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Cloning and expression of lipase gene from native isolate of *Bacillus subtilis*

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Abstract
Lipases are enzymes which catalyze the hydrolysis of triglycerides into fatty acid and glycerol. Chemical catalysts are presently used in the food and detergent industries in several processing steps in the development of many commercial products. Naturally occurring enzymes with more efficiency, energy saving and environmental friendly properties can be promising alternatives to them. The present investigation has thus been carried out with the aim for heterologous expression of lipase gene isolated from *Bacillus subtilis* using gene specific primers in the host *E. coli*. The gene consists of an open reading frame of 639 bp which encodes 213 amino acids. Initially, the isolated lipase gene was cloned in to T/A cloning vector (pTZ57 R/T) and later sub-cloned into the bacterial expression vector pET- 28a (+) and then expressed in *E. coli* BL21. The small scale recombinant protein expression was achieved by inducing with 1 mM IPTG at 37 °C with 4 h. of induction time. The size of the expressed recombinant protein was estimated to be 23 kDa upon electrophoresis with 12% SDS-PAGE. This lipase gene from *Bacillus subtilis* successfully expressed in *E. coli* BL21 strain can be explored for commercial application.

Keywords: *Bacillus subtilis*, lipase, expression

Introduction
Lipases, are the third most widely utilized enzymes, after proteases and amylases (Carbohydrases), due to their multiple applications [1]. Lipase (EC 3.1.1.3) is an enzyme that catalyses the hydrolysis, esterification and transesterification of long-chain triacylglycerols at the oil-water interface. It is also known as fat splitting, glycerol ester hydrolase, or triacylglycerol acylhydrolase [2-3]. These reactions are employed in the processing of fats and oils, detergents and degreasing formulations, food processing, fine chemical and pharmaceutical synthesis, paper production and cosmetics manufacturing [4-5].
The significant rise in wider application of enzymes in various chemical processes has increased over a few decades [6] and is a matter of concern in the field of enzymology and has necessitated for a search of them in different natural sources. Microbes are a good source for this as they are easily accessible, and have the advantages of quick growth on low-cost media and their constant supply due to lack of seasonal oscillations. Microbial enzymes can be genetically manipulated, have a wide range of catalytic activity, can be scaled up to give large yields, easier and safer to synthesize and are more stable than plant and animal enzymes [4]. Hence these enzymes are more valuable than those from plants or animals for commercial use.
Lipase can be produced by a range of bacteria, among which *Bacillus* sp., is the most investigated. *Bacillus* species are attractive industrial organisms for numerous reasons, including their high growth rates, which result in short fermentation cycle times, their ability to secrete proteins into the extracellular medium and have the status of Generally Recognized As Safe (GRAS) as per the Food and Drug Administration (FDA) of the United States [7].
Bacillus lipases are classified into eight families, with lipases from *Bacillus* belonging to the 1.4 and 1.5 subfamilies of genuine lipases and having low molecular weight and structure homology (about 19 kDa) with other enzymes in the family. The amino acid composition located around the active region of most lipases is a Gly-X-Ser-X-Gly (X-any amino acid) pentapeptide and for *Bacillus* lipases it is Ala-X-Ser-X-Gly. The great majority of known lipases include a hydrophobic molecular lid domain and its "open form" is critical for enzyme function. As a result, enzymes must undergo conformational changes that occur in the presence of an interface, such as oil drops to get into an open confirmation and this structure where the lid is dislocated and the enzyme is revealed in its open and active state is referred to

as interfacial activation. There is tremendous interest in achieving various lipases in their "open form" in order to obtain increased activity. However *Bacillus* lipases are always active and do not require interfacial activation since their structure does not include a lid domain covering their active site. *Bacillus* lipases are also appealing to study as they can easily adapt for a variety of industrial processes due to their exceptional structural and biochemical properties (activity over a wide pH range, stability in organic solvents and heat stability) [8].

Furthermore, much is now understood about the biochemistry, physiology, and genetics of *Bacillus subtilis* and other species, allowing for more development and industrial application of these organisms. *Bacillus subtilis*, a gram-positive bacteria family that has been extensively investigated and exploited, offers enormous potential in different disciplines. Currently, the genome of *B. subtilis* has been sequenced, with 53 per cent of gene sequences reported. Lipases from *B. subtilis* have recently got a lot of attention since they have the potential to be employed in the food and chemistry sectors. However, lipase expression efficiency in wild type *B. subtilis* is low, limiting its widespread use. The use of recombinant DNA technology to boost the lipase yield and characterize the lipase of *B. subtilis* has been employed to increase the synthesis of enzyme. As *B. subtilis* lipase is more likely to be folded correctly in the host than in other systems, it has a substantially higher activity. At present there is no acceptable plasmid for expressing lipase in *B. subtilis*, so designing and building a novel plasmid for efficient lipase over expression is not only important, but also urgent [5].

Biotechnology plays a significant role in global industry, particularly in the pharmaceutical, food and chemical sectors. The market for industrial enzymes alone is worth $1.6 billion per year. Proteases, lipases, carbohydrases, recombinant chymosin for cheese production and recombinant lipase for detergent production are all essential [9]. Lipases, on the other hand, account for only around 3per cent of total enzyme sales worldwide. Lipases have gained worldwide attention in attempts to harness their physiology and biotechnological applications due to their vital roles and additional studies concerning lipases released in recent years. Due to the enormous potential of lipase in various applications the present investigation was carried out to clone and express the lipase gene of *Bacillus subtilis* in *E. coli* BL21 codon plus strain.

Material and methods

The study related to the research on "Cloning and expression of lipase gene from a native isolate of *Bacillus subtilis* was carried out in the Department of Plant Biotechnology, University of Agricultural Sciences, Bangalore.

Strains, plasmids, and culture medium

Bacillus subtilis was procured from Department of Agricultural Microbiology, UAS, GKVK, Bengaluru, Karnataka. *E. coli* DH5α and *E. coli* BL21(DE3) were used as cloning and expression host respectively, whereas pTZ57R/T and pET-28a(+) (Fig.2) were used as cloning and expression vector respectively. Luria broth and Luria bertani agar medium were used for cultivating and maintaining *E. coli* at an incubation temperature of 37 °C.

Cloning and sequencing analysis of lipase gene

Total genomic DNA was isolated from *Bacillus subtilis* by using HiPurA^{TM} Bacterial Genomic DNA Purification Kit (Cat No. MB505, HIMEDIA).

Around 20 lipase gene sequences from different strains of *Bacillus subtilis* were downloaded from the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). Upon multiple sequences alignment by Bio-edit software, a set of oligonucleotide primers were designed using FastPCR software 6.3. The sequence of the forward primer F1 was 5′-ATGAAATTTGTAAAAAGAAGGATCATTGCACT-3′ and that of the reverse primer was 5′-TTAATTCGTATTCTGGCCCCCGCCGTTCAG-3'. PCR was performed with an initial denaturation at 95 °C for 5 min, followed by 94 °C for 45 s, 59 °C annealing temperature for 45seconds, 72 °C extension temperature for 1 min, and a final extension at 72 °C for 10 minutes with 40 cycles. A 5 µL volume of PCR product was electrophoresed in 0.8% agarose gel for detection. The PCR product was purified using a DNA purification kit (QIAquick, QIAGEN). The purified fragment was ligated with the pTZ57R/T vector, and the ligated product was transformed into *E. coli* DH5α competent cells using a standard protocol [10].

Positive transformants were selected based on blue white screening and white colonies were selected and confirmed by colony PCR. The PCR and restriction digestion confirmed plasmid was outsourced at Agri genomics Pvt. Ltd for sequencing. Plasmid sequencing result of lipase gene was analyzed with the help of MEGA 7.0 software, Bio-edit and by nucleotide sequence analysis using NCBI database for similarity check.

Sub cloning of lipase gene into expression plasmid

Both the plasmids pTZ57R/T -lip and pET-28a (+) were isolated from *E. coli* DH5α to clone the lipase gene into expression vector. The primers were designed for the amplification of the lipase gene from pTZ57R/T -lip with Sac I and HindIII restriction sites. The sequences of designed primers were follows: F2 (forward primer), 5′-GCGCGGATCCATGAAATTTGTAAAAAGAAGG-3′ and R2, (reverse primer), 5′ACCAAGCTTATTCGTATTCTGGCCCCCG-3′. This recombinant plasmid pTZ57R/T having lipase gene was named as pTNABSL5221 from which the gene was amplified. Both the amplified lipase gene and the plasmid pET-28a (+) were digested with SacI and HindIII; the products were purified and ligated using T4 DNA ligase to form the expression vector pET-28a-lip. The recombinant expression vector was transformed into *E. coli* BL21 competent cells. Positive clones were identified by colony PCR and double restriction digestion. Detailed protocols were from [10].

Expression of lipase gene in *Escherichia coli* BL21

A transformant of *E. coli* BL21 harboring pET-28a-lip was cultured with vigorous shaking at 37 °C overnight in 1 mL LB broth containing 50 µg/ mL kanamycin. The culture was transferred using 10 µL inoculum to 1mL fresh LB broth containing the same concentration of kanamycin; the culture was incubated with shaking at 37 °C until the OD600 reached 0.4 to 0.8; IPTG was then added to a final concentration of 1 mmol/ L. Cells were collected 2, 4, 6, and 8 h after IPTG addition for protein detection by SDS-PAGE. The negative control was prepared by the same method using cells not

subjected to IPTG induction.

Identification of expression product

Samples obtained at different induction times were adjusted such that their OD 600 value was 0.6.Cells from 30 mL culture were collected by centrifugation and resuspended in 20 mM Tris-HCl buffer, pH 8.0. The cell suspensions were sonicated and soluble lipase (crude enzyme) was obtained from the supernatant. The tubes were kept for centrifugation at 6000 rpm for 10 min to harvest the bacterial cells. The supernatant was removed and pellet was dissolved in 60 µL of 7M urea and kept at -20 °C. Sample preparation was done with addition of 5 mL buffer to 20 mL of the crude protein (uninduced and induced at different time) and incubated at 95 °C for 5 min. The samples were then cooled to room temperature and mixed with 6X SDS gel loading buffer before loading onto the gel. Supernatant fractions of 20 µL were subjected to SDS-PAGE analysis.

Results and discussion

Isolation, Cloning and sequence analysis of *Bacillus subtilis* lipase gene

The selected strain of *Bacillus subtilis* procured from Department of Agricultural Microbiology, UAS, GKVK, Bengaluru was grown on Luria Bertani (LB) agar medium. A lipase-encoding gene was amplified from genomic DNA of *B. subtilis* by PCR using specific primers and the amplified gene length of 640 bp was confirmed by agarose gel electrophoresis (Plate 1). The genomic DNA of *Bacillus cereus* was extracted using Wizard Genomic DNA Purification kit (Promega) and the product was then used as template for amplification of lip gene with PCR technique using F-Lip and R-Lip primers [11]. Molecular cloning, nucleotide sequence, purification and some properties of lipase from *Bacillus thermocatenulatus* has been reported [12].

The amplified lipase gene was cloned into TA cloning vector pTZ57R/T and transformed into *E. coli* DH5α. The white colonies were selected and done the colony PCR to confirm the positive clones with 640 bp amplicon (Plate 2). The recombinant plasmids were extracted from PCR positive colonies and confirm the gene by PCR (Plate 3) as wells as by restriction digestion (Plate 4). The PCR and Restriction digestion results have shown the desirable fragment of 640 bp on agarose gel electrophoresis. The confirmed recombinant pTZ57R/T lip clones were sequenced and the comparative analysis showed that the clones had 99.53 per cent similarity with *Bacillus subtilis* strain RI4914 (CP051306.1), *Bacillus subtilis subsp subtilis* strain PJ-7 (CP032855.1), *Bacillus subtilis* strain SRCM103581 (CP035406.1) and *Bacillus subtilis* strain TLO3 (CP023257.1). It was 99.37 and 99.22 per cent with *Bacillus subtilis* strain PU1 (KY643825.1) and *Bacillus subtilis* strain JAAA (CP045425.1) respectively (Fig.3) [13]. Cloned a moderate heat-resistant lipase gene was cloned from *Bacillus subtilis* FS1403. Sequence analysis revealed that the lipase gene encoded a 212-amino-acid protein containing the conserved motif Ala-X-Ser-X-Gly. Phylogenetic analysis suggested that *B. subtilis* FS1403 lipase gene belongs to the family 1.4 of bacterial lipase genes.

[14] functionally expressed a thermo-alkaliphilic lipase from Bacillus subtilis DR8806, as an N-terminal 6xHis-tagged recombinant enzyme in Escherichia coli BL21(DE3) using pET-28a(+) expression vector. Sequence analysis revealed an open reading frame of 639 bp encoding a 212-amino acid protein containing the well-conserved Ala-His-Ser-Met-Gly motif. The sequencing results suggested that the lipase gene from *B. subtilis* was successfully cloned into the recombinant pTZ57R/T vector and named the construct as pTNABSL5221. The sequence confirmed lipase gene was further cloned into prokaryotic expression vector pET28a for its expression studies.

Expression of lipase in *E. coli* BL21

The lipase gene was amplified from construct pTNABSL5221 using the specific primers with restriction sites and cloned in the right direction into pET-28a vector. The ligated product having recombinant plasmid (pET-28a-lip) was transformed into *E. coli* BL21 and plated on media with antibiotic. Transformed colonies were selected and confirmed through colony PCR, which amplified the 640 bp gene fragment (Plate 5). The gene in the recombinant plasmids was then confirmed with restriction digestion (Plate 6) in which a 640 bp fragment of the gene separated and with PCR where a fragment of the same size amplified (Plate 7). The confirmed recombinant pET-28a-lip construct was named aspTNABSL25621

Expression of the lipase gene was driven by the T7 promoter and induced by the addition of IPTG. Protein expression was monitored and analyzed by SDS-PAGE, which revealed the presence of a new protein in the supernatant obtained after induction with IPTG and the sonication treatment, which was highest with 1 mM IPTG and 8 h sonication treatment, further most of the lipase expressed in *E. coli* existed intracellularly and had an approximate molecular weight of 23 kDa, which was not observed in the supernatants obtained without IPTG induction (Plate 8). Similar recombinant protein size was obtained by earlier researches when the lipase gene was cloned and expressed in *E. coli* BL21. Expressed a thermo-alkaliphilic lipase from *B. subtilis* DR8806 was expressed as a N-terminal 6xHis-tagged recombinant enzyme in *E. coli* BL21 using pET-28a (+) expression vector [14]. One-step purification of the His-tagged recombinant lipase was achieved using Ni-NTA affinity chromatography with a specific activity of 1364 U/mg and an apparent molecular mass of 26.8 kDa. A gene encoding extracellular lipase was cloned from *Bacillus licheniformis* [15]. The recombinant protein containing His-tag was expressed as inclusion bodies in *E. coli* BL21 (DE3) cells, using pET-23a as expression vector. Expressed protein purified from the inclusion bodies' demonstrated 22 kDa protein band on 12 per cent SDS PAGE. Similar recombinant protein size was obtained by earlier researches when the lipase gene was cloned and expressed in *E. coli* BL21 the lipase encoding gene from *Bacillus subtilis* DI2 was amplified, using PCR and cloned into a vector system and sequenced [15]. It was further expressed in *E. coli* DH5α, the recombinant enzyme was purified and its molecular weight was determined to be around 28 kDa.

A thermo-alkaliphilic lipase from *B. subtilis* DR8806 was expressed as an N-terminal 6xHis-tagged recombinant enzyme in *E. coli* BL21 using pET-28a (+) expression vector [14]. One-step purification of the His-tagged recombinant lipase was achieved using Ni-NTA affinity chromatography with a specific activity of 1364 U/mg and the molecular weight of purified enzyme had an apparent molecular mass of 26.8 kDa

A gene encoding extracellular lipase was cloned from *Bacillus licheniformis* [16]. The recombinant protein containing His-tag was expressed as inclusion bodies in *E. coli* BL21

(DE3) cells, using pET-23a as expression vector. Expressed protein purified from the inclusion bodies demonstrated 22 kDa protein band on 12 per cent SDS PAGE

SDS confirmed lipase will be characterized biochemically and it will be utilized for production of biofuel from non-edible oil and used cooking oil.

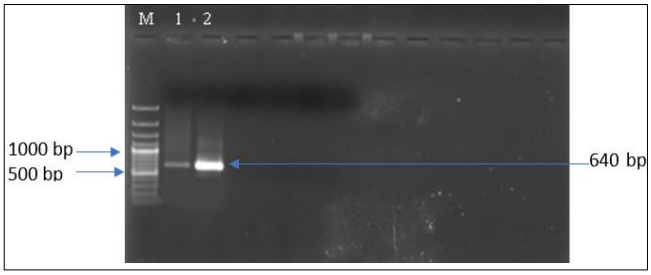
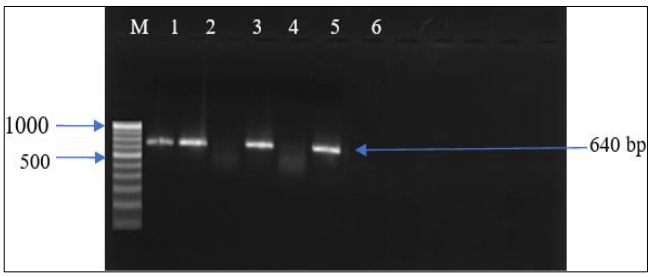


Plate 1: PCR amplification of Lipase gene from *B. subtilis* Genomic DNA, Lane M: 100 bp Ladder, Lane 1: lipase gene, Lane 2: purified lipase gene



P**late 2:** Colony PCR of transformed *E. coli* DH5α containing pTZ57RT + lipase, Lane M: 100 bp Ladder, Lane 1-6: Transformed colonies

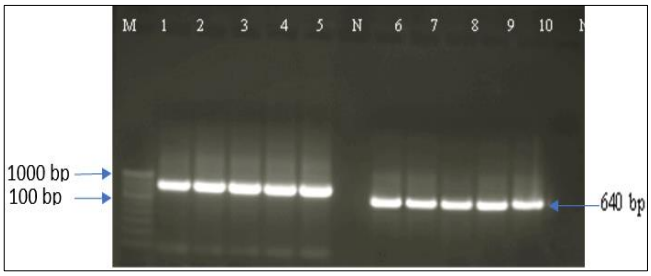


Plate 3: PCR analysis of construct pTNABSL5221, Lane M: 100 bp Ladder, Lane 1-5: PCR products amplified with M13 primers, Lane 6-10: PCR products amplified with gene specific primers, Lane N: Negative control

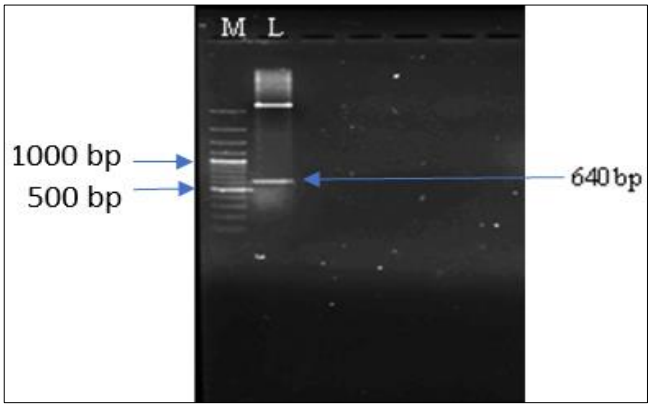


Plate 4: Restriction digestion analysis pTNABSL5221, Lane M: 100 bp Ladder, Lane L: Plasmid digestion with *Sac*I and *Hind* III

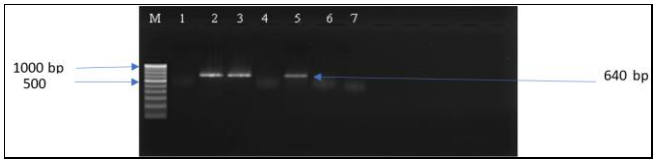


Plate 5: Colony PCR of transformed *E. coli* BL21 containing pTNABSL25621, Lane M: 100 bp Ladder, Lane 1-7: colonies

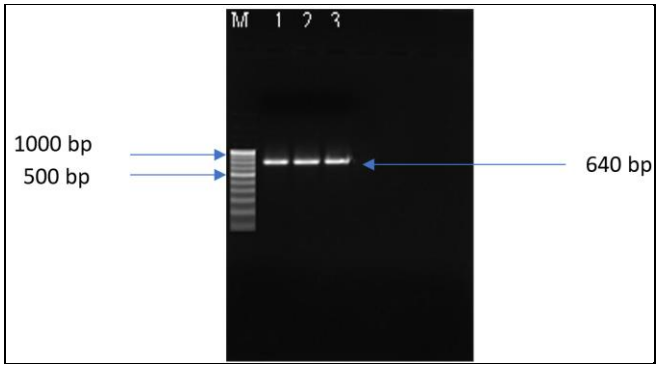


Plate 6: PCR analysis of construct pTNABSL25621, Lane M- 100 bp Ladder, Lane 1-3: plasmid from transformed colony

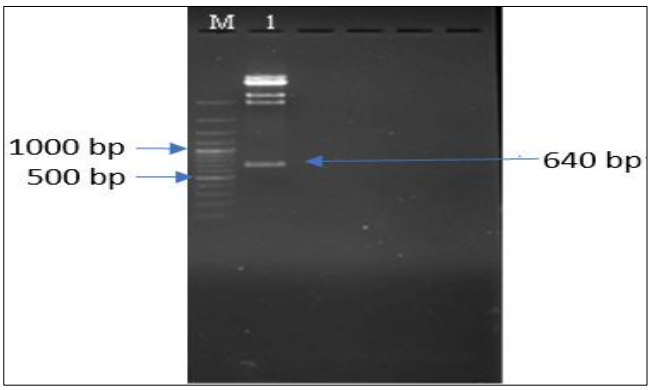


Plate 7: Restriction digestion of pTNABSL25621, Lane M: 100 bp Ladder, Lane 1: vector pET28a+ lipase of *B. subtilis*

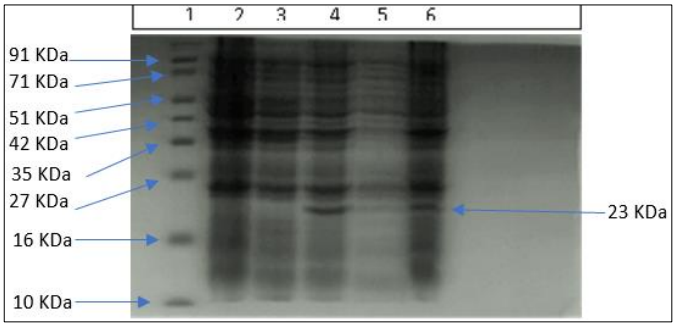


Plate 8: SDS- PAGE analysis of crude protein from bacteria, Lane 1: Pre-stained 4 colour protein ladder (10 - 180 kDa), Lane 2: Empty BL21 kept to growth for 8 hours, Lane 3: Tr. pET28a + lipase, No IPTG addition and kept to growth for 8hrs, Lane 4: Tr. pET28a + lipase, IPTG added and kept to growth for 8 hrs, Lane 5: Tr. pET28a + lipase, IPTG added and kept to growth for 8 hrs (Supernatant fraction), Lane 6: Tr. pET28a + lipase, IPTG added and kept to growth for 8 hrs (Pellet fraction)

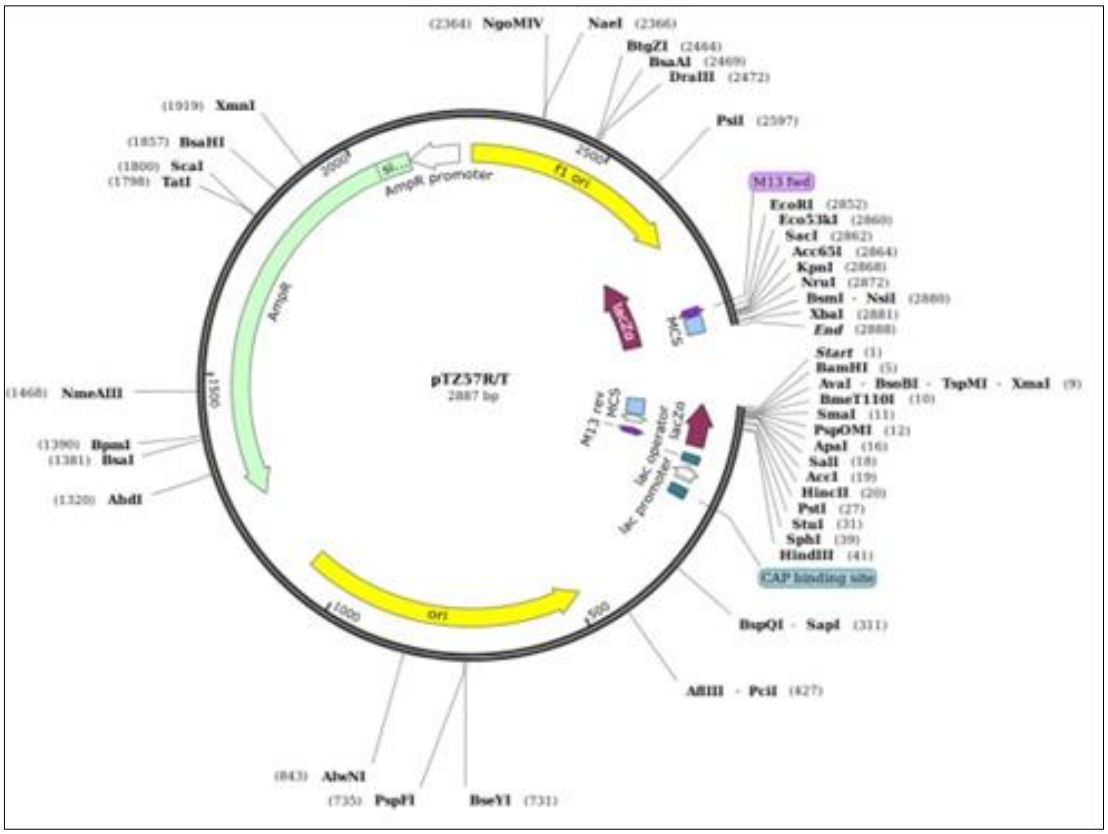


Fig 1: Vector map of T/A cloning vector pTZ57R/T (Thermo scientific, India)

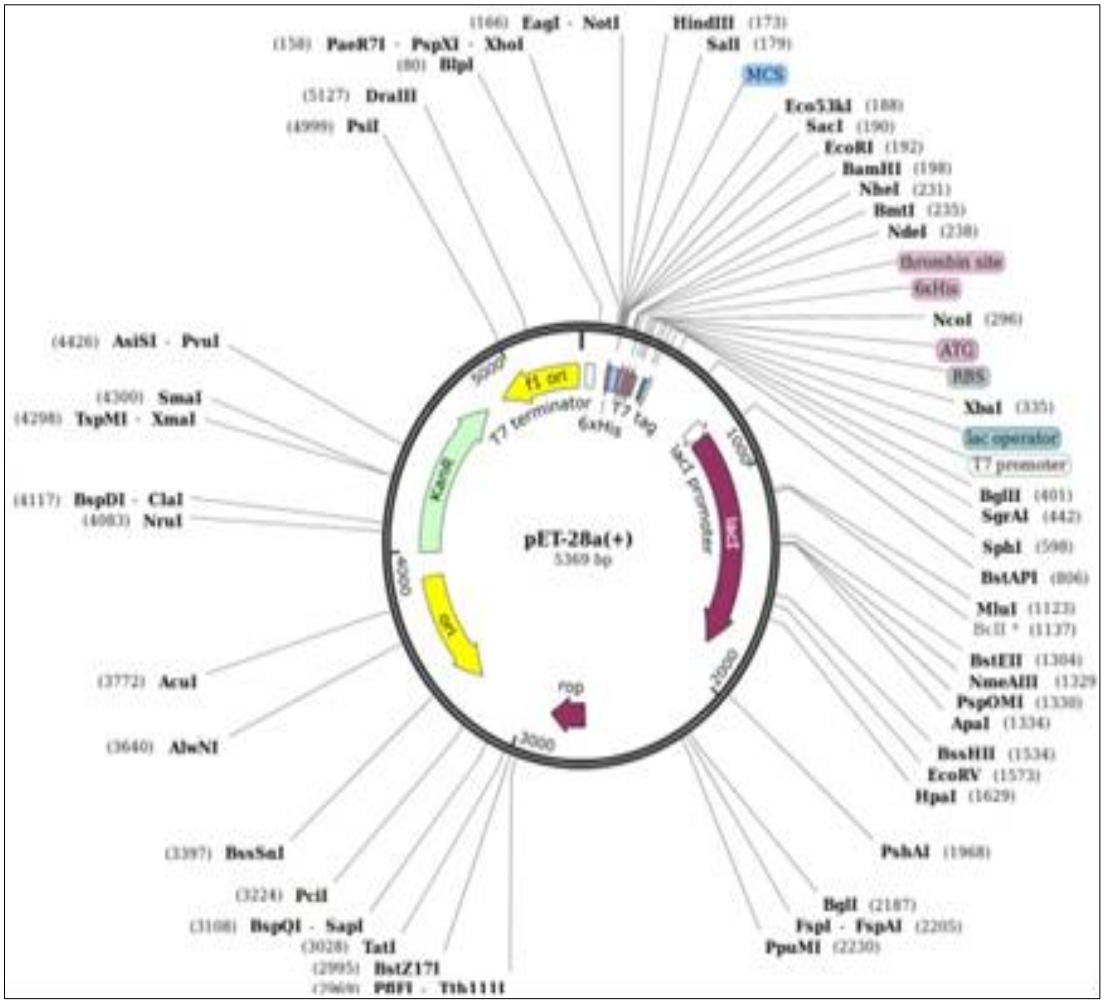


Fig 2: Vector map of bacterial expression vector pET-28a (+) (NEB, England)

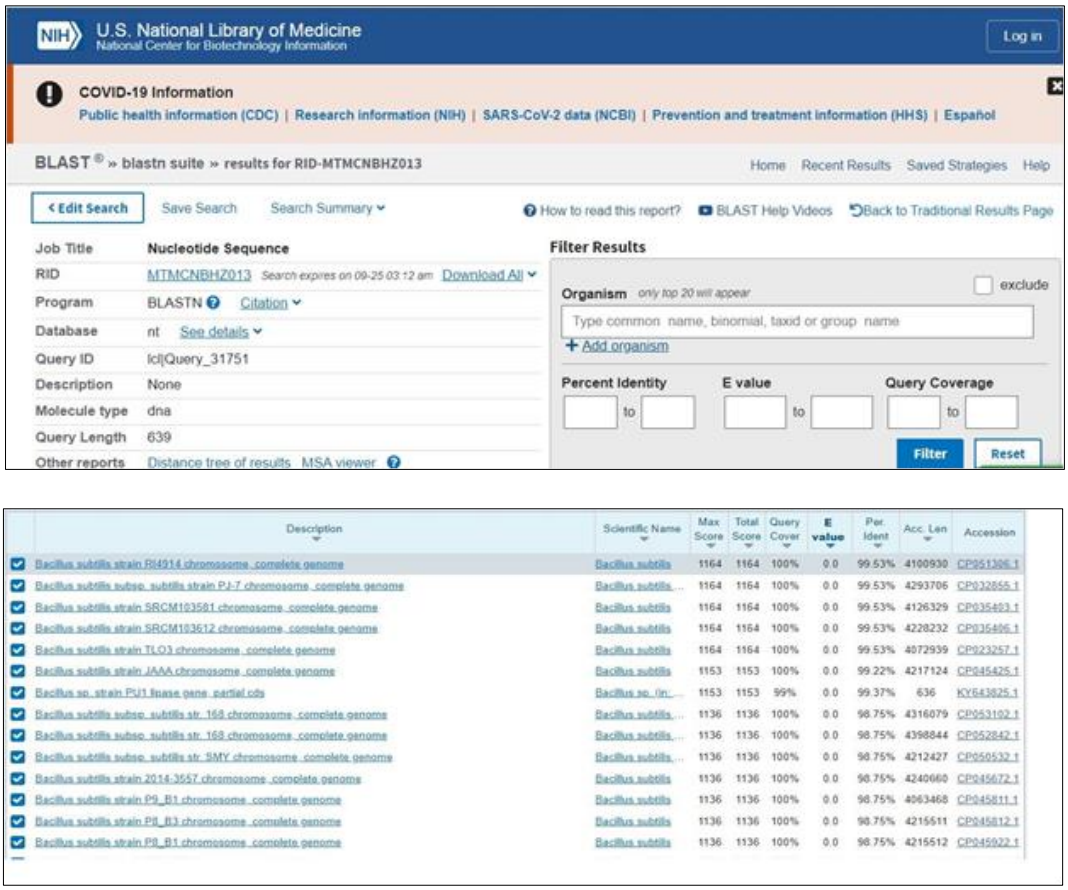


Fig 3: BLAST analysis of lipase gene nucleotide sequence of *B. subtilis* isolate using NCBI database

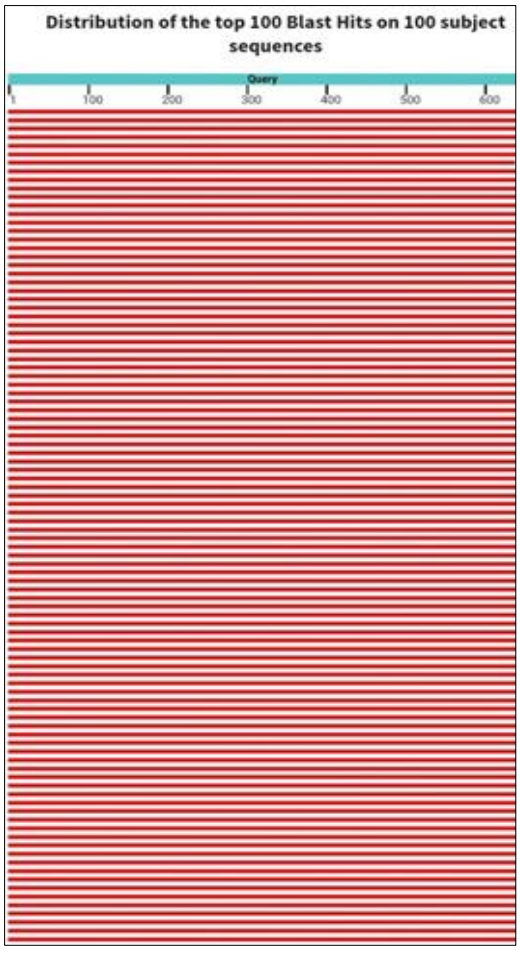


Fig 4: Graphic summary of NCBI blast result, red lines are per cent of similarities of top hundred hits

Conclusion

Our study make way for the production of lipase in large scale and their utilization in industry as well as can be very effectively utilized for the production of biodiesel from used cooked oil or from non-edible oil

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