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Cloning and Expression of a Subfamily 1.4 Lipase from *Bacillus licheniformis* IBRL-CHS2

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Abstract: We report on the cloning of the lipase gene from *Bacillus licheniformis* IBRL-CHS2 and the expression of the recombinant lipase. DNA sequencing analysis of the cloned lipase gene showed that it shares 99% identity with the lipase gene from *B. licheniformis* ATCC 14580 and belongs to subfamily 1.4 of true lipases based on amino acid sequence alignment of various *Bacillus* lipases. The 612 bp lipase gene was then cloned into the pET-15b(+) expression vector and the construct was transformed into *E. coli* BL21 (DE3) for bulk expression of the lipase. Expression was analysed by SDS-PAGE where the lipase was found to have a molecular weight of about 23 kDa.

Keywords: *Bacillus licheniformis*, Lipase, Subfamily 1.4 Lipase, Cloning, Expression

INTRODUCTION

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) act at the interface between a hydrophobic lipid substrate and a hydrophilic aqueous medium to catalyse the hydrolysis of ester bonds in triglyceride molecule to yield free fatty acids, diglycerides, monoglycerides, and glycerol (Verger 1997). This process is termed as interfacial activation where upon contact at a lipid-aqueous bilayer, the lid that caps the active site of the lipase opens and the active site is available for catalysis to take place. Lipases have been reported to possess diverse substrate specificity and are widely used in pharmaceutical, dairy, detergent, cosmetic, oleo chemical, fat-processing, leather, textile, and paper industries (Sharma *et al.* 2001). Therefore, the search for new lipases suitable to be used in the various industries mentioned is always encouraged. It is a known fact that *Bacillus*

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lipases belong to subfamily 1.4 of true lipases which encompasses the lipases from *B. subtilis*, *B. pumilus,* and *B. licheniformis* among others. They share similar traits like the conserved pentapeptide A-H-S-M-G, small in size, demonstrate good activity at alkaline pH and possess broad substrate specificity which are all biotechnologically valuable properties (Eggert *et al*. 2002). The *Bacillus* strain used in this research was identified using molecular approach (16S rRNA sequencing) as *B. licheniformis* (Noor Mazuin *et al.* 2009) and is referred to as *B. licheniformis* IBRL-CHS2. In this study, the lipase gene belonging to *B. licheniformis* IBRL-CHS2 was cloned and the recombinant lipase was consequently expressed in soluble form to lay foundation for future structure-function studies of the enzyme, its characterisation and to exploit its potential as a biocatalyst.

MATERIALS AND METHODS

Cloning of Lipase Gene

The genomic DNA of the bacterium was extracted and the lipase gene was amplified by polymerase chain reaction (PCR) using suitable primers with incorporated restriction sites for *Nde*I and *BamH*I (underlined) in the forward: 5'-CGC CGG CAT ATG CGT CGT CAT TCA TTT TTA-3' and reverse: 5'-TCT ATT GGA TCC TTA TTT CCC GCT GGC GGT-3' primers, respectively. The PCR conditions were as follows: 1 initial denaturation step at 94°C for 1 minute, 30 repeated cycles of thermal denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 1 minute followed by a final extension for 10 minutes at 72°C. The PCR product was purified, ligated into pGEM®-T Easy vector system (Promega®, USA), and transformed into *E. coli* JM109 competent cells. The transformants were spread on LB agar plate supplemented with 100 µg/mL ampicillin, 1 mM IPTG, and 20 µg/mL X-Gal. After overnight incubation at 37°C, a white clone containing the lipase insert was identified with the aid of blue-white screening where positive clones were white while negative clones appeared blue. For further confirmation, the recombinant plasmid (pGEM-lip) was extracted from the clone and sent to First Base Laboratories for DNA sequencing. The sequence obtained was aligned with other *Bacillus* sp. lipases and analysed using MEGA 6 (Molecular Evolutionary Genetics Analysis) software. The nucleotide and amino acid sequences of this gene can be accessed in GenBank (accession numbers: KU984433 and AOT80658)

Over-expression of the Lipase Gene

The lipase gene was digested out from pGEM-lip using the restriction enzymes *Nde*I and *BamH*I, ligated into a similarly digested pET-15b(+) vector, and transformed into *E. coli* BL21 (DE3) host cell. A clone carrying the expression construct (pET-lip) was picked and grown in LB broth supplemented with 100 µg/mL ampicillin and 1% glucose (v/v) at 37°C. When the OD_{600nm} reached 0.8, IPTG was added to a final concentration of 1 mM and the incubation was continued at 15°C and 180 rpm for another 24 hours. Cells were harvested and

re-suspended in 0.1 M Tris-Cl buffer, pH 8.0 and the suspension was subjected to sonication to release intracellular proteins. The fractions were analysed by SDS-PAGE using 12% polyacrylamide gel to observe the expression of the lipase.

RESULTS AND DISCUSSION

Cloning of Lipase Gene

The lipase gene is 612 bp long which codes for 204 amino acids. The size of the protein is deduced to be 23 kDa. Based on the analysis performed by using the MEGA 6 program, the sequencing results revealed that the lipase gene shares 99% similarity with the lipase gene from *B. licheniformis* ATCC 14580 (NCBI accession number CP000002.3). The aligned amino acid sequence of the cloned lipase with other subfamily 1.4 lipases showed that this lipase shares 74% homology with the lipase of *B. sonorensis* (WP006636517.1), 67% homology with lipase A of *B. subtilis* (BAP19127.1), 67% homology with the lipase of *B. amyloliquefaciens* (AGO17775.1), and 63% homology with the lipase of *B. pumilus* (ACA60975.1). The catalytic triad is formed by Ser-107, Asp-159, and His-182. Sequence analysis also suggests that this lipase belongs to subfamily 1.4 of true lipases because aside from being small, it has the conserved pentapeptide unique to this subfamily, Ala-His-Ser-Met-Gly with Ser being part of the catalytic triad (Fig. 1). The nucleotide and amino acid sequences of this gene can be accessed in GenBank (accession numbers: KU984433 and AOT80658).

Over-expression of the Lipase Gene

The expression system in this study is T7 promoter driven where the expression of protein is repressed until the addition of IPTG. However, the key issue in the expression stage was leaky expression of the recombinant lipase that turned out to be toxic to the host. Hence, 1% of glucose was added to overcome the toxicity which led to good expression of the recombinant lipase. Incubation of the culture after induction at a low temperature, i.e., 15°C for a longer period of time (24 hours) also favoured the expression system. Most of the expressed recombinant lipase is in the soluble fraction which is desirable as it makes purification easier (Fig. 2). The size of the recombinant lipase corresponds to the deduced size of 23 kDa. The lipase's small size could be attributed to the lack of lid domain that covers its active site which is typically present in other lipases. This means that the lipase does not undergo interfacial activation and its active site is solvent exposed (Eggert *et al*. 2001).

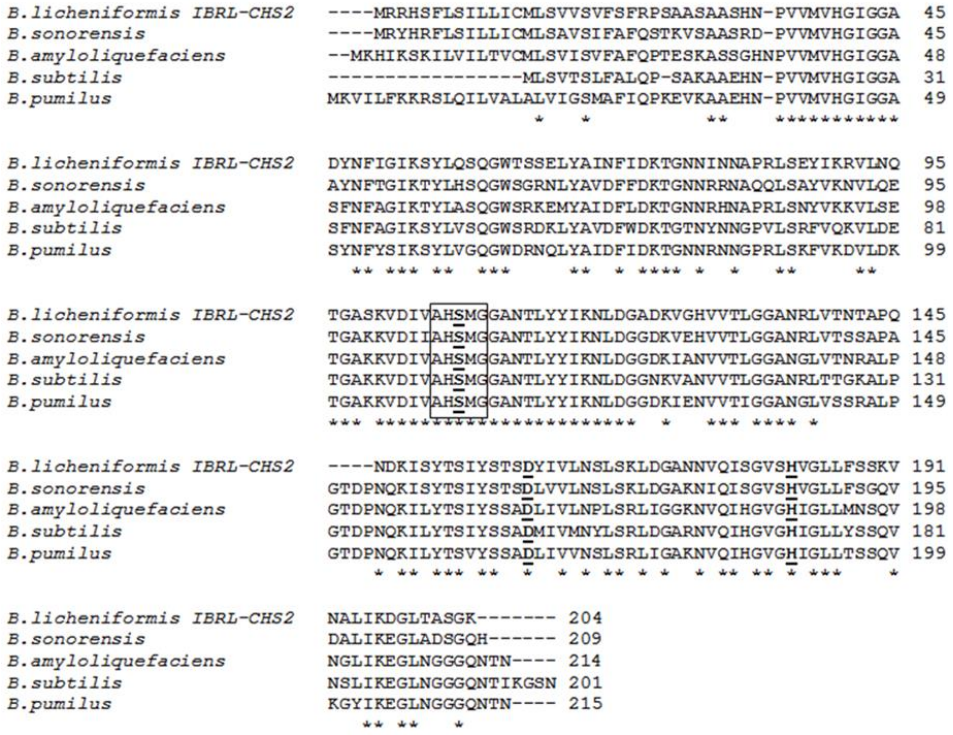

Figure 1: Alignment of *B. licheniformis* IBRL-CHS2 lipase with *B. sonorensis* lipase (NCBI accession number: WP006636517.1), *B. amyloliquefaciens* lipase (AGO17775.1), *B. subtilis* lipase A (BAP19127.1), and *B. pumilus* lipase (ACA60975.1). The conserved pentapeptide is boxed and the catalytic residues of Ser (S), Asp (D), and His (H) are labelled in bold and underlined. Similar amino acid residues in the sequences are marked with asterisk (*).

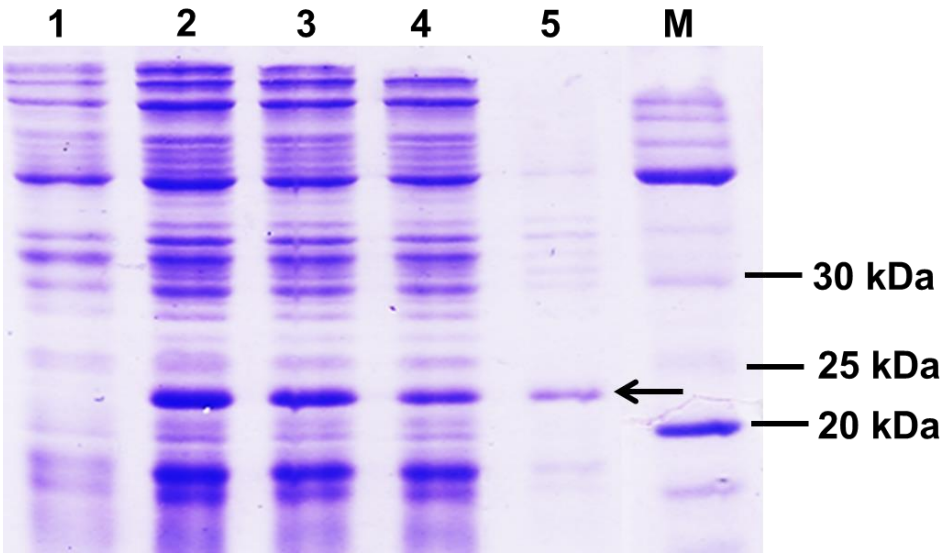

Figure 2: Expression of recombinant lipase by *E. coli* BL21 (DE3) harbouring pET-lip. Expression conditions were at 15°C for 24 hours after induction with 1 mM IPTG. Arrow indicates the recombinant lipase, deduced size of 23 kDa. M: *BenchMark™ Protein Ladder*; 1: uninduced; 2: induced-crude cell extract; 3: induced-total protein after sonication; 4: soluble fraction; 5: insoluble fraction.

CONCLUSION

The lipase gene was cloned and expressed as a 23 kDa protein. Amino acid sequence analysis of the recombinant lipase revealed that it belongs to subfamily 1.4 of true lipases and suggests that it possesses certain biotechnologically important traits that make the study of this enzyme crucial. Purification and characterisation studies of this lipase are underway to further determine its efficacy as a biocatalyst.

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