Isolation of Lipase Gene of the Thermophilic *Geobacillus stearothermophilus* Strain-5

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Abstract: In earlier study a new thermophilic strain *Geobacillus stearothermophilus* strain-5 producing thermostable lipase was isolated and identified based on 16S rRNA sequencing. Phylogenetic analysis revealed its closeness to *geobacilli* especially the thermophilic *Geobacillus stearothermophilus* with optimal growth and lipolytic enzyme activity at 60°C and pH 7.0. In this study thermostable lipase gene from this bacterium was isolated by PCR using degenerate primers. The DNA fragment coding for lipase gene was cloned in the pCR 4-TOPO plasmid and the ligation products were transformed into *Escherichia coli* XL1-blue cells. Partial sequencing of the gene was carried out (accession number DQ923401). Analysis by BLAST program showed some sequence similarity to that, of several lipase genes from thermophilic *Geobacillus* and *Bacillus* submitted to Genbank.

Key words: *Geobacillus stearothermophilus*, gene isolation, lipase gene, thermostable lipase

INTRODUCTION

In the last decade several extreme thermophilic and hyperthermophilic microorganisms that are capable of producing extremely thermostable enzymes were isolated. The most important characteristic of thermophilic microorganisms is their ability to produce thermostable enzymes with higher operational stability and a longer shelf life. The advantages for using thermostable enzymes in industrial processes include the decreased risk of contamination, the increased diffusion rate and the decreased cost of external cooling (Malhotra et al., 2000; Abdel-Fattah, 2002).

Lipolytic enzymes, triglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in food, dairy, detergent, oil and fat, cosmetic and pharmaceutical industries (Gupta et al., 2004a, b). The reasons for the enormous biotechnological potential of microbial lipases include the facts that they are stable in organic solvents, do not require cofactors, possess broad substrate specificity and exhibit a high enantioselectivity (Jaeger and Reetz, 1998). Lipases are ubiquitous in nature, they are found more abundantly in microbial flora comprising bacteria, fungi and yeast. They are also found in the pancreas of mammals such as pigs and humans, in the intestine of fish and in higher plants such as castor bean and rapeseed (Pandey et al., 1999). Lipases of microbial origin represent the most widely used class of enzymes in biotechnological application and organic chemistry (Gupta et al., 2004a, b). They are an excellent alternative to classical organic techniques in the selective transformation of complex molecules (Pandey et al., 1999).

Production of thermostable lipases from thermophilic strains is of importance in industrial processes due to the valuable role of lipases in the enzyme market (Abdel-Fattah, 2002). As most of the industrial processes operate at a temperature exceeding 45°C, lipase should be active and stable at a temperature around 50°C (Sharma et al., 2002). Several thermophiles that produce thermostable lipolytic enzymes were isolated, such as *Bacillus* sp. (Sharma et al., 2002), *B. stearothermophilus* (Sinehaikut et al., 2001), *B. thermooleovorans* (Lee et al., 1999) and *Thermus* sp. (Dominguez et al., 2004).

Recombinant DNA technology represents a very attractive feature that can be used to overcome the cost limitation of industrial application of lipolytic enzymes. Many thermostable lipase genes have been cloned and sequenced (Ewis et al., 2004; Soliman et al., 2007; Abdel-Fattah and Gaballa, 2008). Schmidt-Dannert et al. (1996) reported the first nucleotide sequence of a lipase from thermophilic prokaryote.

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A glycerol-inducible lipase was isolated from the thermophilic *G. stearothermophilus* strain 5 earlier (Berekaa *et al.*, 2009). In this study, *G. stearothermophilus* was isolated from desert soil in Egypt and identified according to 16SrRNA sequence. Lipase gene was isolated and cloned in T/A cloning vector for sequencing.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids:** The isolate used in this study was isolated from desert soil sample and was purified and identified using morphological, biochemical test and 16S rRNA as *Geobacillus stearothermophilus* (accession number: DQ923400). *Escherichia coli* strain XL1-blue was also used in this study. The PCR®-TOP® plasmid (3956 bp) was used for PCR cloning. This plasmid was obtained from Invitrogen Life Technologies, USA.

**Lipase assay:** Lipase activity was routinely determined colorimetrically by p-nitrophenyl palmitate (pNPP) method (Vorderwuelbecke *et al.*, 1992). The assay mixture contained 900 µL of the assay reagent and 100 µL of enzyme solution. The assay reagent was prepared by adding 1 mL of solution (1) to 9 mL of solution (2) dropwise to get an emulsion that remained stable for 2 h. The solutions were prepared as follows: solution (1) contained 90 mg of p-nitrophenyl palmitate dissolved in 30 mL of 2-propanol, solution (2) contained 2 g of Triton-X 100 and 0.5 g gum arabic dissolved in 450 mL buffer (Tris/HCl, 50 mM, pH 8). After incubation of the enzyme solution with substrate for 20 min at 60°C, the liberated p-nitrophenol was measured at 410 nm. One unit of enzyme was defined as the amount of enzyme that releases 1 mol. p-nitrophenol from the substrate per minute.

**Monitoring growth and lipolytic activity:** Growth and extracellular lipolytic activity was monitored on the medium. Two milliliter of an overnight culture were used to inoculate 50 mL of the production medium containing (%): glycerol: 2.25; glucose: 0.76; Tween 80: 0.76; KH₂PO₄: 0.38; yeast extract: 1; peptone: 0.2; (NH₄)₂SO₄: 0.2; MgSO₄.7H₂O; KH₂PO₄: 0.1; CaCl₂: 0.02, pH adjusted to 7.5 and incubated at 60°C. The culture was allowed to grow at 60°C with shaking at 150 rpm. Growth was monitored by measuring the absorbance at 420 nm. At the indicated time, 0.5 mL of the growing cultures was taken and centrifuged in a microcentrifuge at 7500 rpm for 3 min to pellet cells. The supernatants were assayed for extracellular lipase using p-nitrophenylpalmitate as a substrate.

**DNA isolation:** An overnight culture of the strain *Geobacillus stearothermophilus* grown at 60°C was used for the preparation of genomic DNA to be used for the cloning strategy. DNA was isolated from the according to plasmid minipreps of the transformed cells was carried out using Wizard® Plus Miniprep DNA Purification System (Promega, UK). DNA was analyzed using 1% agarose gel by electrophoresis.

**Gene isolation and cloning:** Lipase gene was isolated by Polymerase Chain Reaction (PCR) using highly degenerate lipase gene primers designed from the available thermophilic lipase genes deposited in the GeneBank. The degenerate primers were as follows: Forward primer: 5'-CATATGATGAAAKGCTGYGGGTGT-3' and reverse primer: 5'-GGATCTTTAAGGCCGAAARCTGC-3'. The PCR reaction was as follows: 3 µL of genomic DNA (200 ng), 5 µL of 10X Pfu buffer, 3 µL of each degenerate primers (30 pmole), 3 µL of dNTPs (0.4 mM), 1 µL of Pfu (2 units) and the final volume was adjusted to 50 µL with distilled sterilized water. The PCR condition was 1 cycle at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Finally, the tube was incubated at 72°C for 5 min. The PCR products were analyzed using 1% agarose gel by electrophoresis. To facilitate cloning of the PCR products onto pCR®-TOP® T/A cloning plasmid, the PCR product was treated with Taq polymerase as follow: 10 µL of the PCR product was taken into a 0.5 mL tube to which 2 µL of Taq polymerase buffer and 2 units of Taq polymerase and dATP were added. The final volume was adjusted to 20 µL and the tube was incubated at 72°C for 30 min. This treatment facilitates the addition of T/A to the PCR product to enhance the T/A cloning strategy. To ligate the treated PCR product onto a pCR®-TOP® vector, 2 µL of PCR product was taken in a clean 0.5 mL tube to which 1 µL pCR®-TOP® vector and 1 µL of 10X ligase buffer were added followed by the addition of 2 units of ligase enzyme. The final volume of the ligation reaction was adjusted to 10 µL by the addition of nuclease free water. The tube was incubated at 16°C for 16 h. Transformation of *Escherichia coli* XL1-blue competent cells was carried out according to Sambrook *et al.* (1989).

**Screening of recombinant E.-coli XL1-blue pCR 4-TOPO:** The recombinant *E. coli* XL1-blue cells obtained were screened in selective LB/IPTG/X-Gal/tween 20/agar plates. Moreover, colonies PCR was conducted to screen recombinant bacteria for ligated DNA insert. A small part of each bacterial colony was transferred to a clean sterile end presentation tube, to which the rest of the PCR reaction components was added as described earlier. The PCR condition was as follows; 1 cycle at 95°C for 5 min to
lyse the *E. coli* cells followed by 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. The PCR products were analyzed through 1% agarose gel by electrophoresis.

**Sequencing of the PCR product**: Sequencing of the PCR product cloned onto pCR 4-TOPO vector was carried out using dyeodeoxy chain termination method according to Sanger *et al.* using ABI Prism Ready Reaction dye Terminator Sequencing kit and analyzed on an ABI 377 automated sequencer (Sanger *et al.*, 1977).

**RESULTS AND DISCUSSION**

**Monitoring of lipase production**: In a earlier study (Berekaa *et al.*, 2009) a newly identified *G. stearothermophilus* was characterized and showed an inducible nature of lipolytic enzyme synthesis using glycerol and glucose. Moreover, the inducible lipase showed a great thermostability. In the present study the bacterial growth and the extracellular lipolytic activity of the *G. stearothermophilus* 5 cells were monitored. Bacterial growth was determined by measuring the absorbance of the culture suspension at 420 nm. Lipolytic activity was measured using p-nitrophenyl palmitate as mentioned before. Data of Fig. 1 showed the growth and the level of lipolytic activity through 48 h of incubation. Data showed that, the level of extracellular enzyme production started at late log phase of the bacterial growth and increased gradually with bacterial growth till reached its maximal level about (450 U mL⁻¹) after 36 h. In general, lipolytic enzymes were found to be produced in the late exponential phase and growth-associated (Lee *et al.*, 1999; Gupta *et al.*, 2004b).

**Isolation of lipase gene**: In an attempt to isolate the DNA fragment coding for the lipolytic activity from *G. stearothermophilus*, PCR based technique was used. Lipase gene specific degenerate primers, designed from the available thermophilic lipase genes in the Genebank were employed. These degenerate primers specific conserved sequences at both ends of lipase gene. Polymerase chain reaction was carried out to amplify lipase gene. The size of the PCR product was found to be around 2.4 kbp as shown in Fig. 2. The size of the PCR product was more than enough to encode a protein of a molecular weight of 61 KDa that corresponds to the molecular weight of the lipase produced and purified previously from *G. stearothermophilus* 5 (data not shown). The PCR was widely used to isolate lipase gene from different isolates, a PCR method suitable for the isolation of lipase genes directly from environmental DNA was also described (Bell *et al.*, 2002).

**Cloning of the gene**: To facilitate the sequencing of the purified PCR product, T/A cloning strategy was conducted. Purified PCR product was ligated onto the pCR 4-TOPO plasmid (Fig. 3) and the ligation products was transformed into *E. coli* XL1 blue competent cells. The transformed cells were plated onto LB agar plates supplemented with ampicillin, IPTG, X-Gal and Tween 20 as a substrate for the lipase enzyme. Plates were incubated at 37°C for overnight. Results showed the presence of white recombinant and blue non-recombinant colonies. White colonies (potential positive clones) were tested for the presence of DNA insert using colonies PCR strategies.

![Fig. 1: Monitoring bacterial growth and lipolytic activity of *G. stearothermophilus* 5 cells](image)

![Fig. 2: Agarose gel (1%) electrophoresis of PCR product (Lane 2) generated from *G. stearothermophilus* DNA using lipase gene specific primers. Lane 1 represents 1 kbp pair DNA base pair marker](image)
Fig. 3: Schematic representation of pCR 4-TOPO plasmid that was used for cloning of PCR product fragment

Fig. 4: Agarose gel (1%) electrophoresis of colonies PCR products (Lanes 2-12). Lane 1 represents one kilobase pair DNA base pair marker. Lane 12 showing a positive colony PCR product about 2.5 kbp

Colonies PCR technique was used to screen recombinant bacteria for the presence of DNA insert. The presence of positive PCR product (Fig. 4, Lane 12) was confirmed. The size of the cloned gene (insert) is about 2.4 kbp and this confirmed that there is a clone. Many lipase genes have been cloned and sequenced (Cho et al., 2000; Rahman et al., 2005; Saeed et al., 2006). However, recombinant enzyme production has been limited to a few microbial lipases (Houde et al., 2004).

Fig. 5: Partial coding sequence of the lipase gene form *G. stearothermophilus* 5-strain

**Sequencing of the PCR product:** The partial DNA sequence of the PCR product generated by the forward and reverse primers was carried out. The partial sequence (959 base pairs) (Fig. 5) was deposited in the GenBank sequence database and given the accession number DQ923401. Sequence analysis of the partial sequence revealed high similarity to that of several thermostable lipase genes from thermophilic *Geobacillus* and *Bacillus*.
Table 1: Data analysis of DNA sequence using BLAST search program

<table>
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<tr>
<th>Accession</th>
<th>Description</th>
<th>Total score</th>
<th>E-value</th>
<th>Max ident (%)</th>
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<tr>
<td>U78785.1</td>
<td><em>Bacillus steaethermophilus</em> lipase gene, complete CDs</td>
<td>1168</td>
<td>0</td>
<td>89</td>
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<tr>
<td>FJ392756.1</td>
<td>Uncultured bacterium clone JK01 lipase gene, complete CDs</td>
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<td>DQ009618.1</td>
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<td>987</td>
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<td>86</td>
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<tr>
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<td>82</td>
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<td>664</td>
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Fig. 6: Phylogenetic relation lipase gene sequence of *G. steaethermophilus* strain-5 with lipase gene of different lipases from the database. The dendrogram was generated by the neighbor-joining method using BioEdit software.

and the degree of homology was found to be around the N-terminal part of that gene (obtained from the forward primer alone). Eighty nine percent similarity with was showed with *Bacillus steaethermophilus* lipase (accession no U78785) and Uncultured bacterium clone JK01 lipase (accession no FJ392756) and 88% with *Geobacillus* sp. SF1 lipase gene (accession No. DQ009618) (Table 1). This result confirmed that, the isolated and cloned fragment most likely encode one of the thermophilic lipase gene from *G. steaethermophilus* (Fig. 6). Schmidt-Dannert *et al.* (1996) reported the first nucleotide sequence of a lipase from thermophilic prokaryote. Abdel-Fattah and Gaballa (2008) showed high similarity between the thermostable lipase gene isolated from *G. thermodenitrificans* Toshiki and other thermostable lipase genes deposited in the database.

In conclusion a PCR generated DNA fragment of 959 bp was obtained from *G. steaethermophilus* and based on the sequencing result it was found that the partial coding sequence revealed high similarity to that of other thermostable lipase genes deposited in the GeneBank from thermophilic *Geobacillus* and other *bacilli*. However, more sequencing will be needed to obtain the full length lipase gene in an attempt to express this gene and characterize the lipase in term of kinetic properties towards different substrates and to classify the enzyme.

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REFERENCES


