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Isolation and Identification of a Lipase Producing *Bacillus* sp. from Soil

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Abstract: Lipase production in an indigenous lipolytic *Bacillus* sp. was detected in media containing Tributyrin-Tween 80 and Rhodamine B-Olive oil. The statistical Taguchi model was used to predict the optimum experimental conditions for bacterial growth and lipase production. Partial optimization was carried out for selection of salt base, oil, glucose, NH_4Cl and yeast extract concentrations, inoculum density, pH and agitation. Maximum lipase activity was detected in the cell free supernatants of cultures grown in a medium containing 10 g L^{-1} yeast extract, 15 g L^{-1} NH_4Cl, 3 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgSO_4.7H_2O$, 2 g L^{-1} glucose, 0.6 mM $MgCl_2$ and 15 ml L^{-1} olive oil, pH 8.5 at 30°C for 24 h and low agitation. The amount of lipase produced in the designed medium was in agreement with the predicted values by the statistical method. 16S rRNA cloning and sequencing identified the test organism as *Bacillus pumilus*.

Key words: *Bacillus pumilus*, lipase, Taguchi method, soil isolate, molecular identification, 16S rRNA sequencing

INTRODUCTION

Lipases (Triacylglycerol acylhydrolase; EC 3.1.1.3) are versatile and ubiquitous biocatalysts with a wide range of applications in food, dairy, detergent and pharmaceutical industries (Gupta *et al*., 2004a). These enzymes are active at the interface of aqueous and non-aqueous phases which distinguishes them from esterases (Pandey *et al*., 1999). The activity of lipases is independent from cofactors and they do not catalyze side reactions. Moreover, lipases display exquisite chemoselectivity, regioselectivity and sterioselectivity (Jaeger and Eggert, 2002).

Microbial lipases are commercially important because of their unique properties and the ease of bulk extracellular production compared to lipases from other natural sources (Jaeger and Eggert, 2002). Among the lipase producing bacteria, several species of *Bacillus* such as *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. thermoleovorans*, *B. stearothermophilus* and *B. sphaericus* possess lipases suitable for biotechnological applications (Nthangeni *et al*., 2001; Rahman *et al*., 2003; Ruiz *et al*., 2003). In addition, alkalophilic and thermophilic microorganisms have been the focus of many studies for new sources of lipases that are stable and function optimally at extreme alkaline pH values and high temperatures (Nthangeni *et al*., 2001; Sharma *et al*., 2001).

Lipase production is dependent upon a number of factors including carbon and nitrogen sources, pH, temperature, aeration and inoculum size (Kim *et al*., 1996; Gupta *et al*., 2004a). Recently, statistical designs have been employed to optimize enzyme production and minimize the number of experiments (Krishna *et al*., 2001; Rathi *et al*., 2002; Gupta *et al*., 2004b). In this research, we isolated a lipase producing *Bacillus* from soil and partially optimized lipase production using the statistical method of Taguchi. Identification of the organism was carried out both biochemically and by 16S rRNA sequencing.

MATERIALS AND METHODS

The study was conducted in 2006 at the Microbiology Department of Shahid Beheshti University and the National Institute for Genetic Engineering and Biotechnology, Tehran, Iran.

A number of soil *Bacillus* isolates from a previous collection were screened for lipase production (Eftekhar *et al*., 2003). Four isolates were able to produce lipase, one of which (strain F3) was chosen for the present

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study. Plasmid pTZ57R/T (Ins T/A clone, PCR product cloning kit, #k1214, Fermentas) was used as the cloning vector for 16S rRNA and *Escherichia coli* TG1 was used as the recipient strain for recombinant plasmids.

Screening for lipolytic activity: Lipolytic activity was detected on TW agar plates containing 1% Tributyrin and 1% Tween 80 (pH 8). Lipase/esterase production was detected by observing clear zones around isolated colonies (Akatsuka *et al*., 2003). Lipase activity was then detected by growth on Rhodamine B lipase agar at 30°C for 72 h (Kouker and Jaeger, 1987). Colonies which showed orange fluorescence under UV irradiation indicated true lipase activity and non-lipolytic bacteria formed pink colonies.

Cloning and sequencing of the 16S rRNA: Plasmid and genomic DNA were extracted from overnight grown cultures in LB broth using phenol/chloroform as described before (Sambrook *et al*., 2001). Amplification of 16S rRNA was carried out using primer oligonucleotides fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-TAA GGA GGT GAT CCA GC-3') (Weisburg *et al*., 1991) provided by Gen Fanavaran (Tehran, Iran). DNA amplifications were carried out in 50 μL reaction mixtures containing 5 μL of 10 x PCR buffer, 2 μL dNTP mixture (10 mM), 1.5 μL $MgCl_2$ (50 mM), 2 μL of each primers (10 pmol μL^{-1}), 1 μL of DNA (5-10 ng) and 0.6 μL Taq DNA polymerase (5 U μL^{-1}). Amplification was performed in a thermocycler (Techne Flexigene, Model FFG05TUD, Minneapolis, MN, USA) using the following program. A 5 min denaturation period at 95°C was followed by 30 cycles each; 1 min at 95°C, 1 min at 50°C and 2 min at 72°C with a final extension for 10 min at 72°C. All PCR chemicals were purchased from Cinagen (Tehran, Iran).

The PCR product was purified from the agarose gel and cloned into plasmid pTZ57R/T in *E. coli* TG1 using standard procedures. Recombinant colonies were screened on LB agar supplemented with ampicillin (100 μg mL^{-1}), IPTG (0.5 mM) and X-Gal (20 μg mL^{-1}) as previously described (Sambrook *et al*., 2001). Presence of the cloned 16S rRNA gene in recombinant plasmids was verified using M13/pUC universal primers (Gen Fanavaran, Tehran, Iran). The DNA insert was then sequenced using the dideoxy termination method with ABI automated sequencer at the Research Center for Gastroenterology and Liver Diseases (Taleghani Hospital, Tehran, Iran) and homology was analyzed through BLAST (blastn algorithm) at http://www.ncbi.nih.gov/blast.

Selection of salt basal media: M9 medium consisting of 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 2 mM $MgSO_4.7H_2O$, 1 g NH_4Cl, 2 g glucose and 10 mL olive oil L^{-1} (pH 7) and G4 medium containing 35 g NH_4Cl, 3 g K_2HPO_4, 1 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 2 g glucose, 0.6 mM $MgCl_2$ and 10 mL olive oil L^{-1} (pH 7.0) were used (Sambrook *et al*., 2001; Gupta *et al*., 2004b). Two other media, M9+Y and G4+Y, were prepared by adding 5 g L^{-1} yeast extract to M9 and G4. The media were inoculated with 5% seed culture and bacterial growth and lipolytic activity were measured at 6 h intervals up to 30 h at 30°C.

Selection of oil for lipase induction: Seven oils with different compositions were used for their effects on lipase production. Olive oil, canola oil, sunflower oil and grape seed oil used at 1% and coconut (1.5%) were obtained from commercially available sources. Tributyrin used at 2% and glycerol (10%) were purchased from Merck.

Partial optimization of lipase production by the Taguchi experimental design: The effect of seven factors (oil, glucose, NH_4Cl, yeast extract, inoculum density, pH and agitation) was investigated at two levels shown in Table 3. Design Expert 6.0.10 (Stat Ease Inc., Minneapolis, Minn., USA) was used to generate a set of 8 experimental trials according to the Taguchi orthogonal L_8 array (Table 3). Bacterial growth and lipase production were monitored every 6 h at 30°C for 36 h. All experiments were performed in triplicate and mean values were used for statistical analysis.

Inoculum preparation: M9 without oil was used to prepare inocula for selection of salt basal media and G4+Y was used for the rest of the experiments. Seed cultures were incubated for 18 h at 30°C before use.

Growth estimation and detection of lipase activity: To measure bacterial growth and lipase production, culture samples (1 mL) were removed at designated times and were centrifuged at 5000 g for 10 min. Pellets were resuspended in 1 mL of 0.01 M phosphate buffer (pH 7) and absorbance was measured at 600 nm (Gupta *et al*., 2004b). Culture supernatants were used to determine lipolytic activity using *p*-nitrophenyl palmitate substrate (*p*NPP, Sigma). The substrate was dissolved in 10 mM acetonitrile followed by adding absolute ethanol and 20 mM Tris-HCl (pH 8) at a ratio of 1:4:95 (v/v/v), respectively. Substrate hydrolysis was monitored for 5 min at room temperature and the amount of released *p*-nitrophenol was determined spectrophotometrically by measuring the increase in absorption at 405 nm (Model DU-530 Beckman, USA). One unit of lipase was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute (Cho *et al*., 2000).

RESULTS AND DISCUSSION

Among the *Bacillus* isolates screened for lipase production on TW agar, one (F3) showed lipolytic zones after 48 h at 30°C. When F3 was grown on Rhodamine B lipase agar, orange fluorescence was observed under UV irradiation indicating lipase activity.

Growth and lipase production: *Bacillus* F3 grew rapidly in both M9 and G4 media containing yeast extract (G4+Y and M9+Y). However, lipolytic activity was detected only in G4+Y (Table 1). M9+Y supported bacterial growth shown by high optical density values but no lipolytic activity was observed in M9 with or without yeast extract. Organic nitrogen sources such as yeast extract have been shown to play a crucial role on lipase expression (Gupta *et al*., 2004a; Sharma *et al*., 2001). Maximal lipolytic activity was shown to be 3 U mL^{-1} after 24 h of growth in late logarithmic phase (Table 1). Medium G4+Y was then chosen for further studies. Presence of higher concentration of ammonium chloride (3.5%) in G4 compared to M9 (0.1%) seemed to be another factor responsible for lipase expression.

Lipase production was induced in the presence of olive oil similar to a number of other reports (Sugihara *et al*., 1991; Wang *et al*., 1995; Gupta *et al*., 2004a). As a matter of fact, all long fatty acyl chain triacylglycerol test substrates were as effective inducers of lipase production as olive oil (Table 2). On the other hand, glycerol or tributyrin did not induce lipase production. Glycerol was previously reported to enhance lipase production in a thermophilic *Bacillus* strain (Gupta *et al*., 2004b).

Identification of the lipolytic *Bacillus*: Biochemical characterization of F3 showed it to be a non-motile, catalase positive, indole and VP negative Gram positive rod with central and elliptical spores. It produced acid but no gas from glucose, but no acid was produced from arabinose or mannitol. Growth on anaerobic agar was also detected. Furthermore, F3 hydrolyzed gelatin and casein but not starch. Metabolization of citrate and de-amination of phenylalanine did not occur and lecithinase or NO_2 were not produced. Growth occurred at temperatures between 20 to 55°C at pH range 4 to 10 and up to 7% NaCl concentrations.

Identification of F3 by 16S rRNA amplification showed a 1500 bp fragment which was then cloned in *E. coli* TG1. Eight recombinant clones were selected and their DNA inserts were further amplified using the M13/pUC universal primers. Four recombinant plasmids with 1500 bp inserts were obtained, one of which was chosen for sequencing. The sequencing results revealed 99% homology to *Bacillus pumilus* (Genbank accession No. EU285662).

Optimization of bacterial growth and lipase production: In an effort to optimize bacterial growth and lipase production, the statistical method of Taguchi was used to design culture conditions with different media compositions. Statistical designs have been employed to optimize enzyme production and minimize the number of experiments for a number of organisms including *Bacillus* sp. (Krishna *et al*., 2001; Rathi *et al*., 2002; Gupta *et al*., 2004b; Prakasham *et al*., 2005). In order to enhance bacterial growth and lipase production, seven variables were screened by the Taguchi method using G4+Y medium and olive oil (Table 3).

Table 1: Bacterial growth (OD_{600}) and lipase activity (U mL^{-1}) in basal media during 30 h incubation at 30°C and 250 rpm shaking

	6 h		12 h		24 h		30 h	
Media	OD_{600}	U mL^{-1}	OD_{600}	U mL^{-1}	OD_{600}	U mL^{-1}	OD_{600}	U mL^{-1}
G4	0.13	0.00	0.14	0.00	0.40	0.00	0.06	0.00
G4+Y	0.78	0.00	2.40	1.60	2.36	3.00	1.96	0.00
M9	0.20	0.00	0.22	0.00	0.12	0.00	0.11	0.00
M9+Y	2.10	0.00	2.60	0.00	2.65	0.00	2.30	0.00

Table 2: Bacterial growth (OD_{600}) and lipase activity (U mL^{-1}) in the presence of different oils. Cultures were grown for up to 30 h at 30°C and 250 rpm shaker speed

	6 h		12 h		24 h		30 h	
Oil	OD_{600}	U mL^{-1}	OD_{600}	U mL^{-1}	OD_{600}	U mL^{-1}	OD_{600}	U mL^{-1}
Olive	2.10	0.00	3.00	3.88	2.69	4.00	2.44	0.00
Canola	2.16	0.00	2.08	3.80	2.46	4.10	2.29	0.00
Sunflower	1.99	0.00	2.81	3.80	2.30	4.30	2.10	0.00
Coconut	1.77	0.00	2.92	3.80	3.27	4.00	2.73	0.00
Grape seed	1.81	0.00	2.70	3.40	2.59	3.40	2.22	0.00
Tributyrin	1.51	0.00	1.52	0.00	1.70	0.00	1.80	0.00
Glycerol	0.88	0.00	2.01	0.00	2.37	0.00	2.17	0.00

Table 3: Measurement of bacterial growth and lipase activity using the Taguchi orthogonal L_8 array

Trial No.	A Olive oil (ml L^{-1})	B Inoculum (%)	C Glucose (g L^{-1})	D NH_4Cl (g L^{-1})	E Yeast extract (g L^{-1})	F pH	G Agitation (rpm)	Growth (OD_{600})	Lipase activity (U mL^{-1})
1	5	1	2	15	0	6.0	100	0.34±0.12	0.00
2	5	1	2	35	10	8.5	300	3.37±0.30	4.75±0.46
3	5	10	8	15	0	8.5	300	0.36±0.04	0.00
4	5	10	8	35	10	6.0	100	3.85±0.04	4.75±0.41
5	15	1	8	15	10	6.0	300	3.00±0.03	0.00
6	15	1	8	35	0	8.5	100	0.13±0.03	0.00
7	15	10	2	15	10	8.5	100	4.35±0.27	4.83±0.22
8	15	10	2	35	0	6.0	300	0.50±0.07	0.00

Table 4: ANOVA analysis (partial sum of squares) of bacterial growth for the Taguchi orthogonal array L_8

Source	Sum of squares	df	Mean square	F-value	Prob. > F	
Model	22.94	5	4.59	1716.17	0.0006	Significant
B	0.61	1	0.61	229.80	0.0043	
C	0.18	1	0.18	68.34	0.0143	
E	21.85	1	21.85	8172.18	0.0001	
F	0.035	1	0.035	13.18	0.0682	
G	0.26	1	0.26	97.35	0.0101	
Residual	5.347 E-003	2	2.674 E-003			
Cor total	22.95	7				

*: The F-value of 1716.17 implies that the model is significant. **: Values < 0.1000 for Prob. > F indicate that the model terms are significant (B, C, E, F and G). Letter(s) B, C, E, F and G correspond to Table 3

Table 5: Statistical parameters of bacterial growth for the Taguchi orthogonal array L_8

Terms	Bacterial growth
F-value*	1716.170
Prob. > F**	<0.1000
Standard deviation	0.052
Mean	1.990
Coefficient of Variation (CV)	2.600
R^2	0.999
Adjusted R^2	0.999
Predicted R^2	0.996
Adequate precision	92.938

*: The model F-value of 1716.17 implies that the model is significant. **: Value of Prob. > F less than 0.1000 indicate model terms are significant

Table 6: Contribution of experimental factors on bacterial growth and lipase production obtained by the Taguchi orthogonal array L_8

	Bacterial growth		Lipase production	
Factors	Sum of squares	Contribution (%)	Sum of squares	Contribution (%)
Oil	4.961 E-004	2.162 E-003	2.73	6.37
Inoculum	0.61	2.68	2.92	6.82
Glucose	0.18	0.80	2.92	6.82
NH_4Cl	4.851 E-003	0.021	2.73	6.37
Yeast extract	21.85	95.22	25.67	59.99
pH	0.035	0.15	2.92	6.82
Agitation	0.26	1.13	2.92	6.82

The ANOVA analysis revealed that the inoculum size, glucose and yeast extract concentrations, pH and agitation significantly enhanced bacterial growth. On the other hand, oil concentration above 0.5% and ammonium chloride concentrations above 1.5% were not significant (Table 4). The coefficient of determination (R^2) was 0.999 which was in a reasonable agreement with the predicted R^2 (0.996) and showed a satisfactory adjustment of the quadratic model to the experimental data. Adequate precision measures the signal to noise ratio, a ratio greater than 4 is desirable. An adequate precision value of 92.938 indicates an adequate signal (Table 5).

Contribution of experimental factors on bacterial growth and lipase production are shown in Table 6. Yeast extract at 10% concentration, had the highest contribution on growth (95.22%) and lipase production (59.99%). In comparison to yeast extract, the effect of other factors on lipase production was negligible. In fact, the sum of squares for all other factors showed the Prob. > F-values greater than 0.1 which are not significant at the 90% confidence interval. Indeed, lipase was not produced in the absence of yeast extract regardless of different concentrations of olive oil, glucose, ammonium chloride, starting inocula, pH or agitation. On the other hand, at least 1.5% NH_4Cl was needed for lipase production in G4+Y medium and higher concentrations up to 3.5% did not further enhance lipase production.

Lipolytic activity was not detected with high glucose concentrations and low inoculum density (1%) despite the observed acceptable growth (trial No. 5, Table 3). However, this effect was compensated when high inoculum density (10%) was used (trial No. 4, Table 3). Catabolic repression by glucose has been previously reported for some lipolytic microorganisms (Gowland *et al*., 1987; Rapp, 1995; Gupta *et al*., 2004a).

The effect of agitation on lipase production varies depending on the bacterial strain. Optimum lipase production for most *Bacillus* species has been reported to occur at lower agitation rates (Gupta *et al*., 2004a). However, production of lipase in *Pseudomonas putida*

3SK was maximum at 500 rpm (Lee and Rhee, 1994). It is reasonable to believe that physical parameters such as pH, temperature and aeration most probably influence lipase production by modulating bacterial growth (Gupta *et al*., 2004a). Maximal lipase activity was 4.83±0.22 U mL^{-1} in a medium containing 15 mL olive oil, 2 g glucose, 15 g ammonium chloride and 10 g yeast extract L^{-1} at pH 8.5 and low agitation using an initial inoculum of 10 % (trial No. 7, Table 3).

Lipase production in *B. pumilus* has been reported to occur at low levels. Kim *et al.* (2002) have reported lipase yields as low as 0.5 U mL^{-1}. Maximal lipase production in *B. pumilus* so far has been reported to be 12.8 U mL^{-1} (Möller *et al*., 1991). Our preliminary efforts to produce lipase from *B. pumilus* F3 led to production of 4.83 U enzyme mL^{-1}. Further studies are needed to enhance lipase production in this strain. We are currently in the process of cloning the F3 lipase under a strong promoter to be able to determine molecular properties of the lipase as well as increasing enzyme expression and yields for future industrial applications.

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