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Isolation and Identification of L-Asparaginase Producing Bacteria and from Forest Soil of Western Mazandaran

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ABSTRACT

L-asparaginase is an enzyme that catalyzes the hydrolysis of l- asparagine to aspartic acid and ammonia. It is currently used for the treatment of leukemia and some tumors. L-asparaginase decomposes L-asparagine and deprives cancer cells from this amino acid. This study aimed to isolate and identify the native L-asparaginase producing bacteria. To isolate l-asparginase-producing bacteria, 30 soil samples were collected from forests in west of Mazandaran. The soil samples were cultured in the M9 culture medium and then screened. Bacteria with the highest enzymatic activity were identified through 16S rRNA gene sequencing. The enzymatic activity was measured by the nesslerization method. Two isolated bacteria, namely *Enterobacter cloacae* and *Citrobacter* spp. Showed the highest enzymatic activity of 10.156 and 74.843 U/mL, respectively. The optimal conditions for enzymatic activity of the identified bacteria are similar to the physiological conditions of the human body indicating the need for complementary studies on asparaginase to achieve an effective enzyme.

1. Introduction

As a hydrolase enzyme, L-asparaginase (L-asparagine amidohydrolase E.C:3.5.1.1) is a specific importance enzyme in medicine. By hydrolyzing the amide bond, this enzyme decomposes L-asparagine into L-aspartate and ammonia. L-asparagine is, therefore, the substrate for L-asparaginase so that its hydrolytic activity depends on the presence of this substrate in the culture medium. Due to specificity of the L-asparaginase hydrolytic activity, this enzyme is used as an valuable drug for treatment of leukemia, especially lymphosarcoma and related tumors, and acute lymphoblastic leukemia (ALL) (Bessoumy and Jehan, 2004). The substrate and product of this enzyme play a key role in the metabolism of all living organisms. The significant physiological role of this enzyme and its controlled expression and activity balance amino acids in the human body. On the other hand, an increase in the concentration of ammonia produced by this enzyme causes an increase in pH, which probably controls the buffer system of body liquids (Ramya et al., 2012). Tsuji *et al.* (1957) reported deamidation of L-asparagine by *E. coli* extract leading to the release of ammonia and aspartic acid (Whelan and Wriston, 1969). Mashburn and Wriston (1963) extracted L-asparaginase from *E. coli* extract and found its anti-tumor activity (Yazdani et al., 2012) Numerous sources have been identified for L-asparaginase, but its enzymatic properties differ from an organism to another. Microorganisms are affordable resources for producing this enzyme; therefore, only two species, namely *Erwinia chrysanthemi* and *Escherichia coli*, are currently used for producing large volumes of L-asparaginase (Nouri et al., 2019). L-asparaginase

is extensively used in chemotherapy due to its role in eliminating or reducing L-asparagine in blood. This in turn significantly declines the growth of cancer cells in the absence of L-asparagine (El-Bessoumy et al., 2004). Bacteria are among the most important sources for producing enzymes. This anti-cancer compound can be a good alternative for chemical drugs used in chemotherapy. Accordingly, this study aimed at isolating and identifying L-asparaginase producing bacteria from forest soil in west of Mazandaran, Iran. The identified bacteria can be used as L-asparaginase producing sources to be used in the treatment of acute lymphoblastic leukemia (ALL).

2. Materials and Methods

A total of 30 soil samples with at least 15 km apart were randomly collected from forests in western Mazandaran in the summer. The bacteria were screened through the slightly modified rapid plate assay on M9 culture medium. After preparing the broth culture medium in the autoclaved tube, a single colony was transferred to the medium using a sterile loop. The culture medium was then incubated at 37°C for 48 h. Thereafter, 1000 µL of the microbial suspension was transferred to a sterile micro tube containing 100 µL DMSO. The micro tube cap was tightly closed by para film after vortexing and then transferred to a -70°C freezer. The bacterial DNA was extracted using the Cinna Gen DNA Extraction Kit. One-hundred microliter of protease buffer was added to each isolated sample. The samples were then kept at 95°C for 10 min. Forty microliters of Lysis solution were added to the samples and then vortexed for 20 s. Then, 300 µL of the precipitation solution added to the microtubes and inverted several times, and kept at -20 °C for 20 min. Precipitation by acetone used for concentrating and precipitating L-asparaginase. To this end, increasing percentages of acetone were added to the enzyme and the enzymatic activity was evaluated to determine the percentage of acetone causing maximum enzyme precipitation. The purified enzyme was used for SDS-PAGE and determining the enzymatic activity. After preparing the culture medium, a loop of the single colony of each sample was separately cultured and kept in a shaker incubator at 37 °C and 200 rpm to grow bacteria (the culture medium gradually turned darker). The cultured bacteria were then transferred to sterile falcon tubes, and the raw enzymatic extract was separated by centrifuging at 10000 rpm for 15 min. The enzymatic activity was determined by preparing the test, blank, and standard tubes. The standard tubes containing different concentrations of the standard substrate and the standard blank were used for plotting the standard calibration curve. First, 0.2 mL of Tris-HCl and 1.7 mL of L-asparagine were added to the test and blank tubes, and 0.1 mL of TCA was only added to the blank tube. The tubes were incubated at 37 °C for 5 min (TCA stops the enzymatic activity of L-asparaginase in the blank tube). Then, 0.1 mL of the enzyme produced by the bacterial samples added to the blank and test tubes, and the tubes were incubated at 37°C for 10 min. TCA (0.1 mL) then added to the test tubes and the tubes were centrifuged at 10000 rpm for 5 min. Then 0.5 mL of the supernatant obtained after centrifugation was added to 0.7 mL of distilled water. Finally, 1 mL of Nessler's reagent added to the test and blank tubes, and the tubes were kept at room temperature for 10 min. Absorbance of the samples was read at 480 nm in a spectrophotometer.

3. Results

In the first stage of bacterial isolation, 30 colonies of bacteria were isolated from soil samples and purified on the nutrient agar plates. The L-asparaginase producing colonies were then screened on the M9 culture meduim, and two colonies were isolated; of which, one colony with the highest enzymatic activity surrounded by a pink-red halo was selected for further analysis (Fig. 1). Biochemical tests were performed for the preliminary identification of purified colonies.

The colonies that changed the culture medium color to pink, were stained with Gram staining procedure. The bacteria were divided into Gram-positive and Gram-negative bacteria after staining based on color. Two samples with the highest change into pink in the M9 culture medium were used for Gram staining. These two bacteria were stained by the Gram staining kit and then observed under a bright field microscope. According to the results, both samples were Gram-negative bacteria (Fig. 2).

Colonies that were not Gram-positive bacilli were removed. After two stages of sampling from different regions, a total of 12 L-asparaginase producing colonies with the same morphological characteristics such as consistency, transparency, and geometric shape were isolated. All colonies were strongly catalase-positive. The samples absorbance was calculated according to the Beer-Lambert law. The standard absorbance curve was obtained by plotting absorbance against standard samples concentration (Fig. 3).

The concentration of unknown isolates was calculated based on the slope of the standard curve (Table 1).

In order to obtain a qualitative result, the extracted DNA was taken directly on the 1% gel and the bands were well observed. The results indicated the presence of DNA and the lack of DNA fragmentation in the samples (Fig. 4).

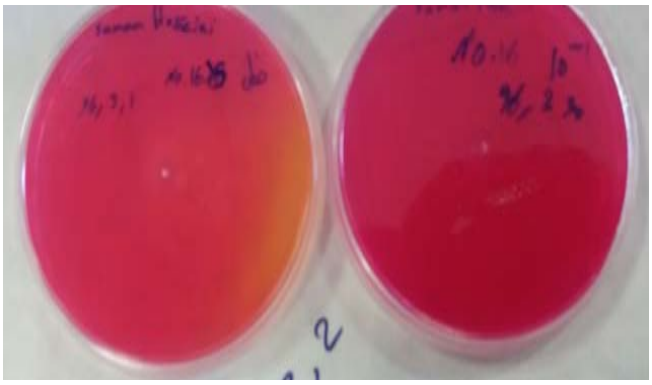

Figure 1. Color change in the M9 culture medium due to production of the L-asparaginase enzyme by some colonies after 48h of incubation at 37°C

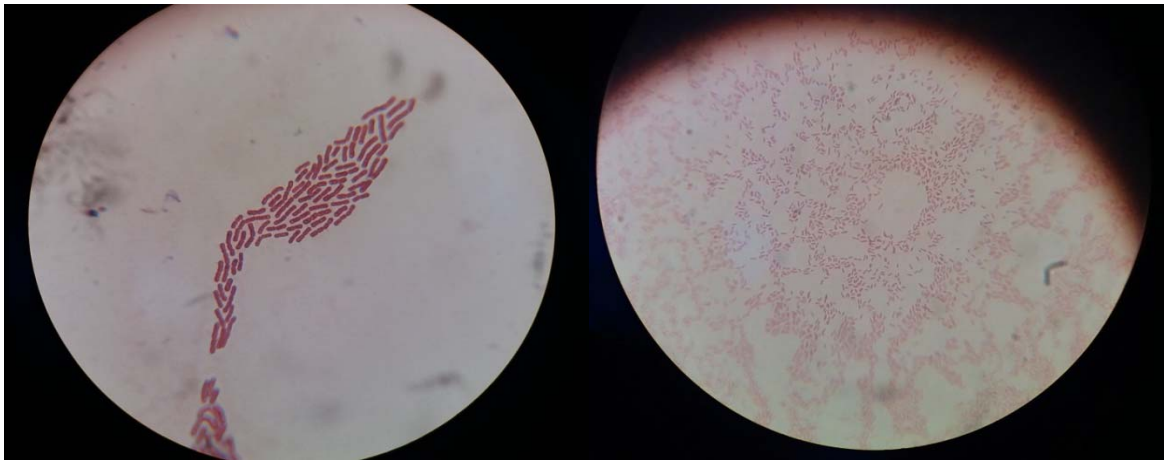

Figure 2. Microscopic images of Gram-negative bacteria.

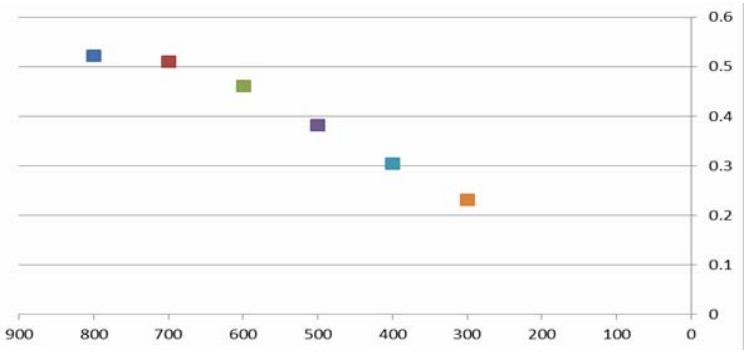

Figure 3. Standard absorbance diagram

Table 1: Absorbance of the samples in terms of U/mL

Sample	Calculated concentration (µm/mL)	Absorbance at 480 nm	U/mL
1	101.56	0.065	**10.156**
2	748.43	0.479	**74.843**

Figure 5 shows the results of the 16S rDNA PCR products electrophoresis in 1% agarose gel.

The homology of the resulting sequences were compared with that of sequences recorded in NCBI databases. The homology was less than 100% in all cases probably indicating new bacterial strains. The sequencing results were eventually blasted on the NCBI website, and the sequences were compared with those available in the gene bank to determine the samples in the phylogenetic tree (Fig. 6).

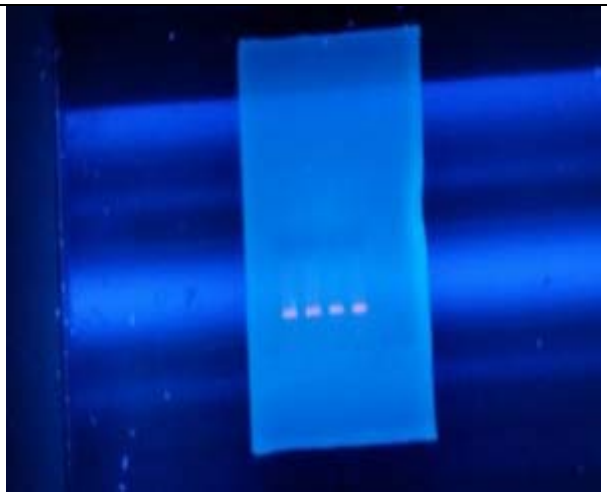

Figure 4: The electrophoresis results in 1% agarose

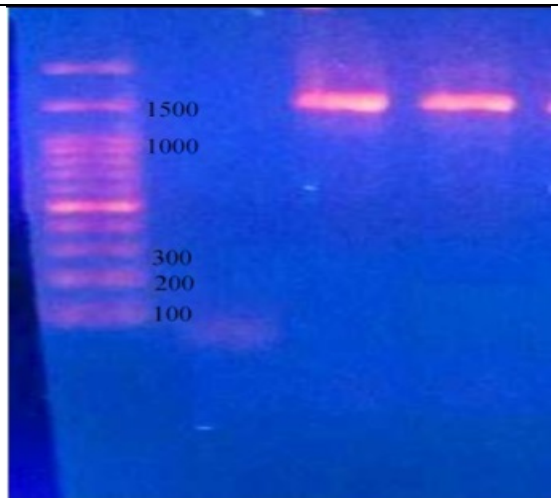

Figure 5: The electrophoresis of the PCR product of two samples in 1% agarose by 1465bp.

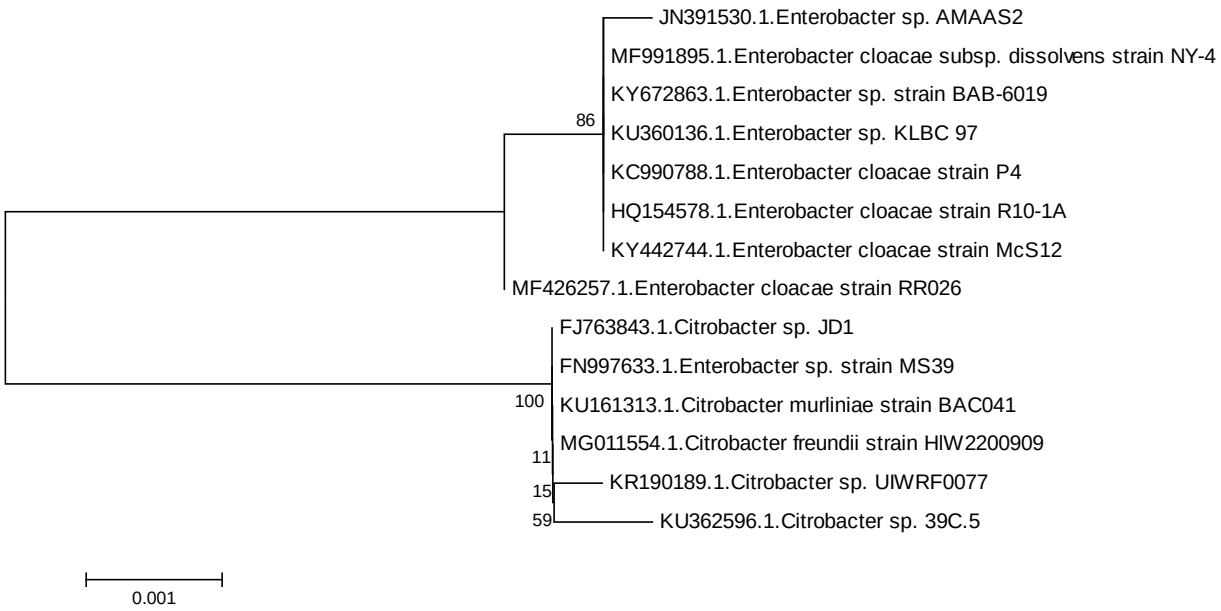

Figure 6. The phylogenetic tree of E15 and E17 strains plotted in MEFA 5 by the neighbor joining method using the bootstrap criterion

4. Discussion

Bacteria are affordable and accessible sources for producing various enzymes. According to the literature, L-asparaginase has been extracted from various bacteria such as *E. coli* (Ghane et al., 2008), *Pseudomonas Aeruginosa* (Bessoumy et al., 2004), bacilli (Moorthy et al., 2010), and *Streptomyces noursei* (Dharmaraj et al., 2011). L-asparaginase is known as an effective medication for the treatment of acute lymphoblastic leukemia (ALL). Despite its higher compatibility with tissues and vital mechanisms of the human body than other drugs used in chemotherapy, this enzyme is associated with some adverse effects like any other drug. These adverse effects may even threaten the patient's life. Accordingly, efforts are made to achieve L-asparaginase with a higher therapeutic capability and fewer adverse effects. Hence, studies are focusing on finding new microorganisms with high enzyme production capacity even with better therapeutic effects. Two cost-effect approaches examined in this study were (Dharmaraj, 2011) manipulation of the enzyme protein to achieve a more desirable enzyme such as PEG-L-asparaginase and (El-Bessoumy et al., 2004) search for finding new asparaginase enzyme in nature. The second approach, *i.e.* finding new microorganisms for producing more desirable enzymes, was used in this study. The forest soil of west Mazandaran, full of pristine areas rich in new microorganisms, was selected for sampling because of the countless number of bacteria in the soil. The two bacteria (*Enterobacter cloacae* and *Citrobacter* spp) isolated in this study showed the highest enzymatic activity of 10.156 and 74.843 U/mL, respectively. A comparison of the enzymatic activity of bacteria with those reported in the literature (Yazdani et al., 2010; Prakasham et al., 2014) revealed that the L-asparaginase enzyme produced by bacteria is an isoenzyme with different properties that should be further studied. so, L-asparaginase has been found (Safary et al., 2019) that production of L-asparaginase depends on various parameters like the concentration of carbon and nitrogen sources, pH of culture medium, temperature, fermentation time and oxygen transfer rate also these parameters differentiate from one organism to another (Orabi et al., 2019) that is consistent with the results of this study. Given that the forest soil in west Mazandaran is rich in bacteria, more tests are recommended to be conducted for producing more effective asparaginase enzymes. The medicinal properties of enzymes produced by bacteria identified in this study are suggested to be compared with commonly used asparaginase drugs.

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Refereces

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