 g/L. The minimum concentration with maximum activity was selected as optimum. Optimum yeast extract concentration of 3 g/L (Paul et al., 2015) and 2 g/L (Hasan-Beikdashti et al., 2012) were reported. It was observed that the lipase activity generally tends to decrease with an increase in yeast extract concentration. The difference might be due to the difference in the organism and their utilization of carbon sources.

3.4.5. Effect of different oils

From the result, the maximum lipase production was found in olive oil followed by rice bran oil, sunflower oil, mustard oil and coconut oil (Fig. 3(V)). The reason might be due to the influence of the higher amount of unsaturated fatty acids present in olive oil (86%) as compared to the other oils used. Paul et al. (2015) shows the effect of different oils as the lipid source in production media. Comparing Lipase production, determined by quantitative lipase activity assay, the production media using olive oil as lipid source shows maximum lipase production compared to sunflower oil and coconut oil.

3.4.6. Effect of concentration of olive oil

The Activity of lipase was found to be increased gradually from 0 to 15 mL/L and decrease with further increase in concentration (Fig. 3(VI)). This might be due to the fact that higher amount of olive oil hinders the microbial growth by restricting oxygen transfer and in turn reduces the lipase production. Paul et al. (2015) optimize the olive oil concentration and found to be approximately 10 mL/L as an optimum value.

3.5. Statistical optimization using RSM

The design layout with the response after the experiment is shown in Table 2. It is observed from the table that the specific activity was varied from 0.16 to 1.05 µmole/min/mg protein. After ANOVA of the quadratic model, the p value (< 0.05) was found to be significant with adjusted R² (0.9701) and predicted R² (0.8796) are in good agreement with each other (Table 3). Multiple regression analysis of experimental data gave the following equation where 'Y' represents enzyme specific activity:

Table 2
RSM design layout with the response after the experiment

Std	Run	Factor 1 A: peptone (g/L)	Factor 2 B: YA (g/L)	Factor 3 C: olive oil (mL)	Response 1 Activity (μ M/min/mg)
1	1	8	5	10	0.31
2	2	12	5	10	0.9
3	3	8	10	10	0.16
4	4	12	10	10	0.75
5	5	8	5	18	0.19
6	6	12	5	18	0.39
7	7	8	10	18	0.13
8	8	12	10	18	0.34
9	9	6.63641	7.5	14	0.47
10	10	13.3636	7.5	14	0.27
11	11	10	3.29552	14	0.9
12	12	10	11.7045	14	0.51
13	13	10	7.5	7.27283	0.5
14	14	10	7.5	20.7272	0.8
15	15	10	7.5	14	1.05
16	16	10	7.5	14	1.05
17	17	10	7.5	14	1.05
18	18	10	7.5	14	1.05
19	19	10	7.5	14	1.05
20	20	10	7.5	14	1.05

Table 3

(a) ANOVA for quadratic model; (b) Fit summary of model

a. Response 1: activity						
Source	Sum of squares	df	Mean Square	F- value	p-value	
Model	0.9224	9	0.1025	69.54	< 0.0001	Significant
A-peptone	0.0101	1	0.0101	6.87	0.0256	
B-YA	0.0233	1	0.0233	15.78	0.0026	
C-olive oil	0.0167	1	0.0167	11.36	0.0071	
AB	0.0171	1	0.0171	11.61	0.0067	
AC	0.2211	1	0.2211	150.02	< 0.0001	
BC	0.0066	1	0.0066	4.49	0.0602	
A ²	0.4130	1	0.4130	280.25	< 0.0001	
B ²	0.1948	1	0.1948	132.17	< 0.0001	
C ²	0.1302	1	0.1302	88.34	< 0.0001	
Residual	0.0147	10	0.0015			
Lack of Fit	0.0147	5	0.0029			
Pure Error	0.0000	5	0.0000			
Cor Total	0.9371	19				

b. Std. Dev.	0.0384	R²	0.9843
Mean	0.7895	Adjusted R²	0.9701
C.V. %	4.86	Predicted R²	0.8796
		Adeq Precision	23.6802

$$Y = 1.05 + 0.09 A - 0.08 B - 0.04 C + 0.0012 AB - 0.09 AC + 0.023 BC - 0.27 A^2 - 0.15 B^2 - 0.17 C^2$$

(Eq. 2)

The model was further processed to understand the interactions among variables followed by optimization to get the maximum specific activity. Effect of peptone and yeast extract, the values were converging and the contour and 3D plot are shown in Fig. 4a. Similarly, in Fig. 4b and Fig. 4c, the effect of peptone and olive oil and yeast extract and olive oil are shown respectively. From Table 4, the optimized values were found to be 10.0 g/L peptone, yeast 7.5 g/L extract and 14 mL/L olive oil and the model predicted maximum specific activity at these optimized conditions as 1.05 μ mole/min/mg protein. After

the experimental run with optimized value, specific activity was obtained as 1.1 $\mu\text{mole}/\text{min}/\text{mg}$ proteins, which is very close to the predicted value, indicating the validity of RSM model.

Table 4: Final concentrations after optimization

Factors	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	Peptone	10.00	8.00	12.00	0.0000	Actual
B	YA	7.50	5.00	10.00	0.0000	Actual
C	Olive oil	14.00	10.00	18.00	0.0000	Actual

3.6. Comparison of specific activity after optimization

From Fig. 5, it is clear that there is a 10% increase in lipase specific activity after single parameter optimization and a 23% increase in lipase specific activity after statistical optimization using RSM.

4. Conclusion

From the results obtained in this present study, it can be concluded that lipase was successfully produced from the indigenously isolated bacteria. The incubation temperature, pH, peptone concentration and yeast extract concentration were optimized. It was observed that lipase production was enhanced in presence of unsaturated oil such as olive oil. After optimization, a 10% increase in lipase specific activity was observed by using single parameter optimization whereas 23% activity was increased by a statistical optimization method. With the optimum conditions, large scale production of lipase can be possible using the isolated bacteria and the product after purification can be used for various hydrolysis and methanolysis reactions and other industrial applications.

Abbreviations

ANOVA

Analysis of variance

BSA

Bovine Serum Albumin

CCD

Central Composite Design

PVA

Polyvinyl alcohol

RSM

Response Surface Methodology

Declarations

- **Ethics approval and consent to participate:** the article is original, has not already been published in a journal, and is not currently under consideration by another journal
- **Consent for publication:** both the authors have consent for publication.
- **Availability of data and materials:** Not applicable
- **Competing interests:** Authors have no conflict of interest
- **Funding:** Not Applicable
- **Authors' contributions:** Prama was involve in experimental work and manuscript writing, Soham was involve in design of experiment, software analysis and manuscript preparation.
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- **Authors' information:** Dr. Soham Chattopadhyay, assistant professor in department of Bioengineering, BIT Mesra, Ranchi. He did his B. Tech and M. Tech in Biotechnology and did his PhD from IIT Kharagpur in Biochemical Engineering and Bioprocess Technology.

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Figures

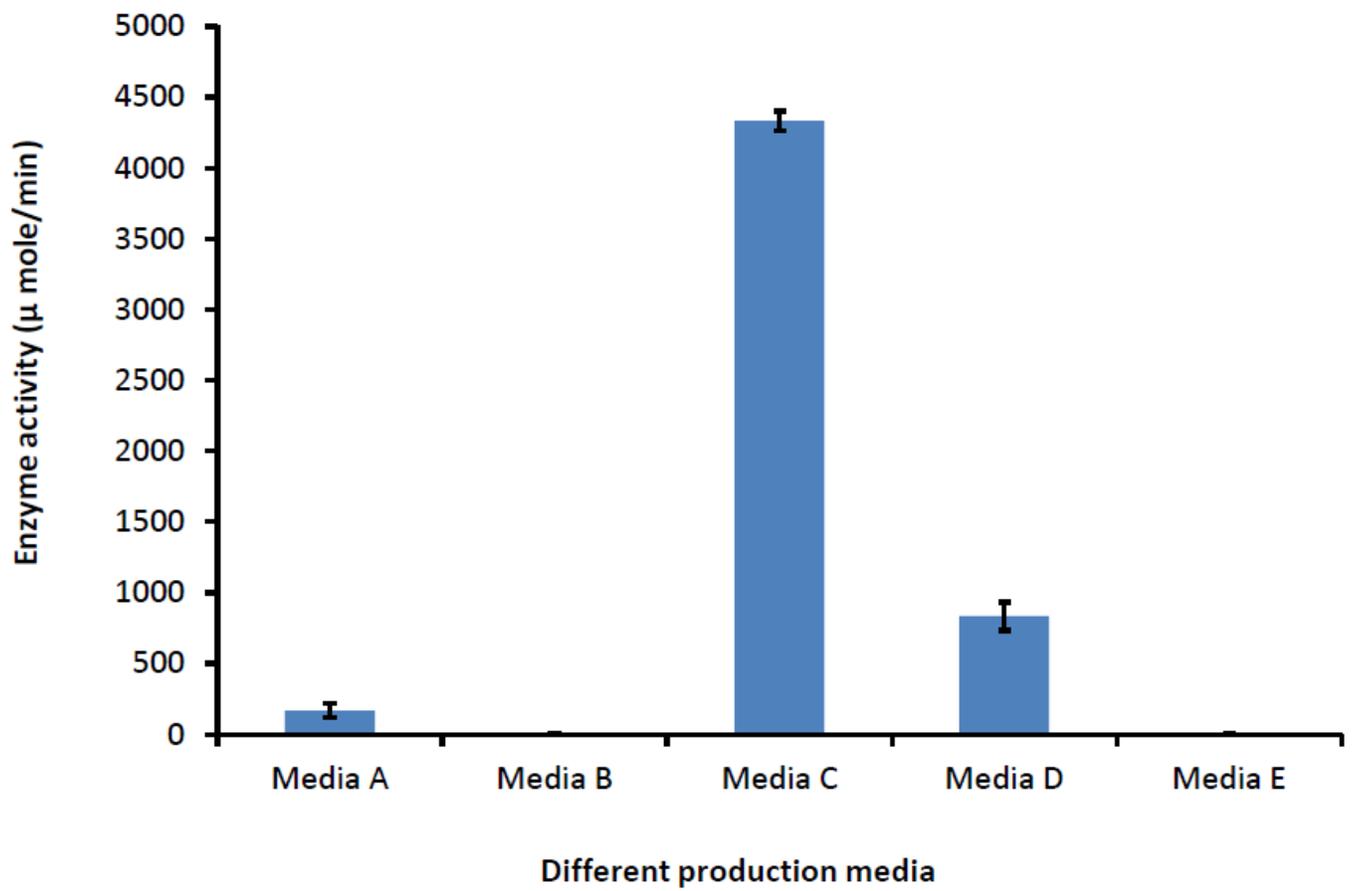


Figure 1

Screening of different media for the production of lipase

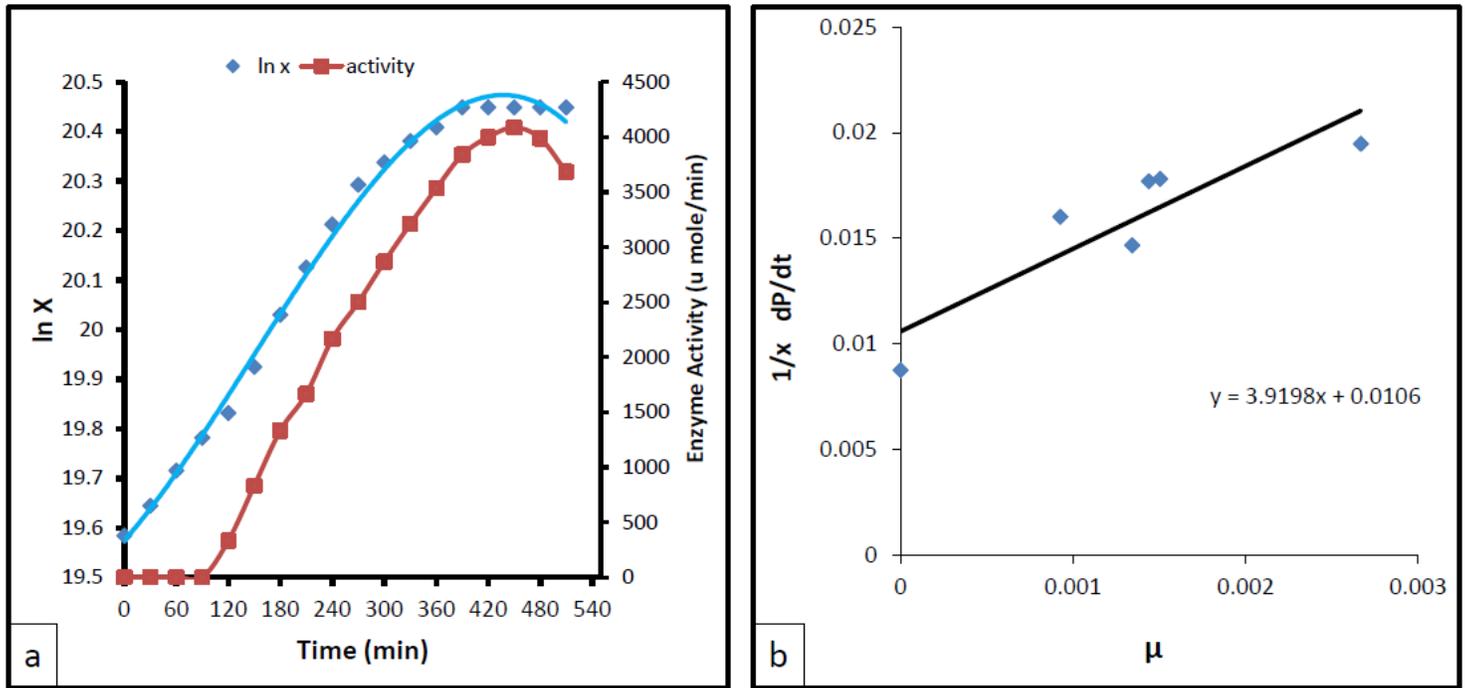


Figure 2

(a) Time profile of bacterial growth and product formation (b) Relation between specific growth rate and product formation rate

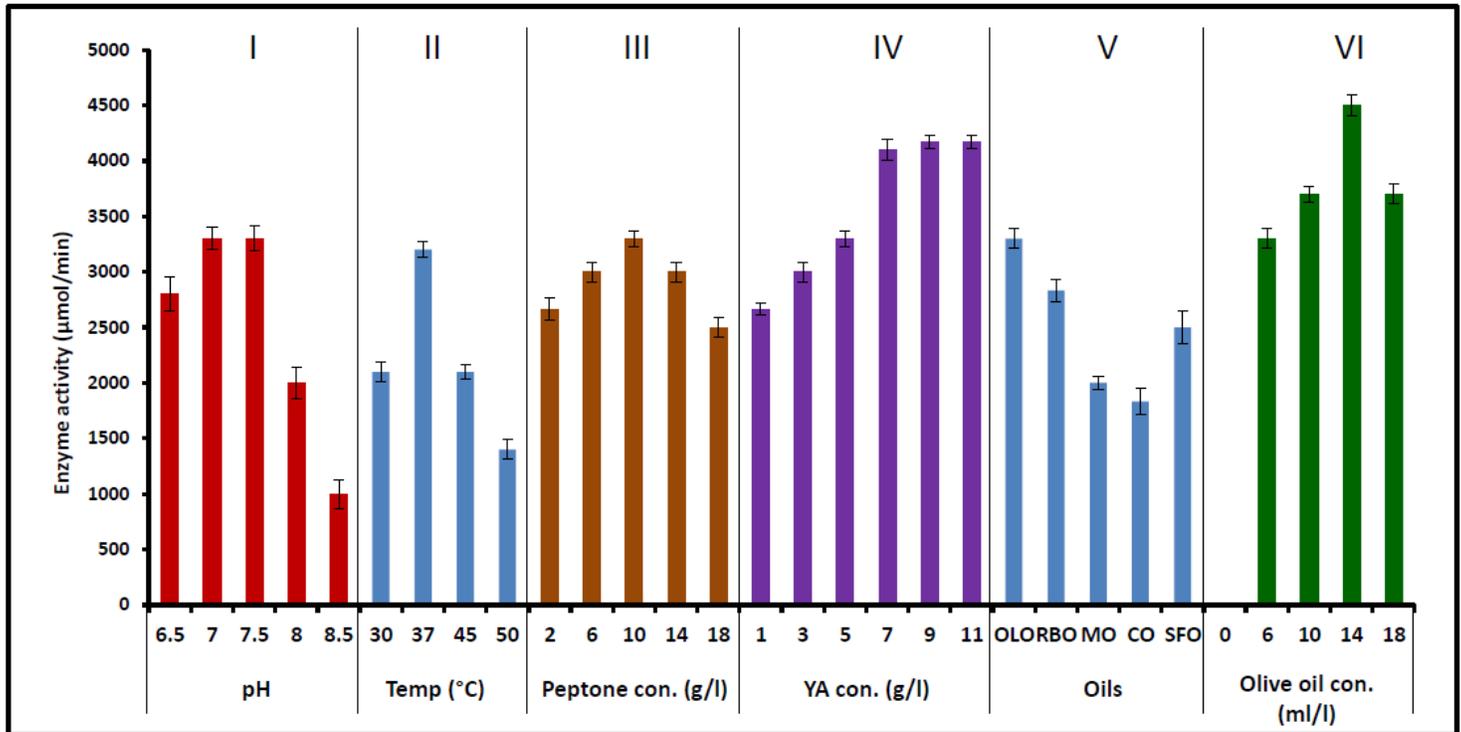


Figure 3

Effect of various parameters on lipase activity. Conditions are as follows: (I) Yeast extract (YA) concentration 5 g/L, temperature 37 °C, Peptone 10 g/L, olive oil 10 mL/L; (II) Yeast extract (YA) concentration 5 g/L, pH 7, Peptone 10 g/L, olive oil 10 mL/L; (III) Yeast extract (YA) concentration 5 g/L, temperature 37 °C, pH 7, olive oil 10 mL/L; (IV) pH 7, temperature 37 °C, Peptone 10 g/L; olive oil 10 mL/L; (V) Yeast extract (YA) concentration 5 g/L, temperature 37 °C, pH 7, Peptone 10 g/L, different oils 10 mL/L, OLO: olive oil, RBO: Rice bran oil, MO: Mustard oil, CO: Coconut oil, SFO: Sunflower oil, (VI) Yeast extract (YA) concentration 5 g/L, temperature 37 °C, pH 7, Peptone 10 g/L

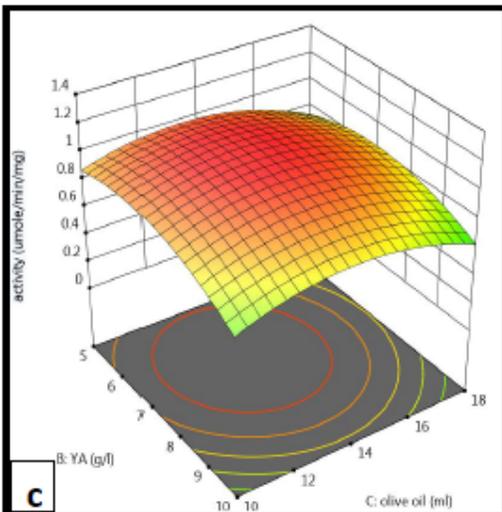
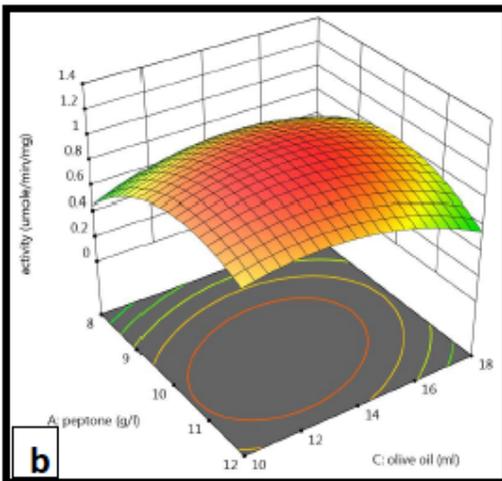
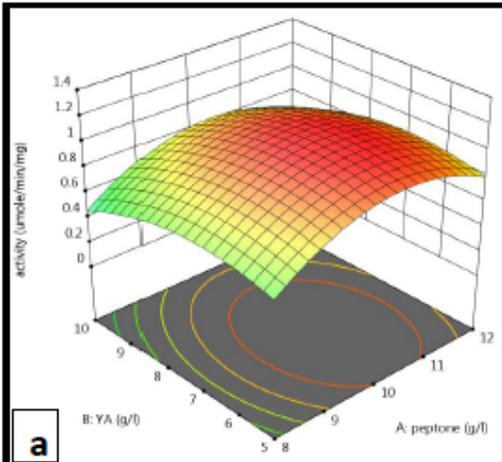


Figure 4

3D surface plot on (a) effect of yeast extract (YA) and peptone; (b) effect of peptone and olive oil; and (c) effect of YA and olive oil, on lipase specific activity.

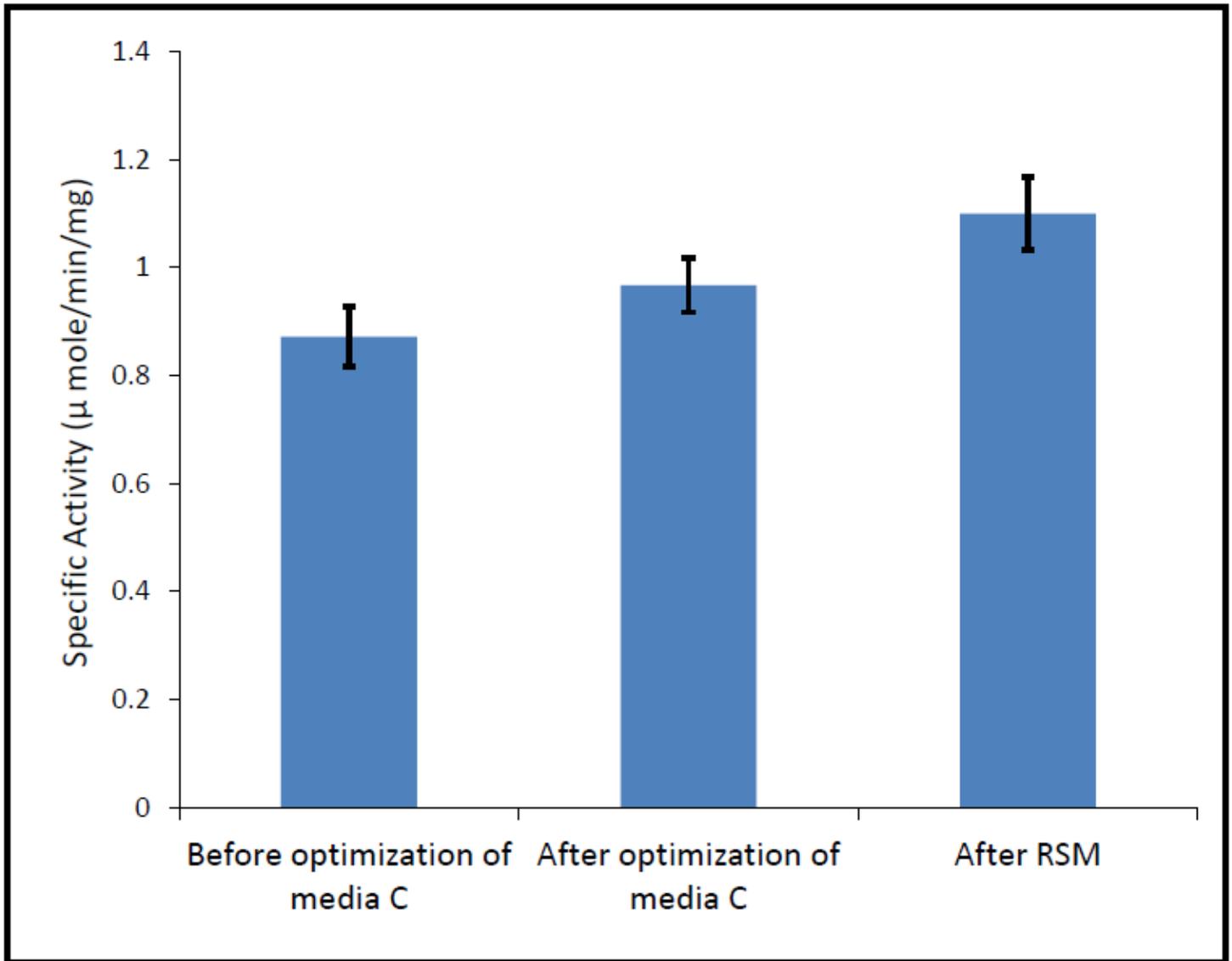


Figure 5

Lipase specific activity before optimization, after single parameter optimization and after statistical optimization using RSM