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Enhanced production of thermostable lipase from *Bacillus cereus* ASSCRC-P1 in waste frying oil based medium using statistical experimental design

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ABSTRACT

This study aim to isolate, identify a bacterial isolate and optimize production medium using frying oil waste for lipase production. Nine strains were isolated from an Egyptian soil samples. Among the isolates, a potent bacterial candidate ASSCRC-P1 was found to be the most potent lipase producer strain at 60 °C. Genotypic identification of ASSCRC-P1 showed 94% similarity with *Bacillus sp.* strains. Phylogenetic tree confirmed that ASSCRC-P1 was nearly similar to *Bacillus cereus.* Therefore, it was given the name *Bacillus cereus* ASSCRC-P1 and its 16S rRNA nucleotide has been deposited in the GenBank Data Library under the accession number: KJ531440. A sequential optimization strategy, based on statistical experimental designs, was employed to enhance the lipase production by this strain. A 2-level Plackett–Burman design was applied to differentiate between the bioprocess parameters that significantly influence lipase production followed by Box-Behnken design to optimize the amounts of variables which have the highest positive significant effect on lipase production. Overall more than 2.15-fold improvement in lipase production was achieved due to optimization compared to that obtained using the basal medium.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are widely distributed among microorganisms, animals and plants and they catalyze the hydrolysis of esters of long chain aliphatic acids from glycerol at lipid and water interfaces (Jensen 1983). Lipases are produced by several microorganisms, *viz*., bacteria, fungi, yeast, actinomycetes, archea, eucarya, *etc*. Microbial genera involved in the commercial production of lipases include: *Candida*, *Mucor*, *Rhizopus*, *Aspergillus*, *Penicillium*, *Geotrichum*, *Rhizomucor*, *Bacillus*, *Pseudomonas* and *Staphylococcus* (Aravindan *et al.*, 2007). Microorganisms with potentials for producing lipases can be found in different habitats, including wastes of vegetable oils and dairy industries, soils contaminated with oils, seeds, and deteriorated food (Sharma *et al*., 2001). Only microbial thermostable lipases are commercially significant for their potential use in industries, such as speciality organic syntheses (Rubin and Dennis 1997), hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing (Kazlauskas and Bornscheuer 1998), resolution of racemic mixtures and chemical analyses (Lee *et al*., 1995). A major requirement for thermally stable lipase would allow enzymatic reaction to be performed at higher temperatures and would be helpful to increase conversion rates, substrate solubility and to reduce the contamination of microorganism. Several lipases have been purified and characterized from thermophilic isolates, mainly from *Bacillus* (Kumar *et al*., 2014). Fried food is a major component of diets in many countries. The increasing demand for fried food has resulted in large quantities of waste frying oil. Kock *et al*. (1996) reported that used frying oils contained more than 30% of polar compounds from the oxidative degradation of triglycerides.

These oils can be considered a high-energy food source, till now used/discharged for animal feed, but they also may be considered as high-energy source for microbial growth and transformation into high-value products (Haba *et al*., 2000).

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Generally, high productivity has been achieved by culture medium optimization. Optimization of the concentration of each compound that constitutes cultivation medium is usually a time-consuming procedure.

The use of by-products as substrates for lipase production, adds high value and low-cost substrates may reduce the final cost of the enzyme (Veerapagu *et al*., 2013). Statistical optimization using Response Surface Methodology (RSM) has been widely used to augment the conventional shake flask method especially when a large number of variables are to be evaluated. It has helped to determine the optimum concentration of selected media components (Sunitha *et al*., 1998). Optimization of lipase production by RSM has been reported in cultures of *Cadida* sp., *Pseudomonas aeruginosa* and *Bacillus pumillus* (Chien-Hung *et al.*, 2006).

The main objective of the present study was to isolate and genotypically identify bacterial strain that efficiently expresses a desirable thermostable lipase. RSM optimization methods were implemented to maximize the enzyme production by using waste frying sunflower oil.

MATERIALS AND METHODS

Sample collection and isolation of thermostable lipase producing bacteria

Soil samples were collected during summer from soil contaminated with oil beside the oil wells in El Ain El Sokhna, open lagoons area in Ras Sedr and from soil around Mosa spring in South Sinai. Soil samples are collected in sterile containers and store in refrigerator at 4°C until reached to the laboratory. Isolation of thermophilic bacteria with lipolytic activity was carried out according to the method adopted by Gowland *et al.* (1987). *In situ*, enrichment was achieved by mixing the soil samples with sterile commercial cocking oil and incubating for 10 days at 65°C. Enriched soil samples were suspended in sterile water in a ratio of 1:1. Sequential enrichment of thermophilic bacteria with lipolytic activity was performed by inoculating 1ml of the soil suspension in a medium consisted of 0.025% yeast extract and 0.1% waste cooking sunflower oil emulsified in aqueous 10% (w/v) gum Arabic (Abdel Fattah 2008). The inoculated flasks were incubated with shaking at 55 ºC. One milliliter of the culture was plated onto agar plates of the same medium for further purification. The single colonies were selected and inoculated in nutrient agar (NA) slants. Bacterial isolates were routinely grown on (NA) medium at 55ºC and preserved at -80ºC in 50% glycerol.

Identification of thermophilic lipase producing bacteria

DNA isolation and PCR amplification

An overnight culture of the promised bacteria grown at 55 ºC was used for the preparation of genomic DNA. The DNA was isolated using Promega kit for preparation of genomic DNA according to the manufacturer's instructions. PCR amplification of 16S rDNA region was carried out according to Sambrook *et al.* (1989). The 16S rDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16S rDNA region. The domain bacteria-specific primer 27F (forward primer) was 5'AGAGTTTGATCMTGGCTCAG3' and universal bacterial primer 1492R (reverse primer) was 5'TACGGYTACCTTGTTACGACTT3' (Edwards *et al*., 1989).

The PCR mixture consists of 30 picomoles of each primer, 10 ng of chromosomal DNA, 200 µM dNTPs and 2.5 Units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles in 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 2 minutes. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis and the remnant was purified using QIAquick PCR purification reagents (Qiagen).

DNA sequencing, phylogenic analysis and tree construction

DNA sequences were obtained using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan), BigDye Terminator Cycle Sequencing. The PCR product was sequenced using the same PCR primers. Automated DNA sequencing based on enzymatic chain terminator technique was done using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan).

The thermal cycling mixture was as follows: 8 µl of BigDye terminator mix, 6 µl of the sequencing primer (10 pmol) and 6 µl of the sample (PCR product) then the reaction was run in the thermal cycler. The cyclic reaction composed of 1 min at 95 ºC, then 49 cycles of 30 sec at 95 ºC, 10 sec at 52 ºC and 4 min at 60 ºC. The products were purified using special column according to the instruction of the manufacturer. The elute were taken and (1:1) volume ratio of high dye formamide was added and run at 95 ºC for 5 min for denaturation, then shock on ice. Afterward, the samples become ready for sequencing in 3130 X DNA sequencer and analysis

Phylogenetic data were obtained by aligning the nucleotides of different 16S RNA retrieved from BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program version 1.8 with standard parameters. The classifier is trained on the new phylogenetically consistent higher-order bacterial taxonomy (Ribosomal Database Project, RDP Classifier) proposed by Wang *et al.* (2007), (http://rdp.cme.msu.edu/classifier/classifier.jsp). Phylogenetic and molecular evolutionary analyses were conducted using BioEdit version 7.0.4.01. A rooted phylogram was derived from the distance matrices using the neighbour-joining method through the TREEVIEW program. All analyses were performed on a bootstrapped data set containing 1000 replicates (generated by the program).

Nucleotide sequence ID

The nucleotide sequence of the 16S rRNA gene of strain ASSCRC-P1 has been deposited in the GenBank under accession number: KJ531440.

Secondary structure prediction and restriction site analysis

The RNA secondary structure of the isolate ASSCRC-P1 was predicted according to Brodsky *et al*. (1995) using Genebee online software by the greedy method and the restriction sites of the strain DNA was analyzed by NEB cutter Version 2.0 according to Vincze *et al*. (2003).

Production of thermophilic lipase

Production of lipase was carried out using medium consisted of (g %): Peptone, 0.3; KH_2PO_4, 0.2; KCl, 0.05; $MgSO_4.7H_2O$, glucose, 1.0 and waste frying sunflower oil (WFO) 1.0). The pH of the medium was adjusted to 7.0 (Akhtar *et al*., 1980). Cultivation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of production medium .The sterilized flasks were inoculated with 2 ml of 24 h old inoculum. The optical density of the inoculum was adjusted to 1.0 at 600 nm. The inoculated flasks were incubated at 45 ºC for 72 h in a rotary shaker adjusted at 200 rpm.

Determination of the lipase enzyme activity

Samples were analyzed according to Huge-Jensen *et al*. (1987). One ml of culture filtrate was mixed with 2.5 ml of deionized water and 1 mL of 0.1 M of tris –HCl buffer (pH 7.5), 3 ml of 10% (v/v) triglyceride emulsion **(**10 % (v/v) of the triglycerides (tributyrin or olive oil) emulsified in 10 % (w/v) Arabic gum in a hot water and homogenized in a top drive homogenizer for 10 min**)**. The mixture was incubated for 2 h at 37 ºC in a shaking water bath. At the end of incubation time 10 ml of 99% ethyl alcohol was added and the resulting mixture was then titrated against 0.05 N NaOH using thymolphthalein as indicator, boiled enzyme samples were used as blank. One unit of lipase activity was defined as the amount of enzyme that produced 1 µmole of free fatty acids per min under the standard assayed conditions. The activities were expressed in U/ml.

Thermal stability of the lipase producing bacteria

Crude lipase enzyme produced by bacterial isolate coded GP1(ASSCRC-P1) was tested for its thermal stability by incubating the crude enzyme solution for 240 minutes in water baths adjusted to 60°C and 65°C (Kumar *et al*., 2005). Samples were taken each 30 minute for lipase activity determination.

Experimental designs

Plackett–Burman design

The Plackett–Burman experimental design was used to evaluate the relative importance of various nutrients for the production of lipase by *Bacillus cereus* ASSCRC-P1 in submerged fermentation. Fifteen components were selected and each variable was represented at two levels, high concentration (+1) and low concentration (−1) in 16 trials as shown in Table 1 (Plackett and Burman, 1946). The tested factors included culture volume, waste frying oil, Tween 80, Triton X-100, peptone, beef extract, K_2HPO_4, KH_2PO_4, NaCl, KCl, $FeSO_4$, $ZnSO_4$, $MgSO_4$, $CuSO_4$, and $MnSO_4$. Each row represents a trial run and each column represents an independent variable.

Plackett Burman experimental design is based on the first order model:

$Y= B_0 + \Sigma B_i x_i$. Eq. (1) Where Y is the response (lipase activity), B_0 is the model intercept and B_i is the variables estimates. The effect of each variable was determined by following equation,

$E(X_i) = 2(\Sigma M_i^+ - M_i^-)/N.$ Eq. (2)

Where $E(X_i)$ is the effect of the tested variable, M_i^+ and M_i^- represent lipase production from the trials where the variable (X_i) measured was present at high and low concentrations, respectively and N is the number of trials in Eq. (2).

The standard error (SE) of the concentration effect was the square root of the variance of an effect and the significance level (*p*-value) of each concentration effect was determined using student's *t*-test

$t(X_i) = E(X_i)/ SE$…… Eq. (3) Where E (X_i) is the effect of variable X_i.

Box-Behnken design

In order to describe the nature of response surface in the experimental region, a Box-Behnken design was applied (Box and Behnken 1960). As presented in (Table 2), three favorable salts were prescribed into three levels, coded -1, 0 and +1 for low, middle and high concentrations (or values) respectively.

The results in Table 2 represent the design matrix of a 15 trial experiment. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and response lipase production. For the three factors the polynomial equation is in the following form:

$Y = ß_0 + ß_1 x_1 + ß_2 x_2 + ß_3 x_3 + ß_{11} x_1^2 + ß_{22} x_2^2 + ß_{33} x_3^2 + ß\, b_{12} x_1 x_2 + ß_{13} x_1 x_3 + ß_{23} x_2 x_3$ Eq.(4)

Where Y is the predicted production of lipase (U/ml), X_1, X_2 and X_3 are the independent variables corresponding to the concentration of waste frying oil, triton X-100 and $MnSO_4$ respectively; $ß_0$ is the intercept, $ß_1$, $ß_2$, $ß_3$ are linear coefficients, $ß_{11}$, $ß_{22}$, $ß_{33}$ are quadratic coefficients, $ß_{12}$, $ß_{13}$, $ß_{23}$ are cross product coefficients. Statistical software SPSS (version 16.0) was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Experiments were performed in triplicate and mean values are given.

RESULTS AND DISCUSSION

Screening of different bacterial isolates for production of lipase enzyme

When the effluent samples were serially diluted and plated in a nutrient medium that enriched lipolytic organisms, nine bacterial strains were isolated as described in experimental part and cultivated in the production medium. All bacterial isolates produce a considerable amount of lipase enzyme but highest lipase activity (160 U/ml) was achieved by bacterial isolate coded GP1 (ASSCRC-P1) (Figure 1). These results were more than that

obtained from several researchers as Guzman and Virginia (2008) who detected lypolytic activity of 0.079 U/ml by *Bacillus pumilus* LV01 isolated from water samples and maximum lipase production of 12 U/ml by *Pseudomonas gessardii* selected from a soil sample (Veerapagu *et al*., 2013). These results were in accordance to Boonmahome and Mongkolthanaruk (2013) who found that *Pseudomonas aeruginosa* isolate NA37 selected from soil contaminated with oil in Khon Kaen region showed high lipase activity (190 mU/ml) detecting with *p*-nitrophenyl palmitate as a substrate. On the other hand, Dhiman and Chapadgaonkar (2013) isolated and identified as *Bacillus* ISC 1, from a site of regular oil spill, showed high lipase production of 25 U/ml.

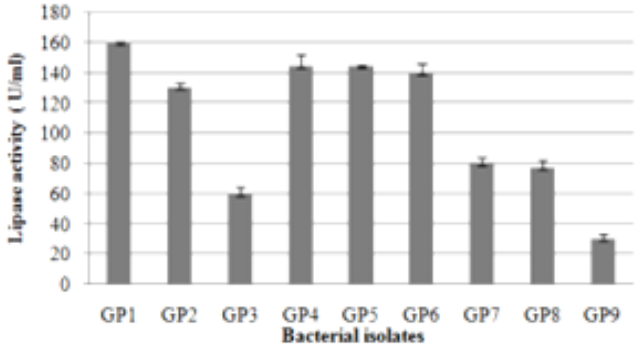

Fig. 1: Screening of bacterial isolates for their ability to produce lipase enzyme

Identification and taxonomic classification of bacterial isolate ASSCRC-P1

The primer specificity, sequencing and phylogenetic analysis

The primer pairs' 27F/1492R amplified the fragments of the expected size (around 1400-1500 in the gel) of the genomic DNA isolated from strain ASSCRC-P1. The amplification capacity of this primer is 27 bp and 1492 bp fragments and the nucleotides ranged between 8 to 1507 bp. The 16S rRNA gene amplified with a set of universal primers 27F and 1492R from ASSCRC-P1 isolate was observed on agarose gel electrophoresis.

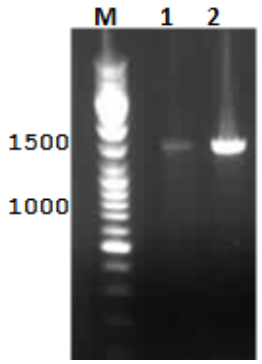

Fig. 2: Agarose gel electrophoresis of PCR products obtained by amplification from genomic DNA isolated from *Bacillus* strains with the primer pairs. Lane 1 M: molecular-weight marker; Lane 2, purified PCR product; Lane 3, PCR product for 16s rDNA (1500bp) of *Bacillus cereus* ASSCRC-P1.

The results in Fig. 2 showed an amplicon band with the size of about 1.5kb. These results were in accordance with that reported by Akhmaloka *et al*. (2006) who found that the PCR products using primers 27F and 1492R should be about 1500 base pairs long. The specificity of the PCR is affected by multiple factors, especially the primer pairs. In this study, the 27F/1492R primer pair was used as a specific primer for bacteria. The primer was examined by PCR amplification using genomic DNA, isolated from strain ASSCRC-P1. The primers were successfully used to amplify genomic DNA from the isolated sample. These results are in agreement with those cited by Edwards *et al*. (1989) who found that these primers are specific for bacteria.

Alignment, phylogenetic tree construction and G+C%

The nucleotide sequence of *Bacillus cereus* ASSCRC-P1 strain was matched with the 16S rRNA reported gene sequences in the gene bank database. The database of NCBI Blast (www.ncbi.nlm.nih.gov/BLAST) was used to compare the *Bacillus cereus* ASSCRC-P1 with those of *Bacillus* species strains. The results showed the high sequence similarity species (94%) with *Bacillus pumilus* and *Bacillus cereus* strains.

The phylogenetic tree of the strain (Figure 3) showed that strain ASSCRC-P1 is most closely related to *Bacillus cereus*. Therefore, it was proposed a name *Bacillus cereus* ASSCRC-P1. The nucleotide sequence of 16S rRNA gene has been deposited in GenBank under accession number: KJ531440.

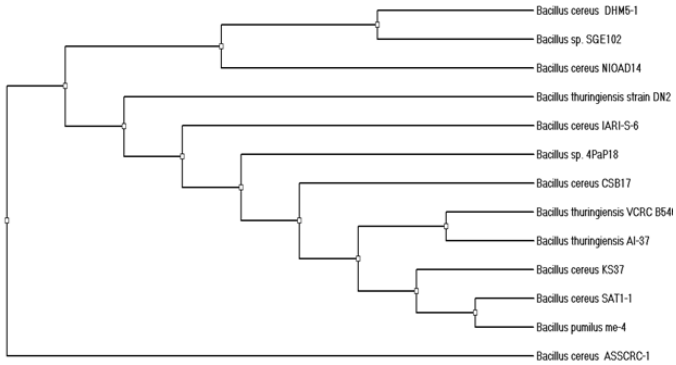

Fig. 3: Neighbor-joining phylogenetic representation of the strains and their closest NCBI (BLAST) relatives based on 16S rRNA gene sequences of *B. cereus* ASSCRC-P1and some known sequences of *B. cereus* sp.

The percentage of G+C is one of many general features used to characterize bacterial genomes. The G+C content of the genomic DNA was 54 mol% obtained from the phylogenetic analysis. These results were in accordance to that reported by Galtier and Lobry (1997); McCutcheon and Moran (2010) who mentioned the percentage composition of the known *Bacillus species* can range from 25% to 75% for G+C richness.

Secondary structure prediction and restriction site analysis

The RNA secondary structure was predicted for 16S rRNA of *Bacillus cereus* ASSCRC-P1 (Figure 4). It showed that the free energy of structure is -24.4 kkal/mol, threshold energy is -4.0 with cluster factor, conserved factor 2, compensated factor 4 and conservatively is 0.8. The prediction of restriction sites of *Bacillus cereus* ASSCRC-P1 strain showed the restriction sites for

various enzymes such as Bfal, Spel, EcoP151, EcoR l, BSPMI and Bsgl etc (Figure 5).

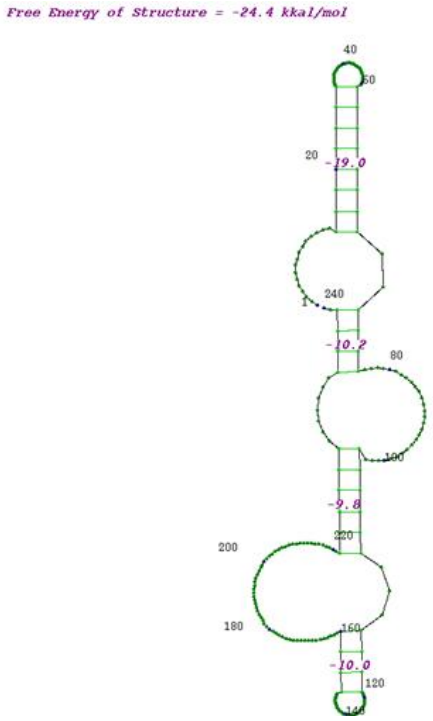

Fig. 4: Secondary structure prediction of 16s rRNA of the strain *Bacillus cereus* ASSCRC-P1 was done using Genebee online software

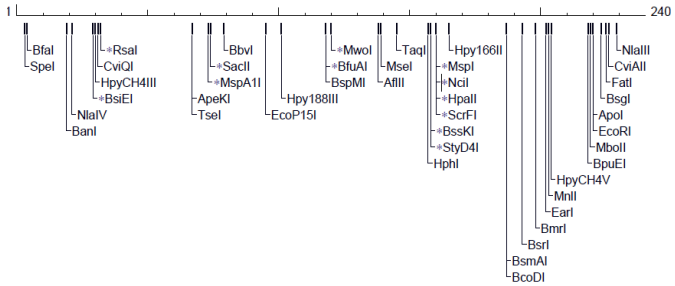

Fig. 5: Restriction sites of the strain *Bacillus cereus* ASSCRC-P1 were predicted using NEB single cutter

Thermal stability of the Bacillus cereus ASSCRC-P1 lipase

For achieving our goal, we tested the thermal stability of lipase produced by bacterial isolate GP1 (ASSCRC-P1) by incubating the crude enzyme solution for 240 minutes in water baths adjusted to 60 and 65 °C. Figure 6 showed that the lipase enzyme can stand up to 180 min at 60°C and lost only 10% of its activity after 210 min while the enzyme can stand at 65°C for about 150 min and its activity retain 100% with slight loss of its activity with the increase of incubation time at the same temperature. The stability of the lipase at high temperatures suggests its usefulness in industrial uses as detergents and treatment of domestic sewage employed at temperatures exceeding 50 °C (Sharma *et al*., 2002). Similar results were reported by Kumar *et al*. (2005). Recently several thermostable lipases have been reported from the genus *Bacillus* such as *Bacillus cereus* (Senthilkumar and Selvakumar 2007), *Bacillus thermoleovorans* (Castro-Ochoa *et al*., 2005). The bacterial isolate ASSCRC-P1 was chosen for further investigations.

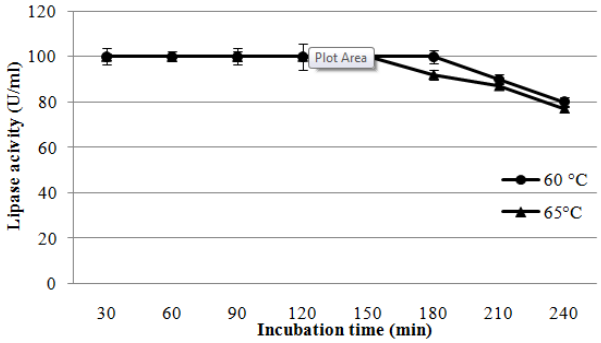

Fig. 6: Thermal stability of *Bacillus cereus* ASSCRC-P1 lipase activity at 60° C and 65°C

Optimization of lipase production by Bacillus cereus ASSCRC-P1

The production optimization of lipase was done by two steps

Evaluation of the factors affecting lipase activity by the Plackett–Burman Design

In the first approach, the Plackett–Burman design was used. Fifteen factors (variables) including culture conditions and medium constitution were chosen to perform this optimization process. The lipase activity averages for the different trials were given in U/ml and shown in Table 1. The main effect of each variable upon lipase activity was estimated as the difference between both measurements averages made at the high level (+1) and at the low level (-1) of that factor. Data in Table 1 showed a wide variation from 18± 1.16 to 192± 2.677 U/ml on lipase activity reflected the importance of medium optimization to attain higher yields. Data analysis of the Plackett–Burman experiments involved a first order model. The main effects of the examined factors on the enzyme activity and their ranking were calculated and presented graphically in Figure 7.

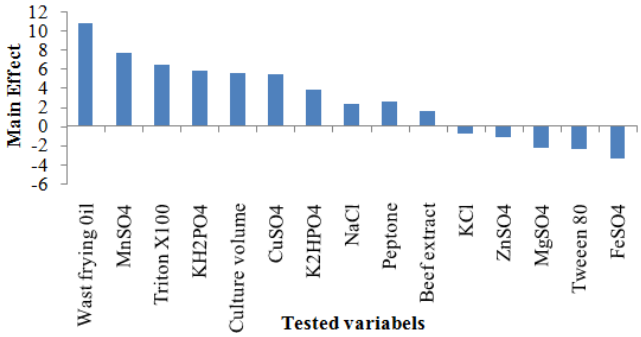

Fig. 7: Effect of different medium composition on lipase activity (U/ml) produced *by B. cereus* ASSCRC-P1

On the analysis of the regression coefficients of the tested variables: waste frying oil, $MnSO_4$, triton X-100, KH_2PO_4, culture volume, $CuSO_4$, K_2HPO_4, NaCl, peptone and beef extract, showed positive effect on lipase activity while $FeSO_4$, Tween 80,

Table 1: Coded levels and real values for Plackett-Burman experimental design.

Trial No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Lipase activity (U/ml)
	Culture Volume	Waste Frying oil	Tween 80	Triton X100	Peptone	Beef extract	K_2HPO_4	KH_2PO_4	NaCl	KCl	$FeSO_4$	$ZnSO_4$	$MgSO_4$	$MnSO_4$	$CuSO_4$	
1	+1(50)	-1(40)	+1(0.50)	+1(0.5)	-1(5)	+1(10)	-1(1)	-1(1)	+1(0.5)	+1(0.5)	+1(0.001)	-1(0)	+1(0.001)	-1(0)	+1(0.001)	34.80 ± 0.669
2	-1(25)	+1(80)	+1(0.50)	-1(0.25)	-1(5)	-1(5)	-1(1)	+1(2)	+1(0.5)	+1(0.5)	-1(0)	+1(0.001)	-1(0)	+1(0.001)	-1(0)	82.86± 3.701
3	+1(50)	+1(80)	-1 (0.25)	+1(0.5)	-1(5)	-1(5)	+1(2)	-1(1)	+1(0.5)	-1(0)	+1(0.001)	-1(0)	+1(0.001)	+1(0.001)	+1(0.001)	125.52± 5.63
4	-1(25)	-1(40)	+1(0.5)	+1(0.5)	+1(10)	-1(5)	+1(2)	+1(2)	-1(0)	+1(0.5)	-1(0)	+1(0.001)	-1(0)	+1(0.001)	+1(0.001)	125.52± 1.378
5	-1(25)	+1(80)	-1(0.25)	-1(0.25)	+1(10)	+1(10)	+1(2)	+1(2)	+1(0.5)	-1(0)	+1(0.001)	-1(0)	+1(0.001)	-1(0)	-1(0)	85.50± 6.1000
6	-1(25)	-1(40)	-1(0.25)	+1(0.5)	+1(10)	+1(10)	-1(1)	-1(1)	-1(0)	+1(0.5)	-1(0)	+1(0.001)	+1(0.001)	+1(0.001)	+1(0.001)	060.00± 5.118
7	+1(50)	-1(40)	-1(0.25)	-1(0.25)	-1(5)	+1(10)	+1(2)	+1(2)	+1(0.5)	-1(0)	+1(0.001)	+1(0.001)	-1(0)	-1(0)	-1(0)	078.42± 2.165
8	+1(50)	-1(40)	+1 (0.50)	-1(0.25)	+1(10)	-1(5)	-1(1)	-1(1)	-1(0)	+1(0.5)	+1(0.001)	-1(0)	+1(0.001)	+1(0.001)	-1(0)	065.28± 1.575
9	+1(50)	+1(80)	+1(0.50)	-1(0.25)	-1(5)	+1(10)	+1(2)	-1(1)	+1(0.5)	+1(0.5)	-1(0)	+1(0.001)	-1(0)	-1(0)	-1(0)	124.86± 0.512
10	-1(25)	+1(80)	+1(0.50)	+1(0.5)	+1(10)	-1(5)	-1(1)	-1(1)	+1(0.5)	-1(0)	+1(0.001)	-1(0)	-1(0)	+1(0.001)	+1(0.001)	151.08± 4.685
11	+1(50)	+1(80)	-1(0.25)	+1(0.5)	-1(5)	+1(10)	+1(2)	+1(2)	-1(0)	+1(0.5)	-1(0)	-1(0)	-1(0)	+1(0.001)	+1(0.001)	**192.00± 2.677**
12	-1(25)	-1(40)	+1(0.50)	-1(0.25)	+1(10)	-1(5)	+1(2)	+1(2)	+1(0.5)	-1(0)	-1(0)	-1(0)	+1(0.001)	-1(0)	-1(0)	110.22± 1.850
13	+1(50)	+1(80)	-1(0.25)	+1(0.5)	+1(10)	+1(10)	-1(1)	+1(2)	-1(0)	-1(0)	-1(0)	+1(0.001)	+1(0.001)	+1(0.001)	+1(0.001)	168.00± 2.047
14	-1(25)	-1(40)	+1(0.50)	-1(0.25)	-1(5)	+1(10)	+1(2)	-1(1)	-1(0)	-1(0)	+1(0.001)	+1(0.001)	+1(0.001)	-1(0)	-1(0)	036.00± 2.402
15	+1(50)	+1(80)	1(60)-	+1(0.5)	+1(10)	-1(5)	-1(1)	+1(2)	-1(0)	+1(0.5)	+1(0.001)	+1(0.001)	-1(0)	-1(0)	+1(0.001)	084.18±1.457
16	-1(25)	-1(40)	-1(60)-	-1(0.25)	-1(5)	-1(5)	-1(1)	-1(1)	-1(0)	-1(0)	-1(0)	-1(0)	-1(0)	-1(0)	-1(0)	018.00± 1.160

Real values (given in parentheses) are in g.

$MgSO_4$, $ZnSO_4$ and KCl were negatively contributed. The first order model describing the correlation between the fifteen factors and the lipase activity could be presented as follows:

$$Y_{activity} = 93.488+23.155X_1+12.808X_2+25.930X_3+12.621X_4+ 2.559X_5–10.325X_6–16.83X_7–23.087X_8–19.201X_9–2.021X_{10}+ 12.343X_{11}+15.143X_{12}+11.101X_{13}+14.449X_{14}+16.845X_{15} \quad (Eq.5).$$

Based on the calculated *t* -test and *p*-values (Table 3), it was evident that the waste frying oil, triton TritonX-100 and $MnSO_4$ were found to be the most significant variables affecting lipase production. The uses of waste frying oil in production of lipase is involved in the recycling of insoluble organic material (Dandik, *et al*., 1993) and is also use as alternative substrates for the production of medium or high-value products presents an advantage over the traditional processes. The recycling of materials leads to what has been called "clean technology", in which materials are systematically used and re-used to bring about the drastic increase in resource activity needed to make human activity sustainable (Veerapagu *et al*., 2013). Lin *et al*. (1995) found that addition of triton X-100 to the fermentation medium of *Pseudomonas pseudoalcaligenes* F-111 increased the alkaline lipase production by 50-fold. Similar results were obtained by Castro-Ochoa *et al.* (2005) for activating lipase production by *Bacillus thermoleovorans* CCR11. On the other hand, addition of $MnSO_4$ enhances the production of lipase by *Acinetobacter haemolyticus* (Clift, 1997).

Other variables with less significant effect were not included in the next optimization experiment, but instead they were used in all trials at their (+1) level, for the positively contributing variables. According to these results, the optimized medium composed of the following in (g/l): KH_2PO_4, 2; K_2HPO_4, 2; peptone, 5; beef extract, 5; $CuSO_4$, 0.001; NaCl, 0.5 was used as a plain medium for further investigations.

Evaluation of the factors affecting lipase activity by Box-Behnken experimental design

In order to search for the optimum concentration of the most significant medium components, (waste frying oil, triton X-100 and $MnSO_4$) showing confidence level 99% and above in the Plackett–Burman design for lipase production, experiments were performed according to the Box-Behnken design. The results obtained were analyzed by standard analysis of variance (ANOVA) as shown in Table 2. The second-order regression equation provided the levels of lipase production as a function of X_1, X_2 and X_3 can be predicted by the following equation:

$$Y_{lipase} = –410.548 +13.479X_1 – 109.823X_2 +187492.197X_3 – 0.090X_1^2 +7.911X_2^2 – 0.0003386X_3^2 + 0.576X_1X_2 – 400.770X_1X_3 – 410.548X_2X_3$$

Where Y_{lipase} is the response (lipase production) and X_1, X_2 and X_3 are the coded values of the test variables (waste frying oil, triton X-100 and $MnSO_4$) respectively. The three-dimensional response surface plots are the graphical representations of the regression equation. They are helpful in understanding both the main and the interaction effects of the factors on the response value.

Table 2: Experimental results of Box-Behnken design for lipase activity by *Bacillus cereus* ASSCRC-P1.

	Factor levels							
	Waste frying oil (X_1)		Triton x100 (X_2)		$MnSO_4$ (X_3)		Lipase activity (U/ml)	
Trial No.	Coded	Actual (g/l)	Coded	Actual (ml/l)	Coded	Actual (mg/l)	Experimental	Predicted
1	-1	60	-1	1	0	2	420	392
2	+1	100	-1	1	0	2	350	310
3	-1	60	+1	4	0	2	170	190
4	+1	100	+1	4	0	2	210	238
5	0	80	0	2	-1	1	250	236
6	-1	60	0	2	-1	1	140	166
7	+1	100	0	2	+1	4	50.0	34.0
8	-1	60	0	2	+1	4	160	142
9	+1	100	-1	1	-1	1	180	210
10	0	80	-1	1	+1	4	210	250
11	0	80	+1	4	-1	1	230	190
12	0	80	+1	4	+1	4	70.0	64.0
13	0	80	0	2	0	2	340	344
14	0	80	0	2	0	2	334	344
15	0	80	0	2	0	2	338	344

Table 3: Statistical analysis of Plackett- Burman design showing coefficient values, *t*-test and *P*- values for each variable on lipase activity.

Variables	Coefficient	*t*-test	P-value	Confidence level %
Intercept	93.488			
Culture volume	23.155	2.60	0.0100	99
Waste frying oil	12.808	5.12	0.0001	99
Tween 80	25.930	-1.12	0.1387	86
Triton X100	12.621	3.028	0.0042	99
Peptone	2.5590	1.225	0.1196	88
Beef extract	-10.325	0.754	0.2311	76
K_2HPO_4	- 16.83	1.784	0.0473	95
KH_2PO_4	-23.087	2.747	0.0075	99
NaCl	-19.020	1.077	0.1492	85
KCl	-2.020	-0.360	0.3617	63
$FeSO_4$	12.343	-1.592	0.066	93
$ZnSO_4$	15.143	-0.551	0.2945	70
$MgSO_4$	11.101	-1.107	0.1428	85
$MnSO_4$	14.449	3.607	0.0013	99
$CuSO_4$	16.845	2.557	0.0191	98

Figure 8(a) showed that the maximum lipase activity was obtained when the concentrations of waste frying oil ranged between 75-95 (g/l) and 1.5-2.5 mg/ml for Triton X-100. Figure 8 (b) showed that maximum lipase activity was achieved with low concentrations of Triton X-100 and 2-3 mg/ml of $MnSO_4$ while Figure 8 (c) showed that maximum lipase activity was obtained at the same concentrations of $MnSO_4$ and all levels of waste frying oil. It is also reported that surfactants increase the accessibility of the substrates. However thermostable lipase activity from *G. stearothermophilus* strain-5 was not enhanced by surfactants and lipase activity and could only retain 80% of its activity after 30 minutes incubation in Triton X-100. In another report, thermoalkaliphilic lipase of *Geobacillus* sp. T1 was significantly enhanced by Tween 80 (188%) followed by Tween 60 (126%) but was inhibited by Triton X-100 (72%) and sodium dodecyl sulfate (SDS) (5%).Thermostable alkaline lipase from *Bacillus* sp. DH4 was activated by Triton X-110 and Triton X-114 with 164% and 148% activity respectively but was inhibited by SDS (Jagtap *et al*., 2010).

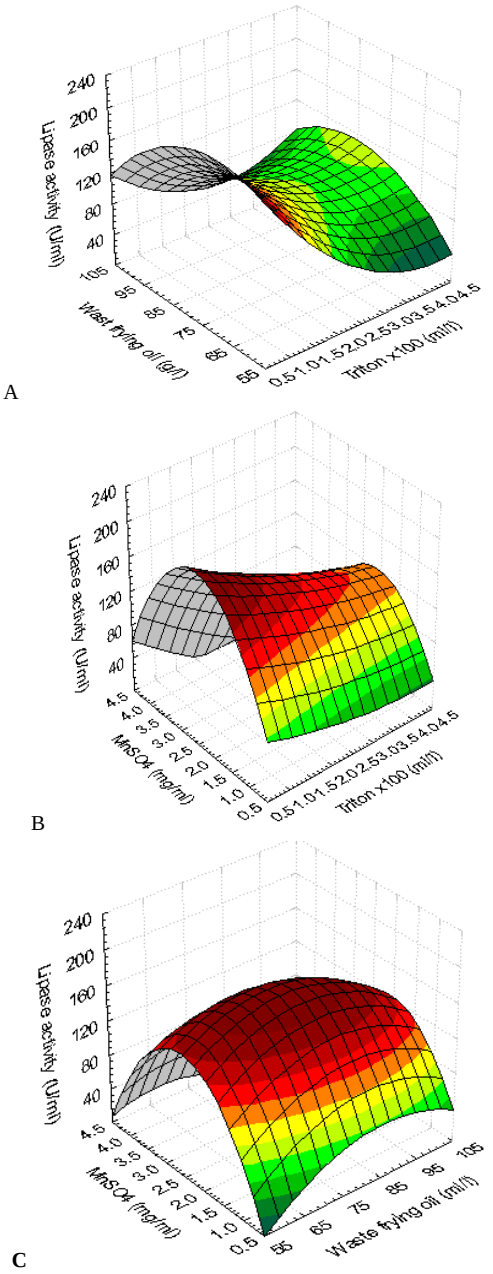

Fig. 8: Response surface plot of lipase production by B. cereus ASSCRC-P1 showing: **(A)** the interactive effects of different concentrations of waste frying oil and triton X-100 at X_3= 0, **(B)** the interactive effects of different concentrations of triton X-100 and $MnSO_4$ at X_1= 0, **(C)** the interactive effects of different concentrations of waste frying oil and $MnSO_4$ at X_2= 0

Table 4: Analysis of variance (ANOVA) test for Box-Behnken experimental design and Model coefficients estimated by multiples linear regression.

Term	Regression coefficient	Standard error	t- test	P-value
Intercept	-410.548	202.685	-2.026	0.089
X_1	13.479	4.696	2.87	0.028
X_2	-109.823	44.68	-2.458	0.049
X_3	187492.2	41773.412	4.488	0.004
X_1^2	-0.09	0.029	-3.114	0.021
X_2^2	7.911	6.204	1.275	0.249
X_3^2	-3.39E+07	5.71E+06	-5.929	0.001
X_1X_2	0.576	0.332	1.737	0.133
X_1X_3	-400.77	359.391	-1.115	0.307
X_2X_3	-410.548	202.685	-2.026	0.089

F value =10.495; P>F= 0.005; R^2= 0.933; R = 0.966; Adjusted R^2=0 .844.

The results obtained by (ANOVA) analysis (Table 4) showed a significant *F*-value (10.495) which implied the model to be significant. Model terms have values of Prob > F (0.0001) less than 0.05, considered significant. The determination of coefficient (R^2) was calculated as 0.933 for lipase activity (a value of R^2 > 0.75 indicated the aptness of the model) which indicates the statistical model can explain 93.3% of variability in the response.

The goodness of the model can be checked by the determination of coefficient (R^2) and correlation coefficient (R). The R^2 value is always between 0 and 1. The closer R^2 value to 1, the stronger the model and the better in the predicted response. The value of R (0.966) for (Eq. 6) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. An overall 2.15-fold increase in lipase was being achieved after application of RSM. This fold increase obtained from our results is more than that obtained by *Staphylococcus arlettae* JPBW-1 through optimization of fermentation variables using response surface methodology recorded 1.8-fold in lipase production as reported by Chauhan *et al*. (2013). In another report, a 1.6-fold increase in lipase production observed in *Arthrobacter* sp. BGCC#490 followed by a 1.4-fold increase by *Enterobacter aerogenes* under optimized conditions of physico-chemical parameters using statistical methods . On the other hand, our fold increase is less than 3.15 and a 5-fold obtained by *Enterococcus faecium* MTCC 5695 and *Burkholderia* sp. respectively using RSM optimization (Ramakrishnan *et al*., 2013).This reflects the necessity and value of optimization process.

Validation of the model

Three repeated experiments were performed under the predicted optimum medium (g/l): waste frying oil, 80 ; KH_2PO_4, 2; K_2HPO_4, 2; peptone, 5; beef extract, 5; $CuSO_4$, 0.001; NaCl, 0.5; $MnSO_4$, 0.002 with addition of 2 ml/l of triton X-100 and culture volume, 50 ml. Under these conditions, lipase activity was 340 U/ml which was in close agreement with the predicted (344 U/ml).

Therefore, these models could be considered as quite reliable for predicting the lipase production from *Bacillus cereus* ASSCRC-P1. Therefore, optimization through statistical experimental design has been applied in the production of many lipolytic enzymes (Kock *et al.*, 1996).

CONCLUSION

In this report we studied the production of thermostable lipase from *Bacillus cereus* ASSCRC-P1 isolated from soil contaminated with oil beside the oil wells in El Ain El Sokhna, Egypt characterized with hot weather. The optimization of the enzyme production by using statistical experimental design was done by using waste frying oil based medium. A highly significant quadratic polynomial equation obtained by the Box-behnken design was very useful for determining the optimal concentrations of constituents that have significant effects on lipase production. A high similarity was observed between the predicted and experimental results, which reflected

the accuracy and applicability of RSM to optimize the process for lipase production.

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