Short Communication

Molecular detection of lipase A gene in putative *Bacillus subtilis* strains isolated from soil

Hamid Mir Mohammad Sadeghi[*1, 2], Mohammad Rabbani[1], Fatemeh Moazen[1], Sareh Homami[1]

[1]*Department of Biotechnology and Isfahan Pharmaceutical Research Center, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, I.R. Iran* [2]*Applied Physiology Research Center, Isfahan University of Medical Sciences, Isfahan, I.R. Iran*

Abstract
The present study was undertaken to screen the soil samples collected in Iran for the presence of the *Bacillus subtilis* lipase A gene. The bacterial colonies obtained from the collected soil samples were examined by physical appearance, biochemical tests and the polymerase chain reaction (PCR). Only four colonies were identified as putative *B. subtilis* strains and all contained the lipase A gene. However, the intensities of the DNA bands were different and correlated with the differences obtained from the biochemical tests. Polymorphism of the lipase gene was also determined in samples using SSCP assay. In conclusion, this study demonstrates an easy and reliable method for detection of the lipase gene in *B. subtilis* strains. Further screening of the soil by this method will enable the detection and identification of industrially more favorable lipases.
Keywords: Lipase A gene; PCR; *Bacillus subtilis*; Soil; Detection

Nowadays there is increased industrial demand for enzymes produced by microorganisms (Gupta *et al*., 2004; Jaeger *et al*., 1999). Among these are lipases, which are glycerol ester hydrolyses, such as true lipases and esterases that break long or short acylglycerols to fatty acids and glycerol, respectively (Brockerhoff and Jensen, 1974). The *Bacillus subtilis* lipase is one of the smallest lipases reported and because of its unique characteristics has commercial and research applications for example, the *B. subtilis* lipase A gene can be expressed in its active form in *Escherichia coli* without the need to co-express any specific chaperones. (Droge *et al*., 2003). Several investigators have focused their attention on the isolation, cloning and mutation of this enzyme (Reetz and Carballeira 2007; Acharya *et al*., 2004). Screening for microorganisms in soil from areas, which have extreme conditions such as very hot climates may lead to the isolation of highly useful enzymes (Masayama *et al*., 2007). In the present study, the first step towards obtaining this goal was investigated, which involved the setting up of a fast and reliable technique for screening collected soil samples for lipase-containing *Bacillus* strains.

Soil samples from different areas of Isfahan in Iran that included river banks, gardens and agricultural lands were collected and dried at room temperature for 48 h. Subsequently, different dilutions of these samples were plated onto nutrient agar plates (incubated for 24 hours at 37ºC) and bacterial colonies were obtained. From these colonies, the ones displaying the colony morphology of *B. subtilis* were separated, streaked onto nutrient agar plates and incubated at 37ºC for 24-48 h. For the biochemical and molecular experiments, *B. subtilis* PTCC 1254 and *Bacillus*

[*]*Correspondence to:* **Hamid Mir Mohammad Sadeghi,** Ph.D.
Tel: +98 311 792 2616; Fax: +98 311 668 0011
E-mail: h_sadeghi@pharm.mui.ac.ir

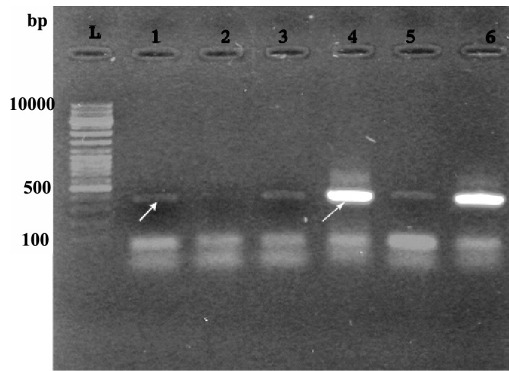

Figure1. PCR amplification of the B. subtilis lipase A gene. In this experiment the DNA templates used were as follows: lanes 1 and 2 are positive and negative controls and lanes 3-6 are putative *B. subtilis* strains isolated from Iranian soil corresponding to samples 3,6,8, and 13 in Table 1, respectively. L: GeneRuler DNA ladder mix (Fermentas, Germany). Arrows show the location of the obtained PCR bands.

licheniformis (PTCC) 1331 obtained from the Persian Type Culture Collection were used as positive and negative controls, respectively.

Biochemical tests for the detection of *B. subtilis* strains were performed according to the procedures reported by Mc Fadden (2000) which included tests for catalase, lecithinase, nitrate, Voges-Proskauer (VP), citrate and maltose. DNA extraction of the chosen colonies was performed using the "high pure template preparation kit" (Roche, Germany). Primers were designed according to the two conserved regions of the *B. subtilis* lipase A gene (Forward: 5´-ATGGTTCACG-GTATTGGAGG-3´ and Reverse: 5´-CTGCTGTAAATG-GATGTGTA-3´). The polymerase chain reaction (PCR) was performed according to a previous procol (Mir Mohammad Sadeghi *et al*., 2008) and the obtained products were electrophoresed on 0.7% (w/v) agarose gel. Based on the *B. subtilis* lipase A gene sequence, the amplified PCR product should be approximately 371 bp. Single strand conformational polymorphism (SSCP) of the amplified PCR products was conducted using procedures described by Sambrook and Russell (2001).

The results of the biochemical tests performed on 15 colonies are shown in Table 1. Based on these tests, four samples were identified as being putative *B. subtilis* (colony numbers 3, 6, 8 and 13).

PCR products obtained after amplification of the lipase A gene are illustrated in Figure 1. Only in the positive control, a weak band of approximately 370 bp was observed. Regarding the samples isolated from Iranian soil, four samples which were identified as putative *B. subtilis* also showed a band of approximately 370 bp. Since the intensity of the obtained bands between these samples was different, optimization of the experimental conditions was performed by altering $MgCl_2$ concentration (1-, 1.5-, 2-, 2.5- and 3 mM) and the annealing temperature (55-, 58.3- and 64ºC). As can be seen in Figure 2, the $MgCl_2$ concentration of 2.5 mM gave the most intense band.

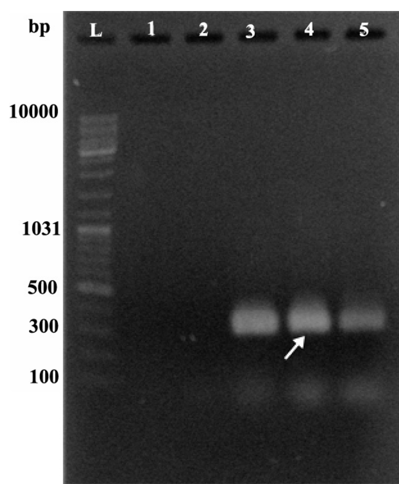

Figure 2. Optimization of $MgCl_2$ concentration for detection of the lipase A gene. Using the genomic DNA of *B. subtilis* 168 as the template, five PCR reactions were performed using the following $MgCl_2$ concentrations: 1-, 1.5-, 2-, 2.5- and 3 mM corresponding to lanes 1-5, respectively. Annealing temperature was 55ºC. L: GeneRuler DNA ladder mix.

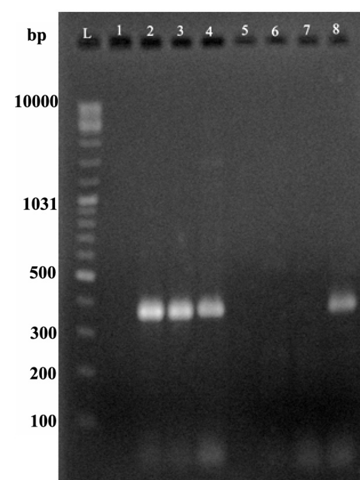

Figure 3. Effect of different annealing temperatures on PCR amplification of the lipase A gene. PCR was performed using the genomic DNA of *B. subtilis* 168 as template, $MgCl_2$ concentrations of 1.5-, 2-, 2.5- and 3 mM and annealing temperatures of 58.3 (lanes 1-4, respectively) and 64ºC (lanes 5-8, respectively). L: GeneRuler DNA ladder mix.

Table 1. Biochemical tests performed on colonies obtained from the soil samples.

Sample number	Maltose test	Citrate test	Voges-Proskauer test	Nitrate test	Lecithinase test	Catalase test
1	-	+	-	+	+	+
2	-	+	-	-	+	+
3	-	+*	+	+	-	+
4	+	+	-	+	-	+
5	+	+	+	+	-	+
6	-	+	+	+	-	+
7	+	+	+	+	-	+
8	-	+*	+	+	-	+
9	-	-	-	-	+	+
10	+	-	-	+	+	+
11	-	+	+	+	+	+
12	-	+	+	+	+	+
13	-	+	+	+	-	+
14	+	+	+	+	-	+
15	+	+	+	+	-	+

*Faster response to citrate test as compared to samples 6 and 13. + means test results were positive, - means test results were negative.

Regarding the annealing temperature, the intensity of the bands decreased when higher annealing temperatures (64ºC) was applied (Fig. 3).

Form the results obtained, the optimum PCR conditions for the detection of the lipase A gene in putative *B. subtilis strains* included an annealing temperature of 55ºC and $MgCl_2$ concentration of 2.5 mM. Thus, the samples were amplified under these optimized conditions and then electrophoresed (Fig. 4). Which showed that the amount of amplified DNA for all samples increased in comparison to previous experiments (Fig. 1). However the differences observed in these samples regarding the intensity of the obtained DNA bands were still present.

The SSCP results are shown in Figure 5. The patterns of the obtained bands for samples 3 and 8 were similar to each other. Sample 13 and the control showed the same pattern (lanes 4 and 5) but were different from samples 3, 6 and 8 (lanes 1-3, respectively).

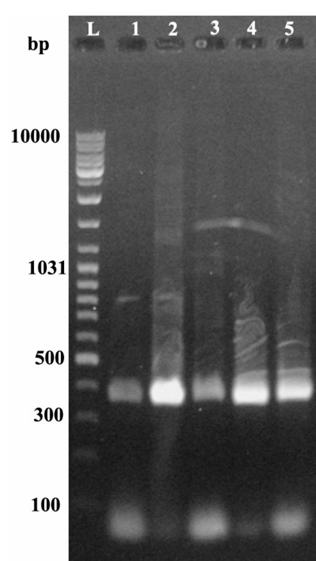

Figure 4. Electrophoresis of PCR products obtained under optimized condition (2.5 Mm $MgCl_2$ and an annealing temperature of 55ºC). DNA extracted from samples 3, 6, 8, 13 (see Table 1) and *B. subtilis* 168 (positive control) were used as templates (lanes 1-5, respectively). L:GeneRuler DNA ladder mix.

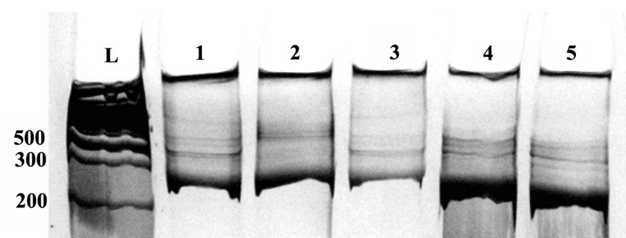

Figure 5. SSCP of the obtained PCR products. Samples 3, 6, 8, 13, (see Table 1) and *B. subtilis* 168 (positive control) were electrophoresed using an 8% (w/v) polyacrylamide gel. The gel was visualized by silver staining. Lanes 1-5 correspond to samples 3, 6, 8, 13 (see Table 1) and control, respectively. L:GeneRuler DNA ladder mix.

The results of this study indicate that the designed primers were suitable for use in molecular identification of the *B. subtilis* lipase A gene. Additionally, when *B. licheniformis* DNA (a bacterium closely related to *B. subtilis*) was used as template, no PCR bands were obtained demonstrating the specificity of these primers.

The putative *B. subtilis* colonies showing a faster response to the citrate test also had less intense PCR bands. This correlation can offer a possible tool for identifying differences in *B. subtilis* strains using the intensity of the PCR bands as an indicator. The SSCP experiments also revealed that the differences in the intensity of the PCR bands could have been due to the polymorphism of this gene.

Pinchuk *et al*. (2002) have studied the genetic diversity and production of isocoumarin production by various *B. subtilis* strains isolated from different habitats however, they did not detect the lipase gene. In another investigation, Ruiz *et al*. (2005) collected samples from three different areas in Argentina and after obtaining colonies, PCR amplification of the lipase gene from the high enzyme producing strains was carried out. The difference in that study was the use of enzyme activity for the screening method rather than the obtained PCR bands. It seems that a combination of this method and those that have been devised in our study can create a successful and easy procedure for isolating *B. subtilis* strains having the desired lipolytic activity.

In conclusion, in this study, a fast and reliable method for the detection of the *B. subtilis* lipase A gene has been developed. Morever, the bacterial strains containing this gene have been identified in the Iranian soil samples. This will help in further screening of soil samples for lipase producing *B. subtilis* strains.

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