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Cloning and Integration of *Bacillus subtilis* Lipase gene into *Pichia pastoris*

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Abstract
Lipases are enzymes which catalyze the hydrolysis of triglycerides into fatty acids and glycerol. Lipases of microbial origin are more stable, enjoy greater industrial importance presenting a fascinating field of future research. The methylotropic yeast *Pichia pastoris* has gained widespread acceptance as a system of choice for heterologous expression. In this study, the lipase gene of 639 bp from *Bacillus subtilis* was amplified and further ligated into expression vector pPICZαA and transformed into competent *E. coli* DH5α cells. Additionally, the recombinant plasmid was PCR amplified and double digested using Kpn I and Not I restriction enzymes for confirmation. The confirmed recombinant plasmid pPICZαA::rLP was designated as pYNAPICBL20622, and it was digested with Sac I to obtain a linearized recombinant plasmid. The linearized construct pYNAPICBL20622 was transformed into *Pichia pastoris* strain X-33 *via* electroporation. The integration of the lipase gene into the *Pichia pastoris* genome was confirmed by PCR analysis using gene-specific primers and AOX primers. The lipase gene confirmed *Pichia pastoris* clones can be utilized for the production of lipase and utilized in various industrial use.

Keywords: *Pichia pastoris*, lipase, PCR

Introduction
Biological expression systems are used for the production of heterologous proteins in industrial and medical present days. These proteins can consist of recombinant vaccines, drugs, and agricultural products [6]. The existing expression systems include bacteria, yeasts, molds, mammals, plants, and insects. Prokaryotic cells such as Gram-negative bacteria are among the first cells used in genetic engineering. One of the most important cells is *Escherichia coli* is one of the most important and widely used systems for cloning recombinant DNA and subsequently, for the production of heterologous proteins [4]. Bacterial expression system has several advantages including rapid multiplication, simple and inexpensive nutritional requirements, high-level expression, and fast and easy transformation. However, this cell factory has some limitations such as intracellular aggregation and misfolding of heterologous proteins, production of lipopolysaccharide, lack of posttranslational modification, and protein degradation due to proteases [11]. Yeasts are eukaryotic cells that are widely used for the expression of several proteins in vaccine and pharmaceutical production. In recent years, to solve the problem of protein expression, methylotrophic yeasts such as *Hansenulla polymorpha* and *Pichia pastoris* (*P. pastoris*; syn. *Komagataella phaffii*) have been developed. Among these, *P. pastoris* has become the most popular for its less cost and expression host system. The Pichia expression system has advantages for the expression of high yields of different recombinant proteins. Another advantage of this system is its high similarity with advanced eukaryotic expression systems such as CHO cell lines. This yeast system is inexpensive, it also has relatively rapid expression time, co-translational and posttranslational processing. The hydrolytic enzymes known as lipases can be utilised in a variety of industrial applications for alcoholysis, acidolysis, aminolysis, and hydrolysis processes. Biodiesel is typically produced by esterifying or transesterifying non-edible oil with methanol. Because of its better selectivity, moderate reaction conditions, and simple downstream processing, lipase-catalyzed biodiesel synthesis is undoubtedly one of the more promising technologies among the several available. The enzymes lipase and phospholipase are key contributors in biodiesel production. Lipolytic enzymes such as esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) catalyze both the hydrolysis and the synthesis of acylglycerides and other fatty acid esters. The true lipases differ from the carboxylesterases in their maximal activity on water-insoluble long-chain esters[1]. A few businesses are commercialising enzymatically generated biodiesel.

Lipase transforms free fatty acids (FFA) and triacylglycerol to fatty acid methyl esters, which are the major component of biodiesel. Mittelbach was the first to report on the generation of biodiesel with lipase. This enzyme can catalyse a wide range of substrates, including free fatty acids. Oil and acyl acceptors such as alcohol are two more important basic ingredients for biodiesel production. With this preview, the present study involved cloning of *lipase* gene of *Bacillus subtilis* into the *Pichia pastoris* integrative vector.

Materials and Methods

All the reagents and chemicals used in the present study were of analytical and molecular grade and purchased either from Sigma-Aldrich, USA or HiMedia Laboratories, India. The Recombinant plasmid pTNABSL5221 having lipase (639 bp) gene of *Bacillus subtilis* available in the laboratory was used as starting material in the present investigation [14].

The antibiotics used in the present study were ampicillin from Sigma-Aldrich and zeocin™, *Pichia pastoris* X-33, Pichia integrative Vector pPICZαA (Fig.1) from Invitrogen, USA. The bacterial host strain used was *E. coli* DH5α and *Pichia pastoris* X-33 was used for lipase expression.

Cloning of lipase gene into pichia integrative vector pPICZαA

The plasmid constructs pTNABSL5221 isolated from freshly cultured *E. coli* DH5α in LB broth with ampicillin was incubated overnight at 37 °C. Plasmid was isolated from this culture using QIAprep ®Spin Miniprep kit and run on 0.8% agarose gel and quantified using a multi-mode microplate spectrophotometer.

The cloning of rLP from the parent vector pTNABSL5221:rLP into the pPICZαA expression system was achieved by amplifying the gene followed by restriction digestion with Kpn I-HF and NotI-HF and ligation into the empty vector backbone pPICZαA. Standard recombinant DNA methods were carried out according to the methods described [2] and [9]. Primers were designed with restriction enzymes sites to amplify the *lipase* gene from construct using sequenced gene. The primers information is as follows;

Primer	Sequence
BsLipF	5'-TGCGCTGGATCCATGAAAAAAGTGTTTTC-3'
BsLipR	5'GAGCGTGAATTCTTTGCAATCACCAATTAAT-3'

A total of 20 µL of PCR reaction mixture was prepared for each sample with PCR programme as follows denaturation at 95 °C for 5 min, 35 cycles (95 °C for 45 s, 65 °C for 40 s, 72 °C for 60 s), finally extension at 72 °C for 5 min. The PCR products were purified by using QIAquick Gel Purification Kit Protocol from Qiagen (USA) and used for the next procedure.

The *Pichia pastoris* integrative vector pPICZαA(Figure 1) was isolated from *E. coli* using Qiagen Plasmid MiniPrep Kit and it was run on 0.8 percent agarose gel electrophoresis to check the plasmid quality. The plasmid DNA concentration and purity (A260/A280) were analyzed in a multi-mode microplate spectrophotometer. The lipase gene was confirmed in pTNABSL5221 construct restriction digestion with KpnI and NotI and PCR using gene-specific primers. The double-digested *lipase* gene was ligated into the KpnI and NotI digested empty pPICZαA vector backbone in an optimal molar ratio of ends of vector: insert (1:3) and the components of ligation mix were added to 0.2 ml micro-centrifuge tubes and incubated at 16 °C for 16 h followed by overnight incubation at 4 °C.

Transformation of the ligated product into competent *E. coli* cells was done using heat- shock method. To propagate pPICZα or to select Zeo^R transformants in *E. coli*, Low Salt LB was required as salt must remain low (< 90 mM) and the p^H must be 7.5 to make sure that drug remains active. After overnight incubation, the recombinant clones from LSLB plates were selected by using zeocin (2.5µg/ml) as selection pressure. *E. coli* DH5α cells grown on zeocin low salt LB plate were checked for the presence of recombinant pPICZαA:: rLP through colony PCR. The recombinant plasmids were isolated from colony PCR-positive clones and the lipase gene was confirmed by PCR and restriction digestion. The confirmed lipase recombinant pPICZαA:: rLP vector was named as pYNAPICBL20622

Integration of lipase gene into *Pichia pastoris* genome by electroporation

pYNAPICBL20622 construct was linearized for integration of lipase gene into Pichia genome through homologous recombination at the AOX site which is present in pPICZαA vector. The linearized recombinant plasmid pYNAPICBL20622 was transformed into *P. pastoris* by electroporation. Freshly prepared competent cells (80 µL) were mixed with ~ 5 µg of lineralized plasmid and transferred to an ice-cold electroporation cuvette (0.2 cm). The cells were incubated for 5 min. on ice and subjected to an electric field as described by the manufacturer (Bio-rad Laboratories, Inc., USA). The charging voltage was 2000V with a capacitance of 25 µF at 2000 resistance which gives field strength of~7500V fern. Immediately after giving the electric pulse (within 8 seconds), 1 mL of ice-cold 1M sorbitol was added to the cuvette and the contents were transferred to a sterile microfuge tube. Incubated the transformed pichia cells in 30 °C for 2 hours without shaking, from this about 100-200 µL of the cells were plated on YPDS agar plates having 400-1000 µg/mL concentrated zeocin and incubated at 30 °C for 48 hrs.

Isolation of genomic DNA of *pichia pastoris* clones to confirm lipase integration.

The zeocin (400 to 1000 ug/ml) resistant Pichia colonies were grown in 10mL of YPDS broth at 30 °C till the A600 of the culture reached 0.6-1.0 (16-18hrs). 5 mL of cells were harvested by centrifugation at 1500xg for 5-10 min at room temperature and washed once with 10 mL of sterile water. Parent host cells (X-33) were also grown in a similar manner and were used as a control.

The cells were suspended in 600µl of freshly prepared Sorbitol buffer containing 2000U of Zymolyase. After thorough mixing, the tubes were incubated at 30 °C for 30min to achieve 80 per cent spheroplasting. Spheroplasts were pelleted by centrifugation at 7500 rpm. Then the cells were lysed by suspending spheroplasts in 180 µl of AL Buffer. The lysed cells were mixed with 200µl of ethanol (95-100 per cent) by vortexing for 15 seconds for better binding. The lysate was loaded in HiPure Miniprep Spin column and centrifuged at 10000rpm for 1 min. The spin column was placed in a new 2.0 mL collection tubes. The column was prewashed with 500 µl of prewash solution (PWB) by centrifugation at 10000 rpm for 1 min. The prewashed column was once again washed with 500 µl wash solution (WS) and

centrifuged at 13000 rpm for 3 min. to dry the column. Finally, the pure yeast genomic DNA was eluted by adding 50 µl of Elution buffer (EB) at 10000 rpm for 1 min. The purity of genomic DNA was checked by 0.8 per cent agarose gel electrophoresis pure genomic DNA was further used to confirm the integration of lipase by PCR.

PCR was carried out with gene specific primers with conditions of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1.0 min after the initial denaturation at 95 ° for 3 min. The products were analyzed by agarose gel (1.0 per cent) electrophoresis along with standard molecular size markers. The colonies showing the amplified DNA of the expected size were selected for further study. PCR positive Pichia clones were further confirmed for lipase integration by AOX primers.

Results and Discussion

Lipases are the hydrolytic enzymes that can be used in various industrial applications for alcoholysis, acidolysis, amynolysis and hydrolysis reactions. Lipases have a central role in the bioenergy sector, particularly in production of biodiesel. The *C. antarctica* lipase gene has been cloned in recent years, and various methods, such as choosing a different expression system, are used to progressively boost the expression level [8] fusion protein expression [12], codon optimization and mutagenesis [7, 16].

In previous research work, 640bp lipase gene from the bacteria *Bacillus subtilis* was cloned into a pTZ57R/T cloning vector and named as pTNABSL5221[14]. In the present investigation, the above-mentioned construct already available in the lab was used as the starting material. The *Bacillus lipase* gene was amplified and cloned into yeast integrative vector pPICZαA and it was linearized to transform into host *Pichia pastoris via* electroporation. Further, lipase protein was induced in Pichia and confirmed by SDS PAGE.

PCR amplification and purification of a *lipase* gene from a plasmid construct pTNABSL 5221

PCR amplification of *lipase* gene from plasmid construct pTNABSL5221 was done using gene specific primers with restriction sites by optimizing the PCR conditions at 65 °C annealing temperature. The amplified product of 640bp was obtained (Plate1), which was purified using a QIAquick PCR Purification Kit and digested with Kpn I and NotI. The pPICZαA vector was isolated, restriction digested with same enzymes and ligated with the lipase gene. Transformation of the ligated product into competent *E. coli* cells was done using the heat-shock method. After overnight incubation, the recombinant clones from low Salt LB plates were identified by using zeocin as selection pressure (Plate 2). The colonies were picked and checked for the presence of an insert.

The colonies from the transformed plate were picked randomly and colony PCR was performed, for which, colonies in lanes with ~640 bp band size in gel electrophoresis, were selected (Plate 3) and inoculated for plasmid isolation. Recombinant plasmid was then isolated and PCR was performed for confirmation (Plate 4). The positive recombinant plasmids were further confirmed by double digestion with KpnI and NotI restriction enzymes as bands at 3.6 kb and ~640 bp were obtained (Plate 5). The lipase gene confirmed in the pPICZαA construct was named as pYNAPICBL20622 (Figure 2). The integrative vector pPICZαA for expression of the cloned CVS rabies glycoprotein gene in *Pichia pastoris* X-33 was used [13].

Integration of lipase gene into *Pichia pastoris* genome

The construct pYNAPICBL20622 was linearized by restriction digestion with SacI enzyme and confirmed by agarose gel electrophoresis with 4.2kbp-sized band (Plate 6). Electroporation was performed by using *Pichia pastoris* X-33 electrocompetent cells, linearized recombinant plasmid (~1µg/µL) and 0.2 cm cuvette using an electroporator. After electroporation, cells were spread on YPDS plates containing 300 µL/mL zeocin and growth of yeast was observed in 2 days in transformed plates.

Similarly, [16] cloned the extracellular lipase gene from *Yarrowia lipolytica* (YlLip2) into the pPICZαA and integrated into the genome of the methylotrophic yeast *Pichia pastoris* X-33 and cloned *Yarrowia lipolytica* lipase LIP2 gene (YlLIP2) into a constitutive expression vector pGAPZαA and electro-transformed into the *Pichia pastoris* X-33 strain.

From transformed plate, colonies were picked randomly for inoculation in YPDS media with 25µL/100mL zeocin and kept at 30 ºC for 18-24 hrs. The cultures with good growth were used for DNA isolation and quality was checked by gel electrophoresis (Plate 7). PCR analysis was performed to verify the integration of the gene into the *Pichia* genome. For this, PCR was kept with two sets of primers namely, AOX1 primers and gene specific primers. By using gene specific primers, amplicon size of about 640 bp on agarose gel (Plate 8) confirmed the integration of *lipase* gene in transformed *Pichia pastoris* clones. Similarly, [10] cloned the synthetic lipase gene from *Thermomyces lanuginosus* into *Pichia pastoris* with its original signal peptide.

With respect to the AOX1 primers, in addition to 640 bp of lipase gene, ~588 bp were contributed from the AOX1 promoter and hence the band corresponding to the size ~1.2 kb (Plate 9) confirms the integration of lipase gene into *Pichia pastoris*. Among these Pichia clones from L3 to L6 showed the positive band where as L7 did not show a band indicating integration of lipase in L3 to L6 clones and not in clones of L7. Similarly a gene encoding thermostable lipase namely *LK1* was successfully cloned into expression vector pPICZαA and integrated into a chromosomal fungal host, *Pichia pastoris* GS115 [3].

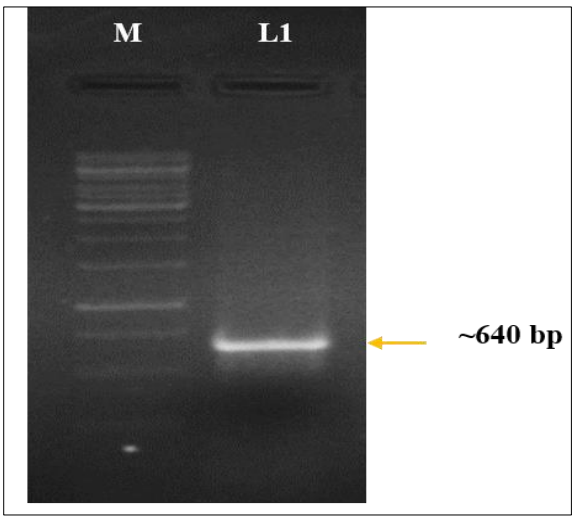

Plate 1: PCR amplification of lipase gene isolated from plasmid construct Ptnabsl5221 M- 1kb Ladder; L1- Amplified lipase gene ~640bp

Plate 2: Transformed colonies of pPICZaA +lipase in *E. coli* DHSa cells

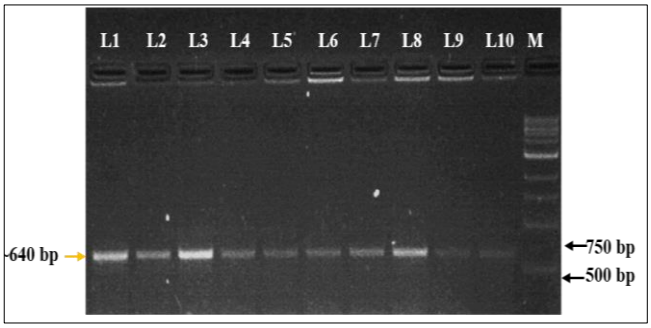

Plate 3: Colony PCR of transformed pPICZaA in *E. coli* DHSa cells M- 1 kb Ladder; Lane 1-10 - Transformed colonies with *lipase* gene

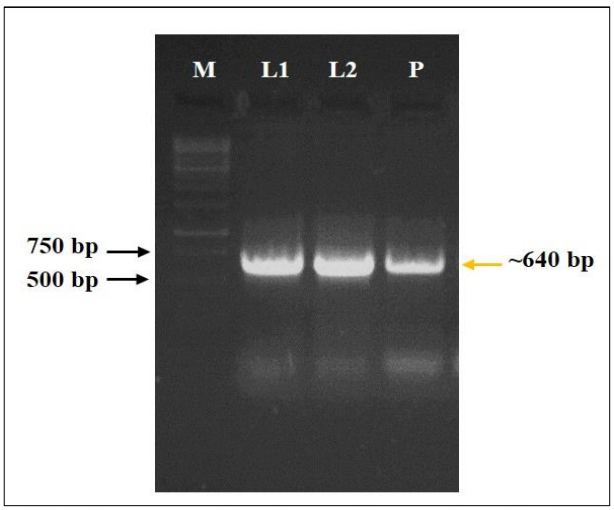

Plate 4: PCR confirmation of recombinant plasmid pPICZaA:: rLP M- 1 kb Ladder; Li and L2- Confirmed *lipase* gene in PCR; P- Positive)

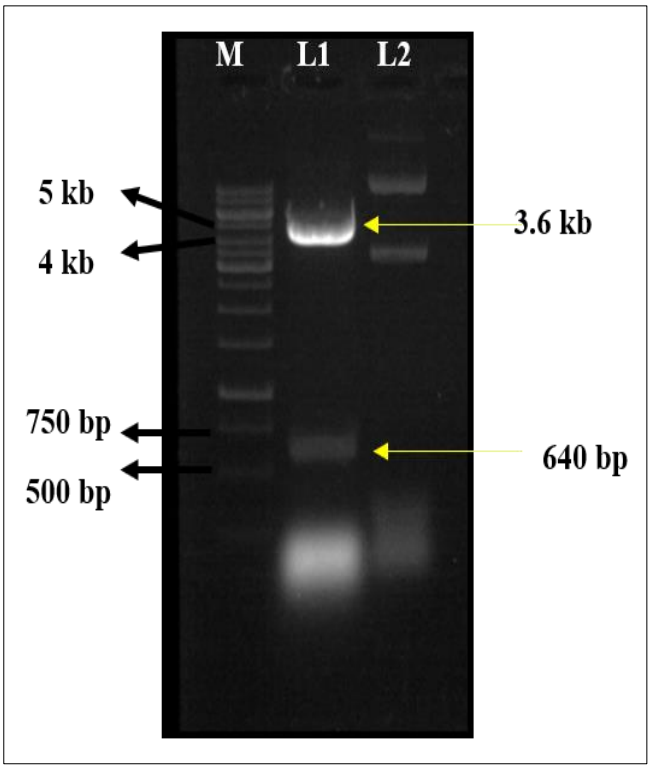

Plate 5: Restriction digestion of recombinant plasmid pPICZaA:: rLP (M- lkb Ladder; LI- Digested; L2- Undigested)

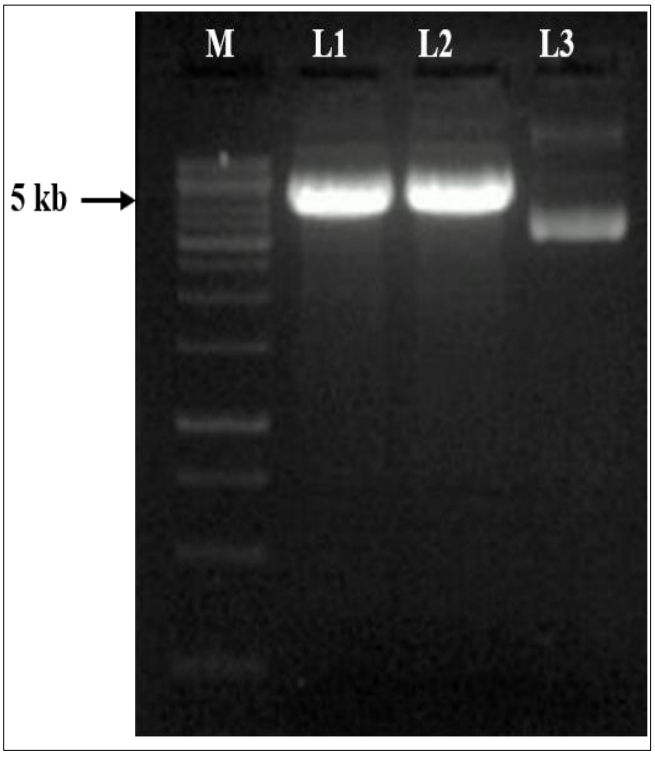

Plate 6: Restriction digestion of recombinant plasmid pYNAPICBL20622 with Sac I (M- lkb Ladder; Li and L2- Digested; L3- Undigested plasmid)

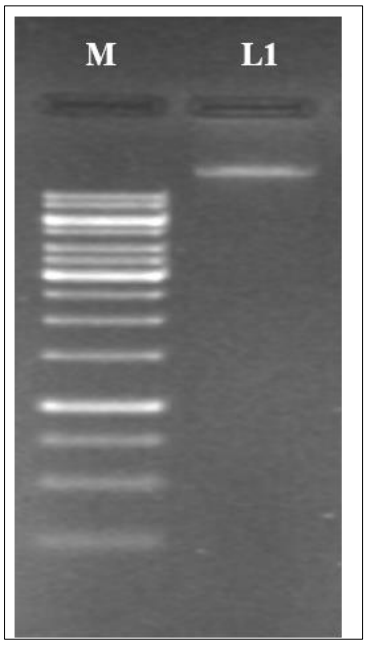

Plate 7: Genomic DNA isolation of *Pichia pastoris* strain X-33 (M- Ikb Ladder; Ll- Genomic DNA of *Pichia pastoris* strain X-33)

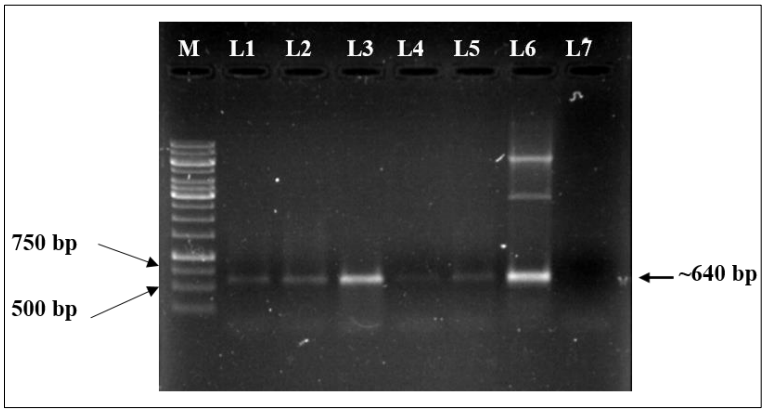

Plate 8: PCR confirmation of *lipase* gene with gene specific primers in *Pichia pastoris* strain X-33 M- ikb Ladder; L1-5 –PCR confirmation of *lipase* gene isolated from DNA of transformed *Pichia pastoris* strain X-33; L6- Positive; L7- Negative)

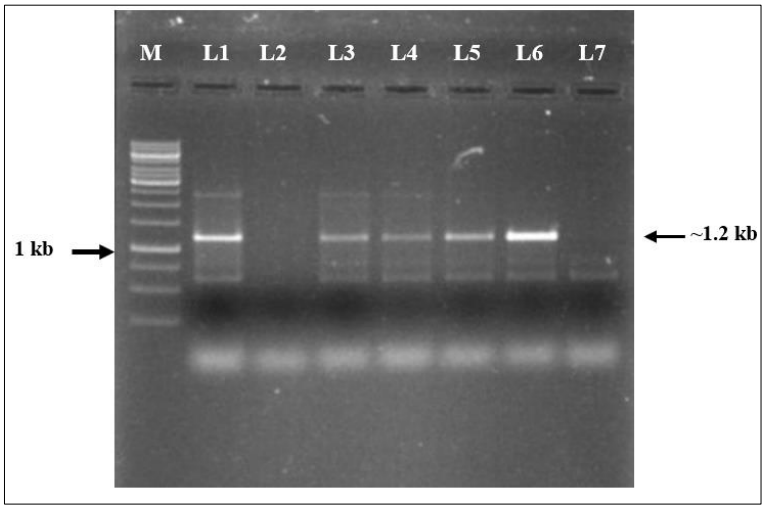

Plate 9: PCR confirmation of lipase gene in *pichia pastoris* X-33 genome by AOX1 primers M- 1kb Ladder; Ll- Positive, L2- Negative, L3 to L7 - *Pichiapastoris* strain
X-33 clones genomic DNA integrated with *lipase* gene.

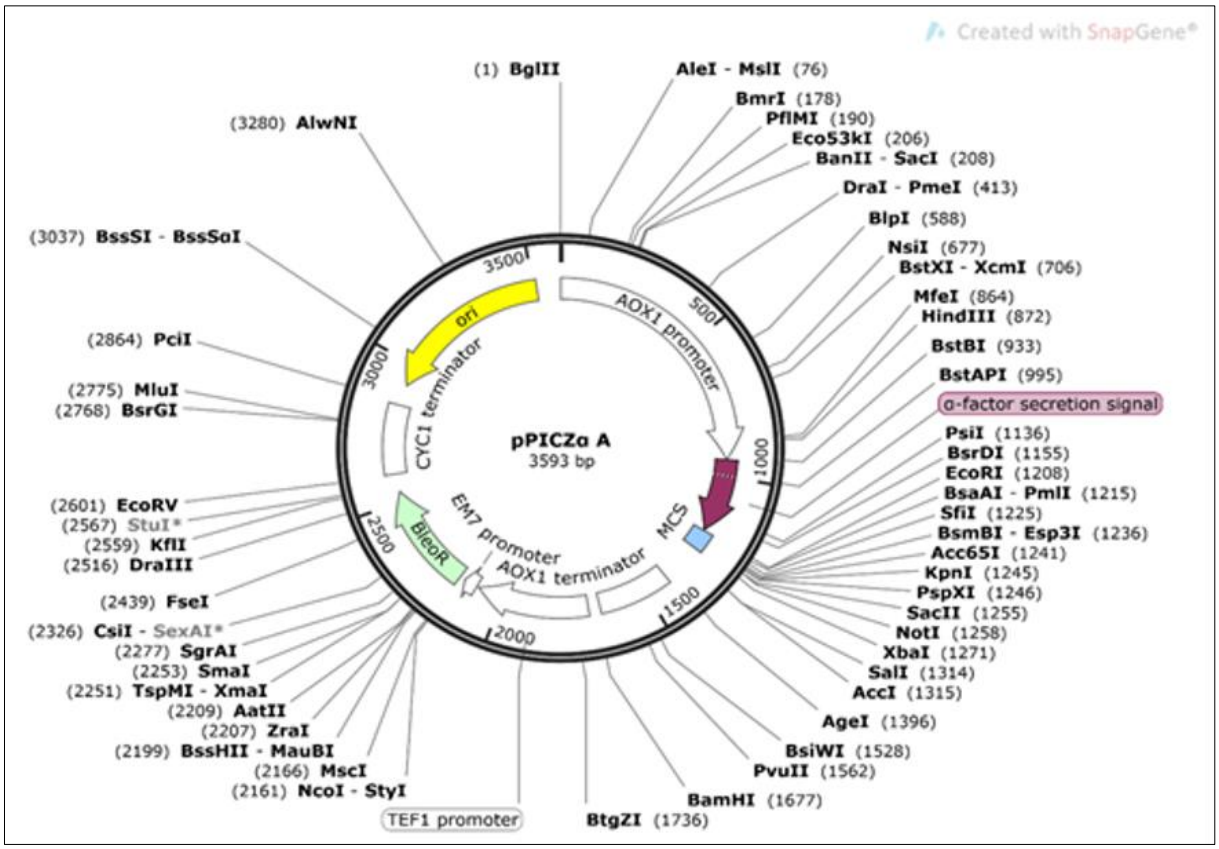

Fig 1: The detailed circular map of yeast integrative vector pPICZazt

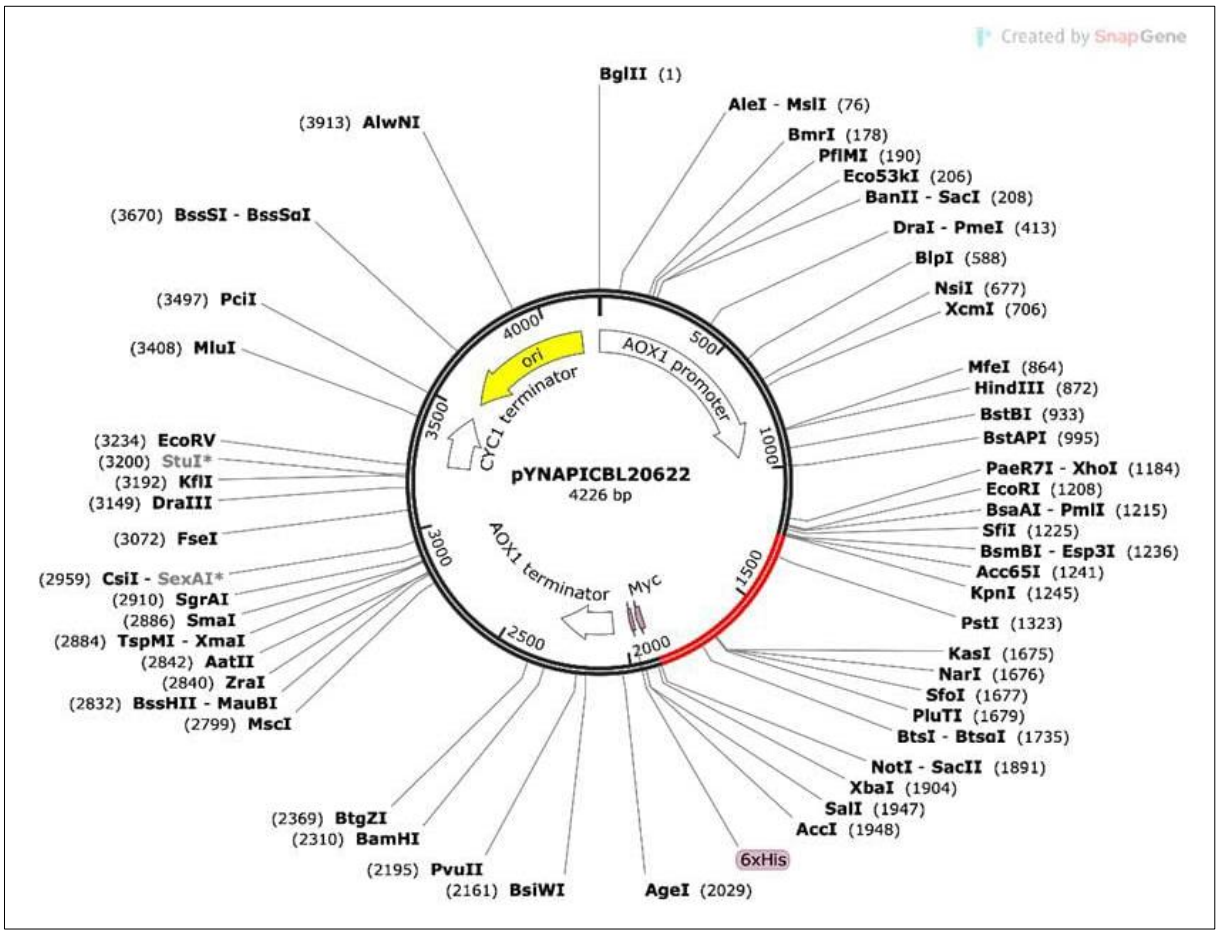

Fig 2: The detailed construct map of pYNAPICBL20622 containing full length *lipase* gene in pPICZaA. The simulation of the vector map was created using SnapGene software.

Conclusion

Pichia pastoris is a suitable eukaryotic host for expression of recombinant proteins due to its good post translation modification, obtaining high cell density and secreting low levels of endogenous proteins into culture media with a strong and tightly regulated promoter AOX1. Lipase is an enzyme of industrial interest which has been cloned into integrative vector pPICZαA and successfully integrated into Pichia genome and it can be effectively utilized for the production of lipase for various applications.

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