World Applied Sciences Journal 11 (3): 354-361, 2010
ISSN 1818-4952

Growth Condition Optimization of the Iranian Thermophilic *Geobacillus* sp. *MKK* with the Aim of Characterizing the DNA Polymerase I Enzyme and its Applications in PCR

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Abstract: The characterization of *T.aquaticus* Taq DNA polymerase followed by the quick popularization of PCR-related technologies was instrumental in the ever-growing interest of the scientific and industrial communities in thermophilic and hyperthermophilic enzymes. Only a few of today's industrial and specialty enzymatic processes utilize thermophilic and hyperthermophilic enzymes hence the need for identification and discovery of such thermostable DNA polymerases has become obvious. In the present study, an Iranian native strain of thermophilic *Geobacillus* sp. *MKK* isolated from a hot spring (pH=6 and 55°C temperature), Ramsar-already shown to produce a thermostable DNA polymerase capable of being used in PCR-has been studied and the conditions for its optimized growth with the purpose of obtaining highest bacterial biomass and therefore higher amounts of the desired DNA polymerase production have been determined. According to the number of cells measured by spectrophometery at OD=600, the optimized conditions for the growth of this strain of *Geobacillus* were determined as the followings: Among lactose, glucose, galactose, fructose and whey; whey was chosen as the best carbon source, Among ammonium sulfate, urea and yeast extract; yeast extract was found to be the proper nitrogen source, pH of 6 was also the best pH among the tested pHs of 4, 5, 6, 7 and 8; 55.C as the optimum temperature among 40°C, 45°C, 50°C, 55°C, 60°C and 65°C and 150 rpm as the optimum rpm out of 120, 150, 170 and 200 rpm for the best growth of the bacteria accordingly to which, the pertaining growth curve was obtained. This could therefore, facilitate the extraction of sufficient amounts of the thermostable DNA polymerase. Cell extract was purified and total protein applied in PCR instead of *Taq* polymerase. The results indicate DNA polymerase of *Geobacillus sp.* MKK can be used in PCR.

Key words: Thermostable DNA polymerase · Taq DNA polymerase · *Geobacillus* sp. *MKK*

INTRODUCTION

Microorganisms are the most important and the main source of production of enzymes. Thermophilic microorganisms are defined as groups of microorganisms which grown at a temperature above 50°C, some of them still actively grow at 80°C [1]. These organisms can easily be found in compost, hot spring, deep vents and other geothermal active regions [2]. Thermophilic microorganisms can be used as sources of thermostable enzymes, which show thermostable properties that fall between those of hyperthermophilic and mesophilic enzymes. These enzymes usually show optimal activity between 60 and 80°C. Active at high temperatures, thermophilic and hyperthermophilic enzymes typically do not function well below 40°C [3]. Thermostable enzymes have a high potential for application as biocatalysts [4] and biotechnological processes can reduce operational costs and increase the reaction rates thus increase the productivity [5]. Only a few of today's industrial and specialty enzymatic processes utilize thermophilic and hyperthermophilic enzymes. The ever-growing number of enzymes characterized from hyperthermophilic organisms and the recent advent of powerful protein engineering tools suggest that thermophilic and hyperthermophilic enzymes will see more and more use in a variety of applications.

Extensive research on the genetics of thermophiles has resulted in the modification of phylogenetic tree. Currently, it is divided into three major groups, which are

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bacteria, archea and eukarya [1]. Recently researches on thermophilic bacteria have extensively been carried out since these organisms offer many advantages either for development of basic sciences or for industrial applications [4]. Isolation of thermophilic bacteria and purification of thermostable enzymes are of significant importance in industry and scientific researches.

Thermostable DNA polymerases are essential enzymes used in biotechnology and biomedical sciences [6]. There is considerable diversity among DNA polymerases and they can be arranged into six major groups of DNA polymerases A, B, C, D, X and Y [7]. The first thermostable DNA polymerase (Taq DNA polymerase) was isolated from thermophilic bacterium *Thermus aquaticus* YT-1 [8, 9, 10]. This polymerase made the polymerase chain reaction (PCR) feasible and introduced a powerful technology that complemented recombinant DNA studies and aided in the diagnosis of inherited and infectious diseases [11, 12]. Since the identification of *Taq* DNA polymerase, a number of thermophilic DNA polymerases have been isolated from both thermophilic eubacteria and archaea sources and characterized [13]. These enzymes have several different notable properties in common [8]. Therefore, taking this fact into account and due to the large number of applications of thermostable DNA polymerase in molecular biology, purification and determination of such enzymes from various thermostable strains, has attracted the increasing attention of researchers worldwide. In this study, growth conditions of *Geobacillus* sp. *MKK* were optimized with the aim of accessing large amounts of thermotable DNA polymerase for use in PCR.

MATERIALS AND METHODS

Bacterial strain *Geobacillus* sp. *MKK* was isolated from a hot spring north of Iran by R. H. Sajedi *et al.* [14] and was kindly supplied for this study.

Initially, the suspension, kept in glycerol, was taken and grown at 55°C, incubated and shaken at 120 rpm (round per minute). After 24 h, 20 µl of suspension was cultured in LB (Luria–Bertani) agar medium containing 1% (w/v) agar. The plates were incubated at 60°C for 24 h.

Gram-staining was performed for single colonies to ensure that there is no contamination. Only *Geobacilli* were observable under the microscope. In the next step, a single colony was taken and transferred to 50 ml of LB broth medium followed by incubation at 55°C, 120 rpm for 24 h. This medium was further used as the pre-culture (OD=1).

Optimization of Growth Condition

Temperature Optimization: 1 ml of the pre-culture was taken and inoculated in 100 ml of the sterilized LB medium containing 1 g of glucose followed by incubation at 150 rpm, pH=7 and the temperatures of 40, 45, 50, 55, 60 and 65°C for 24. Subsequently, the OD (optical density) was read for each temperature, at 600 nm.

pH Optimization: 1 ml of the pre-culture was inoculated in 100 ml of the sterilized LB medium containing 1 g of glucose followed by incubation at 55°C, 150 rpm and pHs of 4, 5, 6, 7, 8 and 9, for 24h. Then, the OD_{600} of each medium with different pHs was read and the bacterial growth was compared in each case.

Round per minute (rpm) Optimization: 1 ml of the pre-culture was inoculated in 100 ml of the sterilized LB medium containing 1 g of glucose followed by incubation at 55°C, pH of 6 and shaken at various rpm: 120, 150, 170 and 200 for 24 h. OD_{600} of each medium was read and the growth pattern compared at different rpms.

Carbon Source Optimization: 100 ml of the sterilized LB medium was again used to inoculate 1 ml of the pre-culture. Each medium was supplemented with different carbon source: glucose 1%, galactose 1%, fructose 1%, lactose 1% and whey 1%, incubated at 55°C, pH of 6, 150 rpm for 24 h and the OD_{600} of each medium was read accordingly.

Ammonium Source Optimization: 1 ml of the pre-culture was inoculated in 100 ml of the sterilized LB medium containing 2 g of glucose followed by incubation at 55°C, pH of 6, rpm of 150 in the presence of different sources of ammonia e.g. yeast extract, ammonium sulfate and urea for 24 h and the OD_{600} of each medium was read. By obtaining the optimized condition for the growth of bacteria, the growth curve was drawn accordingly.

Protein Extraction

Purification of Cell Extract: In order to purifiy the bacterial cell extract, 7 g of the bacterial pellet (biomass) was washed three times using 0.9% physiologic salt for and centrifuged until the supernatant became clear, followed by resuspension in 40 ml of the lysis buffer (containing 50mM of NaH_2PO_4, NaCl 300mM and Imidazol 5mM at the pH of 8) and pipetted. Next, 0.3mM PMSF (phenyl methyl sulphonyl fluoride company [sigma]) was added to this solution followed by sonication 10 times on

ice for 30 min after which the solution was centrifuged at 12000xg, at 4°C for 20 min. The supernatant was taken as the cells extract and kept at -20°C for determination of the total cytoplasmic protein concentration using Bradford's [clause 2.2] technique.

Determination of the Amount of the Protein Using Bradford's Technique: In this method, 1 ml of the Bradford's solution was added to 200 µl of the cellular extract (this indicator is made by dissolving 10 mg of Coomassie Brilliant Blue in 5 ml of ethanol 95% followed by addition of 10 ml of phosphoric acid 85% and with distilled water volume reached to 100 ml). Also, different solutions of Bovine serum albumin (BSA) with concentrations of 0.05, 0.1, 0.25 and 0.5 mg/ml were made as the standard solutions to which 1ml of Bradford's solution was added. After calibration of the spectrophotometer, using the Bradford's solution, the optical density of each standard solution was read at 595nm according to which, the standard curve was drawn and based upon the observed optical absorptions and the standard curve, the concentration of the sample protein was calculated.

Condensations of the Cell Extract Using Ammonium Sulfate: The sample cell extract was poured into a beaker to which ammonium sulfate was added drop by drop so as to reach the saturation of 85% equal to 56.7 g ammonium sulfate in 100 ml. After it was completely dissolved, the dish was kept at 4°C for 5 h, followed by centrifugation at 12000xg for 10 min and the pellet was dialyzed.

Protein Dialysis Process: For this purpose, a dry dialysis sac was transferred to a 1 liter beaker followed by addition of 100mM of NaHCO3 and 10mM of EDTA with the pH adjusted to 7. The beaker was covered by a lid and its content was boiled for 5-10 min, then, the solution was changed with distilled water and the sample was washed three times. To perform the dialysis, the opening of the sac was closed with plastic pins. Then, the ammonium sulfate-condensed-cell extract was added into the dialysis sac with a pipette closing the other opening of the sac. Further, the filled dialysis sac was immersed into the dialysis buffer (buffer A) containing potassium phosphate 20mM, β-mercaptoethanol 10mM, with the pH of 6.5.

The dialysis-sac containing beaker was placed at 4°C and with the buffer A being refreshed every 8 h then the dialysis sac contents transferred into a sterilized falcon and keeping it at -20°C for further analysis.

Performance of the PCR Using the Extracted Proteins: 2µl, 5 µl, 10 µl and 15 µl of the protein samples were removed from the dialysis sac (total bacterial cell proteins) and used in the Polymerase Chain Reaction instead of *Taq* polymerase. and the template in this reaction was the 300bp fragment of the ϕx 174 phage genome.

The ingredients of the PCR were as the follows:

- Template:2µl, Reverse primer:0.5µl, Forward primer:0.5µl, dNTP:0.5µl, Buffer:2.5µl, $MgCl_2$:0.75µl, purified enzyme:10µl (2,5,15µl), H_2O: up to 25µl.

And the PCR was done under the following conditions:

Pre-denaturation for 4 min at 94°C, denaturation for 40 sec at 94°C, annealing for 40 sec at 63°C, extension for 40 sec at 72°C and the final extension for 5 min at 72°C.

RESULTS AND DISCUSSION

Optimization of the Bacterial Growth Conditions: The stock of bacteria stored in 30% glycerol was used as the pre-culture after growth at 55°C for 24 h in LB broth medium. After the 4 stage-growth on the LB medium, mucosal creamy colonies were observed on the medium.

A sample was taken from a single colony followed by smearing and gram-staining and a large number of gram positive rod-like bacteria (*Geobacillus* sp. *MKK*) were observed.

In order to optimize the growth condition, 1 ml of the pre-culture was inoculated in 100 ml of the sterilized Luria–Bertani medium containing 1% glucose and different conditions each time with a different variant were assessed and compared.

Temperature Optimization: The bacterial growth was assessed under the following conditions:

Rpm 150, pH of 7, 1% Glucose (as the carbon source), yeast extract 0.5% (as the nitrogen source), for 24 h and the following temperatures:

- Therefore, the optimum temperature was found to be 55°C.

Table 1: Optical density (absorbance at 600nm) at different temperature in the culture containing yeast extract 0.5%, tryptone 1%, NaCl 1% and 1% glucose.

Temperature(°C)	OD_{600}
40	0.01
45	0.55
50	0.86
55	1.08
60	0.92
65	0.01

Table 2: Optical density (absorbance at 600nm) at different pH in the culture containing yeast extract 0.5%, tryptone 1%, NaCl 1% and 1% glucose

pH	OD_{600}
4	0.01
5	0.01
6	1.37
7	1.07
8	0.87
9	0.01

Table 3: optical density (absorbance at 600nm) at different rpm in the culture containing yeast extract 0.5%, tryptone 1%, NaCl 1% and 1% glucose

Rpm	OD_{600}
120	0.96
150	1.38
170	0.01
200	0.01

Table 4: Optical density (absorbance at 600nm) at different carbon source in the culture containing yeast extract 0.5%, tryptone 1%, NaCl 1%

Carbon source	OD600
glucose (1%)	1.38
galactose (1%)	1.05
lactose (1%)	1.117
fructose (1%)	0.688
whey (1%)	1.261

Table 5: Optical density (absorbance at 600nm) under different concentration of glucose as a carbon source in the culture containing yeast extract 0.5%, tryptone 1%, NaCl 1%

Carbon source	OD_{600}
glucose 1%	1.38
glucose 1%	1.4
glucose 2%	1.44
glucose 3%	1.32

Table 6: Optical density (absorbance at 600nm) under different concentration of whey as a carbon source in the culture containing yeast extract 0.5%, tryptone 1%, NaCl 1%

Carbon source	OD600
whey 1%	1.26
whey 1.5%	1.28
whey 2%	1.3
whey 3%	1.24

Table 7: Optical density (absorbance at 600nm) at different ammonium source in the culture containing 0.5% yeast extract, 1% tryptone, NaCl 1% and 2% glucose

Ammonium source	OD600
Yeast extract (0.5%)	1.44
Ammonium sulfate (0.5%)	1.35
Urea (0.5%)	1.22

pH Optimization: The bacterial growth was assessed under the following conditions:

- Rpm 150, at 55°C, 1% Glucose (as the carbon source), yeast extract 0.5% (as the nitrogen source), for 24 h and the following pHs:
- Therefore, the optimum pH was found to be the pH of 6.

Round per minute (Rpm) Optimization: The bacterial growth was assessed under the following conditions:

- 1% Glucose (as the carbon source), yeast extract 0.5% (as the nitrogen source), pH 6, at 55°C for 24 h and the following rpms:

- The optimum rpm was found to be 150.

Carbon Source Optimization: The bacterial growth was assessed under the following conditions:

- 150 rpm, pH 6, temperature of 55°C, 1%Glucose (as the carbon source), yeast extract 0.5% (as the nitrogen source), for 24 h and the following carbon sources:

- Therefore, the optimum carbon source was found to be glucose. But, since, whey was the most proper carbon source, different concentrations of glucose and whey were compared together and the following results were obtained:

As observed, the best amount of glucose and whey is 2 g per 100 ml LB (2%) and the Glucose 2% is considered as the best carbon source.

Ammonium Source Optimization: Under the growth conditions of pH 6, temperature 55°C, rpm 150 and the carbon source of glucose 2%, the best ammonium source was found to be yeast extract 0.5 %.

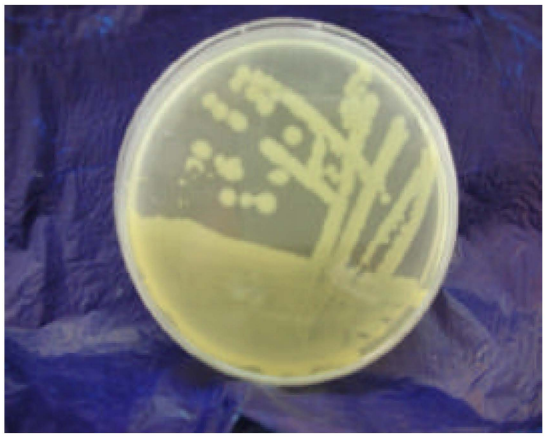


Fig. 1: Mucosal creamy colonies of *Geobacillus* sp. *MKK*

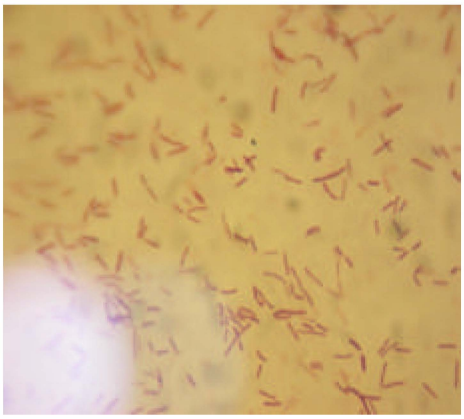
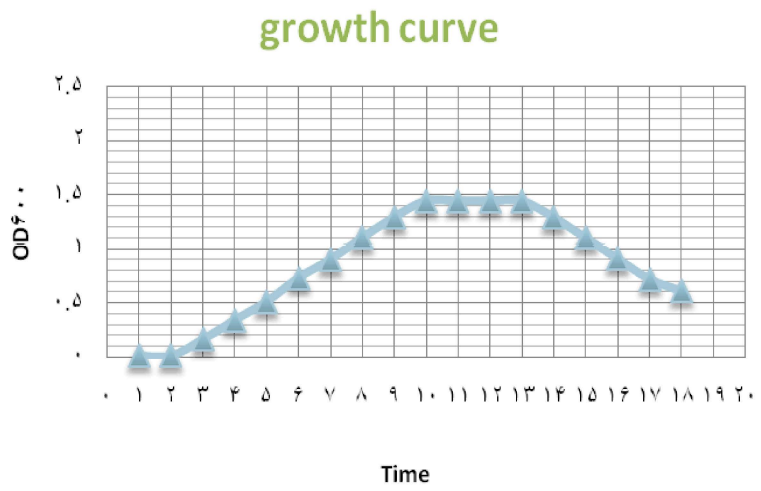
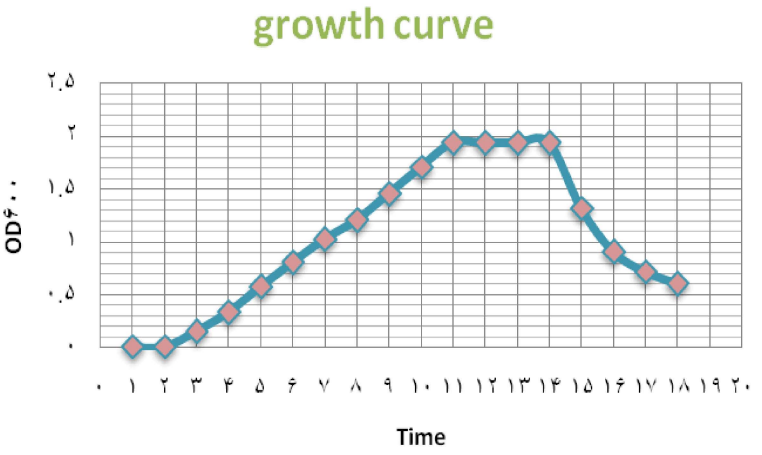


Fig. 2: Morphology of the gram positive rod-like bacteria *Geobacillus* sp. *MKK*



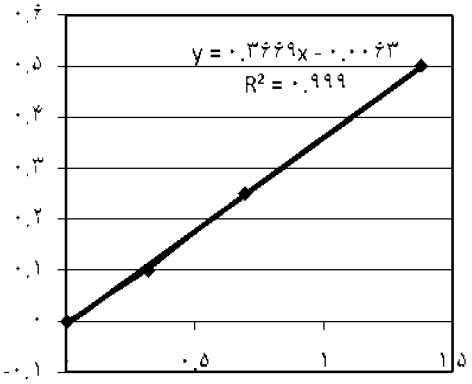
Time	OD_{595}
1	0.01
2	0.01
3	0.16
4	0.34
5	0.51
6	0.73
7	0.9
8	1.1
9	1.29
10	1.44
11	1.44
12	1.44
13	1.44
14	1.29
15	1.1
16	0.91
17	0.72
18	0.61

Fig. 3: Growth curve at 55oC, pH6, rpm150, in culture containing 1% tryptone, 1% NaCl, 0.5% yeast extract and 2%glucose without magnesium sulfate 0.5%



Time	OD_{600}
1	0.01
2	0.01
3	0.16
4	0.34
5	0.58
6	0.81
7	1.03
8	1.21
9	1.46
10	1.71
11	1.94
12	1.94
13	1.94
14	1.94
15	1.32
16	0.91
17	0.72
18	0.61

Fig. 4: Growth curve at 55oC, pH6, rpm150,in culture that containing 1% tryptone, 1% NaCl, 0.5% yeast extract and 2%glucose with magnesium sulfate 0.5%



Concentration of standard solutions(mg/ml)	OD_{595}
0.05	0.158
0.1	0.316
0.25	0.693
0.5	1.377

Fig. 5: Measuring the concentration of total cytoplasmic protein with Bradford's technique

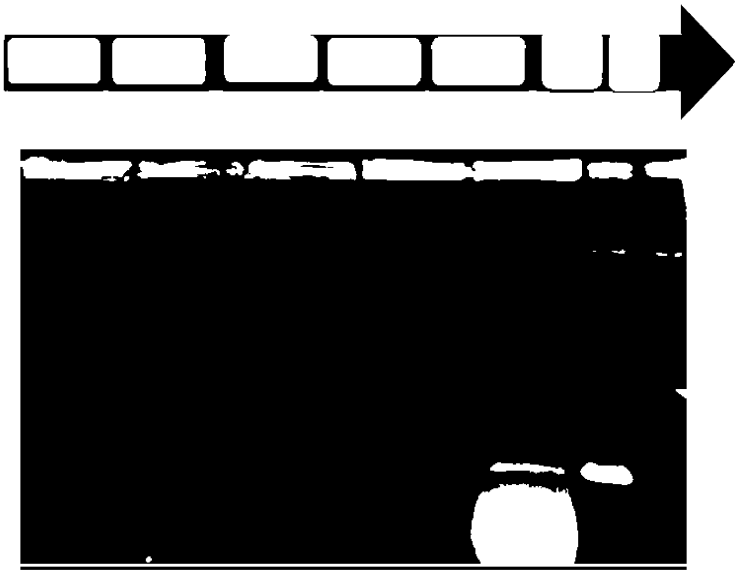


Fig. 6: a: PCR reaction with 1µl of the total protein, b: PCR reaction with 2µl of the total protein, c: PCR reaction with 5µl of the total protein, d: PCR reaction with 15µl of the total protein, e: PCR reaction with 10µl of the total protein, f: Positive control (PCR reaction with 0.2µl Taq enzyme), g: Negative control (PCR reaction with out enzyme).

Since, the purpose of growth optimization of the bacteria was to produce the maximum biomass, therefore obtaining higher amounts of the DNA polymerase I and considering the fact that the DNA polymerase requires Mg^{2+} ion, magnesium sulfate was added to the LB medium (0.5 g per 100 ml LB medium) and enhancement of the bacterial growth was subsequently observed. The OD for the medium containing 2% glucose without magnesium sulfate was 1.44 while the OD for the medium with 0.5% magnesium sulfate reached 1.94 and the optimum growth curve was drawn as below:

Protein Extraction: After the growth of the bacteria in 1000 ml of the LB medium containing 2% glucose and bacteria reached the logarithmic phase, centrifugation was performed at 6000 rpm for 20 min. The supernatant was removed and pellet washed with 0.9% physiologic salt three times followed by another round of centrifugation. The cellular extract was obtained after the lyses of the bacteria with the lyses buffer followed by condensation using the ammonium sulfate method and dialysis for separation of the ions from the proteins within the cell extract.

Further more, the total cellular protein concentration was determined using the Bradford's technique.

The optical absorption of the solutions of bovine serum albumin (BSA), as the standard solutions was used at 595 nm according to which the optical absorption of 200 µl of the cell extract at 595 nm was determined to be 2.3. According to the curve, the concentration of the total cytoplasmic protein was found to be 0.843 mg/ml.

PCR Using the Total Protein: The PCR process was set and performed with total cytoplasmic protein.

Different concentration (1 µl, 2µl, 5 µl, 10 µl and 15 µl) of the total protein was taken but the best result observed with 10 µl of the total protein.

Sample was run on the 1% agarose gel and the enzymatic activity of total protein observed.

As shown in figure 13, 1 µl, 2 µl, 5 µl of total protein did not promote DNA extension activity probably due to low amounts of the enzyme present. The PCR reaction using the 15 µl sample from the total protein was also inconclusive. This indicates that DNA polymerase inhibitor levels in the total protein extract in this volume could prevent the activity of the enzyme. However, 10 µl of the total protein as the source for the enzyme proved to have enough DNA Polymerase to carry out the amplification reaction without effective hindrance from inhibitors.

CONCLUSION

The presence of the observed band is indicative of the significant capability of the DNA polymerase of *Geobacillus* sp. *MKK* in performing the PCR process, in spite of the existence of other proteins in the cellular total protein sample.

In this research, we managed to perform the PCR process using the total cytoplasmic proteins of *Geobacillus* sp. *MKK*, which is a step in paving the way in obtaining a DNA polymerase with high capability for use in polymerase chain reaction.

The enzyme amplified the 300 bp fragment of the ϕx174 phage genome. Previous studies on the DNA polymerase I gene, has indicated that the identified DNA polymerase I, Gsm pol I, as BF (Klentaq1 [15]) possesses the polymerase activity similar to the DNA polymerases family A, but possibly lacking proofreading activity (3' to 5' exonuclease activity). Using the site directed mutagenesis technique utilizing the PCR method, the 3' to 5' exonuclease activity was induced and generated in the enzyme confirmed by the polymerase activity experiments at 72°C. Choice of a DNA polymerase for PCR is usually dependent on its application. For example, DNA polymerases with proofreading activity such as *Pfu* polymerase [16] and Vent polymerase facilitate PCR in cloning, but those lacking proofreading activity are more valuable in PCR for detection purposes or in cases where fidelity and accuracy of the amplified fragments are less important [17]. Therefore, the newly identified DNA polymerase, Gsm Pol I, has potential application not only in PCR and sequencing [6] but facilitates PCR in cloning as well.

Therefore, optimization of the growth conditions of *Geobacillus* sp. *MKK*, with the aim of obtaining high amounts of biomass and consequently, Gsm pol I, was significant. Based upon previous investigations and studies and using data generated in this study, it is suggested that with further investigations, it might be possible to develop an enzyme similar to *Tgo*, *Pfu*, *Pwo* which share the proofreading activity as well.

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