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Tepki Yüzey Metodu ile *Aeromonas caviae* LipT51'den Ekstrasellüler, Termo-Alkali Lipaz Üretiminin İstatistiksel Optimizasyonu

Sümeyra GÜRKÖK[1]*

ÖZET: *Aeromonas caviae* LipT51'den ekstrasellüler termo-alkali lipaz üretimi, tepki yüzey metodu (response surface methodology: RSM) ile istatistiksel olarak optimize edilmiştir. İlk olarak, en yüksek lipaz üretimi için farklı karbon (zeytinyağı, tributirin, ayçiçek yağı, atık kızartma yağı, gliserol, Tween 80, Tween 20, palmiye yağı ve Triton X100) ve azot (pepton, maya özütü, tripton, peynir altı suyu, üre, $NaNO_2$, NH_4NO_3) kaynaklarını taramak üzere tek seferde bir faktör yöntemi uygulandı. Ardından, karbon kaynağı olarak seçilen atık kızartma yağı, azot kaynağı olarak seçilen tripton ve başlangıç pH'sı için optimum değerler Box-Behnken tasarımı (BBD) kullanılarak RSM ile belirlenmiştir. Lipaz üretimi için BBD'nin ikinci dereceden modeli istatistiksel olarak anlamlı ve güvenilir bulundu ($p < 0.0001$, $R^2 = 0.9881$). Maksimum lipaz üretimi (1.6 U mL^{-1}) için doğrulanmış optimum koşullar, % 1.13 atık kızartma yağı, % 1.5 tripton ve pH 7.9 olarak belirlenmiştir. İlk kez bu çalışmada, bir *A. caviae* suşundan lipaz üretiminin optimizasyonu, ucuz atık malzeme kullanılarak optimize edilmiş kültür koşulları altında gerçekleştirildi. Deterjan aktivitesi ile değerli olduğu bilinen lipaz enziminin üretim verimliliği, optimize edilmeyen koşullara göre 2.7 kat arttı.

Anahtar Kelimeler: *Aeromonas caviae*, lipaz, atık kızartma yağı, optimizasyon, tepki yüzey metodu, Box-Behnken tasarımı

Statistical Optimization of Extracellular Thermo-Alkaline Lipase Production from *Aeromonas caviae* LipT51 with Response Surface Methodology

ABSTRACT: Extracellular thermo-alkaline lipase production from *Aeromonas caviae* LipT51 was statistically optimized by response surface methodology (RSM). First, the one factor at a time approach was implemented to screen the sources of carbon (olive oil, tributyrin, sunflower oil, waste frying oil, glycerol, Tween 80, Tween 20, palm oil, and Triton X100) and nitrogen (peptone, yeast extract, tryptone, whey, urea, $NaNO_2$, NH_4NO_3) for the highest lipase production. Then, optimum values for waste frying oil (WFO) selected as carbon source, tryptone selected as nitrogen source and initial pH of the medium were determined by RSM using Box-Behnken design (BBD). The quadratic model of BBD for lipase production was statistically significant and reliable ($p<0.0001$, $R^2 = 0.9881$). The validated optimal conditions for maximum lipase production (1.6 U mL^{-1}) were determined as 1.13% WFO, 1.5% tryptone and pH 7.9. For the first time in this study, optimization of lipase production from an *A. caviae* strain was carried out under optimized culture conditions using cheap waste material. The production efficiency of lipase enzyme, which is known to be valuable with its detergent activity, increased 2.7 times compared to non-optimized conditions.

Keywords: *Aeromonas caviae*, lipase, waste frying oil, optimization, response surface methodology, Box-Behnken Design

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INTRODUCTION

Lipases [EC 3.1.1.3] are a group of enzymes that catalyse the hydrolysis of triacylglycerols into monoglycerides, diglycerides, fatty acids, and glycerol, acting on carboxylic ester bonds. They also catalyse various esterification reactions in non-aqueous media (Amini et al., 2017; Dinanta Utama et al., 2019). Lipases belong to the serine hydrolases and do not require cofactors (Beisson et al., 2000; Chandra et al., 2020). It is one of the enzymes of high biotechnological importance such as proteases and carbohydrases. They have application areas in various industries such as detergent, cosmetics, food, agriculture, pharmaceutical, leather, textile, paper and pulp (Hasan et al., 2006; Bora et al., 2013; Sarmah et al., 2018; Bharathi and Rajalakshmi, 2019).

Microorganisms, plants and animals have the ability to produce lipase. Microbial sources are the most widely used for lipase production in terms of biotechnological applications due to their catalytic activity and stability, high productivity, low production costs, ease of genetic manipulation, regiospecificity and stereospecificity (Hasan et al., 2006; Sharma et al., 2011; Bharathi and Rajalakshmi, 2019). With regard to industrial uses, lipases that are stable in extreme conditions are particularly notable. Microorganisms have the advantage of survival in a wide variety of habitats that can be very hot like hot springs, too cold like glaciers, very basic like soda lakes or very acidic like sulfuric pools, and the enzymes they produce for the maintenance of their metabolism evolve according to the environment in which they live. Thermostable and alkaline lipases have commercial value and are the most preferred enzymes for the detergent, cosmetics, food industry and environmental bioremediation (Bora et al., 2013, Gurkok and Ozdal, 2021).

Bacteria are the most preferred microbial sources for lipase production and have been extensively studied (Bora and Kalita, 2008; Javed et al., 2018). *A. caviae* is a Gram-negative bacterium belonging to the genus *Aeromonas* that occurs in aquatic environments including raw and processed drinking water, soil, foods such as raw meat, milk, fish, vegetables, and clinical samples (Erdem et al., 2011, Miñana-Galbis et al., 2002). *A. caviae* LipT51 has been reported to produce a thermotolerant and alkaline lipase, with optimum 60 °C and pH 9, respectively. This enzyme has been shown to increase the oil removal capacity of detergents when added to washing solution with detergents, and thus is a promising enzyme for the detergent industry (Gurkok and Ozdal, 2021).

Lipase production by bacterial sources is influenced by a variety of nutritional and physico-chemical factors such as the type and concentration of the carbon (C) and nitrogen (N) source, temperature, pH, inoculum size, and agitation speed (Ebrahimpour et al., 2008; Soleymani et al., 2017). The C and N sources of the fermentation medium are referred to as the main parameters in lipase production as in many processes and the choice of the appropriate substrates is crucial for both the growth of the bacteria and the induction of lipase production. The huge demand for lipase enzymes in various industries has forced researchers to look for ways to produce enzymes in cheaper and more favourable fermentation conditions. Inexpensive and efficient sources are extensively preferred to obtain a cost-effective bioprocess since they constitute the largest part of the process cost. In addition to using cheap raw materials, reusing waste materials or using industrial by-products in media preparations is an even better option for this purpose. This perspective also contributes to the elimination of environmental waste (Ozdal et al., 2017). Most commonly used waste materials and by-products such as molasses, castor oil, oil cakes, WFO, seed cake and whey have attracted great attention in terms of minimizing costs and eliminating environmental disposal (Ramachandran et al., 2007; Gururuaj et al., 2016; Ebrahimipour et al., 2017; Ameri et al., 2019; Patel et al., 2020).

In addition to the cheap raw material use, investigation of the optimal values for the other factors in fermentation conditions is essential to obtain high lipase yields at lower costs. Two approaches are used to increase bioprocess efficiency, one is the conventional "one factor at a time" method and the other is statistical optimization techniques. In traditional practice, one variable is changed at a time, and the other parameters are hold at fixed levels. This method cannot reflect the interactive effects between process variables, and it is a laborious, time-consuming, and expensive to evaluate several variables simultaneously. In statistical optimization methods, the interactions of variables are taken into account in creating the optimum process response and are widely used for media optimization. One of these statistical methods is the response surface methodology (RSM). RSM is a statistical optimization technique used for experimental designs, model generation and determination of the optimum conditions affecting process responses. Box–Behnken design (BBD) and central composite design (CCD) are the types of RSM designs, which are frequently used for media optimization in various processes such as enzyme production (Gurkok et al., 2011), pigment production (Ozdal et al., 2017a), biosurfactant production (Ozdal et al., 2017b) and dye adsorbtion process (Ertan et al., 2020).

RSM reduces time and cost by providing less experiments, and also takes into account interactions of variables. RSM reveals the correlations between the factors and responses and has been successfully applied to evaluate and optimize the effect of various parameters on lipase production (Chauhan et al., 2013; Gururaj et al., 2016, Ebrahimipour et al., 2017; Abdel Aziz et al., 2020).

In the first step of the present study, different C and N sources were tested with one factor at a time approach to determine the best sources for lipase production. The next step was performed to determine the optimum concentrations of the selected C and N sources and initial pH of the medium, taking into account their interactions. Optimization of C and N sources and initial pH were performed statistically by using BBD of RSM for enhanced industrially valuable extracellular thermo-alkaline lipase production by *A. caviae* LipT51.

MATERIALS AND METHODS

Microorganism and Growth Conditions

A. caviae LipT51 (GenBank ID: MN818567.1) previously isolated from a hot spring was used for lipase production (Gurkok and Ozdal, 2021). *A. caviae* LipT51 was cultivated in 25 mL Nutrient Broth medium in 100 mL flasks at 30 °C and 150 rpm overnight for inoculum preparation.

Production of Lipase

Basal medium for lipase production was tributyrin broth (TBB) media containing peptone (0.5%), yeast extract (0.3%), and tributyrin (1%), pH 7. Bacterial pre-culture [1% (*v*/*v*) of OD_{600nm} = 1] was inoculated into 100 mL TBB in 250 mL flasks and incubated at 30 °C and 150 rpm for 2 days.

Lipase Activity Assay

Spectrophotometric lipase assay was performed using *p*-nitrophenol palmitate (*p*NPP) as substrate (Gurkok and Ozdal, 2021). The supernatant of the fermentation medium was used as the crude enzyme. Reaction was prepared by mixing crude enzyme (0.25 mL), 4 mM *p*NPP (0.25 mL) and 50 mM Tris-HCl (0.5 mL). Reaction mixture was kept at 25 °C for 10 min and was terminated by the addition of 2 mL 0.5 N Na_2CO_3. Absorbance of released *p*-nitrophenol was measured in a spectrophotometer at 410 nm. *p*-Nitrophenol standard graph was prepared for calculation of lipase activities. One unit of lipase activity was referred as the enzyme amount which liberates 1 µmol *p*-nitrophenol in 1 min. Lipase assays were carried out in triplicate. Control reaction mixture was prepared with 0.25 mL of dH_2O instead of crude enzyme.

Screening of the Carbon and Nitrogen sources

To determine the effects of different C sources on lipase production, olive oil, sunflower oil, WFO, glycerol, Tween 80, Tween 20, palm oil, and Triton X100 were tested. C sources at a concentration of 1% was supplemented separately in lipase production medium instead of tributyrin. WFO was obtained from sunflower oil, which was used five times in French fries. Whatman filter paper (No. 1) was used to remove any remaining particles from WFO.

To determine the effects of different N sources on lipase production, peptone, yeast extract, tryptone, whey, urea, $NaNO_2$ and NH_4NO_3 were tested. N sources at concentrations of 1% was supplemented separately in medium containing only 1% tributyrin.

Optimization by Response Surface Methodology Using Box–Behnken design

RSM was performed by using BBD to evaluate independent variables, WFO (%), tryptone (%) and initial pH at three levels [low (-1), medium (0), high (+1)] for the optimization of dependent variable (*Y*), lipase production (U mL^{-1}) according to the experimental design given in Table 1. The ranges for the factors were 0.5–1.5% for WFO and tryptone, 7–9 for initial pH.

Table 1. BBD of three variables (WFO, tryptone and initial pH) and experimental response (*Y*) (lipase activity) using RSM

Run	Variables			Response (*Y*) Lipase (U mL^{-1})
	WFO (%)	Tryptone (%)	pH	
1	1.5	0.5	8	0.50
2	0.5	1.0	9	0.88
3	1.0	0.5	7	0.60
4	1.5	1.5	8	1.60
5	1.5	1.0	7	0.95
6	1.0	1.5	7	1.45
7	0.5	1.0	7	0.90
8	0.5	0.5	8	0.60
9	1.0	1.0	8	1.53
10	0.5	1.5	8	1.30
11	1.0	1.0	8	1.45
12	1.5	1.0	9	0.95
13	1.0	1.5	9	1.35
14	1.0	1.5	7	1.55
15	1.0	1.5	9	1.40
16	1.0	0.5	9	0.62
17	1.0	0.5	7	0.63
18	1.5	1.0	9	0.92
19	1.5	1.0	7	1.00
20	0.5	1.5	8	1.25
21	0.5	1.0	7	0.90
22	1.5	0.5	8	0.55
23	0.5	1.0	9	0.82
24	1.0	0.5	9	0.60
25	1.0	1.0	8	1.35
26	1.0	1.0	8	1.35
27	1.0	1.0	8	1.48
28	1.0	1.0	8	1.39
29	1.5	1.5	8	1.50
30	0.5	0.5	8	0.65

Minitab® Statistical Software Release 19 (State College, PA) was used for experimental design, creation of quadratic model and evaluation of the data. A set of 30 experimental runs was carried out and the corresponding measurement of each response variable was reported in Table 1.

A second-order polynomial model was fitted to the data to relate the lipase production (Y) to the amount of WFO, tryptone, and initial pH. The quadratic model equation for a three-factor system (Eq. 1) was as follows:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

In Equation 1, the predicted response is Y; the intercept is β_o; independent variables are $X_i X_j$, the linear coefficient is β_i, the quadratic coefficient is β_{ii}, and the interaction coefficient is β_{ij}.

The confidence level is 95% and significance level alpha (α) is considered as 0.05 in regression analysis. p (probability) value less than α (typically ≤ 0.05) is regarded as statistically significant.

RESULTS AND DISCUSSION

In the present study, optimization of lipase production by *A. caviae* LipT51 was discussed. This lipase has recently been shown to be a promising enzyme for the detergent industry, due to its thermotolerant and highly alkaline nature and its ability to remove oil stains (Gurkok and Ozdal, 2021). Firstly, the best C and N sources that provide maximum lipase production were determined by one factor at a time method. WFO was selected as the inexpensive C source, and tryptone was the choice of N source giving the highest lipase yield among the tested N sources. Then, their optimum concentrations of C and N sources and the initial pH of the medium were statistically optimized by RSM.

Selection of C and N Sources

Although evaluating several variables by the one factor at a time method is laborious and time consuming, it is still widely used for optimization of culture conditions, especially for screening best C and N sources (Lima et al., 2003; Soleymani et al., 2017). Preliminary screening of different C and N sources for enhanced lipase production by *A. caviae* LipT51 was performed using this approach. Since it is known that higher lipase production is obtained in the presence of oil or fatty acid-containing C sources or their analogues (Soleymani et al., 2017; Ilesanmi et al., 2020), these types of C sources were screened in the present study.

Tributyrin in the basal medium was substituted with other C sources including olive oil, sunflower oil, WFO, glycerol, Tween 80, Tween 20, palm oil, and Triton X100 (Figure 1a). The highest yields (*c.* 0.8 U mL^{-1}) were observed in the production media containing olive oil, sunflower oil, and WFO. The difference in enzyme yields obtained in the presence of these C sources were statistically insignificant ($p > 0.05$) and for a more cost-effective production, WFO was chosen as the sole C source to be used in following optimization studies.

Effect of N sources on lipase production was investigated using the production media consisting of tributyrin as the C source. The type and concentration of N sources in the basal medium (0.5% peptone and 0.3% yeast extract) was replaced or modified with other N sources, including peptone, yeast extract, tryptone, whey, urea, $NaNO_2$ and NH_4NO_3 at a concentration of 1% (Figure 1b). Although it is aimed to reduce the enzyme production cost by using cheap inorganic N sources or a by-product such as whey, the expected result could not be achieved with these N sources. Since the highest lipase production (0.87 U mL^{-1}) was obtained with tryptone among other N sources, it was chosen as the N source for following optimization studies.

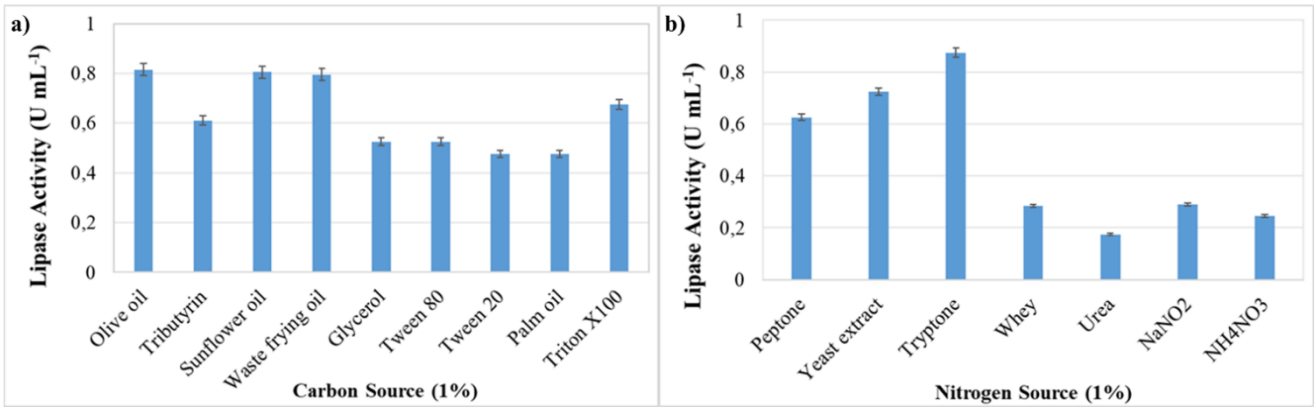

Figure 1. The impacts of carbon (**a**) and nitrogen sources (**b**) on lipase production by *A. caviae* LipT51 incubated for 2 days, at 150 rpm at 30 °C. Results are the average of triplicate assays

Statistical Optimization of Lipase Production by RSM using BBD

RSM was applied to evaluate optimal levels of the parameters (WFO, tryptone, and initial pH) and their interactions for maximum lipase production by *A. caviae* LipT51. The design of the variables and the observed responses (lipase yield) from each run are given in Table 1.

The regression coefficients determined by ANOVA (analysis of variance) of results for lipase production are shown in Table 2a. According to ANOVA, a quadratic model was generated for the prediction of lipase production. Predicted response, lipase yield (*Y*), expressed in terms of WFO (A), tryptone (B), and initial pH (C) is presented in the second order polynomial equation in Equation 2:

$$Y\ (\text{U mL}^{-1}) = -16.38 + 1.834A + 2.221B + 3.858C - 1.0825A^2 - 0.6425B^2 - 0.2394C^2 + 0.3750AB + 0.0050AC - 0.0600BC \quad (2)$$

The significance of the model terms is determined by their *p* value, and terms with $p<0.05$ are considered significant and marked with an asterisk. Coefficients given in Table 2a showed that all the variables significantly affect the lipase production. It is clear that tryptone has a higher effect than WFO and initial pH on lipase production with *p*:0.000 and higher magnitude of the model coefficient. Among the variables, only pH had a negative linear effect and all variables had negative quadratic effects, which refers a better response was achieved at lower levels of the variables. The interactive effect of WFO (%)*tryptone (%) and WFO (%)*pH were positive, while the tryptone (%)*pH has negative interactive effects (Table 2a). A positive interaction value means that the response increases if both variables change to the same level, low or high. The interactions between WFO (%) and pH, and tryptone (%) and pH are statistically insignificant ($p>0.05$) and these terms can be excluded from the final polynomial equation.

ANOVA for lipase production was given in Table 2b. The significance of the regression model was determined by the *F*-test, which showed that the model was apparently significant with high *F* value and quite low *p* value (*F*:184.38 and *p*:0.000). The insignificant lack-of- fit value (*p*:0.973) indicated that the model has no inability to predict lipase production.

The coefficient of multiple determination (R^2) is used to analyse the variations. R^2 value gives an idea of how many data points are among the results of the line that the regression equation creates, and the closer this value is to 1, the stronger the model. For lipase production, the experimental data and the regression model equation were adequately fitted with a very high R^2 value of 0.9881, which means 98.81% of the variations in the response can be explained by the model equation. The adjusted R^2 of 0.9827 and the predicted R^2 value of 0.9772 were in a strong correlation, which showed a reasonable agreement between the predicted and experimental values for lipase production and reliability of the model.

Table 2 Regression coefficient estimates (a) and analysis of variance (b) of quadratic polynomial model of RSM for the production of lipase by *A. caviae* LipT51.

a

Term	Coefficient	Standard Error	*t*	*p*
Constant	1.4250	0.0198	72.07	0.000*
WFO (%)	0.0419	0.0121	3.46	0.002*
Tryptone (%)	0.4156	0.0121	34.33	0.000*
pH	-0.0275	0.0121	-2.27	0.034*
WFO (%)*WFO (%)	-0.2706	0.0178	-15.18	0.000*
Tryptone (%)*Tryptone (%)	-0.1606	0.0178	-9.01	0.000*
pH*pH	-0.2394	0.0178	-13.43	0.000*
WFO (%)*Tryptone (%)	0.0937	0.0171	5.48	0.000*
WFO (%)*pH	0.0025	0.0171	0.15	0.885
Tryptone (%)*pH	-0.0300	0.0171	-1.75	0.095

b

Source	Degree of Freedom	Sum of Squares	Mean Squares	*F*	*p*
Model	9	3.89247	0.43250	184.38	0.000*
Linear	3	2.80406	0.93469	398.48	0.000*
Square	3	1.01085	0.33695	143.65	0.000*
Interaction	3	0.07756	0.02585	11.02	0.000*
Residual Error	20	0.04691	0.00235		
Lack-of-Fit	3	0.00061	0.00020	0.07	**0.973**
Pure Error	17	0.04630	0.00272		
Total	29	3.93939			

S: 0.0484317; R^2: 98.81%; R^2(adjusted): 98.27%; R^2(predicted): 97.72%

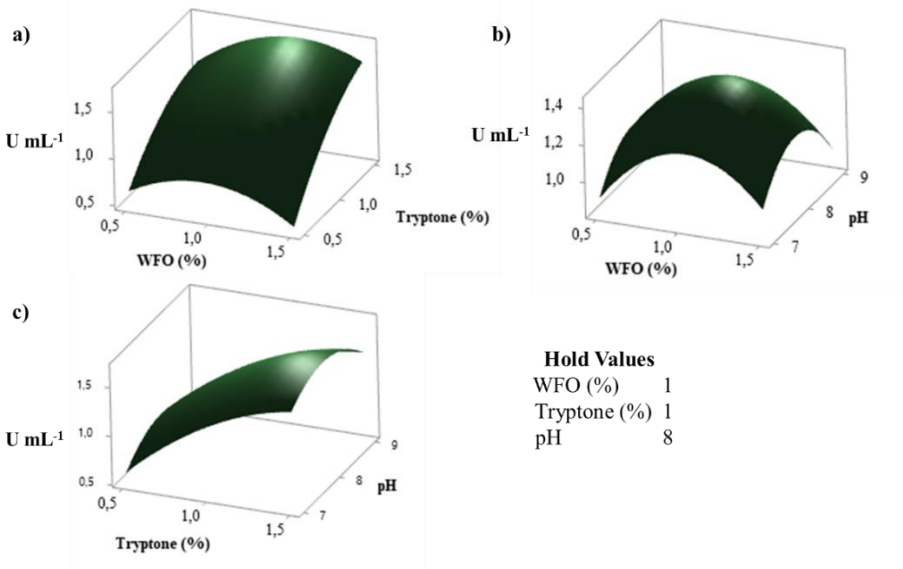

Figure 2. Response surface plots showing the effect of tryptone and WFO (a), pH and WFO (b), and pH and tryptone (c) on lipase production by *A. caviae* LipT51. The third factor was kept constant at the centre point (i.e. WFO 1%, tryptone 1%, and pH 8, respectively) in all cases

Response surface plots in Figure 2 show that the variations in the level of variables strongly influence the lipase yield. The peaks seen on the 3D response surface plots show the optimum levels of variables at which the maximum responses are obtained. It is clear that as the variable levels increases, the production of lipase increases to a certain level. After a certain point, the enzyme yield decreases

and the most likely reasons for this decrease could be C or N catabolite suppression or toxic effects, especially of WFO.

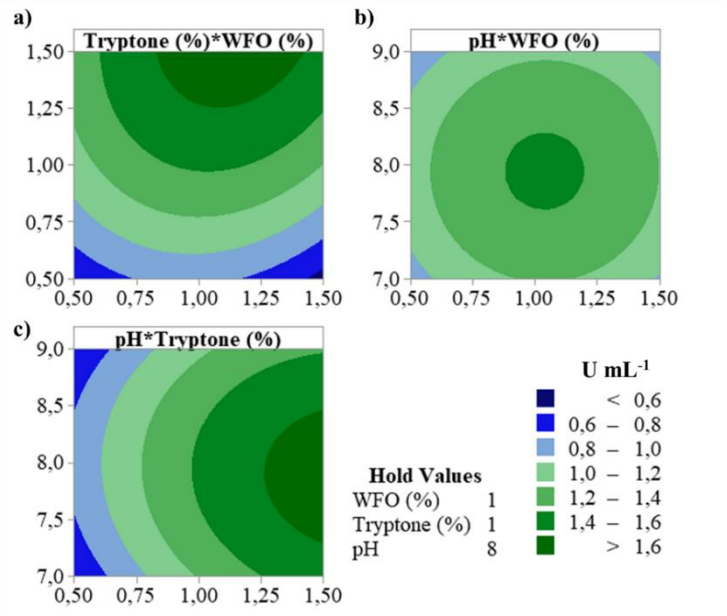

Figure 3 Contour plots showing the interactions of tryptone and WFO (a), pH and WFO (b), and pH and tryptone (c) on lipase production by *A. caviae* LipT51. The third factor was hold constant at the centre point (i.e. WFO 1%, tryptone 1%, and pH 8, respectively) in all cases

2D contour plots in Figure 3 shows the interactive effects and optimum levels of independent variables. Contour plots are generated from a pairwise interaction of the independent variables, holding the third variable constant at its centre value. The circular or elliptical shape of the contour plot shows whether the interactions between variables are negligible or significant, respectively. Elliptical contour plot in Figure 3a indicates a significant interaction between WFO and tryptone, which can also be confirmed from the Table 2a with $p<0.0001$. In Figure 3b and 3c, the contour plots are circular and the interactive effects between pH and WFO (b) and pH and tryptone (c) are statistically insignificant and negligible, with p:0.885 and p:0.095, respectively.

Validation of the Optimized Process Conditions

Validation of the model was performed with the use of response optimizer facility of Minitab 19. According to the model prediction, the maximum lipase production by *A. caviae* LipT51 could be achieved at 1.13% WFO, 1.5% tryptone, and pH 7.9. A well correlation between predicted and measured response was obtained in validation experiments. The prediction (1.7 U mL^{-1}) was validated experimentally in triplicate and resulted in 1.6 U mL^{-1} lipase production, which was 2.7-fold higher than the yield obtained in non-optimized initial basal medium.

This study reported that the lipase production of *A. caviae* LipT51 was maximal at slightly alkaline pH, consistent with other alkaliphilic lipase producing bacteria preferring pH 7 or higher, and the optimum concentrations for C and N sources were also consistent with the requirements of other lipase producer bacteria (Gupta et al., 2004; Dutta and Ray, 2009; Ameri et al., 2019; Ilesanmi et al., 2020).

RSM is an effective optimization technique that is quite popular and frequently used in optimizing enzyme production, as in many other aforementioned processes. Vasiee et al. (2016) used RSM for the optimization of the culture conditions for lipase production by *Bacillus cereus*. They applied coriander seed extract/yeast extract ratio of 16.9 w/w, olive oil (2.37 g L^{-1}) and $MgCl_2$ (24.23 mM) and achieved 1.83-fold increase in lipase yield. Ebrahimipour et al. (2017) optimized lipase production by

Ochrobactrum intermedium Strain MZV101, they achieved 3.46-fold increase under optimized conditions of 1 g L^{-1} molasses, 50–60 °C and pH 10.5–11. Patel et al. (2020) reported the use of RSM for the optimization of lipase production by *Acinetobacter sp*. UBT1. They replaced olive oil in the medium with low cost deoiled seed cakes and determined optimum concentrations of glucose (0.025%), calcium chloride (0.002%), and potassium di-hydrogen phosphate (0.2%), which yielded higher enzyme production.

CONCLUSION

The production of an extracellular, thermotolerant, and alkaline lipase enzyme from *A. caviae* LipT51 was statistically optimized by BBD of RSM. Firstly, the best C and N sources that provide maximum lipase production were determined by classical one factor at a time method. WFO was selected as an effective and inexpensive C source, and tryptone was the choice of N source giving the highest lipase yield among the tested sources. Then, the optimum concentrations of the C and N sources and the initial pH value of the medium were statistically optimized by considering their interactions. Under optimized conditions (1.13% WFO, 1.5% tryptone and pH 7), a 2.7-fold increase in the production of the industrially valuable lipase (1.6 U mL^{-1}) was achieved successfully and cost-effectively.

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Conflict of Interest

The author declares that there is no conflict of interest.

Author's Contributions

The author declares that all the work in the article was performed by Sumeyra Gurkok.

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