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Isolation and molecular identification of bacteria producing alkaline phosphatase enzyme from environmental sources

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ABSTRACT

Alkaline phosphatase (ortho phosphate, monoester hydrolase phosphoinositide E. C. 3.1.3.1) is a non-specific metalloproteinase enzyme that is located inside periplasmic space of bacteria. This enzyme is used to measure freshwater sediment in genetic engineering for cleaning water. Isolation and molecular identification of the bacteria producing alkaline phosphatase and comparison of its production rate in samples collected from different environmental sources including soil, wastewater and yogurt were the objectives of this study. Different environmental samples including soil, wastewater, stool and dairy products were cultured on the specific medium of phenolphthalein phosphate. Then, the bacteria producing the enzyme were isolated and identified based on the colony morphology, biochemical tests and finally PCT test and ribotyping. After the incubation, only the medium cultured with wastewater showed colonies that were discolored and pinkish. BLAST results of the samples confirmed the existence of *Enterococcus durans*, *Shewanella putrefaciens* and *Shewanella xiamenensis*; *Shewanella putrefaciens* with the highest concentration rate (87.48 u/l) was selected as the most superior strain. Investigating the enzyme concentration using a spectrophotometer at wavelength of 405 nm determined that the wastewater mediums were the only medium has potential to produce alkaline phosphatase enzyme among environmental sources of soil, wastewater, stool and yogurt. Other environmental samples did not show any potential for enzyme production.

1. Introduction

Enzymes, as accelerators of chemical reactions, play an important role in cell life. Targeted application of enzymes as an intervention in trend of chemical and biochemical reactions is one of the subjects that has been considered in industry and medicine. Alkaline phosphatase (EC.3.1.3.1) is a kind of phosphatase that hydrolyzes organic esters of phosphoric acid at alkaline pH of 9-10.5 and releases phosphate. This enzyme hydrolyzes a series of phosphate esters at pH of 7.5 to 11 and thus is called alkaline. Phosphatases are regarded as important enzymes for the survival of organisms and create inorganic phosphate (Pi) by analyzing phosphate esters. In bacteria, alkaline phosphatase has been placed in periplasmic space, which is out of cellular membrane. Since this space is exposed to environmental factors more than real internal space of cells, bacterial alkaline phosphatase is more active and has a relatively higher resistance to inactivation-denaturation–degradation (Smith et al., 1988).

Alkaline phosphatase enzyme has many applications considering its high thermal

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resistance to denaturation. This enzyme is used as an important tool for cloning and sequencing of DNA as well as ELISA. Among other applications of this enzyme are genetic engineering, measuring freshwater sediments (for treating water) and restoring sedimentary systems that have been damaged (Ziba et al., 2010). Since some gram-negative and gram-positive bacteria produce this enzyme, different environmental samples such as soil, sewage and dairy products like yogurt were studied in the present work in terms of the presence of bacteria producing alkaline phosphatase. Isolation and molecular identification of bacteria producing alkaline phosphatase and comparison of its production rates in the above mentioned samples were the goals of this research.

2. Material and Methods

2.1. Selecting environmental resources and sampling

The samples were collected from the environmental resources that had the highest probability of containing the bacteria including soil, sewage, feces and local yogurt. Two-hundred meters was considered as the minimum distance between two sampling zones. Samples were collected by spatula and other sampling tools and were transferred to the laboratory in the clean plastic bags. For this purpose, sampling was carried out in 5 collection groups including sewage, feces, soil and manufacturers of traditional yoghurt. After being transferred to the laboratory and sample dilution, the proper dilute of each sample was cultured on phosphate phenolphthalein agar overnight at 37°C. Followed by adding few drops of ammonia solution into the plate lid, as colonies turn bright pink within a few minutes indicated production of alkaline phosphatase enzyme. At the next step, all bright pink colonies were isolated and separately were sub-cultured on the nutrient agar medium for biochemical and molecular identification tests.

2.2. Identifying the isolates

To identify positive alkaline phosphatase, the isolates were inoculated onto SIM, MR-VP, Simmon Citrate and urea agar differential media after gram staining and performing oxidase and catalase tests. TSI biochemical test, urease of DNAs, hydrogen sulfide production and mobility, starch hydrolysis, and gelatin analysis were also performed. All the culture media and tests were incubated at 37˚C for 24 h and the results were studied. For molecular diagnosis of the bacteria, PCR-based molecular identification test was performed.

2.3. PCR-based molecular identification

In this study, molecular identification was performed based on 16S rRNA gene. For this purpose, DNA was extracted from the bacteria by phenol-chloroform method (Ausubel, 1997) and the extracted DNA quality was analyzed via agarose gel electrophoresis and absorption at 260 nm wavelength by spectrophotometry. The extracted genomic DNA was stored at -20˚C until used. The 16S rRNA gene was amplified using conventional PCR with universal primers (forward, AGA GTT TGA TCC TGG CTC AG and reverse, ACG GCT ACC TTG TTA CGA CTT), producing an amplicon of approximately 1,500 bp (Weisburg, 1991). PCR was made in 50 µl of a reaction mixture consisting of 0.25 µM each primer, 0.2 mM deoxynucleoside triphosphates (Fermentase, USA), 1.5 mM MgCl2, 5µl of 10µ Taq buffer, 1.5 units of Taq DNA polymerase (Fermentase, USA), and 5 µl of extracted DNA. PCR was performed on an iCycler thermal cycler (BioRad Laboratories, Hercules, California, USA) with a pre-incubation step of 95°C for 5 min and 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, followed by a final extension step of 72°C for 10 min.

PCR products were sent to Macrogen Company (South Korea) for sequencing. The sequences were recorded using Chromas 2.33 software (Gholamian, 2012 and aligned through NCBI website.

2.4. Spectrophotometer test

In order to measure the activity of alkaline phosphatase enzyme, first, 1 ml of general medium, from which dilution was prepared, was

mixed with 0.25 ml of Toluene and 4 ml of phosphate buffer at pH=11. Then, 1 ml of paranitrophenol substrate solution was added and samples were incubated at 37˚C for 1 h.

Afterwards, the solution was filtered using Whatman filter paper, 4 ml of 0.5 molar sodium hydroxide and 1 ml of 0.5 molar calcium chloride were added to it for completing enzyme activity and has been shaked properly. The samples were measured by a spectrophotometer at the wavelength of 405 nm and calculated as paranitrophenol µg/g of general medium during the 1 h of incubation. One unit of alkaline phosphatase hydrolyzes 1 µM of phosphate paranitrophenol to paranitrophenol at pH=9.8 and 37 ˚C in 1 min (Tabatabaei et al., 1969).

3. Results

In this study, sampling was done from four general environmental resources including soil, feces, sewage and yoghurt. Bacterial identification included culturing in general and specific media, PCR test and BLAST tool. To obtain monocolony, the suspensions containing diluted samples were prepared and these dilutions were used in phenolphthalein phosphate agar medium. As a result, only those bacteria that were able to produce alkaline phosphatase and consume phosphate as the only energy source grew and created colored (pinkish) colony. Among the general media of soil, sewage, feces and yoghurt, pink color change was clearly found in sewage medium. Only one sample showed a mild color change in terms of yoghurt, which was no longer observed in repetition. The number of samples relating to sewage medium was 17, of which the best culturing media were selected in terms of quality of color change and used for the next tests.

3.1. Gram staining and biochemical tests

Gram staining results showed the presence of both gram-negative and gram-positive bacilli. In one case, gram-positive cocci were observed. After separating bacilli to two gram-positive and gram-negative groups, starch, gelatin, citrate, MR and VP tests were performed for gram-positive bacilli and oxidase, catalase, TSI, citrate, SIM, urea, MR and VP tests for gram-negative ones. The results are given in Table 1.

3.2. Enzymatic activity

By combining a reaction containing 300 µl of diluted enzyme in 1 M of deethanolamine buffer at pH=9.8, 0.5 mM MgCl2, 0.5 mM CaCl2 and 150 mM phosphate paranitrophenol at final volume of 3 ml, the enzymatic activity was observed only in the sewage medium with primary color change. It means that only the bacteria in sewage medium were able to produce alkaline phosphatase enzyme.

3.3. Molecular identification

In this study, molecular identification was performed on three bacterial isolates with higher alkaline phosphatase activity (Figure 2). Comparison between submitted 16S rRNA gene sequences in this study and those recorded on Genbank are shown in Table 2.

This Comparison showed that isolate Y1 had 99% similarities to *Shewanella xiamenensis*, isolate Y2 had 100% similarities to *Shewanella putrefaciens*, isolate Y3 had 100% similarities to *Enterococcus durans*. The results were consistent with those obtained by biochemical tests.

4. Discussion

Studies have shown that several enzymes play roles in food elements cycles to convert organic materials to mineral foods, of which is alkaline phosphatase plays a role in production of mineral phosphor and breaking phosphorus esters in soil. The role of this enzyme is important as 90% of organic phosphor is in monoester form. It has been used in many studies due to its high resistance denaturation and inactivation as well as its high activity compared to Eukaryote alkaline phosphatase enzyme. In this research, phosphate was directly collected from general environmental resources including soil, sewage, feces and yogurt using phenolphthalein specific medium. After the incubation, only the medium cultured with sewage changed to pink color.

Table 1. Results of biochemical tests for gram-negative bacilli in general sewage environment

Sample of selected culturing media	Oxidase	Catalase	TSI	Citrate	SIM	Urea	MR	VP
F7	+	+	Acid/acid negative H2S negative gas	+	-	-	+	-
F11	+	+	Acid/acid positive H2S negative gas	-	-	-	+	-
F8	-	+	Alkaline/acid positive H2S negative gas	-	+	+	-	-
F3	+	+	Alkaline/acid negative H2S negative gas	-	-	+	+	-
F5	-	+	Alkaline/acid negative H2S negative gas	-	+	+	-	-

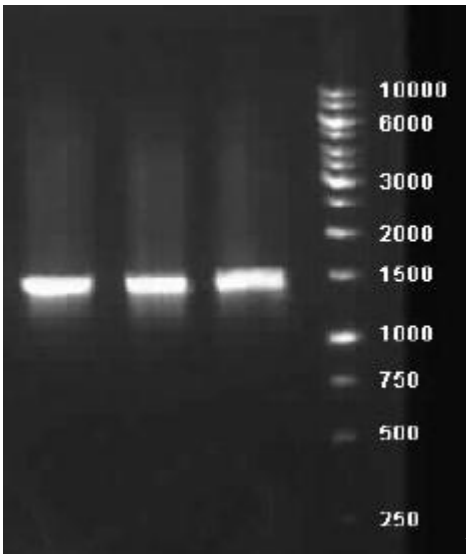

Figure 2. 16S rDNA Amplicon bands of Y1 to Y3 samples

Table2 . Alignment partial 16s rDNA three isolate to high alkaline phosphatase activity.

NO.	Species	Identities (%)
Y1	*Shewanella xiamenensis*	99
Y2	*Shewanella putrefaciens*	100
Y3	*Enterococcus durans*	100

Based on DNA extraction results by observing band, accuracy of extraction and lack of DNA breakage were ensured. Also, BLAST results specified the presence of *Shewanella putrefaciens*, *Shewanella xiamenensis* and *Enterococcus durans* bacteria in the samples. Results of BLAST test showed that none of cases, except one, had 100% homology with recorded sequences, which indicated novelty probability of the strains. On the other hand, studying the enzymatic concentration using a spectrophotometer at wavelength of 405 nm determined that the samples obtained from general sewage environments had high concentration of the enzyme. Based on the definition, a unit of this enzyme is the volume that 1 µM of phosphate paranitrophenol converts to paranitrophenol in 1 min at pH=9.8 and 37˚C. In this regard, all samples, except one, consumed high concentration of substrate and, as a result, showed higher concentration of enzyme in terms of unit per µl. Results of this study suggested that, among soil, sewage, feces and yoghurt, the samples collected from sewage had the potential to produce alkaline phosphatase enzyme, while other samples did not show this potential. Therefore, results of this research introduce sewage as the best general environment for growth of the bacteria producing alkaline phosphatase enzyme and *Shewanella* and *Enterococcus* strains as the enzyme producing bacteria.

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