Int. J. Adv. Eng. Pure Sci. 2022, 34(2): 217-228
DOI: 10.7240/jeps.984858

RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Determination of the Significance of the Most Effective Nutrients on Lipase Production from *Cryptococcus albidus* D24

Cryptococcus albidus D24'ten Lipaz Üretimi Üzerindeki En Etkili Besin Maddelerinin Öneminin Belirlenmesi

Abdussamed URAS[1], Orkun PİNAR[1], Dilek KAZAN[1]

[1]*Marmara University, Faculty of Engineering, Department of Bioengineering, 34854, Maltepe, Istanbul, Turkey.*

Abstract
In this study, the optimization of the medium components used for the production of lipase enzyme from *Cryptococcus albidus* D24 was performed using the Plackett-Burman statistical design method (PBD), and the most important nutrients affecting the production of lipase enzyme from D24 strain were determined as the first step. According to PBD, the highest lipase activity (19.34 U/ml/min) was obtained with medium including Tween 80 (X2) 2.5% (v/v), and (g/L) Peptone (X4) 8.0, Yeast Extract (X6) 7.5, Beef Extract (X7) 7.5, Malt Extract (X8) 7.5, NH_4Cl (X9) 6.0, $NaNO_3$ (X10) 1.5, $(NH_4)NO_3$ (X12) 6.0, $(NH_4)HCO_3$(X13) 6.0, $MgSO_4.7H_2O$ (X15) 1.0, and KH_2PO_4 (X16) 2.0 at the end of 144 h cultivation. Regarding the concentration effect (CE) values obtained from PBD, NH_4Cl (CE=7.1587), olive oil (CE=3.5544), $(NH_4)HCO_3$ (CE=3.0747), and tryptone (CE=2.1427) were evaluated as more effective nutrients among the sixteen compounds studied. After that, the optimum concentrations of these effective compounds were determined by Response Surface Methodology (RSM). Experimental results showed that the medium containing olive oil (X3), tryptone (X5), NH_4Cl (X9), and $(NH_4)HCO_3$ (X13) yielded maximum lipase activity (12.03 U/ml/min) with 1.5% (v/v), 3.0 g/L, 7.5 g/L, and 12.0 g/L respectively, and a cost reduction for raw materials can be achieved to obtain one (1) unit of enzyme activity.
Keywords: *Cryptococcus albidus* D24, lipase, Plackett-Burman, Response Surface Methodology

Öz
Bu çalışmada, *Cryptococcus albidus* D24'ten lipaz enzimi üretimi için kullanılan besiyeri bileşenlerinin Plackett-Burman İstatistiksel Tasarım Yöntemi (PBD) kullanılarak optimizasyonu gerçekleştirilmiş ve ilk adım olarak D24 suşundan lipaz enzimi üretimine etki eden en önemli besin maddeleri belirlenmiştir. PBD'ye göre en yüksek lipaz aktivitesi (19,34 U/ml/dk), Tween 80 (X2) %2,5 (h/h) ve (g/L) Pepton (X4) 8.0, Maya özütü (X6) 7.5, Et ekstraktı (X7) 7.5, Malt Ekstrakt (X8) 7.5, NH_4Cl (X9) 6.0, $NaNO_3$ (X10) 1.5, $(NH_4)NO_3$ (X12) 6.0, $(NH_4)HCO_3$ (X13) 6.0, $MgSO_4.7H_2O$ (X15) 1.0 ve KH_2PO_4 (X16) 2.0 bileşenlerini içeren ortam ile 144 saat sonunda elde edilmiştir. PBD'den elde edilen konsantrasyon etkisi (CE) değerlerine göre incelenen on altı bileşik arasında, NH_4Cl (CE=7.1587), zeytinyağı (CE=3.5544), $(NH_4)HCO_3$ (CE=3.0747) ve tripton (CE=2.1427) daha etkili olarak bulunmuştur. Daha sonra belirlenen bu etkili bileşiklerin optimum konsantrasyonları, Yanıt Yüzey Yöntemi (RSM) ile belirlenmiştir. Deneysel sonuçlara göre, zeytinyağı (X3), tripton (X5), NH_4Cl (X9) ve $(NH_4)HCO_3$ (X13) bileşenleri, sırasıyla %1,5 (h/h), 3.0 g/L, 7.5 g/L ve 12.0 g/L konsantrasyonlarında maksimum lipaz aktivitesi (12.03 U/ml/dak) elde edildiği ve bir (1) ünite enzim aktivitesi elde etmek için, hammadde maliyetinde bir azalma sağlanabileceği gösterilmiştir.
Anahtar Kelimeler: *Cryptococcus albidus* D24, lipaz, Plackett-Burman, Yanıt Yüzey Yöntemi

I. INTRODUCTION

Lipases (triacylglycerol ester hydrolyzes, EC 3.1.1.3), one of the most crucial industrial enzymes, have the ability to catalyze the hydrolysis of triglycerides to different monomers including fatty acids, diglycerides, monoglycerides, and glycerol. Besides hydrolysis reactions, their ability to form ester bonds facilitates them to catalyze various reactions including esterification, inter-esterification, and transesterification [1-3].

The first lipase from the pancreas was identified by J. Eberle in 1834. Then, various sources, including microorganisms, animals, and plants have been investigated for lipase production [4]. Among all these sources, yeasts were found to be more attractive producers compared to other sources due to easy production, ease of genetic manipulation, high production yield, low production cost. Additionally, their lipases are also applicable to various areas such as detergent, pharmaceutical, biofuel, pulp and paper, and chemical industries [2, 5-8]. For that reason, different yeast species such as *Rhizopus delemar*, *Rhizomucor miehei*, *Candida antarctica*, *Candida rugosa*, *Candida cylindracea*, and *Saccharomycopsis lipolytica* have been investigated for their ability to produce lipases [9, 10]. Although different yeast strains have different lipase production capacities, environmental

Corresponding Author: Orkun PİNAR, Tel: 0 216 777 3573, e-posta: orkun.pinar@marmara.edu.tr
Submitted: 19.08.2021, **Revised:** 25.03.2022, **Accepted:** 25.03.2022

conditions including the type and concentration of medium components, pH, temperature, incubation time, inoculation volume, aeration rate, and agitation speed are the main factors that affect enzyme production [11].

The first step for enhancing enzyme production capacity is the determination of the medium components and their concentrations since the selection of favorable ingredients as carbon and nitrogen sources and inorganic salts may influence cell growth and productivity [12-17]. Moreover, different experimental design methodologies were applied to investigate the significance of a large number of medium constituents and their combinatorial interactions on lipase production [18]. The one-factor-at-a-time method is a classical optimization method that comprises the changing of a single variable while keeping others constant. In this method, a limited number of values are evaluated to understand the effect of each component and it is very hard to obtain reliable data at the end of the limited number of experiments to understand the interactions among the components as well as their effects on production [19]. On the other hand, statistical methods help us to overcome the drawbacks of the classical optimization methods. They minimize the margin of error and reduce the total number of experiments. Additionally, along with the rapidity of resulting the process, it reduces the time consumed and intense labor while giving reliable and accurate data compared to classical methods [20]. Among the other statistical methods, the Plackett–Burman design (PBD) and response surface methodology (RSM) are the most popular statistical design approaches used for bioprocessing [18]. PBD is applied to find the main factors from numerous variables in order to fix or eliminate for further optimization processes, while response surface methodology (RSM) is used for obtaining the optimal conditions of a multivariable system [21].

To select crucial media components for optimum lipase production, Salihu et al. (2013) [20] applied Plackett-Burman design using eleven components. Six components, including sucrose, $(NH_4)2SO_4$, Na_2HPO_4, $MgSO_4$, Tween-80, and olive oil indicated positive effects on lipase production with maximum enzyme activity.

Furthermore, Maharana and Ray (2014) [22] determined not only the most effective carbon sources including glucose, lactose, sucrose, xylose, fructose, maltose, and organic nitrogen sources, but also inorganic nitrogen sources including beef extract, peptone, yeast extract, mineral chlorides such as KCl, NaCl, $CaCl_2$, $MnCl_2.4H_2O$, $MgCl_2$ and $BaCl_2.2H_2O$ and various solid substrates like sugarcane bagasse, wood chips, and coconut oil cake on lipase production from *Pseudomonas* sp. AKM-L5 by using PBD. Based on nitrogen sources, the maximum effect on lipase production was observed using calcium nitrate, while it was found that inorganic nitrogen sources have greater stimulation on lipase production compared to organic nitrogen sources. Additionally, lactose as carbon source, $MnCl_2.4H_2O$ as mineral chlorides and ground nut oil cake as solid substrates provided maximum positive effects.

Moreover, Lanka and Latha, (2015) [23] applied Plackett-Burman statistical tool to determine various physical and nutritional variables including not only temperature, pH, and incubation period but also medium components such as Gum Arabic, for lipase from *Emericella nidulans* NFCCI 3643. After PBD medium optimization, the most effective variables were selected for RSM using a three-level Box-Behnken design.

Samaei-Nouroozi et al., (2015) [24] also used PBD to determine the effect of ten medium components such as glucose, inorganic salts, and olive oil, and they used CCD to evaluate the level of significant factors for lipase produced from *Alkalibacillus salilacus* SR-079 Halo. In these factors, olive oil-KH_2PO_4 showed square effects while interaction effects were observed by using olive oil with glucose and with NaCl for lipase production.

In light of these explanations, PBD and RSM statistical design approaches were applied within the scope of this work to determine the effect of nitrogen sources and waste cooking oil on lipase production from *Cryptococcus albidus* D24 [25]. Therefore, the significant carbon and nitrogen sources were determined by using the PBD method, and further optimization was achieved by RSM methodology to obtain the concentration level of the investigated ingredients for lipase production from *C. albidus* D24.

II. MATERIAL AND METHODS

2.1. Strain and chemicals

C. albidus (D24), deposited at Ege University, Faculty of Science, Department of Biology, Basic and Industrial Microbiology Section, was kindly supplied from Assoc. Prof. Dr. Tansel Yalçın. Most of the chemicals were of analytical grade and supplied either by Merck (Darmstadt, Germany) or Sigma (St Louis, MO) along with a few chemicals that were supplied from BioLife (Port Charlotte, USA) unless otherwise noted.

2.2. Preculture preparation

Preculture with an initial pH of 6.2 was prepared in 100 mL Erlenmeyer flasks with the final working volume of 20 mL. It was sterilized in autoclave at 1.06 bar and 121°C for 15 minutes and after sterilization, the medium was inoculated with a single colony of *C. albidus* (D24). Incubation was carried out in an orbital shaker at 180 rpm and 28 °C for 16 h. At the end of the cultivation, cells were harvested by centrifuge at 4000

rpm for 6 min under aseptic conditions. Then, wet cell pellet was resuspended in the basal medium and transferred to the lipase production medium to adjust the initial optical density (OD) of 1.0 at 600 nm. In all experiments, initial optical density (OD) was kept at the constant value of 1.0.

2.3. Determination of the most effective nutrients

Sixteen medium components including waste oil (X1) and olive oil (X3) as carbon sources, Tween 80 (X2) as a surfactant, peptone (X4), tryptone (X5), yeast extract (X6), beef extract (X7), and malt extract (X8) as organic nitrogen sources, and ammonium chloride (NH_4Cl) (X9), sodium nitrate ($NaNO_3$) (X10), ammonium sulfate ($(NH_4)_2SO_4$) (X11), ammonium nitrate ($(NH_4)NO_3$) (X12), ammonium hydrogen carbonate ($(NH_4)HCO_3$) (X13), and ammonium bicarbonate ($(NH_4)HCO_3$) X(14) (purchased from local market) as inorganic nitrogen sources, magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (X15) and potassium dihydrogen phosphate (KH_2PO_4) X(16) as minerals (Table 1) with three dummy variables were evaluated in twenty sets of experiments (Table 2) using Plackett-Burman statistical design (PBD).

Table 1. The medium components proposed in Plackett-Burman experiments and their corresponding low and high concentration levels.

Variable Code	Medium Ingredients	Low Level (g/L) (-1)	High Level (+1)
X1	Waste oil	0.0% v/v	2.5% v/v
X2	Tween 80	0.5% v/v	2.5% v/v
X3	Olive oil	0.0% v/v	2.5% v/v
X4	Peptone	2.0	8.0
X5	Tryptone	0.0	7.5
X6	Yeast Extract	2.0	7.5
X7	Beef Extract	0.0	7.5
X8	Malt Extract	0.0	7.5
X9	NH_4Cl	0.0	6.0
X10	$NaNO_3$	1.5	6.0
X11	$(NH_4)_2SO_4$	0.0	6.0
X12	$(NH_4)NO_3$	0.0	6.0
X13	$(NH_4)HCO_3$	0.0	6.0
X14*	$(NH_4)HCO_3$	0.0	6.0
X15	$MgSO_4.7H_2O$	0.25	1.0
X16	KH_2PO_4	0.5	2.0

* X14 is purchased from the local market with the lowest price as a cost-effective inorganic nitrogen source alternative for industrial production. Although X13 and X14 have the same molecular formula, X14 has impurities compared to X13.

Each medium was experimented twice, while the lipase activity for each run was performed as triplicate. Since the assessment of wastes from the food industries to produce value-added compounds would be very attractive for both economic and environmental standpoints, waste cooking olive oil was used as a carbon source while olive oil was also used as the control. PBD is a two-level factorial design and allows the screening of k-1 variables, where k is the number of the set of experiments. Dummy variables are not the components used in experiments, but they can be used to determine the measurement error. The effect of the concentrations and the estimation of the effect of the dummy variable were achieved by using the following equation described by Rajput et al., (2016) [26] (Equation 1).

$$CE(X_i) = 2\,(\Sigma\, N_i^{+} - \Sigma\, N_i^{-}) / k, \quad (1)$$

where $CE(X_i)$: The concentration effect of the variable; N_i^{+} and N_i^{-}: The lipase production of the trials, where the variable X_i estimated at its higher and lower concentrations, respectively; k: The number of trials in this equation. The following equation is used to calculate the variance of dummy variables in order to estimate experimental error. (Equation 2);

$$Veff = \Sigma\,(ED)^2 / n, \quad (2)$$

where Veff: The variance of the concentration effect (experimental error); ED: The concentration effect of the dummy variable; n: The number of dummy variables. The square root of the variance of dummy variables was used to calculate the standard error (SE) of the concentration effect. Student's t-test given below was used to establish the significance level (p-value) of each concentration effect. (Equation 3):

$$t(X_i) = E(X_i) / SE, \quad (3)$$

where $E(X_i)$ is the effect of the variable X_i.

Equation 4 was used to obtain the statistical confidence level of each variable (Equation 4).

$$\text{Statistical confidence} = (1 - p) \times 100. \quad (4)$$

The statistical confidence level of 95%, which was considered as a highly significant effect on lipase production corresponds to a value of p = 0.05. Confidence level of 70-95% was evaluated to be effective, whereas confidence level below 70% was accepted as insignificant.

2.4. Statistical experimental design

For the development, improvement, and optimization of bioprocesses, RSM including, mathematical and statistical methods is used for modeling and analysis of problems. These problems are the response of the interest affected by a large number of variables and the main goal is the optimization of this response [27-29].

Therefore, after using PB design to determine the most effective nutrients on lipase synthesis, Response Surface Methodology (RSM) was used to obtain the optimum concentration of the key medium components that affect lipase production. The most effective components with their concentrations were prepared to investigate the optimum conditions on lipase production and their effects.

The highest and lowest level of each source were given in Table 3, while the statistical design for the most effective components and their concentrations were shown in Table 4.

Table 2. PB Design for 16 medium components & 3 dummy variables; '-' means to use the lowest level of concentrations in different set of experiments.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	D1	D2	D3
1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1
2	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1
3	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1
4	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1
5	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1
6	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1
7	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1
8	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1
9	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1
10	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1
11	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1
12	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1
13	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1
14	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1
15	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1
16	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1
17	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1
18	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Table 3. The most effective medium components used on statistical experiments.

Variable Code	**Medium Ingredients**	**Concentration of nutrients**	
		Low level (g/L)	**High Level(g/L)**
X3	Olive Oil	0.5% (v/v)	2.5% (v/v)
X5	Tryptone	3	15
X9	NH_4Cl	3	12
X13	NH_4HCO_3	3	12

Table 4. Statistical Design for the most effective components and their concentrations ('-1' means to use lowest, '0' means to use middle and '1' means to use the highest level of concentrations in different set of experiments).

RUN	X3	X5	X9	X13
1	0	0	-1	-1
2	0	0	1	-1
3	0	0	-1	1
4	0	0	1	1
5	-1	-1	0	0
6	-1	1	0	0
7	1	-1	0	0
8	1	1	0	0
9	0	-1	0	-1
10	0	1	0	-1
11	0	-1	0	1
12	0	1	0	1
13	-1	0	-1	0
14	1	0	-1	0
15	-1	0	1	0
16	1	0	1	0
17	-1	0	0	-1
18	1	0	0	-1
19	-1	0	0	1
20	1	0	0	1
21	0	-1	-1	0
22	0	1	-1	0
23	0	-1	1	0
24	0	1	1	0
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0

Lipase production was carried out in 500 mL Erlenmeyer flasks including 100 mL of the production medium at 250 rpm and 28 °C.

2.5. Determination of Lipase Activity

The method explained by Yalçın et al., (2014) [25] was used to determine the activity of D24 lipase with triplicate measurements. 100 µL of enzyme solution and 2 mL of the pNPP solution prepared in 0.05 M sodium acetate were mixed. Then, the reaction mixture was incubated for 3 min at 37 °C. After incubation, 150 µL of 1 M Na_2CO_3 was added to stop the reaction. Finally, the absorbance of the reaction mixture was measured at 410 nm spectrophotometrically against substrate-free enzyme as the blank. One unit of lipase activity (U) was described as the enzyme that releases 1 µmol p-nitrophenyl for 1 min at 37 °C and pH 5.6.

III. RESULTS AND DISCUSSION

3.1. Determination of the most effective nutrients for D24 lipase production using Plackett-Burman statistical approach

As shown in Table 5, the highest lipase activity of 19,34 U/ml/min was obtained using Medium 6, which includes Tween 80 (X2) (v/v) 2.5%, and (g/L) peptone (X4) 8.0, yeast extract (X6) 7.5, beef extract (X7) 7.5, malt extract (X8) 7.5, ammonium chloride (X9) 6.0, sodium nitrate (X10) 1.5, ammonium nitrate (X12) 6.0, ammonium hydrogen carbonate (X13) 6.0, $MgSO_4.7H_2O$ (X15) 1.0, and KH_2PO_4 (X16) 2.0.

To analyze the effect of each ingredient, concentration effects, t-value, *p*-value, and the significance level of each component are calculated, and results are shown in Table 6. The standard error of 2.9078 calculated for each component was the same. According to concentration effect data, Tween 80 (X2), olive oil (X3), tryptone (X5), yeast extract (X6), malt extract (X8), ammonium chloride (X9), ammonium nitrate (X12), ammonium hydrogen carbonate (X13), $MgSO_4.7H_2O$ (X15), and KH_2PO_4 (X16) had a positive effect, while waste oil (X1), peptone (X4), beef extract (X7), sodium nitrate (X10), ammonium sulfate (X11), and ammonium bicarbonate (X14) had a negative effect on lipase production.

Furthermore, the Pareto chart, which illustrates the order of significance of all variables affecting lipase production from *C. albidus* D24 (Figure 1) was drawn. The results revealed that ammonium chloride (X9) is the best component with the highest contribution to increase the lipase activity by more than 20%. Olive oil (X3), which follows ammonium chloride, enhanced lipase production by more than 10%.

Generally, the production of an extracellular lipase might require an inducer such as fatty acids. Among the various fatty acids, oleic acid was evaluated to be the best inducer for lipase production [30]. Therefore, compounds containing oleic acid are mostly used for lipase production. Olive oil containing a high amount of oleic acid is one of the carbon sources widely used for lipase production [31]. Additionally, triolein, a triacylglycerol containing three oleic acid molecul*es*, also includes up to 83% of olive oil [32]. Similar to literature, olive oil used for D24 lipase production showed the great significance level as 96% (Table 6). The highest significance level of olive oil reported by Rajendran et al., (2007) [14] is in agreement with our results.

Nitrogen sources, in addition to carbon sources, have an impact on lipase production. Thus, the effect of different nitrogen sources on the production of D24 lipase was examined. Among the nitrogen sources evaluated, ammonium chloride showed 99% of significance. The next most important nitrogen source

that increased D24 lipase was ammonium hydrogen carbonate with 94% of significance. Additionally, while tryptone, yeast extract, and sodium nitrate showed significance between 80 and 90%, other components such as beef extract, malt extract, ammonium nitrate, Tween 80, waste oil, KH_2PO_4, and $MgSO_4.7H_2O$ showed significance below 80%.

Regarding the concentration effect (CE) values obtained from PB design (Table 6), ammonium chloride (CE=7.1587), olive oil (CE=3.5544), ammonium hydrogen carbonate (CE=3.0747), and tryptone (CE=2.1427) were evaluated as the more effective nutrients among the (sixteen) 16 compounds studied.

Mainly, using the olive oil concentration at an optimized level in the production medium is critical for an improved production. Since using too much oil in the production medium prevents the growth of microorganisms by forming a bi-phasic barrier between microorganisms and its physicochemical environment. As a result of this situation, oxygen transfer and nutrient utilization by microorganisms could be blocked [33-34].

For that reason, the concentration of olive oil together with the most effective inorganic and organic nitrogen sources were optimized by using RSM in order to find their best concentrations of these four nutrients to achieve the highest lipase production.

Table 5. Concentration of each component (g/L) used in the production medium and average lipase activities determined.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	Lipase Activity (U/ml/min)
1	2.5	2.5	0.0	2.0	0.0	2.0	7.5	0.0	6.0	1.5	6.0	6.0	6.0	6.0	0.25	0.5	4.26
2	2.5	0.5	0.0	2.0	0.0	7.5	0.0	7.5	0.0	6.0	6.0	6.0	6.0	0.0	0.25	2.0	5.63
3	0.0	0.5	0.0	2.0	7.5	2.0	7.5	0.0	6.0	6.0	6.0	6.0	0.0	0.0	1.0	2.0	3.88
4	0.0	0.5	0.0	8.0	0.0	7.5	0.0	7.5	6.0	6.0	6.0	0.0	0.0	6.0	1.0	0.5	3.67
5	0.0	0.5	2.5	2.0	7.5	2.0	7.5	7.5	6.0	6.0	0.0	0.0	6.0	6.0	0.25	2.0	18.03
6	**0.0**	**2.5**	**0.0**	**8.0**	**0.0**	**7.5**	**7.5**	**7.5**	**6.0**	**1.5**	**0.0**	**6.0**	**6.0**	**0.0**	**1.0**	**2.0**	**19.34**
7	2.5	0.5	2.5	2.0	7.5	7.5	7.5	7.5	0.0	1.5	6.0	6.0	0.0	6.0	1.0	0.5	6.74
8	0.0	2.5	0.0	8.0	7.5	7.5	7.5	0.0	0.0	6.0	6.0	0.0	6.0	6.0	0.25	0.5	1.35
9	2.5	0.5	2.5	8.0	7.5	7.5	0.0	0.0	6.0	6.0	0.0	6.0	6.0	0.0	0.25	0.5	14.60
10	0.0	2.5	2.5	8.0	7.5	2.0	0.0	7.5	6.0	1.5	6.0	6.0	0.0	0.0	0.25	0.5	15.07
11	2.5	2.5	2.5	8.0	0.0	2.0	7.5	7.5	0.0	6.0	6.0	0.0	0.0	0.0	0.25	2.0	0.100
12	2.5	2.5	2.5	2.0	0.0	7.5	7.5	0.0	6.0	6.0	0.0	0.0	0.0	0.0	1.0	0.5	13.35
13	2.5	2.5	0.0	2.0	7.5	7.5	0.0	7.5	6.0	1.5	0.0	0.0	0.0	6.0	0.25	2.0	9.610
14	2.5	0.5	0.0	8.0	7.5	2.0	7.5	7.5	0.0	1.5	0.0	0.0	6.0	0.0	1.0	0.5	2.180
15	0.0	0.5	2.5	8.0	0.0	7.5	7.5	0.0	0.0	1.5	0.0	6.0	0.0	6.0	0.25	2.0	0.100
16	0.0	2.5	2.5	2.0	7.5	7.5	0.0	0.0	0.0	1.5	6.0	0.0	6.0	0.0	1.0	2.0	12.27
17	2.5	2.5	0.0	8.0	7.5	2.0	0.0	0.0	0.0	6.0	0.0	6.0	0.0	6.0	1.0	2.0	3.680
18	2.5	0.5	2.5	8.0	0.0	2.0	0.0	0.0	6.0	1.5	6.0	0.0	6.0	6.0	1.0	2.0	10.69
19	0.0	2.5	2.5	2.0	0.0	2.0	0.0	7.5	0.0	6.0	0.0	6.0	6.0	6.0	1.0	0.5	3.730
20	0.0	0.5	0.0	2.0	0.0	2.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.25	0.5	5.330

Table 6. Calculated concentration effect, t-value, *p*-value, and the significance level of each component.

Components	Concentration Effects	t-value	p-value	Significance
Waste Oil (X1)	-1.1938	-0.4106	0.3183	68.1665
Tween 80 (X2)	1.1914	0.4097	0.3191	68.0854
Olive Oil (X3)	3.5544	1.2224	0.0380	96.2027
Peptone (X4)	-1.2242	-0.4210	0.3082	69.1761
Tryptone (X5)	2.1427	0.7369	0.1215	87.8456
Yeast Extract (X6)	1.9692	0.6772	0.1436	85.6445
Beef Extract (X7)	-1.5168	-0.5216	0.2266	77.3407
Malt Extract (X8)	1.4600	0.5021	0.2404	75.9600
Ammonium chloride (X9)	7.1587	2.4619	0.0056	99.4386
Sodium nitrate (X10)	-1.7582	-0.6047	0.1770	82.3042
Ammonium sulfate (X11)	-2.6303	-0.9046	0.0783	92.1695
Ammonium nitrate (X12)	0.0460	0.0158	0.9662	3.3772
Ammonium hydrogen carbonate (X13)	3.0747	1.0574	0.0544	94.5640
Ammonium bicarbonate (X14)*	-2.9916	-1.0288	0.0581	94.1944
$MgSO_4.7H_2O$ (X15)	0.5671	0.1950	0.6103	38.9721
KH_2PO_4 (X16)	1.2866	0.4425	0.2885	71.1463

*Although a similar effect was expected both from X14 and X13 because of the identical molecular formula, X14 showed a negative effect because of impurities compared to X13.

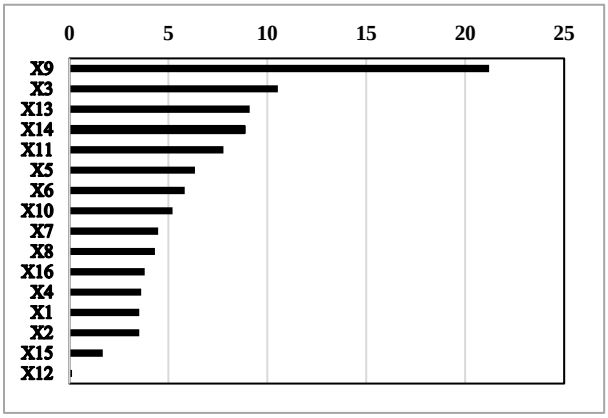

Figure 1. Pareto chart representing the contribution percentage of the medium components (**X1:** Waste Oil, **X2:** Tween 80; **X3:** Olive Oil, **X4:** Peptone, **X5:** Tryptone, **X6:** Yeast Extract, **X7:** Beef Extract, **X8:** Malt Extract, **X9:** Ammonium chloride, **X10:** Sodium nitrate, **X11:** Ammonium sulfate, **X12:** Ammonium nitrate, **X13:** Ammonium hydrogen carbonate, **X14:** Ammonium bicarbonate, **X15:** $MgSO_4.7H_2O$, **X16:** KH_2PO_4).

3.2. Optimization of the concentration of the most effective substrates using statistical experimental design

After the determination of the most effective components, Response Surface Methodology (RSM) was used to find out the best combination of independent variables to increase lipase activity. The combinations of four compounds and lipase activity obtained from these combinations are summarized in Table 7.

To understand the simultaneous effect of the most effective components as olive oil (X3), tryptone (X5), NH_4Cl (X9), and NH_4HCO_3 (X13) on lipase activity, main effect plot (Figure 2) was constructed between mean lipase activity and the concentrations of four components. An interesting situation is observed by comparing main effect plots of the inorganic nitrogen source, Figure 2 (a) and (b), respectively. It is clear from Figure 2 (a) that lipase activity was decreased with increasing the concentration of X13 up to (w/v) 7.5%. Above this value, the positive effect of NH_4HCO_3 (X13) on lipase production was observed. Just the opposite of the X13 effect, lipase production was enhanced up to (w/v) 7.5% NH_4Cl (X9). However, over this concentration, lipase production was reduced (Figure 2 (b)). On the other hand, the presence of tryptone (X5), as an organic nitrogen source, did not cause a difference in lipase production at a concentration below (w/v) 9.0% (Figure 2 (c)). However, increasing tryptone amount in the medium caused a decrease in lipase activity. When olive oil's main effect (Figure 2 (d)) on the mean lipase activity (U/mL/min) was investigated, it is clear that olive oil has a positive impact on lipase synthesis from *C.*

albidus D24 at all concentrations investigated.

The situation mentioned above is further analyzed by looking at how these four most effective compounds interact with each other to enhance D24 lipase production (Figure 3). The first raw of Figure 3 (a), (b), and (c)) shows how NH_4HCO_3 (X13) interacts with NH_4Cl (X9) (Figure 3 (a)), tryptone (X5) (Figure 3 (b)) and olive oil (X3) (Figure 3 (c)) for lipase production. It is clear that the lower and higher concentrations of NH_4HCO_3 have opposite effects on lipase production in the presence of NH_4Cl (Figure 3 (a)) and tryptone (Figure 3 (b)). At low NH_4HCO_3 concentration (w/v, 3%), lipase activity decreased up to (w/v) 7.5% of NH_4Cl and then increased, while at higher NH_4HCO_3 values, the opposite effect was observed. For tryptone (Figure 3 (b)), the interaction of NH_4HCO_3 with tryptone is exactly the opposite of the interaction of it with ammonium chloride (X9). When the interaction between NH_4HCO_3 (X13) and olive oil is examined (Figure 3 (c)), at all olive oil concentrations studied, lower amounts of X13 yielded lower lipase activity. But lipase activity consistently showed an increasing trend in all olive oil concentrations independent of X13 concentration.

Interestingly, at constant ammonium chloride (X9) concentration, although the lipase activity was higher at higher NH_4Cl concentrations, raising the concentration of three variables caused a decrease lipase production at both higher and lower ammonium chloride concentrations (Figure 3 (d), (e), and (f)). The situation observed for ammonium chloride was completely reversed for NH_4HCO_3, NH_4Cl, and olive oil at constant tryptone concentrations (Figure 3 (g), (h), and (i)).

As for olive oil, lipase production was enhanced at higher olive oil concentration (Figure 3 (j), (k), and (l)). When the interaction of olive oil with inorganic and organic nitrogen sources was investigated, at low olive oil concentrations, lipase activity was slightly changed with the increment of NH_4HCO_3, NH_4Cl, and tryptone (Figure 3 (j), (k), and (l)). However, dramatic and opposite changes were observed with NH_4HCO_3 and NH_4Cl (Figure 3 (j) and (k)) at the presence of the higher amount of olive oil. As seen from Figure 3 (j), lipase activity was decreased with raising NH_4HCO_3 up to 7.5% concentration. Above this value, lipase activity was elevated as a higher concentration of NH_4HCO_3 has a positive effect. Opposite to NH_4HCO_3, enzyme activity was raised with increasing ammonium chloride concentration (Figure 3 (k)) up to the same concentration of ammonium hydrogen carbonate and then decreased.

Overall, olive oil, and NH_4Cl have a positive impact on lipase production from *C. albidus* D24, while the effect of tryptone and NH_4HCO_3 depends on the concentration of other nutrients studied.

Table 7. Concentrations (g/L) of olive oil (X3), tryptone (X5), NH_4Cl (X9), and ammonium hydrogen carbonate (X13) used in RSM and average lipase activities determined for each run.

Run	X3	X5	X9	X13	Activity (U/ml/min)
1	1.5	9	3	3	8.060
2	1.5	9	12	3	11.27
3	1.5	9	3	12	3.040
4	1.5	9	12	12	0.860
5	0.5	3	7.5	7.5	1.680
6	0.5	15	7.5	7.5	2.520
7	2.5	3	7.5	7.5	5.440
8	2.5	15	7.5	7.5	4.970
9	1.5	3	7.5	3	4.000
10	1.5	15	7.5	3	1.040
11	1.5	3	7.5	12	12.03
12	1.5	15	7.5	12	10.77
13	0.5	9	3	7.5	2.680
14	2.5	9	3	7.5	1.100
15	0.5	9	12	7.5	4.860
16	2.5	9	12	7.5	3.240
17	0.5	9	7.5	3	3.260
18	2.5	9	7.5	3	7.950
19	0.5	9	7.5	12	4.760
20	2.5	9	7.5	12	11.63
21	1.5	3	3	7.5	1.370
22	1.5	15	3	7.5	0.650
23	1.5	3	12	7.5	6.590
24	1.5	15	12	7.5	2.840
25	1.5	9	7.5	7.5	5.760
26	1.5	9	7.5	7.5	3.530
27	1.5	9	7.5	7.5	6.230

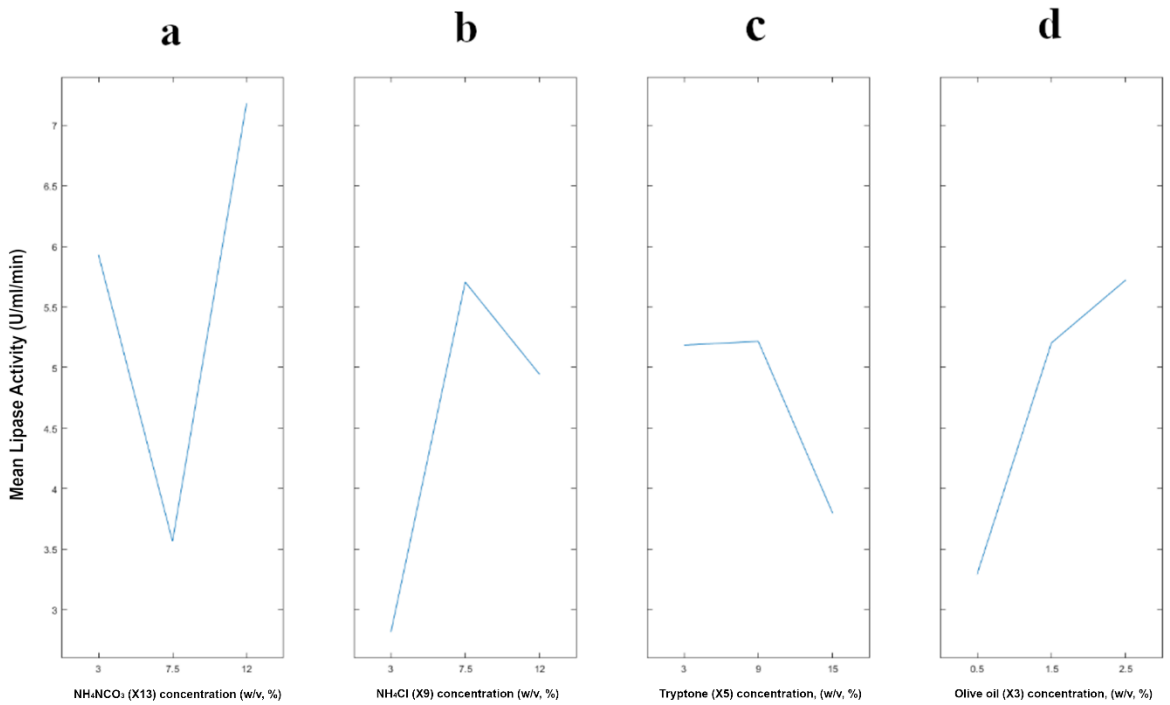

Figure 2. Main effects plot for lipase activity

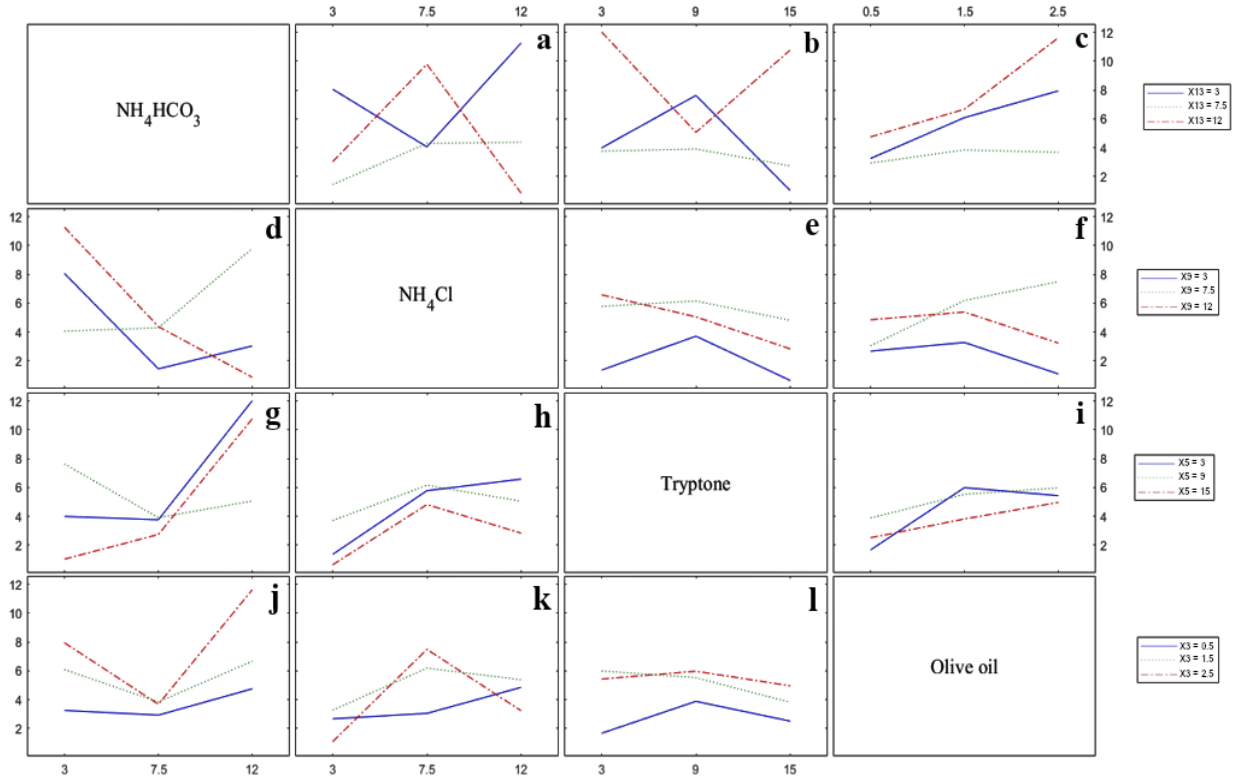

Figure 3. Interaction of the effect of the most effective compounds for lipase production.

IV. CONCLUSIONS

Enzymes are widely used in different areas such as detergent, food, agricultural, and pharmaceutical industries to obtain the desired product in an efficient, fast, and environmentally friendly manner. However, their usage is restricted because of their stability under extreme conditions of the processes. Therefore, different attempts have been made to find microorganisms that have an ability to produce enzyme having excellent properties.

Depending on our preliminary experimental studies, the newly isolated *C. albidus* D24 from petroleum sludge produces organic solvent stable specifically acetone stable lipase enzyme. To produce lipase from *C. albidus* efficiently, this thesis aimed to screen the most important medium components by using PB statistical design. After the determination of most effective nutrients, their responses and optimum concentrations were optimized by Response Surface Methodology (RSM) to develop cost-effective process.

According to experimental results, Tween 80 was the best surfactant compared to Triton X-100 and Tween 20. The optimum concentration of Tween 80 was determined as (v/v) 2.5%. However, in the literature, the enhancement of lipase activity up to 13 times with 2.0% Tween 80 was reported, we first decided to carry out experimental work to determine the most effective nutrients for lipase production from *C. albidus* D24.

According to PB statistical design, *C. albidus* D24 produced crude enzyme having 19.34 U/ml/min lipase activity in the medium including Tween 80 (X2) (v/v) 2.5%, (g/L) peptone (X4) 8.0, yeast extract (X6) 7.5, beef extract (X7) 7.5, malt extract (X8) 7.5, ammonium chloride (X9) 6.0, sodium nitrate (X10) 1.5, ammonium nitrate (X12) 6.0, ammonium hydrogen carbonate (X13) 6.0, $MgSO_4.7H_2O$ (X15) 1.0, and KH_2PO_4 (X16) 2.0 at the end of 144 hours of cultivation period at 28°C and pH 7.0.

Among the sixteen different nutrients studied, olive oil was the best carbon source for D24 lipase production. As for nitrogen sources, tryptone have higher concentration effect compared to other organic nitrogen sources investigated. Besides organic sources, inorganic nitrogen sources were also analyzed. Ammonium chloride and ammonium hydrogen carbonate were the best inorganic sources studied. The concentration effect values of ammonium chloride olive oil, ammonium hydrogen carbonate, and tryptone were 7.1587, 3.5544, 3.0747, and 2.1427, respectively.

When, RSM was applied to determine the optimum concentration of these four effective compounds, the optimum concentration of olive oil (X3) tryptone (X5), ammonium chloride (X9), and ammonium hydrogen carbonate (X13) were determined as (v/v) 1.5%, 3.0 g/L, 7.5 g/L, and 12 g/L, respectively.

To decide the economic feasibility of the media, the cost of the medium components required to produce one unit enzyme activity was calculated.

In the production medium, where the highest activity (19.34 U/ml/min) was obtained with the PDB method, 4 (four) organic and 4 (four) inorganic nitrogen sources, 2 (two) mineral salts, and Tween 80 as a carbon source were used. In this medium, the raw material cost required to obtain one (1) unit of enzyme activity was determined as 0.104 Euro, considering the costs of all media components. In the RSM study conducted with four (4) most effective media components (Olive oil (X3), Tryptone (X5), Ammonium chloride (X9), and Ammonium hydrogen carbonate (X13)) determined according to PDB, maximum lipase activity (12.03 U/ml/min) was achieved through optimization. Although, activity was obtained with only four (4) components as a result of the optimization made with RSM was slightly decreased compared to activity obtained with medium 6 in PDB, the raw material cost required to obtain one (1) unit of enzyme activity was found to be 0.0277 Euro which is lower compared with PDB. In other words, a reduction in raw material cost was observed with the medium optimized by the RSM method. To our best knowledge, this is the first research work to optimize the lipase production from *C. albidus* D24.

ACKNOWLEDGEMENTS

This work was supported by Marmara University, Scientific Research Projects Committee [grant number FEN-C-YLP-060510-0142], and Scientific and Technological Research Council of Turkey (TUBITAK) with 1007 - Public Institutions Research Funding Program (KAMAG) [grant number 115G079].

REFERENCES

[1] Divakar, S. and Manohar B. (2007). In: Industrial Enzymes: Structure, Function and Applications. Use of Lipases in the Industrial Production of Esters. J. Polaina and A. P. MacCabe (eds.), Dordrecht, Springer Netherlands: p 283-300.

[2] Anvari, M. (2015). Extraction of lipase from *Rhizopus microsporus* fermentation culture by aqueous two-phase partitioning. *Biotechnol. Biotechnol. Equip.*, 29(4), 723-731.

[3] Venditti, I., Palocci, C., Chronopoulou, L., Fratoddi, I., Fontana, L., Diociaiuti, M., & Russo, M. V. (2015). *Candida rugosa* lipase immobilization on hydrophilic charged gold nanoparticles as promising biocatalysts: Activity and stability investigations. *Colloids Surf. B.*, 131, 93-101.

[4] Ping, L., Yuan, X., Zhang, M., Chai, Y., & Shan, S. (2018). Improvement of extracellular lipase production by a newly isolated *Yarrowia lipolytica* mutant and its application in the

biosynthesis of L-ascorbyl palmitate. *Int. J. Biol. Macromol*., 106, 302-311.

[5] Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme Microb. Technol.*, 39(2), 235-251.

[6] Anbu, P. (2014). Characterization of an extracellular lipase by *Pseudomonas koreensis* BK-L07 isolated from soil. *Prep. Biochem. Biotechnol.*, 44(3), 266-280.

[7] Priji, P., Unni, K. N., Sajith, S., Binod, P., & Benjamin, S. (2015). Production, optimization, and partial purification of lipase from *Pseudomonas* sp. strain BUP 6, a novel rumen bacterium characterized from Malabari goat. *Biotechnol. Appl. Biochem.*, 62(1), 71-78.

[8] Vakhlu, J. (2006). Yeast lipases: enzyme purification, biochemical properties and gene cloning. *Electron. J. Biotechnol.*, 9(1), 69-85.

[9] Patel, R. N. (2008). Synthesis of chiral pharmaceutical intermediates by biocatalysis. *Coord. Chem. Rev*., 252(5-7), 659-701.

[10] Singh, A. K., & Mukhopadhyay, M. (2012). Overview of fungal lipase: a review. *Appl. Biochem. Biotechnol*., 166(2), 486-520.

[11] Dong, H.N., Zhao, X.M., Yuan, M.A.Y., Zhang, M.H. (2012) Optimization of a synthetic medium for ethanol production by xylose-fermenting *Zymomonas mobilis* using response surface methodology., *Chin. Sci. Bull.*, 57, 28-29.

[12] Maia, M. M. D., Heasley, A., De Morais, M. C., Melo, E. H. M., Morais Jr, M. A., Ledingham, W. M., & Lima Filho, J. L. (2001). Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation. *Bioresour. Technol.*, 76(1), 23-27.

[13] Fickers, P., Nicaud, J. M., Gaillardin, C., Destain, J., & Thonart, P. (2004). Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*. *J. Appl. Microbiol.*, *96*(4), 742-749.

[14] Rajendran, A., Thirugnanam, M., & Thangavelu, V. (2007). Statistical evaluation of medium components by Plackett-Burman experimental design and kinetic modeling of lipase production by *Pseudomonas fluorescens*. 6, 469-478.

[15] Salihu, A., & Alam, M. Z. (2012). Production and applications of microbial lipases: a review. *Sci. Res. Essays*, 7(30), 2667-2677.

[16] Soleymani, S., H. Alizadeh, H. Mohammadian, E. Rabbani, F. Moazen, H. M. Sadeghi, Z. S. Shariat, Z. Etemadifar & M. Rabbani (2017). Efficient Media for High Lipase Production: One Variable at a Time Approach. *Avicenna J. Med. Biotechnol*. 9(2), 82.

[17] Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., & Tripathi, C. K. M. (2017). Strategies for fermentation medium optimization: an in-depth review. *Front. Microbiol.*, *7*, 2087.

[18] Abdel-Fattah, Y. R., Soliman, N. A., Yousef, S. M., & El-Helow, E. R. (2012). Application of experimental designs to optimize medium composition for production of thermostable lipase/esterase by *Geobacillus thermodenitrificans* AZ1. *J Genet Eng Biotechnol*., 10(2), 193-200.

[19] Chauhan, K., Trivedi, U., & Patel, K. C. (2007). Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. *Bioresour. Technol*., 98(1), 98-103.

[20] Salihu, A., Bala, M., & Bala, S. M. (2013). Application of Plackett-Burman experimental design for lipase production by *Aspergillus niger* using shea butter cake. Int. Sch. Res. Notices, 2013, 1-5.

[21] Al Mamun, M. A., Mian, M. M., Saifuddin, M., Khan, S. N., & Hoq, M. M. (2017). Optimization of fermenting medium by statistical method for production of alkaline protease by *Bacillus licheniformis* MZK05M9. *J. Appl. Biol. Biotechnol.*, 5(6), 24-28.

[22] Maharana, A. K., & Ray, P. (2014). Application of Plackett-Burman Design for improved cold temperature production of lipase by psychrotolerant *Pseudomona*s sp. AKM-L5. *Int. J. Curr. Microbiol. App. Sci*, 3(4), 269-282.

[23] Lanka, S., & Latha, J. N. L. (2015). Response surface methodology as a statistical tool for fermentation media optimization in lipase production by palm oil mill effluent (POME) isolate *Emericella nidulans* NFCCI 3643. *Int. J. Innov. Res. Sci. Eng.. Technol.*, 4(4), 2535-2545.

[24] Samaei-Nouroozi, A., Rezaei, S., Khoshnevis, N., Doosti, M., Hajihoseini, R., Khoshayand, M. R., & Faramarzi, M. A. (2015). Medium-based optimization of an organic solvent-tolerant extracellular lipase from the isolated halophilic *Alkalibacillus salilacus*. *Extremophiles*, 19(5), 933-947.

[25] Yalçın, H. T., Çorbacı, C., & Uçar, F. B. (2014). Molecular characterization and lipase profiling of the yeasts isolated from environments contaminated with petroleum. *J. Basic Microbiol*., 54(S1), S85-S92.

[26] Rajput, K. N., Patel, K. C., & Trivedi, U. B. (2016). Screening and selection of medium components for cyclodextrin glucanotransferase production by new alkaliphile *Microbacterium terrae* KNR 9 using Plackett-Burman design. *Biotechnol. Res. Int.*, 2016, 1-7.

[27] Montgomery, D. C. (2017). Design and analysis of experiments, 9th edition, Hoboken, New Jersey, John Wiley & Sons, Inc, p. 1-731.

[28] Baş, D., & Boyacı, I. H. (2007). Modeling and optimization I: Usability of response surface methodology. *J. Food Eng.*, 78(3), 836-845.

[29] Myers, R. H., Montgomery D. C., and C. Anderson-Cook (2009). Response surface methodology. Hoboken, New Jersey, John Wiley & Sons, Inc, p. 38-44.

[30] Obradors, N., Montesinos, J. L., Valero, F., Lafuente, F. J., & Sola, C. (1993). Effects of different fatty acids in lipase production by Candida rugosa. *Biotechnol. Lett.*, *15*(4), 357-360.

[31] Royer, A., Gerard, C., Naulet, N., Lees, M., & Martin, G. J. (1999). Stable isotope characterization of olive oils. I—Compositional and carbon-13 profiles of fatty acids. *J. Am. Oil Chem.' Soc*., 76(3), 357-363.

[32] Boskou, D., Blekas, G., & Tsimidou, M. (2006). Olive oil composition. In: Olive Oil, D. Boskou (ed.), 2nd edition, Academic Press and AOCS Press, p 41-72.

[33] Pau, H. S., & Omar, I. C. (2004). Selection and optimization of lipase production from Aspergillus flavus USM A10 via solid state fermentation (SSF) on rice husks and wood dusts as substrates. *Pak. J. Biol. Sci*, 7, 1249-1256.

[34] Mukhtar, H., Khursheed, S., Mumtaz, M. W., Rashid, U., & Al-Resayes, S. I. (2016). Optimization of lipase biosynthesis from *Rhizopus oryzae* for biodiesel production using multiple oils. *Chem. Eng. Technol*., 39(9), 1707-1715.