



Optimization of a PCR Method for Detection of Lipase Gene in *Bacillus subtilis*

Hamid Mir Mohammad Sadeghi*, Maryam Yazdanian, Fatemeh Moazen

Department of Biotechnology and Isfahan Pharmaceutical Research Center, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Lipases catalyze hydrolysis and synthesis of triacylglycerols. These enzymes, especially the microbial ones, have wide commercial and industrial usage. *Bacillus subtilis* lipase has interesting properties such as small size and tolerance to basic pH; therefore, developing techniques for its recognition and isolation is the focus of research in many laboratories. In the present study, two factors i.e. $MgCl_2$ concentration and annealing temperature were manipulated for the optimization of the polymerase chain reaction (PCR) condition. As for the $MgCl_2$ concentration, 2.5 mM produced the best results when the annealing temperature was kept at 55 °C. Regarding the annealing temperatures, the best amplification was obtained at 63.9 °C. It can be concluded that the PCR conditions for the detection of lipase gene in *Bacillus subtilis* has been optimized and can be used for the screening and isolation of these bacterial strains.

Keywords: *Bacillus subtilis*; Lipase; Optimization; PCR.

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1. Introduction

Lipases [triacylglycerol acyl hydrolases; (EC 3.1.1.3)] are a class of enzymes which are able to hydrolyze ester bonds of triacylglyceride substrates at oil-water interfaces [1-3]. Microbial lipases vary widely in enzymatic properties and substrate specificities. Consequently, they are currently receiving much attention because of their potential applications in various industrial and pharmaceutical processes. For example alkaline pH-resistant lipases are currently used as additives in detergents [4-6].

Bacillus subtilis 168 lipase (EC-000964) has become an attractive enzyme for industrial applications because:

a) It could be cloned and expressed in *E. coli* showing a broad substrate range which includes various-chain-length *p*-nitro phenyl esters and triglycerides.

b) Nucleotide and deduced amino acid sequences predict a remarkably alkaline pH of 9.73 and a very small size. In fact the *B. subtilis* enzyme is one of the smallest lipases described so far [2, 7, 8]. Additionally, interest in lipases has been increased recently due to their recognition as important virulence factors and their biotechnological potentials [3]. As for the bacteria, *B. subtilis* has long been used in industry for the production of secretory

*Corresponding author: Hamid Mir Mohammad Sadeghi,
Department of Biotechnology, Faculty of Pharmacy, Isfahan
University of Medical Sciences, Isfahan, Iran.
Tel: (+98)311-7922616, Fax: (+98)311-6680011
E-mail: h-sadeghi@pharm.mui.ac.ir

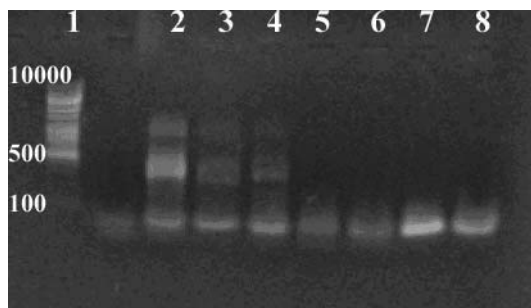


Figure 1. Electrophoresis of the obtained PCR products using different annealing temperatures. $MgCl_2$ concentration was kept constant at 2 mM. Column 1: DNA size marker (1kb ladder); columns 2-8: annealing temperatures of: 63.9, 62.2, 59.9, 56.2, 53.7, 52.0, 51.0 °C, respectively. A PCR product of 371 bp was expected. The bands observed below 100 bp are nonspecific (possibly primer dimmers).

proteins. Because of its nonpathogenic nature and high secretion capacity and existence of a great deal of fermentation technology, *B. subtilis* has been regarded as an attractive host for the secretion of endogenous as well as heterologous proteins [2, 9]. Therefore, it is evident that finding new strains of *B. subtilis* and isolation of industrially favorable lipases is of extreme importance. For this purpose, the present study was undertaken for the optimization of the polymerase chain reaction (PCR) method for the detection of lipase gene from *B. subtilis* 168.

2. Materials and methods

2.1. Materials

Ethidium bromide, loading buffer, 10X PCR buffer, and dNTP were purchased from CinnaGen, Iran. Primers were obtained from FazaBiotech, Iran. Taq polymerase (5 U/ μ l) was purchased from Biotools, Spain. Gen Ruler (1kb DNA Ladder 250 μ g) was from Fermentas, Poland. High Pure PCR Template preparation kit was purchased from Roche, Germany, and Thermal cycler-MyCycler was from Bio-Rad, USA.

2.2. DNA extraction

Bacterial DNA from *B. subtilis* was extracted by high pure PCR template preparation kit. In this method, the bacterial sample was centrifuged and phosphate

buffered saline (PBS) solution was added to the pellet. The mixture was centrifuged and buffer containing lysosyme was then added. After incubation for 15 min. at 37 °C, protease solution was added and the mixture was incubated for 10 min. at 70 °C. Then isopropanol was added and this solution was transferred to a filter tube and centrifuged. After a couple of washes, separating buffer (warmed to 70 °C) was added and centrifuged with the extracted DNA collected in an eppendorf tube. This DNA was kept at -20 °C for future use.

2.3. PCR reaction

The following primers were used for the amplification of the lipase A gene (targeting conserved regions of lipase coding sequence).

Primer1: Sense 5'-ATGGTTCACG-GTATTGGAGG-3'

Primer2: Antisense 5'-CTGCTG-TAAATGGATGTGTA-3'

PCR reactions were performed according to the following conditions: Template DNA

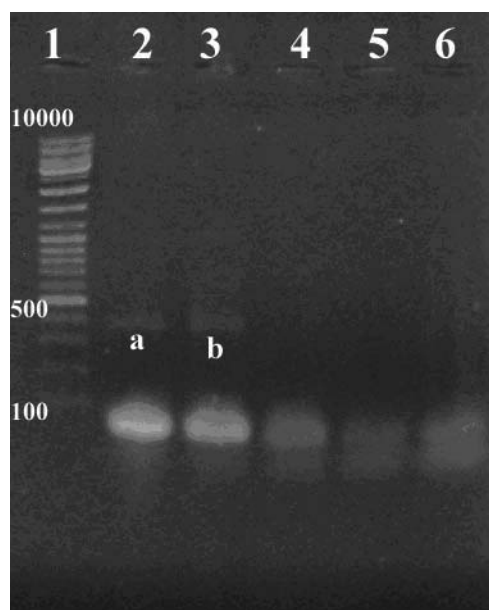


Figure 2. Electrophoresis of the obtained PCR products using different $MgCl_2$ concentrations. The annealing temperature was kept constant at 55.0 °C. Column 1: DNA size marker (1 kb ladder); columns 2-6: $MgCl_2$ concentrations of: 3.0, 2.5, 2.0, 1.5 and 1.0 mM, respectively. Letters **a** and **b** show the expected band (371 bp). The bands observed below 100 bp are nonspecific (possibly primer dimmers).

(50 ng) was added to 10X PCR buffer (5 μ M), Taq polymerase (5U/ml), dNTP (100mM), forward and reverse primers (25 μ M) and (1, 1.5, 2, 2.5 and 3 mM) MgCl₂ in a final volume of 50 μ l.

PCR conditions were as following: One cycle of (94 °C, 5 min.); thirty five cycles of denaturation (94 °C, 1 min.), annealing (different temperatures: 51.0, 52.0, 53.7, 56.2, 59.5, 62.2 and 63.9 °C, 2 min.), and extension (72 °C, 3 min.). The PCR product was electrophoresed on a 0.7% agarose gel. Based on the sequence of lipase A gene, a band of 371 bp was expected.

3. Results

Electrophoresis of PCR products in different annealing temperatures showed that the optimal annealing temperature was 63.9 °C (keeping the MgCl₂ concentration constant at 2 mM for all these experiments). When annealing temperatures of 56.2 °C and below were used, the lipase gene could not be consistently amplified (Figure 1). Using different concentrations of MgCl₂ (keeping the annealing temperature constant at 55 °C) demonstrated that the optimal concentration was 2.5 mM. At lower MgCl₂ concentrations no DNA bands could be detected (Figure 2).

4. Discussion

Detection and isolation of lipase from new strains of bacteria is industrially and commercially very important. In the present study PCR conditions for the detection of lipase A gene from *B. subtilis* was optimized. Since higher annealing temperatures produced more intense bands, it can be concluded that the used primers were specific for the lipase A gene. In fact because a highly conserved region of this gene was used for the primer design, it seems that the optimized PCR conditions can be used for the detection of lipase A gene from any *B. subtilis* strain. Therefore, this method can be used for the screening of various resources such as soil for the detection and isolation of *B. subtilis* strains.

In one study, the annealing temperature of 45-50 °C was used for the detection of lipase gene [10]. However, in that study, the template used was a mutated lipase sequence present in a plasmid. In another study, the DNA from *B. pulmilis* was used for the amplification of lipase gene using the annealing temperatures of 50 °C and 65 °C [5]. However, the experimental conditions for the detection of lipase gene had not been optimized.

In conclusion, for the first time, we have optimized the experimental conditions for the detection of lipase gene in *B. subtilis* which can be used for finding and isolation of new strains of these bacteria as sources for the production this enzyme.

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