Isolation and Identification of Lipolytic Enzyme Producing *Pseudomonas* sp. OME and Optimization of Cultural Conditions

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Lipolytic enzyme-producing bacteria were isolated from edible oil mill effluents on tributyrin agar medium. The shake-flask-scale studies yielded a promising isolate and it was identified as *Pseudomonas* sp. An OME using various microbiological observations such as cultural, microscopic, and biochemical tests was undertaken and confirmed using PIBWIN bacterial identification software. Lipolytic enzyme production was screened with oils such as sunflower, caster, coconut, tributyrin, and olive. Amongst these, olive oil showed an increased lipase production 6.1 U/ml. In view of the highest lipolytic enzyme production with olive oil, further optimizations were carried out using olive oil as a carbon source. Lipolytic enzyme production was optimized by a conventional 'one variable at a time' approach and the significant factors were further analyzed statistically using response surface methodology (RSM). The effect of physical factors such as incubation time, temperature, initial medium pH, and nutritional factors such as concentration of olive oil and yeast extract were examined for lipase production. Lipolytic enzyme secretion was strongly affected by three variables (incubation time, concentration of yeast extract and olive oil). Therefore, the interaction of these three factors was further optimized using response surface methodology. The optimized conditions of lipase production using response surface methodology yielded a maximum of 9.62 U/ml with optimum conditions for incubation, yeast extract and olive oil concentrations were found to be 48 hr, 0.3 g. and 0.9 ml. respectively.

Key words : Oil mill effluents (OME), isolation, lipolytic enzyme, identification, *Pseudomonas* sp. OME, RSM, optimization

Introduction

Historically, the selection of microorganisms that produce exoenzymes has been empirical, starting with samples from very diverse environmental sources. Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry. Microbial lipases have been the subject of many important studies because of their availability and the large number of reactions they catalyze. Lipases (E.C. 3.1.1.3) are enzymes that are primarily responsible for the hydrolysis of acylglycerides. The industrial importance is the hydrolysis of vegetable oils, such as olive oil or coconut oil, to produce fatty acids and glycerol, both of which find widespread applications, especially in soaps and detergents, others such as in cosmetics, pharmaceuticals, food, organic synthesis, dairy, fat and oil modification [1,8,15,17,22]. Most research has concentrated on extracellular lipases that are produced by many different microbial species. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains [9,18]. The potent isolates of the genus belonging to *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium and Pseudomonas* are most extensively used for lipase porudction [9]. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications [2,9,19]. The synthesis and secretion of lipases by bacteria is influenced by a variety of environmental factors like ions, carbon sources, or presence of non-metabolizable polysaccharides. The secretion pathway is known for *Pseudomonas* lipases with *P. aeruginosa* lipase using a two-step mechanism and *P. fluorescens* lipase using

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a one-step mechanism [9]. Optimization of cultivation conditions is expected to improve the enzyme production. In order to attain higher enzyme titres, a number of factors need to be optimized, including a suitable organism and the process [10,12]. The optimization of physical and nutritional factors for submerged and solid state enzyme production is an important problem in the development of economically feasible bioprocesses. Combinatorial interactions of medium components with the production of the desired enzyme are numerous and the optimum processes may be developed using an effective experimental design procedure. RSM which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of bioprocesses [4,5,6,11,20,26]. The goal of present study is to achieve a high level of lipolytic enzyme lipase production by newly isolated promising *Pseudomonas* sp. OME by optimization of physical and nutritional factors using a statistical experimental design.

Materials and Methods

Screening and selection of high lipase producing bacterial isolate

Lipolytic bacteria were isolated from oil mill effluents around Tirupati, India. The ability to produce extracellular lipase was determined by culturing the bacterial isolates on tributyrin agar medium at 37°C for 24-48 hr [13]. A total of 9 bacterial colonies were selected (designated as OME i.e. oil mill effluent I to IX) and tested for enzyme production in shake-flask-scale studies.

Fermentation

The liquid broth (yeast extract 2.5 g/l, K_2HPO_4 1.0 g/l, $MgSO_4.7H_2O$ 0.05 g/l) was amended with olive oil (1%, v/v) and the final pH adjusted to 7.0. The 50 ml liquid broth medium was prepared in 250 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. The 5 hr old cultures (0.2 $OD_{600\ nm}$) inoculated at 5% (v/v) and the flasks were incubated at 30°C for 48 hr with 120 rpm in an orbital shaking incubator.

Measurement of bacterial growth and assay of lipase activity

The bacterial growth in the fermented broth medium was determined using a spectrophotometer at 600 nm. The lipase activity in the fermented broth medium was measured by centrifugation of fermented culture at 10,000 rpm for 15 min at 4°C and the crude centrifugal supernatant was used as enzyme source. Determination of lipase activity was done by the titrimetric method [14,16]. The emulsion of olive oil (10%, v/v) was prepared in arabic gum (5%) in sodium phosphate buffer (0.1 mol/l pH 7.0). The assay mixture contained 2 ml of emulsified olive oil as substrate and appropriately diluted enzyme in phosphate buffer (0.1 M, pH 7.0) at 50°C for 30 min and the enzyme activity was stopped immediately by incubating into boiling water bath for 3 min. Phenolpthalein indicator added to the hydrolyzed mixture and titrated against 0.05 M NaOH solution. One unit of lipase activity was defined as 1.0 μmol of fatty acid liberated per min.

Statistical analysis

Data presented are the averages of three replicates. The statistical analysis for standard deviation was carried out using Instat+v3.33 and SPSS 10.0 soft-ware packages.

Identification of bacterial isolate

Amongst the primary isolates, the promising one selected by plate assay and shake flask culture was used for identification. The selected promising bacterial culture used for preliminary microbiological studies such as cultural, microscopic and biochemical observations.

Optimization of lipase production by 'one variable at a time'

The factors were studied by modifying the medium composition according to the conditions and factors, which were studied. The effect of different oils such as sun flower, caster, coconut, tributyrin and olive on lipase production was screened in shake-flask-scale culture studies by supplementing oils at 1% to the liquid medium and the enzyme extraction and assay carried out as described above. The effect of different physical and nutritional factors for lipase production by *Pseudomonas* sp. OME was investigated by culturing in liquid broth supplemented with 1% olive oil at 30°C under shaking condition, during cultivation lipase activity was monitored. The effect of incubation period on lipase production was observed at regular 12 hr interval, over a period of 72 hr. In the view of maximum lipase production with olive oil at 48 hr of incubation, further optimizations were carried out at this point. The effect of incubation tem-

perature from 30-50°C and the effect of initial medium pH was determined by preparing the Luria broth medium in buffer solution (0.1 M) from 4-8. The effect nutritional factors such as concentration of olive oil (0.3-1.6) and yeast extract (0.1-0.5) on lipase production was screened.

Experimental design using RSM

The significant factors influencing lipase production from 'one variable at a time' method was further optimized by RSM. Three factors selected from 'one variable at a time' approach, were further optimized and used in designing the experiment: Incubation time (A), Olive oil (B) and Yeast extract concentration (C). The ranges of the variables investigated in this study are given in Table 4. A central composite quadratic design containing 20 experiments (20 runs) was generated using Design-Exeper® software (7.1.6 version), lipase production was taken as an independent variable or response.

The results of RSM were used to fit a second order plynominal equation (1) as it represents the behaviour of response [12].

$$Y=\beta_o+\beta_1 A+\beta_2 B+\beta_3 C+\beta_1\beta_1\ A^2+\beta_2\ \beta_2\ B^2+\beta_3\ \beta_3 C^2 +\beta_1\ \beta_2 AB+\beta_1\ \beta_3\ AC+\beta_2\ \beta_3\ BC \quad (1)$$

Y=response variable, β_o=intercept, β_1, β_2, β_3=linear coeeficients, $\beta_1\beta_1$, $\beta_2\ \beta_2$, $\beta_3\ \beta_3$=Suqared coeeficients, $\beta_1\ \beta_2$, $\beta_1\ \beta_3$, $\beta_2\ \beta_3$=interaction coeficients, and A, B, C, A^2, B^2, C^2, AB, AC, BC=level of independent variables.

Results and Discussion

Screening, selection and identification of potent bacterial isolate

The bacterial isolates used in this study were isolated from oil mill effluent samples. The bacterial cultures were grown on tributyrin agar medium 24-48 hr at 30°C and the formation of clear hydrolysis zone gave an indication of lipolytic enzyme producing cultures (Fig. 1). Amongst 9 isolates selected and they were designated as OME (oil mill effluent) I to OME IX. The cultural, microscopic and plate assay characteristics of lipolytic enzyme producing bacterial isolates were given in Table 1. The flask scale culture studies resulted one of the promising bacterial isolate (OME V) with lipase production 5.6 U/ml and it was used for further studies (Fig. 2). The selected bacterial isolate was identified by cultural, microscopic and biochemical tests as *Pseudomonas* sp. OME using PIBWIN (Probabilistic Identification of Bacteria for Windows) bacterial identification software with identification score 64%, the results of these observations are given in Table 2 [3].

Optimized conditions by 'One variable at time method'

The results of lipase production and bacterial growth with

Fig. 1. Screening of lipase production by selected bacterial isolates using tributyrin agar plate assay method.

Table 1. The colony, microscopic and plate assay characteristics of selected bacterial isolates for lipase production

S.No.	Strain No	Colony color	Microscopic observation	Colony diameter (mm)	Activity Zone (mm)
1	OME I	Colour less	Gram +ve, Spore forming	4	-
2	OME II	Colour less	Gram +ve, Spore forming	3	6
3	OME III	Colour less	Gram +ve, Non spore forming	5	-
4	OME IV	Colour less	Gram +ve, Spore forming	3	5
5	OME V	Green	Gram -ve, Non spore forming	4	13
6	OME VI	Green	Gram -ve, Non spore forming	3	5
7	OME VII	Green	Gram -ve, Non spore forming	4	2
8	OME VIII	Green	Gram -ve, Non spore forming	3	-
9	OME IX	Colour less	Gram +ve, Spore forming	2	-

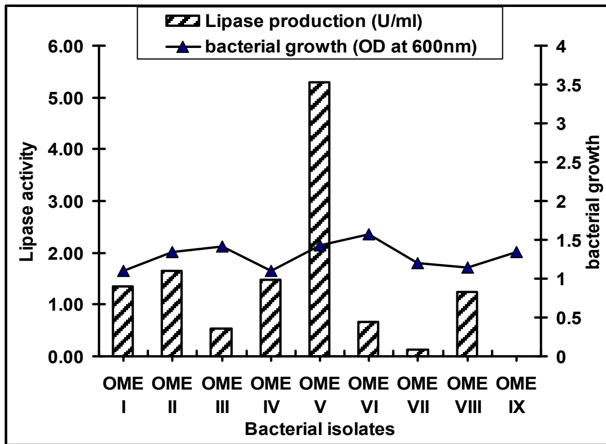

Fig. 2. Screening and selection of promising isolate for lipase production by flask scale culture. OME, Oil mill effluent.

Table 2. Macroscopic and microscopic and certain biochemical tests performed to promising bacterial isolate

Characterstics	Result
Colony size	2-3 mm
Spore staining	- ve
Gram's staining	- ve
Motility	+ ve
Pigment production	+ve, Green colour
Capsule staining	- ve
Indole production	-ve
Methyl red reaction	+ve
V .P. reaction	- ve
Citrate hydrolysis	-ve
Starch hydrolysis	-ve
Casein hydrolysis	-ve
Gelatin hydrolysis	-ve
Urea hydrolysis	-ve
Catalase test	+ve
Oxidase test	+ve
O/F test	-ve
TSI test	-ve
H_2S Test	-ve

different oils such as sunfloweroil, castor oil, coconut oil, tributyrin oil and olive oil by *Pseudomonas* sp. OME shown in Fig 3. High yield of lipase found with olive oil, th ideal substrate for inducing the enzyme production by *Pseudomonas* sp. OME. The effect of physical factors such as incubation time, temperature and initial medium pH on lipolytic enzyme production was shown in Table 3. The enzyme production was increased with incubation time from 12-48 hrand further increase decreased the enzyme activity. The optimum incubation temperature and initial medium pH for enzyme production was found to be at 30°C and pH 7 respectively, with ~7 U/ml (Table 3). The optimum nutritional factors for olive oil and yeast extract concentration found to be at 1.2 and 0.3% (Table 4). The data suggested that the incubation period and the concentrations of olive oil and yeast extract was significantly influenced enzyme production when incubation temperature and medium pH kept constant.

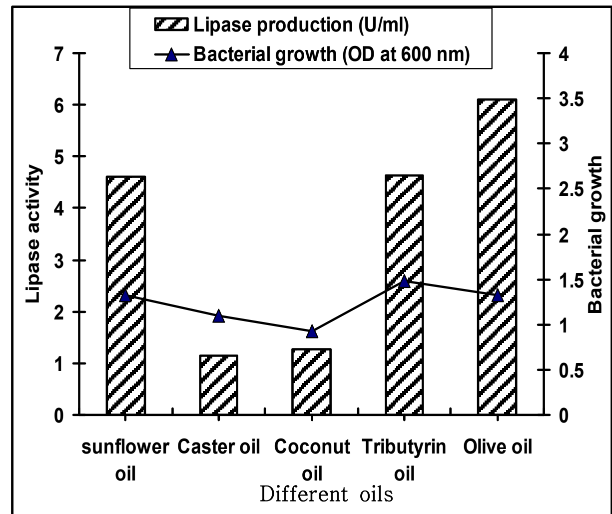

Fig. 3. Bacterial growth and lipase production using different oils by *Pseudomonas fluorescence*.

Table 3. Effect of incubation time, temperature and initial medium pH on lipase production

Incubation time (hr)	U/ml	SDE
12.0	0	0
24.0	1.5	0.08
36.0	4.5	0.14
48.0	6.8	0.18
60.0	3.1	0.11
72.0	2.7	0.07
Temperaure (°C)	**U/ml**	**SDE**
30	6.8	0.22
40	2.1	0.12
50	0	0.00
Initial medium pH	**U/ml**	**SDE**
4	0	0.00
5	2.4	0.08
6	4.2	0.12
7	7.7	0.24
8	0	0.00

Values in the table are means of 3 replicates ±SDE, standard deviation error.

Table 4. Effect of yeast extract and olive oil concentration on lipase production

Olive oil concentration (%, v/v)	U/ml	SDE
0.3	5.1	0.11
0.6	6.3	0.02
1.2	7.1	0.07
1.6	6.2	0.18
Yeast extract concentration (%, w/v)	**U/ml**	**SDE**
0.1	3.3	0.14
0.2	5.1	0.08
0.3	7.1	0.11
0.4	7.0	0.06
0.5	6.8	0.13

Values in the table are means of 3 replicates ± SDE, standard deviation error.

Optimization of lipase production by 'RSM' (Multivariate analysis)

The response surface graphs were prepared for the generated experimental data for lipolytic enzyme production. Overall significant model terms could be explained as the maximum enzyme production. In the interaction studies of incubation time, olive and yeast extract concentration, a peak in lipase production (9.3 U/ml) was attained with incubation time 48 h, oilive oil concentration 0.9% and the yeast extract concentration 0.3% by *Pseudomonas* sp. OME. The significance of regression equation was determined by comparing the F value of the model equation and the lack of fit with the tabulated F value. Regression analysis was performed to fit the response (lipase production) with the experimental data. The values of the analysis of variance (ANOVA) for the fitted model are given in Table 6. The coefficient of determination (R^2) for lipase production, as a function of independent variables, was 81%, which indicated that the model correlated well with the measured data and was statistically significant. The probability of the model equation implying that the model is significant. ANOVA for the three variables indicated that lipolytic enzyme production could be described well by a polynomial model with a relatively high coefficient of determination. The quadratic model for predicting the optimal point is expressed in equation (1), and the model predicted the response satisfactorily for enzyme production. The interaction between factor A (incubation time, h), factor B (olive oil, %) and factor C (yeast extract, %) on lipase production was shown in response surface graphs. The interaction between factor A and factor B

Table 5. The level of factors chosen for the central composite quadratic design for lipase production

Factor	Units	Low Actual (Coded)	High Actual (Coded)
Incubation time : A	hr	24 (-1.0)	72(1.0)
Olive oil : B	%, v/v	0.5 (-1.0)	1.6 (1.0)
Yeast extract : C	%, w/v	0.1 (-1.0)	0.5 (1.0)

Table 6. The central composite quadratic design for lipase production for three independent factors

Run Order	Incubation time	Olive oil	Yeast extract	Actual value	Predicted value
1	0.00	0.00	0.00	9.25	9.58
2	1.00	-1.00	1.00	3.20	2.94
3	0.00	0.00	0.00	9.15	9.58
4	-1.00	1.00	1.00	6.85	7.16
5	-1.00	1.00	-1.00	5.52	4.59
6	0.00	1.57	0.00	9.15	8.98
7	-1.68	0.00	0.00	0.00	1.49
8	0.00	0.00	-0.50	11.2	8.69
9	0.00	-1.28	0.00	2.10	5.87
10	1.00	0.00	-0.50	4.53	5.63
11	0.00	0.00	1.00	8.68	8.30
12	1.00	1.00	1.00	4.35	4.70
13	0.00	0.00	-1.50	1.12	3.82
14	-1.00	-1.00	-1.00	2.24	0.70
15	0.00	-0.57	0.00	9.19	8.40
16	1.00	1.00	-1.00	3.14	2.88
17	1.00	-1.00	-1.00	4.23	2.45
18	0.00	-0.57	0.00	9.12	8.40
19	0.00	0.00	0.00	9.48	9.58
20	-1.00	-1.00	1.00	3.14	1.93

Table 7. Analysis of variance and regression coefficient of the model representing relationship between lipase production

Source	Mean Squares	F- Value	p-value Prob > F	
Model	19.69	4.77	0.011	significant
A-Incubation (hr)	*0.33*	*0.08*	*0.782*	
B-Olive oil (%, V/V)	*24.58*	*5.95*	*0.034*	
C-Yeast extract	*6.44*	*1.56*	*0.240*	
AB	*6.00*	*1.45*	*0.255*	
AC	*0.29*	*0.06*	*0.797*	
BC	*0.89*	*0.22*	*0.652*	
A2	*76.38*	*18.5*	*0.001*	
B2	*11.01*	*2.67*	*0.133*	
C2	*28.20*	*6.83*	*0.025*	

R^2=81%

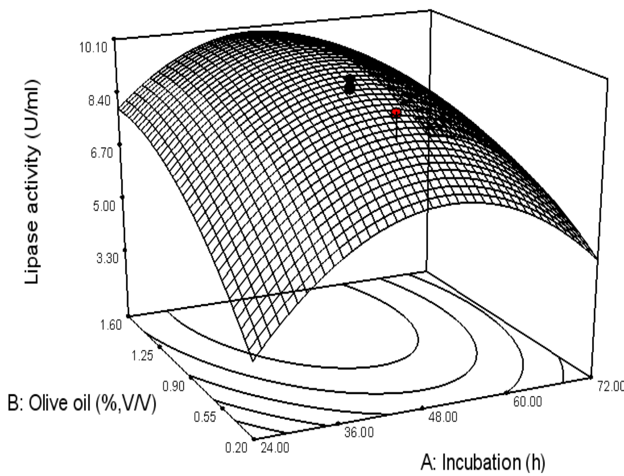

Fig. 4. Response surface for lipase production. The interaction between concentration of olive oil (%) and incubation time (hr).

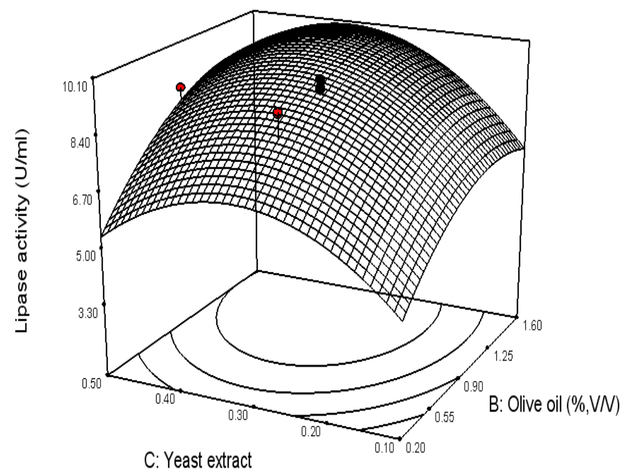

Fig. 6. Response surface for lipase production. The interaction between concentration of yeast extract (%) and olive oil (%).

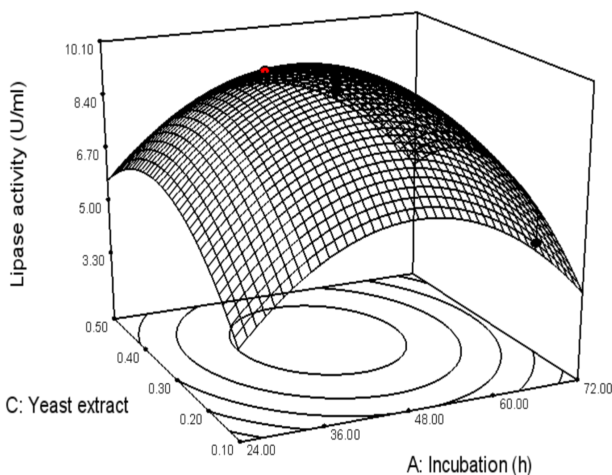

Fig. 5. Response surface for lipase production. The interaction between concentration of yeast extract (%) and incubation time (hr).

was shown in Fig. 4, the interaction between factor A and factor C was shown in Fig. 5, the interaction between factor B and factor C was shown in Fig. 6.

Final equation in terms of coded factors:

$$\text{Lipase activity} = 9.58-0.18A+1.41B+0.76C-2.97A2-1.14\ B2 -2.05C2-0.87AB-0.19\ AC+0.33BC$$

With the advent of new frontiers in biotechnology, the spectrum of lipase application has extended in many other fields. Many different bacterial species produce lipases which hydrolyze esters of glycerol with preferably long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. A characteristic property of lipases is called interfacial activation, meaning a sharp increase in lipase activity observed when the substrate starts to form an emulsion,

thereby presenting to the enzyme an interfacial area [9]. The lipase production in the optimized medium, such as wild-type strain of *Pseudomonas fluorescens* isolated from soil with 1.4 U/ml [24], a low-cost medium has been developed for growth and lipase production (8.3 U/ml) from *Bacillus* THL027 at 65°C and pH 7.0 [7]. The 'one variable at a time' approach for optimizing microbial products may be effective in some situations, but is inadequate as it is a time consuming process and it further neglects the interaction between variables and does not guarantee attaining optimal points [23]. The statistical design is efficient and effective because it provides a good coverage of the experimental space with as few measurements as possible. The collection of statistical techniques for experiment design, model development, evaluation factors, and optimum conditions search. Thus the use of RSM in biotechnological processes is gaining immense importance for the optimization of enzyme production and several other processes [20,21,25]. Production of lipase using various oils was found to have several advantages, such as higher productivity as well as lower operational and capital costs. Because the cost of the enzyme is the major factor for broad application, approaches that either decrease the medium cost or increase production efficiency should be investigated. Since lipase production on a variety of edible oils, the cost of lipase production can be reduced greatly.

In conclusion the current demand of lipases with improved properties has initiated a continuous search for microorganisms producing novel lipases for industrial applications. Statistical optimization of the enzyme production is required to overcome the limitations of classical empirical method and proved to be a powerful and useful tool for the optimization of enzymes.

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초록 : 지방분해효소 생산균 *Pseudomonas* sp. OME 의 분리 동정 및 배양조건 최적화

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폐식용유에서 지방분해효소를 생산하는 세균을 분리하였고, PIBWIN 세균동정 방법으로 생리 생화학적 특성을 조사하여 확인한 결과 *Pseudomonas* sp. OME로 동정하였다. 여러 기질로 지방분해효소 생산을 조사한 결과 올리브유에서 6.1 U/ml의 생산력을 나타내었다. 물리적 인자인 배양시간, 온도. pH 및 올리브유와 효모 추출액의 영양인자에 의한 지방분해효소 생산 조건을 조사 하였다. 효소의 분비는 배양시간. 올리브유 와 효모 추출액의 농도에 강한 영향을 받았으며, RSM을 이용한 최적화는 이들 인자를 가지고 조사하였다. RSM을 이용한 지방분해효소 생산은 배양시간. 올리브유와 효모 추출액의 농도가 48 hr, 0.3 g, 및 0.9 ml에서 최적 생산조건을 나타냈다.