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Cloning and Expression of Lip-A Gene From Streptomyces Coelicolor to Escherichia Coli

KEYWORDS: Lip A gene, restriction digestion, transformation, lipases

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ABSTRACT The exponential increase in the application of lipases in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement. Strain improvement is an essential part of process development for fermentation products. Developed strains can reduce the costs with increased productivity and can possess some specialized desirable characteristics and such improved strains can be obtained by mutation. Thus in the present investigation the E.coli DH5α were cloned with LipA gene isolated from Streptomyces species using a PUC 18 plasmid. The transformation was confirmed by culturing the cloned cells on lipase medium and restriction digestion of the recombinant cells and un cloned cells by HIND III restriction enzyme where in the E.coli DH5α from the wild strains were not cloned. The cloned E.coli DH5α could be one of the sources for lipolytic enzymes for the industries

INTRODUCTION

Lipases catalyze both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. These enzymes usually exhibit broad substrate specificity and degrade acyl *p*-nitrophenyl esters, Tweens, and phospholipids, often with positional selectivity, stereo selectivity, and chain length selectivity. Lipase's are the enzyme that have been proved to play a very important role in oil degrading activity.

The versatility of lipases have resulted in their emergence as one of the leading biocatalysts with definite potential to contribute in exploitation of the less utilized lipid technology and multibillion dollar versatile industrial applications (Joseph *et al.,* 2008). Lipases occur widely in nature, but only microbial lipases are commercially significant.

Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification (Chowdary et al., 2001; Hamsaveni et al., 2001; Kiyota et al., 2001; Krishna and Karanth, 2001; Rao and Divakar, 2001), transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides (Ducret et al., 1998; Zhang et al., 2001) and other chemicals (Therisod and Klibanov, 1987; Weber et al., 1999; Berglund and Hutt, 2000; Liese et al., 2000; Azim et al., 2001). The expectation is that lipases will be as important industrially in the future as the proteases and carbohydrases are currently.

Streptomyces species are known to be lipolytic (Sztajer *et al.*, 1988) and two different types of lipase genes have been cloned from this genus. lipase genes of *Streptomyces exfoliatus* M11 (Pérez *et al.*, 1993) and *Streptomyces albus* G (Cruz *et al.*, 1994), and shown that these highly similar to a psychrophilic lipase from *Moraxella sp.* TA144 (Feller *et al.*, 1990). A second type of lipase gene encoding lipase has been related to group II *Pseudomonas* lipase that has been recently cloned from *Streptomyces cinnamomeus* (Sommer *et al.*, 1997).

MATERIALS AND METHODOLOGY

Isolation of Genomic DNA

Streptomyces culture was procured from NCIM Pune. The mycelia mat of the pure culture was homogenized in a mortar and pestles along with the addition of CTAB extraction buffer. The homogenate was incubated for 30 minutes at 65°C using water bath. After incubation equal volume of chloroform isoamylalcohol was added and centrifuged for 10 minutes. The process was repeated twice. The aqueous phase was separated to which isopropanol was added and incubated overnight.

The contents were then transferred to eppendorf tubes and centrifuged at 4°C at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet was precipitated with 70% ethanol and kept for air drying. The pellet was then dissolved with TE buffer and stored in refrigerator until further use. The DNA was qualitatively determined by running on 1% agarose gel electrophoresis.

Isolation of plasmid from *E.coli* by alkaline lysis method

1.5 ml of overnight culture of *E.coli* (TBI) carrying plasmid (pUC18) was centrifuged for 10 minutes at 5000 rpm. The pellet was suspended in 1 ml of solution A and 2ml of solution B. they were then incubated on ice for 30 minutes till the solution becomes viscous. 0.5 ml of solution c was added, kept on ice for 60 minutes. The suspension was the centrifuge for 10 minutes at 4°C. Phenol: chloroform (1:1) was added to the supernatant then it was centrifuge for 10 minutes at 10,000 rpm. The supernatant was discarded and 10µl of 70% ethanol was added to the pellet for removing other contaminants. The ethanol was then air dried and pellet was re-suspended in 30 µl of TE buffer. The obtained plasmid (pUC18) was analysed by electrophorising in 1% agarose gel electophoresis

Restriction digestion of the Genomic DNA and plasmid

The restriction of the genomic DNA and the plasmid was carried out by using Hind III restriction enzyme. The fractions obtained were determined on agarose gel and the ligation was carried out.

Ligation of Genomic DNA and plasmid

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The reaction mixture required for ligation was mixed in ice cold condition. The ligase buffer, the digested plasmid (pUC 18) and digested genomic DNA was thawed and kept in ice. To the fresh sterile 1.5 ml eppendorf tube 8.1 µl of sterile distilled water was taken. To this 1.5µl of 10x ligase buffer was added. 3 µl of digested genomic and 1.5 µl digested plasmid pUC 18 was mixed gently by tapping. Finally 3µl (1 unit) of T_4 DNA ligase was added and mixed gently. The ligation mixture was incubated at 20°C for overnight and later used for *E.coli* (DH 5α) transformation.

Transformation

24 hours overnight culture of *E.coli* (DH5α) was taken. From that 0.1 ml was taken and inoculated in 25 ml of fresh LB medium. The culture was allowed to grow till its OD reaches 0.5-0.6 at 540 nm .The culture was chilled in ice for 30 minutes. Cells were harvested by centrifugation at 5000 rpm for 10 minutes. Supernatant was discarded and the pellet was suspended in 5 ml of 100mM $MgCl_2$. Once again the pellet was kept in ice for 60 minutes. Culture was spinned, discarded the supernatant and the pellet was suspended in 1 ml of 100mM $MgCl_2$ From this 0.2 ml of culture was taken and 50µl of plasmid pUC18 was added. Suspension was kept in ice for 30 min. Heat shock is given at 42°C for 2 minutes. Then it is again transferred to ice and kept for 5 minutes. Then 2 ml of sterile LB broth was added to the above culture and incubated at 37°C for 1 ½ hour. 0.5 ml of these solutions was taken and plated it on agar plates containing ampicillin (50µg/ml). The plates were then incubated at 37°C overnight. For control 0.2 ml of the culture with only buffer and not with plasmid and sterile LB was added. After incubation 0.5 ml of the control culture was plated on the selective plate. The plates were then incubated at 37°C along with the test plates. After incubation colonies that were formed on the ampicillin plates were observed. In the control plate, no colonies were observed.

Plasmid DNA isolation from cloned cells: 1ml of overnight culture was taken into an eppendorf tube and was centrifuged at 12000rpm for 1minute. The pellet was dissolved in 100µl of double distilled water by gently tapping at the bottom of the tube. 100µl of lysis buffer was added. The eppendorf tubes top was wrapped with parafilm and punctured with a fine needle to avoid popping of the solution. The mixture was kept immediately in boiling water bath for 2 minutes. 50µl of 1 M $MgCl_2$ was added, mixed by tapping and kept on ice for two minutes. The mixture is centrifuged at 12000 rpm for two minutes. 50µl of 5M potassium acetate was added to the same tube and mixed without disturbing the pellet, by tapping gently and is kept on ice for two minutes. It was centrifuged for two minutes at 12000rpm. The supernatant was taken (leaving the bottom portion that contains the pellet) and transferred to a fresh tube. To this 600µl isopropanol was added. Mixed by inverting slowly and the tubes were kept on ice for two minutes. Centrifuged at 12000 rpm for two minutes and the supernatant was discarded. The pellet was washed with 1ml 70% alcohol and centrifuged for 10 minutes 12000rpm. The pellet were dried completely and re suspended the pellet in 50µl water containing 20µg/ml RNase A.

Restriction Digestion of the plasmid isolated from cloned cells: The following mixture was prepared for restriction of the plasmid in a sterile 1.5 ml eppendorf and tube kept in ice cold condition:

Recombinant Plasmid (pUC18) (0.2-1.0µg)	2µl
10 x Restriction buffer	2µl
Restriction enzyme Hind III (3 units / µg plasmid)	1µl
Distilled water	20 µl

Table-1 Reaction Mixture

The eppendoff tubes were incubated the tube at 37°C for 3 hr. The reaction was stopped by adding 0.5 M EDTA (pH 8.0) to give a final EDTA to 20 µl reaction volume. The restricted plasmid was analysed on agarose gel.

RESULTS

Isolation of Genomic DNA: The Genomic DNA was isolated from *Streptomyces ceolicolor* by following CTAB method. The isolated DNA was preserved in TE buffer at 4°C and it was electrophorised for qualitative estimation (Fig-1)

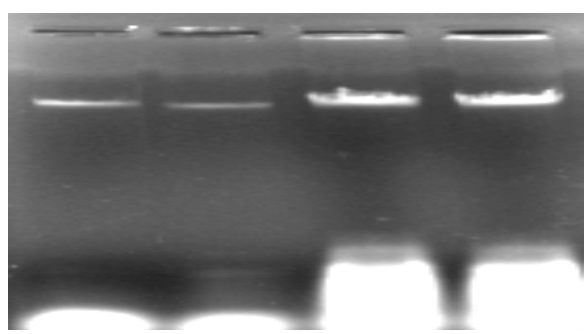

Figure-1: Qualitative estimation of Genomic DNA.

Isolation of plasmid from *E.coli*: Plasmid Puc18 was isolated from E.coli(TB1) by alkaline lysis method. The isolated plasmid was stored at 4°C in TE buffer and electrophorised in 0.8% agarose gel to determine its purity (Fig-2).

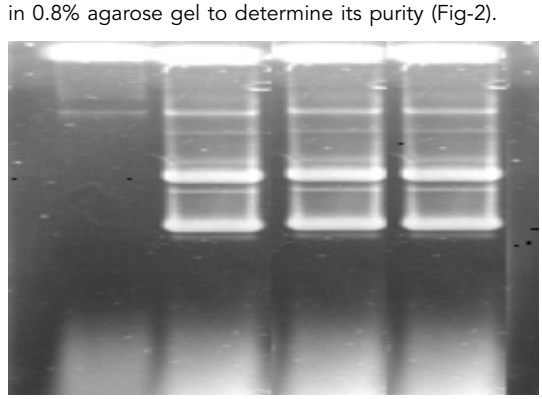

Figure-2: PUC18 Plasmid isolated from *E.coli*

Restriction Digestion and ligation of the Genomic DNA and Plasmid: The fragments were obtained by restriction digestion of the genomic DNA by Hind III enzyme and were ligated into the plasmid DNA using T4 DNA ligase such that the plasmid carries the plasmid carries the gene of interest, that was transformed into *E.coli*

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Transformation: The transformation was carried out by competence cell preparation of *E.coli,* these competent cells were then inserted with the cloned plasmid. The cells were the cultured on specific media plate to determine the cells with transformed plasmid.

Lipolytic activity of the recombinant cells: The recombinants cells were plated in lipolytic medium and lipolysis was occurred on the plates indicates the successful transformation of target gene, where native wild type *E.coli* (DH5α) does not show lipolytic action.

Restriction digestion of the cloned plasmid from the recombinant E.coli: The isolation of the plasmid was carried out from the recombinant cells and restriction digestion was carried out with Hind III enzyme and was determined on agarose gel electrophoresis to indicate the presence of the gene in the recombinant cells (Fig-3).

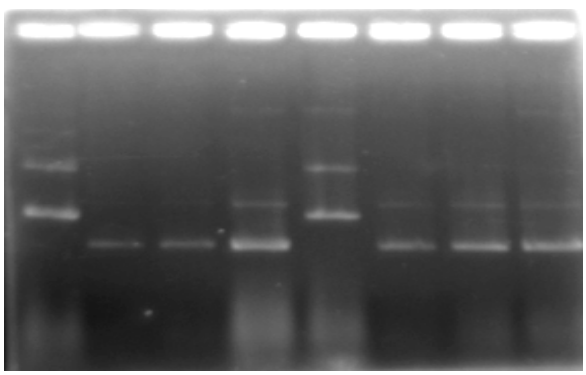

Figure-3: Restriction Digestion of the recombinant cells. (Lane 1- Recombinant plasmid (pUC18-1), Lane 2 and 3-Non recombinant plasmids, Lane 4 - Control plasmid (pUC18), Lane 5- Recombinant plasmid (pUC18-2), Lane 6,7&8 - Non recombinant plasmids.)

DISCUSSION

Henne, A et al., 1999 constructed DNA libraries for *E.coli* DH5α from soil samples as a host and pBluescript SK (1) as a vector. The gene studies was carried out and was found that the product of *lip* (281 amino acids) is 30% identical (35.9% similar) to the lipase (Lip) of *S. albus*. Similar amino acid identities were obtained for the lipases of *Streptomyces coelicolor* (Valdez, F et al., 1999) and a *Moraxella* sp. (Feller, G et al., 1990).

There are several examples where the presence of a restriction system in the recipient is found to have a marked effect on the conjugal transfer of plasmids from *E. coli* to other bacteria. The 12- fold reduction in *S. coelicolor* exconjugant frequency observed with pDJ50 would be consistent with that reported for other intergeneric conjugation systems (that is, - 15-fold reduction per restriction site (Elhai, J et al., 1997).

Chung et al, 1991, isolated lipase gene from *P. fluorescens* SIK W1 which consisted an open reading frame 1347 base pairs long commencing with an ATG start codon encoding a polypeptide of 499 amino acid residues and a TGA stop codon.

REFERENCE

1. Joseph B., P. W. Ramteke and G. Thomas, 2008. Cold active microbial lipases: Some hot | issues and recent developments. Biotechnology Advances. 26: 457–470. | 2. Henne, A., R. Daniel, R. A. Schmitz, and G. Gottschalk. 1999. Construction of environmental DNA libraries in Escherichia coli and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. Appl. Environ. Microbiol. 65:3901–3907. | 3. Valdez, F., G. Gonzales-Ceron, H. M. Kieser, and L. Servin-Gonzalez. 1999. The Streptomyces coelicolor A3 (2) lipAR operon encodes an extracellular lipase and a new type of transcriptional regulator. Microbiology 145:2365– 2374. | 4. Feller, G., M. Thiry, and C. Gerday. 1990. Sequence of a lipase gene from the antarctic psychrotroph Moraxella TA144. Nucleic Acids Res. 18:6413. | 5. Elhai, J., Vepritskiy, A., Muro-Pastor, A.M., Flares, E. And Wolk, C.P. (1997) Reduction of conjugal transfer efficiency by three restriction activities of Anabena sp. strain PCC 7120 J.Bacteriol 179. 1998-2005. | 6. Chung, G.H., Lee, Y.P., Yoo, O.J., and Rhee, J.S. 1991. Cloning and nucleotide sequence of thermostable lipase gene from Psudomonas fluorescens SIK W1. Agricultural and Biological Chenistry, 55: 2359- 2365. | 7. Sommer P., Bormann C. & G tz F. (1997).Genetic and biochemical characterization of a new extra cellular lipase from Streptomyces cinnamomeus. Applied and environmental microbiology. 63: 3553-3560. | 8. Sztajer H, Maliszewska I, Wieczorek J. Production of exogenous lipase by bacteria, fungi and actinomycetes. Enzyme Microb Technol 1988; 10:492–7. | 9. Chowdary GV, Ramesh MN, Prapulla SG. Enzymic synthesis of isoamyl isovalerate using immobilized lipase from Rhizomucor miehei: multivariate analysis. Process Biochem 2001; 36:331–9. | 10. Hamsaveni DR, Prapulla SG, Divakar S. Response surface methodological approach for the synthesis of isobutyl isobutyrate. Process Biochem 2001; 36:1103–9. | 11. Kiyota H, Higashi E, Koike T, Oritani T. Lipase-catalyzed preparation of both enantiomers of methyl jasmonate. Tetrahedron: Asymmetry 2001; 12:1035–8. | 12. Krishna SH, Karanth NG. Lipase-catalyzed synthesis of isoamyl butyrate. A kinetic study. Biochim Biophys Acta 2001; 1547:262–7. | 13. Rao P, Divakar S. Lipase catalyzed esterification of a-terpineol with various organic acids: application of the Plackett–Burman design. Process Biochem 2001; 36:1125–8. | 14. Ducret A, Trani M, Lortie R. Lipase catalysed enantioselective esterification of ibuprofen in organic solvent under controlled water activity. Enzyme Microb Technol 1998; 22:212–6. | 15. Zhang LQ, Zhang YD, Xu L, Li XL, Yang XC, Xu GL, Wu XX, Gao HY, Du WB, Zhang XT, Zhang XZ. Lipasecatalyzed synthesis of RGD diamide in aqueous water-miscible organic solvents. Enzyme Microb Technol 2001; 29:129–35. | 16. Therisod M, Klibanov A M. Regioselective acylation of secondary hydroxyl groups in sugars catalyzed by lipases in organic solvents. J Am Chem Soc 1987; 109:3977–81. | 17. Weber N, Klein E, Mukerjee KD. Long chain acyl thioesters prepared by solvent free thioesterification and transesterification catalyzed by microbial lipases. Appl Microbiol Biotechnol 1999; 51:401–4. | 18. Liese A, Seelbach K, Wandrey C, editors. Industrial biotransformationsWeinheim: Wiley-VCH, 2000. | 19. Berglund P, Hutt K. Biocatalytic synthesis of enantiopure compounds using lipases. In: Patel RN, editor. Stereoselective biocatalysis. New York: Marcel Dekker, 2000. | 20. Azim A, Sharma SK, Olsen CE, Parmar VS. Lipase catalysed synthesis of optically enriched a-haloamides. Bioorg Med Chem 2001; 9:1345–8. |