# APPLICATION OF RESPONSE SURFACE METHODOLOGY FOR STATISTICAL OPTIMIZATION OF LIPASE PRODUCTION BY *Penicillium* sp. EMPLOYING SOLID STATE FERMENTATION

# APLICACIÓN DE LA METODOLOGÍA DE SUPERFICIE DE RESPUESTA PARA LA OPTIMIZACIÓN ESTADÍSTICA DE LA PRODUCCIÓN DE LIPASA POR *Penicillium* sp. EMPLEANDO FERMENTACIÓN DE ESTADO SÓLIDO

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## Abstract

This study was conducted to determine the economical and optimal in vitro growth requirements of *Penicillium* sp. for augmented production of lipases. The optimization of eco-cultural parameters was carried out for solid state fermentation. Preliminary batch testing was performed to find out the favourable conditions for enhanced lipase productivity. The results showed maximal lipase productivity when 10g of the sunflower hulls was utilized as a basal substrate for 3 ml inoculum of *Penicillium* sp. For obtaining the highest enzyme yields the moisture content of the medium was adjusted at 40% with phosphate buffer (pH 6.0), which was in favour of the fermentation performed at 30 ºC for 72 h. The best quantified production of extracellular lipases was recorded with Tween-80 as the extraction medium. As per Response surface methodology (RSM) model, Box-Behnken experimental design was employed in order to analyse the interactive effects of critical medium components on lipase yield obtained from *Penicillium* sp. The optimum concentration of olive oil as additional oil (5%), Tween-80 (3%) and peptone (1%) resulted in maximal enzyme productivity (100.45 U/mg), approximately three times greater than that observed in basal medium. The analysis of variance showed that the devised model was significant ($p < 0.05$).

*Keywords*: lipases, *Penicillium* sp, solid state fermentation, Box-Behnken design.

## Resumen

Este estudio se realizó para determinar los requisitos de crecimiento in vitro económicos y óptimos de *Penicillium* sp. para la producción aumentada de lipasas. La optimización de los parámetros ecoculturales se llevó a cabo para la fermentación en estado sólido. Se realizaron pruebas preliminares de lotes para conocer las condiciones favorables para una mayor productividad de lipasa. Los resultados mostraron una productividad máxima de lipasa cuando se utilizaron 10 g de cáscaras de girasol como sustrato basal para 3 ml de inóculo de *Penicillium* sp. Para obtener los mayores rendimientos de enzimas, el contenido de humedad del medio se ajustó al 40% con tampón de fosfato (pH 6,0), lo que favoreció la fermentación a 30 ºC durante 72 h. La mejor producción cuantificada de lipasas extracelulares se registró con Tween-80 como medio de extracción. Según el modelo de metodología de superficie de respuesta (RSM), se empleó el diseño experimental de Box-Behnken para analizar los efectos interactivos de los componentes críticos del medio sobre el rendimiento de lipasa obtenido de *Penicillium* sp. La concentración óptima de aceite de oliva como aceite adicional (5%), Tween-80 (3%) y peptona (1%) dio como resultado una productividad enzimática máxima (100,45 U / mg), aproximadamente tres veces mayor que la observada en el medio basal. El análisis de varianza mostró que el modelo diseñado fue significativo ($p < 0.05$).

*Palabras clave*: lipasas, *Penicillium* sp, fermentación en estado sólido, Diseño Box-Behnken.

# 1 Introduction

Lipases (triacyl-glycerol-acyl hydrolases, EC 3.1.1.3), catalysing the lipid based compounds at the lipid-water interface, are widely present in nature (Treichel *et al.*, 2010). Yet, lipases isolated from the microbes are the most resourceful enzymes that control multiple bioconversion reactions such as alcoholysis, aminolysis, hydrolysis, esterification and interesterification (Hasan *et al.*, 2006). Microbial lipases can withstand extreme temperature and pH conditions, and are able to work in organic solvents where they display chemo-, region-, and enantioselectivity (Houde *et al.*, 2004). Bacterial genera such as *Bacillus*, *Pseudomonas* and *Burkholderia* have been reported to produce lipases with such abilities. Similarly, fungal genera are also well known to secrete lipases such as *Aspergillus*, *Penicillium* and *Rhizopus*. Lipases may also be isolated from various yeast species of *Zygosaccharomyces*, *Pichia*, *Lachancea*, *Kluyveromyces*, *Saccharomyces*, *Candida*, and *Torulaspora* (Barros *et al.*, 2010, Gupta *et al.*, 2004, Singh and Mukhopadhyay, 2012). It is an omnipresent enzyme with tremendous physiological importance. Lipases have received significant attention lately due to their widespread use in food (modification of aromas), cosmetics (skin and suntan creams constituent), medical (production of various intermediates of drugs), agrochemicals (herbicides such phenoxypropionate), fuel industries (biodiesel production) and others (Hasan *et al.*, 2010, Houde *et al.*, 2004, Ribeiro *et al.*, 2011).

Microbial lipases, produced mainly by submerged fermentation, may also be obtained through solid-state fermentation, which in general is a much cheaper and well adapted process compared to the earlier one (Silva *et al.*, 2017). It gives a wide spectrum of bio products and comparatively a high yield recovery of enzymes with industrial value (Gutarra *et al.*, 2005). The composition of the media plays a vital role during production of lipases through fermentation as the production relies on the optimization of various parameters such as pH, substrate concentration, inoculum level and inducer concentration (Lo *et al.*, 2012). A common way to enhance the lipase production is to change one factor at a time, whereas holding others constant in a shake flask system. However, this technique does not portray the combined effects of all the factors involved and hence it fails to pinpoint the optimal parameters for the reaction. Nevertheless experimental statistical approaches such as response surface methodology (RSM) is an effective substitute to address this concern of biological system based applications (Facchini *et al.*, 2016, Lo *et al.*, 2012, Papagora *et al.*, 2013, Ruchi *et al.*, 2008). RSM can predict the relationships amid the factors and responses along with the ideal level of selected media components (Awad *et al.*, 2015). Optimization of the lipase production by RSM has been described in different cultures of *Candida* sp., *Rhizopus* sp., *Pseudomonas aeruginosa* and *Bacillus pumillus*. *Penicillium* sp. used in this investigation was isolated from the oil contaminated soil sample. Biosynthesis of alkaline lipase from *Penicillium* spp has been well documented. Preliminary studies have indicated that medium components greatly influence the lipase productivity of *Penicillium* sp. (Kempka *et al.*, 2008). In this investigation, after outlining the appropriate physical and chemical parameters of solid state fermentation, a box-behnken design of response surface approach was employed to optimize the interactive effects of olive oil, Tween-80 and peptone for maximum lipases yield.

# 2 Materials and methods

## 2.1 Microorganism and medium

*Penicillium* sp. used in this study was isolated from the soil sampled from oil contaminated sites. The isolate was identified based upon macroscopic and microscopic morphological features. The qualitative screening for lipase activity was performed using plate assay described by Gupta *et al.* (Gupta *et al.*, 2003). In this regard, Rhodamine B and olive oil were added into the potato dextrose agar (PDA) medium. Production of lipases lead to lipolysis of olive oil. This result in formation of free fatty acids forming a fluorescent complex with Rhodamine B. The lipase-producing colonies thus give a fluorescent halo when observed under UV light. The selected isolate was then grown on PDA medium, incubated at 30 °C for 72 h, maintained at 4 °C and sub cultured after every 15 days.

Table 1. Levels of the parameters analysed for optimal production of extracellular lipases from *Penicillium* sp.

| Sr. No | Parameter | Varied Conditions |
|--------|-----------|-------------------|
| 1 | Basal Substrates | Canola Oilseed Cake, Wheat Bran, Rice Bran, Peanut Shells and Sunflower Hulls |
| 2 | Amount of Basal substrate | 5, 10, 15, 20 and 25 g |
| 3 | pH | 5.0, 6.0, 7.0, 8.0 and 9.0 |
| 4 | Moisture content | 20, 30, 40, 50 and 60 % |
| 5 | Incubation temperature | 30, 40, 50, 60 and 70 ºC |
| 6 | Incubation period | 24, 48, 72, 96 and 120 h |
| 7 | Inoculum size | 1, 2, 3, 4 and 5 ml |
| 8 | Extractants | Distilled H$_2$O, phosphate buffer at pH 6.0, 7.0, 8.0 |

## 2.2 Solid-state fermentation and enzyme extraction

Solid state fermentation was performed for the determination of lipase productivity. Different agro-industrial byproducts were collected from the local markets of Lahore, Pakistan and were processed accordingly. 5 g of each substrate was moistened with phosphate buffer (30%) at pH 6.0, sterilized, and inoculated with 1 ml of spore suspension in favour of fermentation. The fermentation flasks were then incubated at 30 ºC for 72 h for lipase production. For enzyme extraction, 100 ml of phosphate buffer (pH 7.0) was dispensed in to each culture flask which were then placed in a shaking incubator at 180 rpm for 60 min under ambient conditions (Iftikhar *et al.*, 2010). The crude extract obtained after filtration was assayed for lipase activity.

## 2.3 Assay for lipase activity

Lipase activity was determined through olive oil preparation formulated as follows: the reaction mixture containing 10 mL of 10% olive oil, 10% gum acacia, 2 ml of 0.6% CaCl$_2$, 5 ml of phosphate buffer (pH 7.0) and 1 ml of enzyme extract (effluent from fermented medium) was kept at 37 °C for 1 h with orbital shaking. The system was then disrupted by acetone-ethanol mixture (1:1 v/v) and the released fatty acids were titrated against 0.1 N NaOH using phenolphthalein as indicator (Balaji and Ebenezer, 2008). The lipase activity was calculated using Eq. (1):

$$lipase\ activity = \frac{\Delta V \times N}{V_{(sample)}} \times \frac{1000}{60} \qquad (1)$$

where $\Delta V$ is the difference between $V2$ and $V1$. $V1$ is the volume of NaOH used against control flask and $V2$ is the volume of NaOH used against experimental flask. $N$ refers to the normality of NaOH and $V_{(sample)}$ is the volume of enzyme extract. One unit of lipase activity was defined as the amount of enzyme which liberated 1 $\mu$mol of fatty acids per minute/ml under specified assay conditions.

## 2.4 Total extracellular protein quantification

Estimation of total protein concentration was carried out according to Bradford (Bradford, 1976).

## 2.5 Optimization of culture conditions for enhanced lipase production

The enzyme production was optimized through small scale testing by studying the effects of various parameters such as amount of substrate (g), pH, moisture content (%), temperature (ºC), the incubation period (h), size of inoculum (ml) and type of extractant (Table 1). Only one variable was varied at a time while others were kept constant during each analysis. The experimental design included three replicates.

## 2.6 Response surface methodology

Box-Behnken design was applied, generating a set of 17 experimental runs using Design Expert software (Version 10.0). The combined effect of independent variables viz., olive oil as an enhancer, Tween-80 as an inducer and peptone as nitrogen source (at three different levels, low (−1), medium (0) and high (+1) was observed in the specific lipase activity, the dependent variable ($\gamma$).
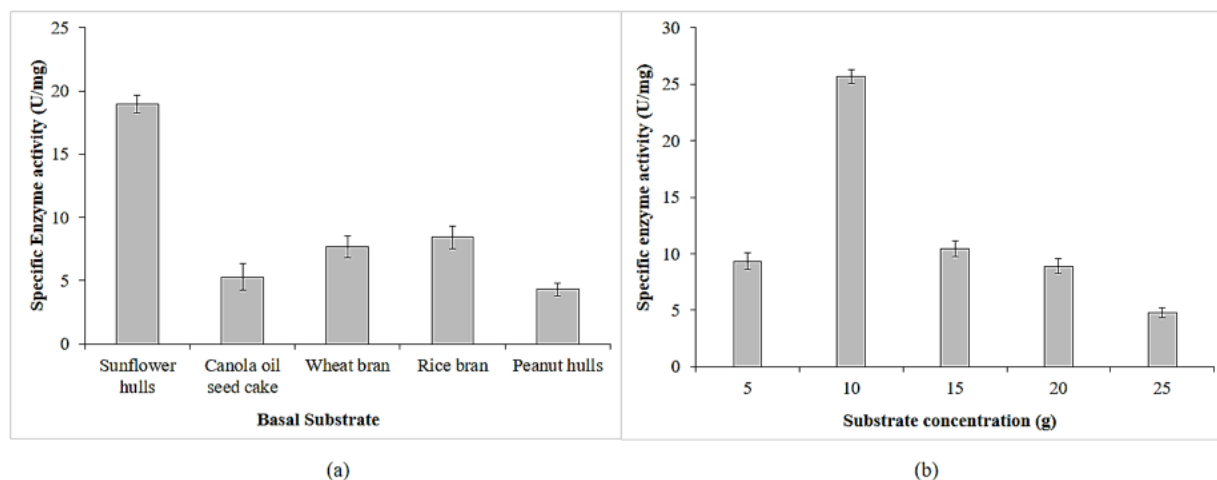
Fig. 1. Effect of basal substrate on extracellular lipase production by *Penicillium* sp. (a) Specific enzyme activity as affected by different substrate types (b) Specific enzyme activity as affected by various substrate levels.

A quadratic polynomial regression model (Eq. 2) was contrived to explain the relationship between dependent and independent variables:

$$\gamma = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \tag{2}$$

Where "$\gamma$" is the predicted response (lipase activity), $\beta_0$ is the intercept, $\beta_1, \beta_2, \beta_3$ are the linear coefficients, $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ are the squared coefficients, $\beta_{12}$, $\beta_{13}$, $\beta_{13}$ are the interaction coefficients. The model was statistically analysed and Analysis of Variance (ANOVA) was performed to validate the goodness of fit for the model.

# 3 Results and discussion

## 3.1 Optimization of physico-chemical factors for enhanced lipase productivity

This study aimed at the identification of the optimal level of input variables that positively influence the experimental response and result in high enzyme yield. In this regard, the effect of different basal substrate of various concentrations, moisture content, pH, incubation temperature, incubation period, inoculum size and extractant was analysed. Each bar signifies the mean of three values and error bars represent the standard deviation in the bar graphs.

## 3.2 Effect of basal substrate on enzyme productivity

Solid state fermentation (SSF) is currently attracting great attention owing to the prospect of consuming inexpensive agro-industrial wastes as substrates. Various types of byproducts were utilized as basal substrates for the extracellular lipase production by *Penicillium* sp. Fig. 1a graphically depicts the effect of different substrates on the specific lipase activity. Maximum lipase productivity (18.94 ± 0.71 U/mg) was observed when sunflower meal was used as basal substrate. Minimum extracellular lipase productivity was, however recorded in the case of peanut shells (4.3 ± 0.49 U/mg).

Substrate concentration also plays a vital role in SSF. The amount of substrate per unit area of working volume of the culture vessels affects the porosity and aeration of the substrate. Various concentrations of sunflower meal were tested and 10 g was found out to be the most appropriate, accounting for highest enzyme yield (25.67 ± 0.65 U/mg) in the range studied (Fig. 1b).

This may be contributed to the better permeation of the microbial mass in low substrate levels. Low Lipase productivity has been observed at increased substrate concentrations due to difficulty in penetration of the substrate by the organism (Singh *et al*., 2010).
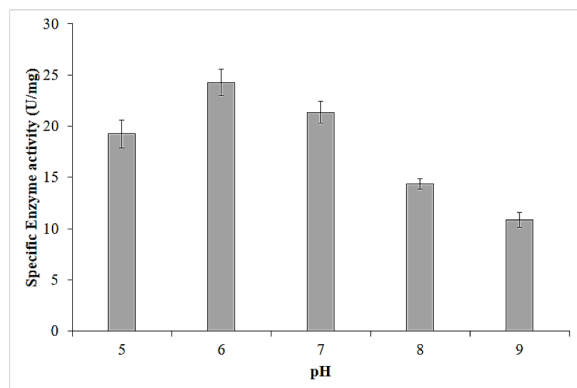
Fig. 2. Effect of pH on lipase production by *Penicillium* sp.



Fig. 3. Effect of moisture content on lipase production by *Penicillium* sp.

### 3.3 Effect of pH on enzyme productivity

Lipase production at different pH conditions was analysed and the results obtained revealed that varying this physical parameter significantly affected the enzyme activity (Fig. 2). In the pH range of 5.0 to 9.0, enzyme production was maximum at 6.0 with specific activity of $24.27 \pm 1.29$ U/mg. However, as the pH escalated above 6.0, the lipase production declined gradually revealing that the lipase production by *Penicillium* sp. favours acidic environment. Filamentous fungi have the ability to withstand a wide range of pH under solid substrate fermentation owing to the high buffering capacity of solid substrate (Amin *et al.*, 2008, Shankar and Mulimani, 2007, Sun and Xu, 2008). In the present investigation, *Penicillium* sp. exhibited this characteristic by growing well under acidic pH and producing enzyme even under alkaline conditions.

### 3.4 Effect of moisture content on enzyme productivity

To examine the effect of moisture content on the ability of *Penicillium* sp. to secrete lipases, different amounts of phosphate buffer (20-60%) at pH 6 were added into the production medium. Fig. 3 depicts the maximum lipase production at 40% moisture content while indecent activity was witnessed at moisture levels higher and lower than this value. In solid state fermentation, the microbial growth is supported on moist solid substrates without any free flowing water. Suitable moisture content regulated by an appropriate diluent is therefore, a key parameter since it contributes to the success of the fermentation process.
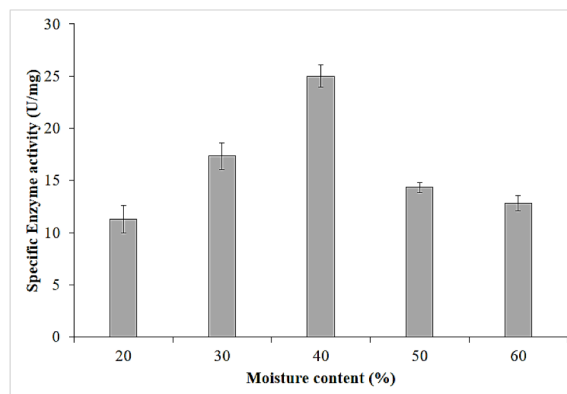
The moisture requirements of microorganisms may vary from one another and depends upon the metabolic activity, heat evolution and environmental factors as well as the type of substrate. The results of the present investigation revealed that changes in the moisture content have considerable impact on the production of lipases. Phosphate buffer being the optimum diluent was able to maintain the stickiness, reduced the porosity, and upheld the oxygen transfer in the substrate at optimum moisture content of 40%, while the other amounts might have cut down the nutrient solubility in the substrate (Contesini *et al.*, 2010).

### 3.5 Effect of temperature on enzyme productivity

The analysis of enzyme productivity post incubation at different temperatures (30-70 °C) revealed the optima at 30 °C and the production dropped on both sides of this value (Fig. 4). High temperature favours the production of the considerable amount of metabolic heat due to which the fermenting substrate temperature escalates and microbial growth and enzyme formation is inhibited (Bhatti *et al.*, 2007). An increase in the temperature may intensify the number of effective substrate and enzyme collisions to form the complex, but as the temperature rises above the optimum value enzyme denaturation takes place. Temperature also affects secretion of extracellular enzymes by altering the physical attributes of the cell membrane (Amin and Bhatti, 2014). It may be associated with the amplified production of proteases at increased temperatures which results in lipase deactivation (Palma *et al.*, 2000).
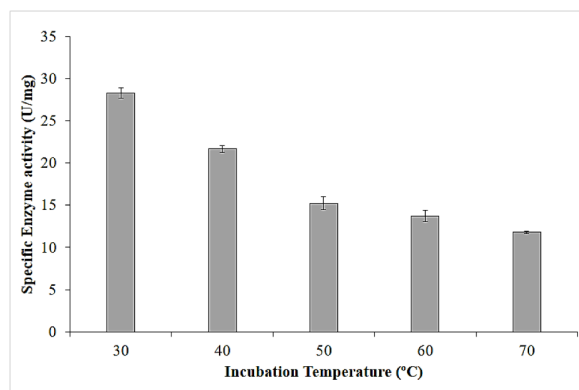
Fig. 4. Effect of incubation temperature on lipase production by *Penicillium* sp.



Fig. 5. Effect of incubation period on lipase production by *Penicillium* sp.

It was reported on account of Rani and Panneerselvam (2009) who observed that maximum lipase production (19.2 U/ml) from cultures of *Aspergillus terreus* at 30 ºC. *Trichoderma reesei* have shown to produce the maximum lipases production (3.5 U/ml) at 30 ºC (Rajesh *et al.*, 2010). Similar findings have been observed by other groups of researchers who documented highest lipase activity at 30 ºC from *Fusarium oxysporum* (17.0 U/ml), *Aspergillus heteromorphus* (23.0 U/ml) and *Gliomastix indicus* (10.8U/ml) (Kapoor *et al.*, 2012, Rifaat *et al.*, 2010, Sneha *et al.*, 2011). In the present study, the lipase enzyme also suggested a tendency towards thermotolerance since substantial lipase productivity was observed even at 40 ºC. Thermophilic lipases hold great prospects for detergent and food industries (Jaeger and Reetz, 1998). The present isolate may therefore be exploited in this respect.

## 3.6 Effect of incubation period on enzyme productivity

In order to determine the effect of incubation period, extracellular lipase production was examined at 24 hours intervals up to 120 hours. The analysis of time course profiles showed the maximum enzyme yield (27.08 ± 1.08 U/mg) after 72 h of incubation (Fig. 5). These findings indicated that the enzyme production was associated with the growth phase as well as availability of nutrients in the media. The subsequent accumulation of byproducts and enzyme proteolysis might also be contributing factors. Similar results have been reported by Kapoor *et al.* (2012) and Roy *et al.* (2004) who studied the lipase production from *Gliomastix indicus* and *Corynebacterium* spp respectively, and obtained the maximum yield after 72 h of culturing.
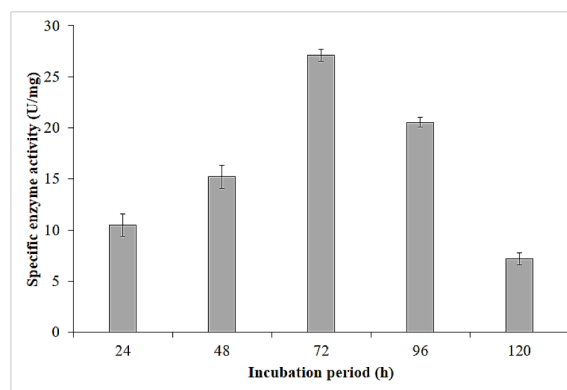
Hee-Yeen *et al.* (2007) also observed the effect of incubation period on lipase activity from *Penicillium chrysogenum* and recorded the maximum enzyme yield on the 5th day (120 h) of incubation. Lima *et al.* (2003) have reported the best yield (13 U/ml) of lipase from *Penicillium aurantiogriseum* after 72 hours.

## 3.7 Effect of inoculum size on enzyme productivity

The effect of inoculum size on the ability of fungus to produce the enzyme was also investigated (Fig. 6). The results revealed that the enzyme production ranged from 27.26 ± 1.57 U/mg to 12.37 ± 1.09 U/mg. The highest enzyme units (27.26 ± 1.57 U/mg) were obtained with 3 ml inoculum size while the lowest (12.37 ± 1.09 U/mg) were credited to an inoculum size of 5 ml. Higher inoculum levels may increase spore concentration, but also raise the water content of the solid substrate. As a result, fungal growth and enzyme induction may be negatively affected in solid state fermentation. On the contrary, lower inoculum levels need longer time for fermenting the substrates in solid state fermentation still cultures. Consequently, inoculum size should be dispersed homogeneously in sufficient amounts for the appropriate growth of microorganism. In view of that, it may be put forward that lower inoculum levels in this study instigated a slow lag phase due which lower enzyme production was obtained (Ramachandran *et al.*, 2004). On the other hand, higher inoculum sizes proved to be inhibitory in nature and lead to oxygen and nutrient deprivation in the culture media thus upsetting the general productivity (Rahman *et al.*, 2005).

Table 2. Box-Behnken design of medium components in coded and actual units for lipase production.

| Std | Run | X1 (Olive oil) | | X2 (Tween 80) | | X3 (Peptone) | | Activity |
|-----|-----|-------|--------------|-------|--------------|-------|--------------|-----------|
| | | Coded | Actual (%) | Coded | Actual (%) | Coded | Actual (%) | (U mg$^{-1}$) |
| 7 | 1 | -1 | 1 | 0 | 3 | +1 | 5 | 50.52 |
| 14 | 2 | 0 | 3 | 0 | 3 | 0 | 3 | 72.15 |
| 12 | 3 | 0 | 3 | +1 | 5 | +1 | 5 | 31.4 |
| 1 | 4 | -1 | 1 | -1 | 1 | 0 | 3 | 43.5 |
| 10 | 5 | 0 | 3 | +1 | 5 | -1 | 1 | 71.57 |
| 6 | 6 | +1 | 5 | 0 | 3 | -1 | 1 | 100.45 |
| 13 | 7 | 0 | 3 | 0 | 3 | 0 | 3 | 74.17 |
| 15 | 8 | 0 | 3 | 0 | 3 | 0 | 3 | 60.44 |
| 3 | 9 | -1 | 1 | +1 | 5 | 0 | 3 | 20.2 |
| 4 | 10 | +1 | 5 | +1 | 5 | 0 | 3 | 53.02 |
| 16 | 11 | 0 | 3 | 0 | 3 | 0 | 3 | 68.7 |
| 8 | 12 | +1 | 5 | 0 | 3 | +1 | 5 | 54 |
| 5 | 13 | -1 | 1 | 0 | 3 | -1 | 1 | 62.22 |
| 11 | 14 | 0 | 3 | -1 | 1 | +1 | 5 | 39.36 |
| 17 | 15 | 0 | 3 | 0 | 3 | 0 | 3 | 70.04 |
| 9 | 16 | 0 | 3 | -1 | 1 | -1 | 1 | 52.62 |
| 2 | 17 | +1 | 5 | -1 | 1 | 0 | 3 | 37.3 |

## 3.8 Effect of extractant on enzyme productivity

The efficacy of extraction medium is of outmost importance. In this regard, the effect of distilled water, 0.1% Tween 80, 0.5% Triton X100, 20% glycerol, and sodium phosphate buffer (100 mM pH 7.0) was observed on the extraction of lipase (Figure 7). The results indicated that Tween-80, non-ionic surfactant, was the best extractant accounting for 20.27 ± 1.29 U/mg of specific lipase activity. However, the lowest activity (10.0 ± 1.07 U/mg) was observed with Triton X100. Tween 80, a non-ionic surfactant, ascertained as an efficient enzyme extractant for the reason that its hydrophobic portion strongly attracts the lipases, stimulating their release from the fermented solids, resulting in better extraction. It also exhibited excellent results since micelles it forms, surround the lipases facilitating in the protein extraction. Similar results have been reported by Silva *et al*. (Silva *et al*., 2014).

## 3.9 Effects of critical medium components using response surface methodology (RSM)

It is fairly challenging to predict the precise effects the involved parameters have on biological processes since they might have multiple interactions with one another. To overcome this RSM was used to create an empirical model for simulation of the lipase activity. Interaction of various independent variables plays crucial role towards the production of enzymes particularly extracellular lipases. Several studies have indicated that olive oil, peptone and Tween-80 greatly influence the production of extracellular lipases.

The interactive effect of three significant variables i.e., A (olive oil as additional oil), B (Tween-80), C (peptone) on the response production (extracellular lipases) was determined statistically using RSM. Since lipases are inducible enzymes, the addition of oils has a domino effect on enzyme yield. Peptone, provided as a nitrogen source, contains rich amounts of minerals and ions which have also reported to upshot lipase production. Tween-80 acting as an effective surfactant lowers the interfacial tension amid oils and water thereby, increasing the cell permeability and hence facilitates in the secretion of enzyme.

A series of experiments were conducted with the possible combinations of independent variable in order to see the effects on the extracellular lipases (Table 2). The observed maximum extracellular lipase activity by the RSM was 100.45 U/ml. Experimental design is described in the table which demonstrates all the actual & coded values of the interacting variables.
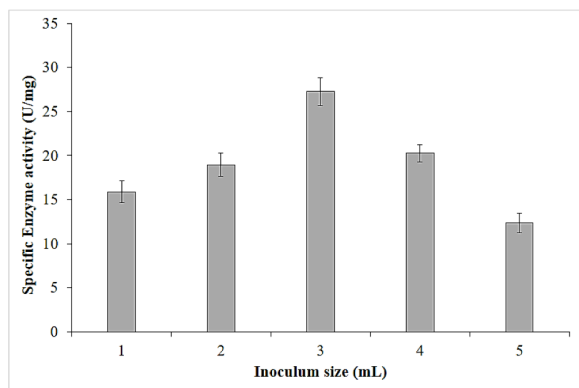
Fig. 6. Effect of inoculum size on lipase production by *Penicillium* sp.



Fig. 7. Effect of extractant medium on lipase production by *Penicillium* sp.

The obtained results were then subjected to the analysis of variance (ANOVA) to establish a response surface quadratic model. As formulated in Eq. 1, lipase activity ($\gamma$) was expressed as a function of concentration of olive oil (X1), Tween-80 (X2) and peptone (X3).

$$\gamma = +3.91750 + (+12.87125)A + (+34.43375)B$$
$$+ (-1.36000)C + (+2.43875)A^2 + (-2.17188)B^2$$
$$+ (-1.68188)C^2 + (-1.56687)AB + (-6.08187)AC$$
$$+ (+0.99125)BC$$

$$(3)$$

As shown in Table 3, the value of "*Probability > F*" less than 0.05 (*Fvalue* = 24.16) suggested that model terms are significant and lipase activity could be well explained by this model. The lack of fit measures the unfitness of the model to represent data within the experimental region. Therefore, the "Lack-of-fit F value" of $p = 0.89$ implies that lack of fit is insignificant relative to the pure error. This also ensures that the equation was suitable for simulation of lipase production with any combination of three variables. The R-squared value was 0.9688 showing a relatively high correlation between experimental and predicted values.

Table 3. Analysis of variance (ANOVA) for quadratic model of lipase activity of *Penicillium* sp.

| Source | Sum of Squares | DF | Mean Square | F-Value | Probability > F | |
|---|---|---|---|---|---|---|
| Model | 5759.09 | 9 | 639.90 | 24.16 | 0.0002 | *Significant* |
| A-Olive oil | 195.62 | 1 | 195.62 | 7.39 | 0.0299 | |
| B-Tween 80 | 1400.07 | 1 | 1400.07 | 52.86 | 0.0002 | |
| C-Peptone | 2.18 | 1 | 2.18 | 0.082 | 0.7823 | |
| AB | 380.64 | 1 | 380.64 | 14.37 | 0.0068 | |
| AC | 301.89 | 1 | 301.89 | 11.40 | 0.0118 | |
| BC | 181.04 | 1 | 181.04 | 6.84 | 0.0347 | |
| $A^2$ | 165.40 | 1 | 165.40 | 6.24 | 0.0411 | |
| $B^2$ | 2491.90 | 1 | 2491.90 | 94.08 | < 0.0001 | |
| $C^2$ | 66.19 | 1 | 66.19 | 2.50 | 0.1579 | |
| Residual | 185.40 | 7 | 26.49 | | | |
| Lack of Fit | 74.35 | 3 | 24.78 | 0.89 | 0.5178 | *not significant* |
| Pure Error | 111.05 | 4 | 27.76 | | | |
| Corrected Total | 5944.49 | 16 | | | | |

Model F-value = 24.16, R-squared = 0.9688, Adjusted R-squared = 0.9287, Predicted R squared = 0.7707, Adequate Precision ratio = 19.594.
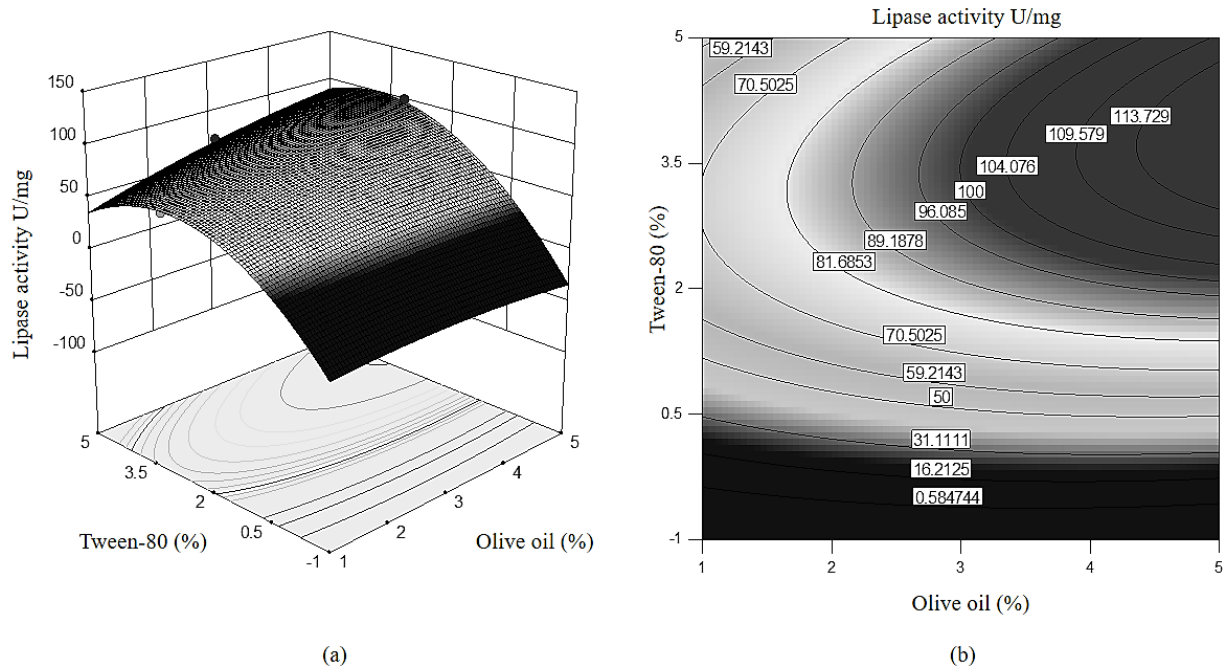
Fig. 8. (a) Response surface curve and (b) Contour plot showing the influence of Olive oil and Tween-80 on the extracellular lipase production by *Penicillium* sp.
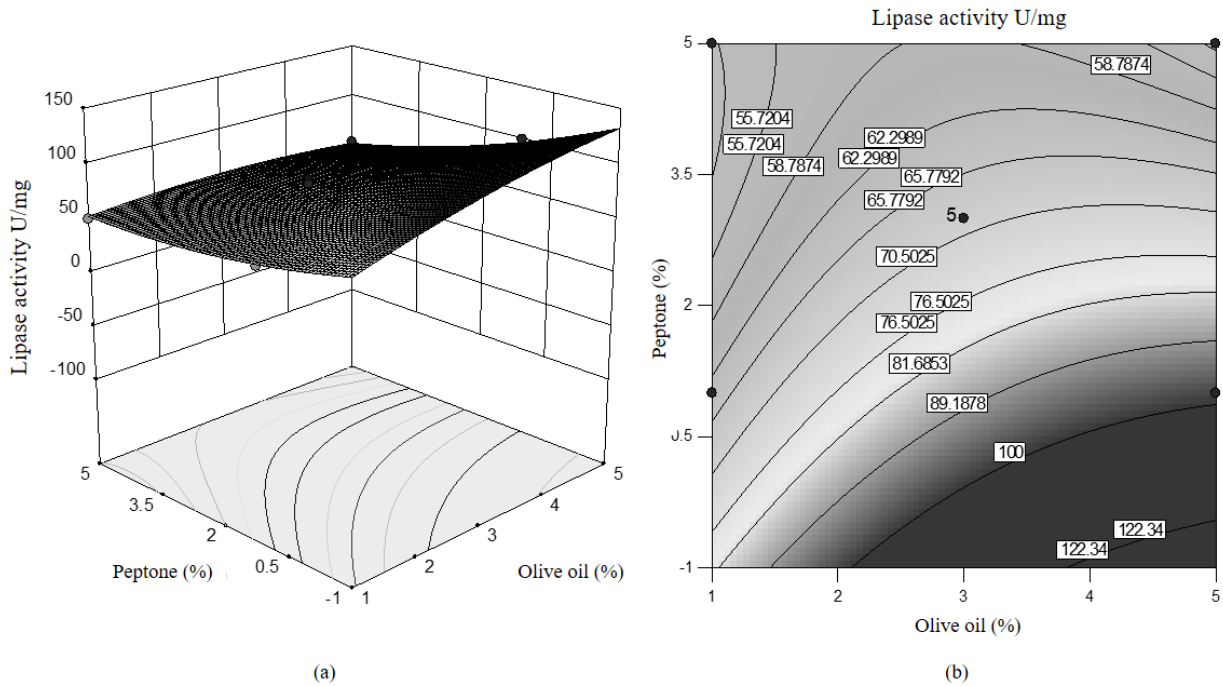


Fig. 9. (a) Response surface curve and (b) Contour plot showing the influence of Olive oil and peptone on the extracellular lipase production by *Penicillium* sp.
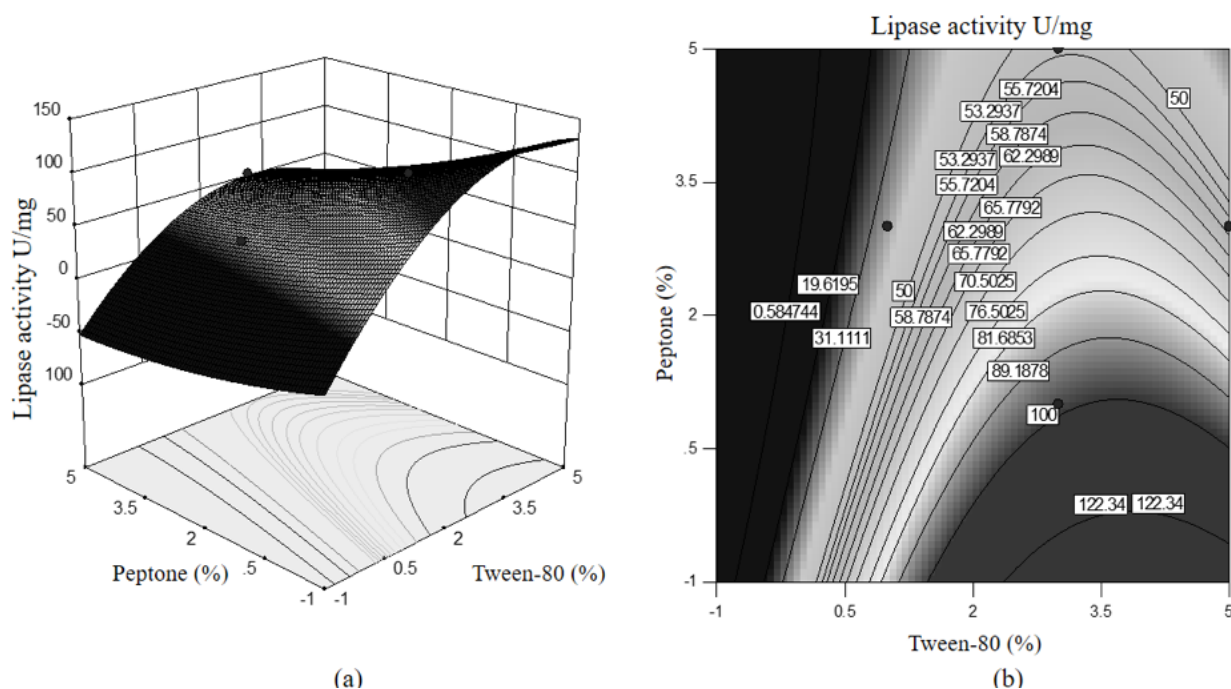
Fig. 10. (a) Response surface curve and (b) Contour plot showing the influence of peptone and Tween-80 on the extracellular lipase production by *Penicillium* sp.

When expressed as a percentage, $R^2$ indicates that the total variation of 96.88% for enzyme activity is credited to the independent variables and only about 3.12% of the total variation cannot be described by the model. The predicted $R^2$ of 0.7077 was in accordance with the adjusted $R^2$ of 0.9287 i.e. the difference is less than 0.2, also supporting that the regression model could be used to describe the response trends. The adequate precision which measured the ratio of signal-to-noise was 19.594, higher than 4, again confirming the model adequacy. Thus, this model could be used to navigate the design space satisfactorily. The three-dimensional response surface curves and contour plots reveal the interactions amongst olive oil, peptone and Tween-80. The 3D response surface curves with the corresponding contour plots presented in Fig. 8, 9 and 10 are based on the function of concentrations of two factors with the other one kept at its optimum level. The significance of the interactions between the corresponding factors is described by an elliptical or saddle nature of the contour plots. Fig. 8 represents the interaction of Tween-80 and olive oil depicting a somewhat synergistic effect on lipase production. In the range studied, the interaction of these variables at high concentrations resulted in higher enzyme yield. This was in accordance with the previous findings in which higher lipase activities could be achieved in the presence of oils and peptone (Gupta *et al.*, 2007, Lima *et al.*, 2003, Lo *et al.*, 2012). Fig. 9 depicts the relationship between peptone and Tween-80. An inverse effect was witnessed in this instance i.e., increased concentration of Tween-80 (3%) with the lowest values of peptone resulted in better lipase productivity of *Penicillium* sp. However, as the concentration of Tween-80 increased from the 3% to 5%, the lipase production decreased. It might be due to the fact that increased in fatty acid accumulation through substrate hydrolysis impeded the rate of enzyme synthesis. Earlier studies reported on the lipase production by *Bacillus pumilus*, *Bacillus* spp, *Rhizopus oligosporus* and *Burkholderia* sp have recorded positive effects of Tween-80 in the range that goes from 0.5% to 2.0% (Iftikhar and Hussain, 2002, Kumar *et al.*, 2011, Lo *et al.*, 2012, Sidhu *et al.*, 1998). As for correlation between peptone and olive oil shown in Fig. 10, a similar behaviour was observed. The lipase productivity increased with the increase in olive oil concentration, but decreased with the increase in peptone concentration.

The optimum olive oil concentration, Tween-80 and peptone as obtained from the maximum point of the polynomial model were 5.0% (v/v), 3% (w/v) and 1.0% (v/v), respectively. In turn, the maximum activity obtained was 100.45 U/mg. The optimization of critical medium components leads to three fold increased in enzyme production when compared to the optimum activity in basal substrate.

# Conclusions

Medium component considerably affects the profits in industrial scale fermentation, owing to the effect on feedstock cost and product yield. The present study showed that sunflower hulls could be effectively used in lipase production by *Penicillium* sp. The RSM was effectively applied to specify the required levels of critical medium components for best enzyme yield by analyzing the relationship between medium factors and their combined contribution. This work may prove to be vital for researchers interested in lipase production. The way forward should be the production of lipase in a bioreactor under optimized conditions. The isolated strain could be potential candidate at the industrial level for conversion of agricultural byproducts in to valuable products like enzyme and thus making the process economical.

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