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Using Submerge Fermentation Method to Production of Extracellular Lipase by *Aspergillus niger*

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***Abstract*—**In this study, lipase production has been investigated using submerge fermentation by *Aspergillus niger* in Kilka fish oil as main substrate. The Taguchi method with an L9 orthogonal array design was used to investigate the effect of parameters and their levels on lipase productivity. The optimum conditions for Kilka fish oil concentration, incubation temperature and pH were obtained 3 gr./ml 35°C and 7, respectively. The amount of lipase activity in optimum condition was obtained 4.59IU/ml. By comparing this amount with the amount of productivity in the olive oil medium based on the cost of each medium, it was that using Kilka fish oil is 84% economical. Therefore Kilka fish oil can be used as an economical and suitable substrate in the lipase production and industrial usages.

***Keywords*—**Lipase, *Aspergillus niger*, Kilka Fish oil, Submerge Fermentation method.

I. Introduction

LIPASE (triacylglycerol acylhydrolases, EC 3.1.1.3), as a biocatalyst catalyzes the hydrolysis of esters and triacylglycerol to glycerol and fatty acids by esterification, trasense-esterification and hydrolysis reactions. Lipases are serine hydrolases which has α/β-hydrolase fold [1], [2]. The catalytic triad is composed of Ser- Asp/Glu-His around the active site [3]. The growing tendency for commercial application of lipase is its capability in maintaining catalytic activity in organic solutions and conversion of excess fats and oils to the products with high value [3], [4]. Lipase is used in dairy products in order to create a different taste, production of detergents with high capability of detergency, biodiesel, especially in peptide synthesis and production of bio-surfactant [2], [5]. Lipase can be produced from plants, alga, animals, and microorganisms. microbial sources by having traits such as capability of using inexpensive substrate, low production cost, possibility of using mutagenesis techniques in microorganism in order to achieve high productivity species are more suitable than plant and animal lipase for industrial applications[6], [7]. Studies have shown that, fungi have high lipase production efficiency in comparison to the other sources. Among these studies, *Aspergillus niger* had more capability in lipase production than other mold strains [8]-[10]. In order to produce lipase by microorganism in the media with high efficiency and stability, the submerge fermentation method can be used. Using vegetable or animal oil in this fermentative method had been necessary for lipase production; therefore it can be referred to Kilka fishoil [11]-[13] (Table I).

TABLE I
THE COMPOSITION OF FATTY ACIDS IN KILKA FISH OIL [13]

Fatty Acid	Amount (%)
C14	4.29
C16	8.22
C 16:1	23.57
C18	2.26
C 18:1	29.33
C 18:2	4.67
C 18:3	--
C 20:4	1.14
C 20:5	5.59
C 22:6	9.4
$\frac{EPA}{DHA}$	0.839
EPA + DHA	14.99

Kilka fish oil is a by-product of Kilka fish meal factories. It is used in leather industries, feeding farmed fish and poultry food. Because of having highest content of oleic acid as lipase inducer in comparison to the other fatty acids in kilka fish oil, it can produce lipase enzyme. In this paper, the production of lipase was investigated in kilka fish oil media as a main and economical substrate in submerge fermentation by *Aspergillus niger* in different levels of three factors like; pH, incubation temperature and kilka fish oil concentration. The Taguchi experiment design method was employed to optimize the experiment conditions of lipase production.

II. Materials and Methods

A. Materials

In this research, the fungal strain *Aspergillus niger* ATCC 9142 was used. This strain was stored and maintained in Potato Dextrose agar slants in 4°C. The Fermentation medium for lipase production was included: Kilka fish oil (bought from Babolsarkilka fish oil and meal factory 581), glucose, yeast extract, KH_2PO_4, KCl, and $MgSO_4.7H_2O$ (German Merk Company).

B. Method of Designing Experiment

In this study, the optimum process conditions for lipase

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production with Kilka fish oil as main substrate were determined according to the Taguchi method. Based on the Taguchi experimental design method, Experimental parameters and their levels to be studied, which were determined in light of preliminary tests, are given in Table II. All experiments was performed two times under identical conditions and their mean values have been reported. An L9 orthogonal array (OA) had been chosen based on the number of factors and levels mentioned in Table II [14].

TABLE II
PARAMETERS AND THEIR LEVELS USED IN THE TAGUCHI METHOD

Parameters	Level1	Level2	Level3
pH	5	7	9
Kilka fish oil concentrate (gr/100ml)	1	2	3
Temp (8°C)	25	35	45

TABLE III
EXPERIMENTAL LAYOUT USING AN L9 ORTHOGONAL ARRAY

Test \ Parameters	pH	Kilka fish oil concentrate (gr/100ml)	Temp (°C)
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

The pH Parameter was chosen in order to determine lipase tolerance in acidic, neutral and alkaline levels. Also the concentration of Kilka fish oil and the temperature for determiningthe best levels of lipase production and achieving the highest lipase production efficiency were used. To analyze the results, Taguchi method has used a statistical measurement of performance called the signal-to-noise (S/N) ratio. The S/N ratio depends on the criterion for the quality characteristic to be optimized. In this study, the Bigger the best has been used

C. Lipase Production

Fermentation medium for lipase production included; glucose, yeast extract, KH_2PO_4, KCl, $MgSO_4.7H_2O$ respectively as 1, 0.05, 0/2, 0.05, 0.05 (g/100ml). Also Kilka fish oil concentrations had designed variables and changed according to experimental design (Table III). After sterilization of Fermentation medium in 121°C for 15 minutes, 1 ml spore suspension containing 10^8 spore/ml was added to each Erlenmeyer flask which contained 100 ml of the fermentation medium. Then the inoculated media incubated in a shaker incubator with the 170 rpm for 96 h in the three temperature levels (45-35-25°C). [9], [15] At the end of fermentation, mycelium was filtered by Whatman's filter paper. Then for transparency and removing impurities, samples were centrifuged at 3500 rpm for 20 minutes in the room temperature. Finally the clear supernatant obtained was called crude enzymatic extract [15].

D. Lipase Activity Measurement

Lipase activity was measured titrimetrically by using reactive media which contained 1 gr. olive oil with 1 ml of extracted enzyme and 4 ml Phosphate buffer M 0.1 in different levels of pH. The solution was kept in a shaker incubator (130 rpm) at temperature of 30˚C for 60 min. A control sample was separately 1 mL of olive oil and 1 mL of enzymatic extract was titulated as the same way. The activity was determined by titulation of released fatty acids with 50 mM potassium hydroxide using phenolftaleine as indicator. One international unit (IU) of lipase activity was defined as the amount of lipase which catalyzes the release of 1 µmol of oleic acid per min under the assay conditions [16].

$$U = \frac{(Va-Vb)\times M\times 1000}{Vc\times t} \quad (1)$$

III. DISCUSSION AND RESULTS

In order to demonstrate the capability of lipase productivity by Kilka fish oil based on the mentioned method, at first, an experiment was performed by 1 gr. Kilka oil.

The enzyme activity compared with control sample was 3/26 IU/ml. It was shown that Kilka fish oil as the main substrate had the capability in lipase production. S/N Ratio response observed in 9 performed experiments (Table IV).

According to the Table V and Fig. 1 for determination of main Parameter and level, the results shown that , the best Parameter and level of experiments had been the level 2 from pH Parameter which was in the neutral range (pH= 7). Also the Level 3 of Kilka fish oil concentration Parameter (3 gr.) was determined as the best main substrate. Because of having high nutrients in 3 gr in compared with 1-2 gr of main substrate, it had been the appropriate and optimized conditions for growing *Aspergillus niger* and production of lipase. Fig. 1 presents the S/N response observed in the experiments with respect to these variables. This figure shows the influence of each individual factor on the mean S/N ratio. Regarding the best determined factors and levels by S/N ratio which were not included in the 9 designed experiments, an experiment was performed based on the optimized conditions. High lipase activity is the main indicator of optimum conditions in this study.

TABLE IV
S/N RATIO

Test	Kilka fish oil concentrate (gr/100ml)	pH	Temp (˚C)	Enzyme Activity IU/ml 1th Test	Enzyme Activity IU/ml 2th Test	S/N Ratio
1	1	5	25	2.416	3.6	9.057
2	2	5	35	3.08	3.04	9.713
3	3	5	45	3	2.916	9.417
4	1	7	35	4.5	4.6	13.158
5	2	7	45	3.16	3.08	9.88
6	3	7	25	4.08	4.2	12.337
7	1	9	45	2.83	2.83	9.035
8	2	9	25	2.5	2.45	7.87
9	3	9	35	4	4.03	12.073

TABLE V
MAIN EFFECTS FOR S/N RATIO TO DETERMINE THE OPTIMUM CONDITION

Parameters	Level1	Level2	Level3	L2-L1
pH	3.396	11.792	9.659	2.395
Kilka fish oil concentrate (gr/100ml)	10.417	9.154	11.276	-1.263
Temp (°C)	9.754	11.648	9.444	1.894

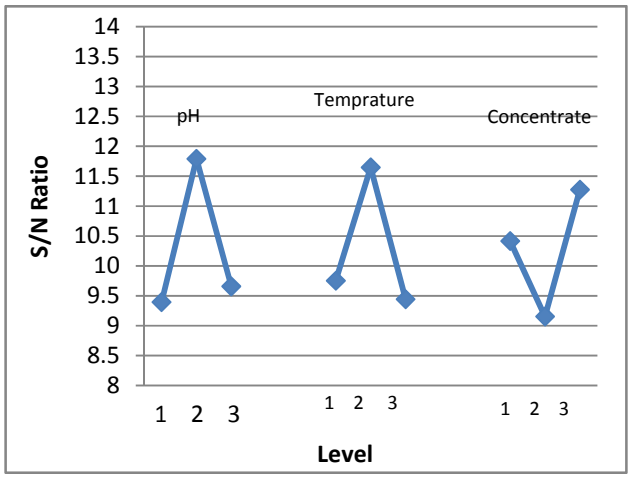

Fig. 1 Main effects plot for S/N ratio versus level of parameters

In Taguchi's method, analysis of variance (ANOVA) has been used to analyze the results of the OA experiments and determine how much variation each parameter has contributed. Analysis of variance of the results showed that, pH with 39.815% is the most effective parameter. Temperature with 32.7 % and kilka oil concentrate with26.144 are the less effective ones (see Table X).

TABLE X
ANALYSIS OF VARIANCE (ANOVA) FOR PH, KILKA OIL CONCENTRATE AND INCUBATION TEMPERATURE

Parameters	(DOF)	Sum of squares	Variance	F-Ratio	Percent [%]
pH	2	10.358	5.179	126.602	39.815
Fish oil	2	6.829	3.414	83.476	26.144
Temp	2	8.64	4.27	104.383	32.771
Error [–]	2	0.08	0.04	--	1.27
Total	8	25.809	--	--	100%

According to the low amount of error with 1.27%, this designing Experiment would be significant and acceptable. In order to evaluate the predicted result by ANOVA, in optimum condition confirmation experiment was done. Result Expended at Optimum Condition was determinate in 5.099 IU/mL. The result of confirmation experiments in optimum conditions showed the lipase activity were 4.59 IU/ml. So the experimental result obtained under optimum conditions for lipase production was in good agreement with predicted values. Hosseni pour and et al. studied on lipase production by *Aspergillus niger* NCIM 584 on soya flour. The highest lipase activity in optimized condition at 30◦C in pH=2 at 7.5% of soya flour amount was obtained 3253 U/I. Also, the optimized concentration for olive oil with 12 gr./l was reported as 4455/84 U/I [15]. Pera and et.al reported that, the highest enzyme activity was obtained in the optimized conditions with pH= 6.5 in the medium containing 2% olive oil in the 37◦C [17]. Other independent work by Falony and et.al, showed that the highest enzyme activity as 1.46 IU/ml was in the medium containing 2 % olive oil and 2% glucose, and the optimum pH was observed between 6.5 to 7.5 [9]. By comparing the studies of the similar researches and the results of this experiment, it can be concluded that by increasing the concentration of this temperature range and pH, the highest amount of enzyme activitywas observed. In accordance with the specified optimized conditions (oil concentration 3%, incubation temperature 35°C and pH=7), in order to estimate the cost of Kilka fish oil with olive oil, an experiment under this condition for the mentioned oil was done. (Table XI and Fig. 2).

TABLE XI
PREDICTIVE AND EXPERIMENTAL RESULTS IN OPTIMIZED CONDITIONS

Main substrate	Enzyme Activity IU/ml
Kilka Fish Oil	4.59
Olive Oil	5.46

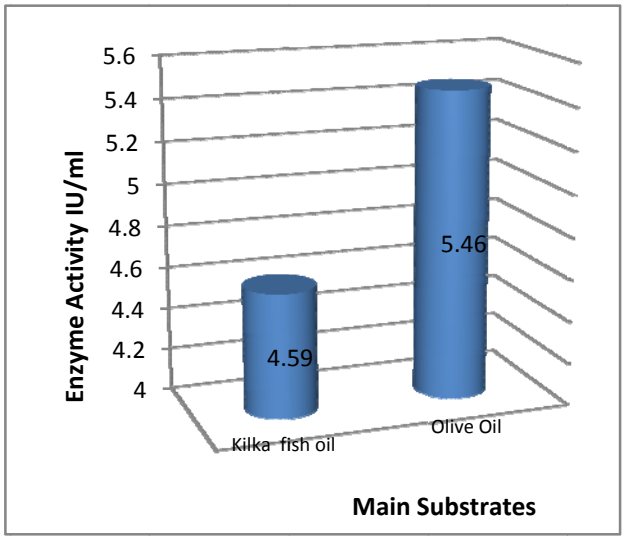

Fig. 2 Comparison between the enzymatic activity of Kilka fish oil and Olive oil as main substrates

The comparison of the enzyme activity for these two oils showed that there is a little difference between the obtained enzyme activity values. Regarding the cost for each kilogram of olive oil (10$)[1] and Kilka fish oil (0.15$), the cost for 3 gr. Kilka fish oil is equal to 0.000345$ and for olive oil is equal to (0.03$). In accordance with the (0.000345$) for 4.59 IU/ml of kilka fish oil enzyme activity, the cost of olive oil enzyme activity (5/46 IU/ml) by Kilka fish oil is equal to 0.000353$ which was too much less than the cost of olive oil (0.03 $). As a result, the cost of kilka fish oil as main substrate for lipase production would be 85% of olive oil cost which using kilka fish oil as a lipase production substrate can have economical

[1] - 1$=30000IRR

value.

IV. Conclusion

In this study, Lipase was produced by *Aspergillus niger* in the submerge fermentation by Kilka fish oil as the main substrate. According to the optimized conditions, the maximum lipase activity was estimated 4.59 IU/ml. ANOVA results indicated that the concentration of kilka fish oil had an influence on lipase activity. Increasing the concentration to a specified amount can facilitate reaching more nutrients for growing *Aspergillus niger* which can be considered as an effective parameter **f**or lipase production with high activity. Also the desired pH and temperature were 7 and 30°C, respectively. In order to estimate the lipase productivity from Kilka fish oil and comparing with olive oil, another experiment was performed with olive in the optimized conditions. The amount of activity was obtained to 5/46 IU/ml. The little difference between them, can be resulted from the percentage of oleic acid (83 %) in olive oil compared with Kilka oil (29.33%). It should be notified that the oleic acid is an effective fatty acid in the lipase production. Regarding the very low price of Kilka fish oil than olive oil, and 84% efficiency, high enzyme activity in the media containing olive oil is justified. Consequently kilka fish oil can be a good and economical media for lipase production.

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