Original article

Induction of mutation in *Bacillus subtilis* lipase gene using error-prone PCR

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Abstract

Introduction and objective: Directed evolution is an incredibly powerful tool for altering the properties of enzymes. This approach uses a "sloppy" version of PCR, in which the polymerase has a fairly high error rate to amplify the wild-type sequence. The aim of this study was to produce the *Bacillus subtilis* mutant lipase which can be active at low temperatures using the error-prone PCR method.

Materials and methods: The pGEM-T vector containing *B. subtilis* lipase gene was transformed into the *Escherichia coli* DH5α. The lipase gene was mutated using the error-prone PCR technique. Screening of the mutants was carried out using both microtiter and Luria-Bertani plates containing 1% tributyrin and 100mg/ml ampicillin. Ten out of 1300 clones which showed the lipase activity at 10ºC were isolated. Among them, three desirable mutants named pFJK1, pFJK2 and pFJK3 were selected. Clones containing the wild type and the mutated lipase gene were both purified and characterized. The enzyme assay at different temperatures was carried out.

Results: The optimum temperature for the activity of lipase was achieved at 10ºC and 30ºC for the mutants and control, respectively. The relative enzyme activity of pFJK1, pFJK2 and pFJK3 at 10ºC was more than two times of their activity at 30ºC. The enzyme retained 70% of its activity at 10ºC and there was a drastic decline in enzyme activity at 50ºC.

Conclusion: Based on the present findings, the enzyme was characterized as a cold adapted lipase which showed the highest activity at 10ºC.

Significance and impact of the study: Cold adapted lipase can be developed for industrial applications such as additives in detergents.

Keywords: Error-prone PCR; Lipase; Cold adapted lipase

Introduction

There is increased industrial demand for enzymes produced by microorganisms. Among these are lipases, which are glycerol ester hydrolyses that break long or short acylglycerols to fatty acids and glycerol, respectively [1,2]. Many cold-adapted microorganisms, called psychrophiles, can grow at 15^{o}C or lower [3]. The cold adaptation of these microorganisms is likely to be due to, at least in part, their ability to produce cold-active enzymes which exhibit higher catalytic activities at low temperatures than their mesophilic and thermophilic counterparts do [4].

The 'cold activity', might be the key to success in some of their biotechnological applications. These applications include their use as catalyst for organic synthesis of unstable compounds at low temperatures. They include additives in detergents, food industry, environmental bioremediations, biotransformation and heterologous gene expression in psychrophilic hosts [5]. Several investigators have focused their attention on the isolation, cloning and mutation of lipases [6,7].

Bacillus subtilis is the best-characterized member of the Gram-positive bacteria. It has proven highly amenable to genetic manipulation, and has therefore become widely adopted as a model organism for laboratory studies. *B. subtilis* secretes two lipases, LipA and LipB, into the culture medium depending on the composition of the growth medium: LipA is produced in rich and in minimal medium, whereas LipB is present only in rich medium [8]. Most lipase sequences include the conserved region [9].

The *B. subtilis* lipase is one of the smallest lipases reported and its unique characteristics have commercial and research applications. The *B. subtilis* lipase A gene, for example can be expressed in its active form in *Escherichia coli* without the need to co-express any specific chaperones [9].

Directed evolution has become a powerful tool both for improving the utility of enzymes in industrial processes and generating the variants that illuminate the relationship between enzyme sequences, structure, and function. The method most often used to generate variants with random mutation is error-prone PCR (ep PCR). It is a technique in which mistakes are introduced into a gene, and hence a protein [10]. Error-prone PCR is modification of standard PCR methods, designed to alter and enhance the natural error rate of the *Taq* DNA polymerase which is commonly used because of its naturally high error rate.

The method introduces random copying errors by imposing imperfect, and thus mutagenic, or 'sloppy', reaction conditions (e.g. by adding Mn^{2+} or Mg^{2+}) to the reaction mixture [11,12]. This technique has the advantage of enabling the development of new enzymatic properties without a structural understanding of the targeted enzyme. Recently, Khurana *et al.* [13] explored ep-PCR to improve the thermal stability of lipase. Kohno *et al.* [14] also succeeded in creating a lipase that could maintain its activity at a high temperature by random mutagenesis with the ep-PCR. However, in the present study we produce the mutant lipase which can be active at lower temperatures (10ºC) using epPCR method. The wild type lipase which is considered as a control group is active at 30ºC.

Materials and methods

The host strain *E. coli* DH5α was used for expressing the lipase gene. The pGEM-T vector containing *B. subtilis* lipase gene was kindly provided by Dr. Jagdeep Kaur of Biotechnology department of Panjab University, Chandigarh, India.

Random mutagenesis

The epPCR was applied for random mutagenesis according to Leung *et al*. [12]. The cloned lipase gene containing plasmid was amplified using FWS3LIPJ (5'-CTA CGC GCA TGG GTG AAA AAA GTA CTT A-3') and RWLIPJ (5'- CTA CGC AGA TCC TTA ATT TGT ATT GAG G-3') primers which were designed with Gene Runner software. The reaction mixture (50µl) contained 3.5mM $MgCl_2$ (3.5µl), 0.4µM forward and reverse primers (2µl), 5µl of 1x PCR buffer (200mM Tris-Hcl, 500mM Kcl), 3U of *Taq* polymerase (0.6µl) and individual solutions of dNTPs.

Unequal concentration of individual dNTPs was prepared in four tubes. For example, 0.1mM dATP (0.5µl), 0.1mM dCTP (0.5µl), 0.5mM dGTP (2.5µl) and 0.5mM dTTP (2.5µl) were added to the first tube. These concentrations changed randomly in the second, third and fourth tubes as well. The DNA was amplified in a DNA thermal cycler (Bio-Rad, USA) at 95ºC for 5mins followed by 25 cycles at 95ºC for 30 seconds, 59.2ºC for 30 seconds, 72ºC for 30 seconds and one time final extension at 72ºC for 10mins.

Purification and cloning of PCR product

The PCR products were then purified by PCR purification kit (QiaGen, Germany). The PCR products and the pGEMT vector were digested with *Nco I* and *Bam H I* restriction enzymes (Fermentas, Canada). The digestion reaction was as follows: 15µl of ep-PCR product, 2µl of 10X buffer, 1µl of Bam HI, 1µl of Nco I and 1µl of autoclaved double distilled water. The digestion reaction mixture was incubated overnight in water bath at 37ºC. The pGEMT vector was digested in the same condition as the ep-PCR product.

The digested PCR products were ligated into the digested pGEMT vector with T4 DNA ligase (Bangalore Genei, India) using 3µl of pGEMT vector, 8µl of ep-PCR product, 2µl of T4 DNA ligase, 2µl of T4 DNA ligase buffer in a total volume of 20µl. The ligation reaction mixture was incubated overnight in water bath at 16ºC. This recombinant plasmid was then transformed into the electro competent *E. coli* DH5α. 2µl of ligation mixture was added to the 60µl of electro competent cells and these cells were given the 2.5KV pulse by electroporater. The 960µl of and Luria-Bertani broth (LB, Himedia, India) was added to the cells and revived at 37ºC in a shaker for 2h. The mixture was spread on LB-Amp plate and grow overnight at 37ºC.

Preparation and selection of the mutants

The transformed colonies which grow on LB-Amp plate were selected and cultured on Luria-Bertani (LBT, Himedia, India) plate containing 100mg/ml ampicillin and 1% tributyrin as a substrate to check the expression of the lipase gene. The colonies which developed clear zones around their growth area were considered as lipase positive and selected to do enzyme activity assay. Screening of mutants was also carried out on the microtiter plate. After overnight growth on LBT-Amp plate, colonies were picked with sterile toothpicks and grown in 96-well plates containing LB-Amp growth medium (250µl) along with the wild type lipase. After overnight growth at 37ºC, the cells were pelleted by spinning at 12000g for 5mins and 10µl of supernatant was used to perform the enzyme activity assay.

Enzyme activity assay

The enzyme activity was determined according to the modified method of Sigurgisladottirr *et al*. [15]. The assay was performed at 4-40ºC for the colonies that show activity on LBT plates and the control which contained the wild type lipase gene. The assay was carried out using

paranitrophenyl laurate (pNP) as a substrate. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1µmol of pNP-laurate in 10mins.

The overnight culture was centrifuged at 12000rpm for 5mins and the supernatant containing enzyme was transferred to a new tube. 850µl of the phosphate buffer was added to another tube and incubated at the temperature at which the assay was supposed to be done for 5mins. Then, 100µl of pNP-laurate (2mg/ml) and 50µl of the supernatant were added to the tube and incubated at the desired temperature for 10mins. 250µl of the stop solution (sodium carbonate) was added and mixed. The tubes were then centrifuged at 12000rpm for 10mins. The amount of pNP-laurate produced was estimated by taking OD at 420nm.

Thermal analysis assay

The thermal analysis assay was done by standard protocol both for the mutants and control at 50ºC to check the thermal stability. The overnight culture was centrifuged at 12000rpm for 10mins and the supernatant of each mutant was incubated at 50ºC for 30 and 60mins, respectively. Enzyme activity assay was performed to check the thermal stability of the mutants.

Results

Isolation and characterization of the randomly mutated lipase

An improvement in the lipase activity at low temperature was achieved by means of phenotypic selection from recombinant cells carrying a randomly mutated gene. The screening of the mutants was carried out in the microtiter plate at 4-10ºC (in cold room). Nearly 1300 clones were screened. Ten clones that showed the higher activity at 10ºC than 30ºC in comparison with the control were selected. Three out of 10 mutants, i.e. mutants number 8, 9 and 10, which demonstrated the highest lipase activity were selected and named as pFJK1, pFJK2 and pFJK3, respectively (Fig. 2). The relative enzyme activity of pFJK1, pFJK2 and pFJK3 at 10ºC was two times more than that of their activity at 30ºC (Table 1). The T-Test has shown that the differences between enzyme activity of mutants and controls at 10ºC and 30ºC were significant (p value 0.01).

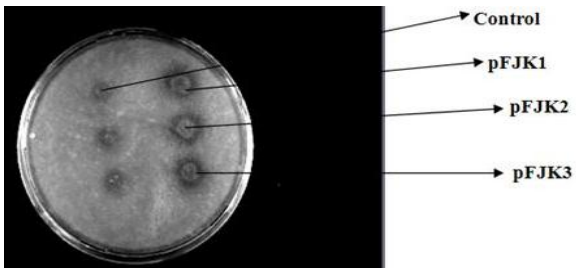

Fig. 1: Pick pock of pFJK1, pFJK2 and pFJK3 on LBT Amp plate (incubation at 10°C for 24h)

Table 1: Screening of cold adapted lipase in microtiter plate

Mutant	Total enzyme activity (unit/ml)	
	10ºC	30ºC
1	300.4	291
2	125.3	123.6
3	254.1	143.3
4	350.2	279.8
5	146.8	123.6
6	225.7	118.4
7	276.4	166.5
8*	575.5	240.3
9*	613.3	240.7
10*	503.8	239.6
Control	279.8	350.02

*: mutants with the highest enzyme activity

The recombinant strains producing the randomly mutated lipase and the wild type were cultured on LBT Amp plate. The optimum temperature for the lipase activity of the randomly mutated enzyme was 10ºC, being 20ºC higher than that of the wild-type enzyme. There was no colony in the LBT-

Amp plate containing pGEM-T vector without lipase gene. The stability of the lipase activity at 50ºC was also measured but all these mutants and control have lost their enzyme activity after incubation at 50ºC for 30mins. There was 72.85% loss of activity for pFJK1, 94.66% and 60.32% for pFJK2 and pFJK3, respectively.

Discussion

This study aimed to induce a mutant of *Bacillus subtilis* with the nature of lipase activity at low temperature. To achieve the target, ep-PCR method was employed using the different recipes of PCR master mix. The results were then compared with those of the control group. We succeeded in creating a lipase protein that could maintain its activity at low temperature by random mutagenesis with the epPCR technique. The lipase retained 70% of its activity at 10ºC and there was a drastic decline in enzyme activity at 50ºC.

Several studies have shown that ep-PCR is a simple and rapid method to generate variants with random mutations in different genes. Using ep-PCR, Ryu *et al.* [16] could induce a mutant of *Photobacterium lipolyticum* with the maximum lipase activity (75%) at temperature of 25ºC. In contrast, the optimum temperature for the activity of the lipase enzyme produced by the wild type was about one and three degrees centigrade. *P. lipolyticum* species was originally isolated from a marine habitat in an environment with a temperature of 3ºC. Using the same method as Ryu *et al.* [16], we were able to develop a number of *B. subtilis* mutants with the ability to produce lipase with the maximum activity at 10ºC. This value was 15ºC lower than that of the Ryu *et al.* [16] study. Although the species in these two studies were different, the methodology was the same.

Recent studies have shown that the mutation of isoleucine to threonine should be a stabilizing mutation as it replaces a hydrophobic residue on the surface of the helix with a hydrophilic residue and it is the major cause of stability of the lipase enzyme activity in thermophilic bacteria such as *Bacillus* species [13]. It has been observed that in the predicted structure of the mutant, at position 56, as threonine replaces isoleucine, the side chain OH (OG1) is at a potential hydrogen bonding distance from the carbonyl oxygen of asparagine 53. Thus, there might be an additional hydrogen bond in the helix as isoleucine is replaced with threonine in this thermostable mutant [13]. It has been proposed that minimal change in the protein structure can provide thermostability and the thermostability could be attributed to few additional hydrogen bonds [17].

Based on Khurana *et al.* [13] and our findings, it is assumed that the ep PCR has made the cold active mutants in which the content of hydrophobic amino acids and hydrophobic bonds in the peptide chains of lipase protein are lower than that of the wild type. Therefore, they would not be activated at high temperatures. However; in order to determine the precise location of the mutated gene, nucleic acid sequencing should be performed.

Conclusion

In conclusion, ep-PCR is recommended as a method of choice in genetic engineering to induce the mutants with the relatively distinctive characteristics.

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Conflict of interest statement: All authors declare that they have no conflict of interest.

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