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Manufacture of L-Asparaginase by Actinobacteria Isolated from Rhizosphere of Plants in Tehran with Strong Anti-cancer Activity

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L-ASPARAGINASE is an anti-neoplasm agent. This agent is classified as an enzyme compound. L-asparaginase is used in medicine as well as in the food industry. Microbial strains that are producing L-Asparaginase can be found in many habitats such as soil. The aim of this study was to find isolates producing L-asparaginase enzyme with high ability to inhibit cancer cells. Bacteria of the Actinobacter family were isolated from the soil samples around the roots of the plants in the actinomycet isolation medium. Then, the bacteria were isolated and extracted by screening in M9 medium. Isolates were identified using16S PCR sequencing method. Three isolates of ASP and B and C showed the highest enzyme production, which showed a molecular weight of 43 kDa and in terms of enzymatic activity112. 572 IU/ml, 99.706 IU/ml, 138.572 IU/ml respectively. These three isolates with sequence analysis was similar to *Streptomyces flavoviridis* and *Streptomyces griseus* and *Streptomyces hypolyticus* respectively. Isolate C showed good effects in inhibiting cell MCF-7, HepG-2. There are various isolates enzyme production power in nature and Actinobacter family has shown more ability to produce various enzymes and metabolites and the isolates isolated in this study are used for industrial enzyme production.

Keywords: L-Asparaginase, Anti-cancer activity, Actinobacteria, Rhizosphere, Plants.

Introduction

L-asparaginase (L-a) is an anti- neoplasm agent which is widely isolated from microbial sources with specific physicochemical properties. Lymphoblastic leukemia (ALL) is a leukemia only ripens in infant [1,2]. The enzyme L-asparaginase is used to treat ALL [3]. Seek evacuation From asparagine, regular cells are synthesized through synthesis [4,5]. Cancer cells still lack asparagine synchronization, Ultimately causes deficit of asparagine in these cells leads to the unavailability of asparagine for synthesis of protein become to their death [6-8]. L-asparaginases are widely distributed and are the main origin of L-asparaginase for the testing of *Escherichia coli* and *Erwinia bacteria* [9,10]. L-asparaginase has been observed in many bacterias, the purification and characterization of this enzyme from bacteria around plant roots are important because in plants, L-a is the famous store of nitrogen and the presence of enzyme-producing bacteria around plant roots is higher [11,12].

Therefore, screening of samples from new and diverse sources is required to isolate microbes that have the ability to produce enzymes with new properties [13,14]. The aim of this study was to isolate bacteria from different soil sources and finally produce L-asparaginase from it for anti-cancer purposes.

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Materials and Methods

Isolation of Actinobacteria (From the soil)

To conduct this study, first from a depth of 30 to 40 cm was sampled from farmland. Samples were transferred to sterile tubes for microbial analysis. Then, 1 gr of soil was mixed in 9 ml of physiological saline and incubated at 100 rpm at 37 ° C for 28±2 hours. The supernatant was collected and sampled and serially diluted with physiological salt. 1 ml aliquots of 10^4-10^9 dilutions were placed on Actinomycete agar agar plates isolated for Actinobacteria isolation (by spreadsheet technique under sterile conditions). Colonies were purified by repeated subcultures [15,16].

Screening for L-asparaginase (Plate method assay)

Screening test for L-asparaginase (Plate method assay) was performed based on studies and methods of Fatima et al. (2019) and Shukla et al. (2014) [17,18].

L-asparaginase activity

Method of L-a efficacy done accordingly the method of Poongothai et al. (2017). For this purpose, positive isolates were selected by screening method (for production). These isolates were cultured on asparagine glucose by fast plate agar (GAA) method and the flasks were shaken at room temperature at 120 rpm for 7 days. Lowry procedure was operated to measurement the amount of protein in crude dialysis supernatant. L-asparaginase efficacy was measured using the procedure of Imada et al., (1973). Absorption of the supernatant using a UV-Spectrophotometer at 480 nm. The released ammonia content was determined. One part of asparaginase is the amount of enzyme that catalyzes the formation of 1 micromole of ammonia/min (at 37 ° C) [19].

$$\text{Units/ml} = \frac{\text{Micromoles ammonia released}}{\text{10 minutes x ml enzyme in reaction}}$$

Determination of molecular weight

SDS PAGE done by lamellar procedure (Pradhan et al., 2013). In this experiment, 10% to acrylamide gels and gels from 5% containing 0.1% SDS were used. The gel was then stained with coomassie and a solution of water with acetic acid and methanol in a ratio of 5: 1: 4. The molecular weight of pure L-asparaginase was compared with standard markers of molecular weight and bovine. Serum albumin (66 kDa) was determined [20].

Anticancer activity

The purified anti-cancer effect of purified L-asparaginase on MCF-7, HepG-2 cells was determined by MTT test. HeLa cervical cancer cells were well maintained in DMEM cells with FBS (10%) and specific antibiotics in a CO2 (5%) barley supplement at 37 °C. Then, suspended cells were stained using trypan blue in order to differentiate between dead and living cells. Living cells were counted using a homocytometer. Live cells 103 5 in 5 were plated in 100 µl per well of one plate 96 wells. After overnight incubation at 37 °C, the culture medium was removed and fresh medium with different concentrations of pure L-a was added again. The wells were incubated at 37 °C for 48 hours. Finally, platinum and untreated cells were considered as a positive control and a negative control [21].

$$\%\text{Inhibition} = \frac{100(\text{ Control- Treatment})}{\textit{Control}}$$

16S rRNA typing

In this research, Actinobacteria that had the maximum L-asparaginase efficacy were analyzed (16S rRNA analysis). 16S rRNA sequencing using universal primer and PCR was utilized for the final identification and detection species (Table 1). The sequences obtained were consigned in the DNA Data Bank of NCBI [22].

Results

Primary screening

Based on the screening results, From of 67 isolates, only 3 had the potential to produce L-asparaginase. For further studies among the isolated colonies, a microbial strain with a pink colony was used. Three colonies (ASP +, B +, C +) isolated their pink plate, indicating their strong L-asparaginase activity (Fig. 1).

TABLE 1. Primers used in this study

Primer Name	MW	TM	Seq.(5-3)	mer
27F	5834	57.5	GAGTTTGATCCTGGCTCAG	19
1541R	6081	59.96	AGTCCCGCAACGAGCGCAAC	20

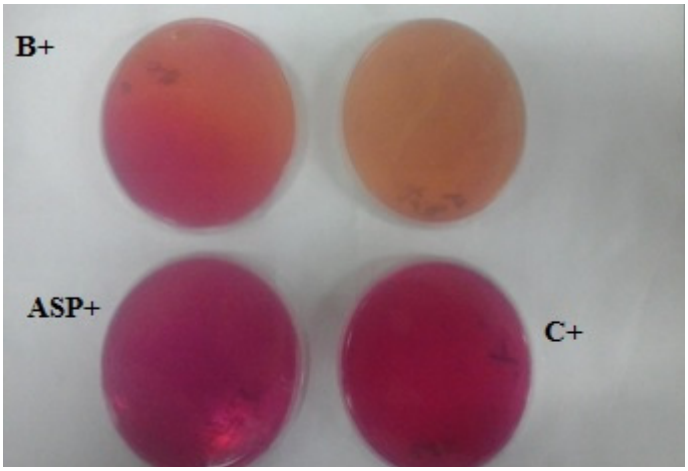

Fig. 1. Developed prominent pink colur surrounding the microorganism growing in the modified M9 medium

Secondary screening

The L-a produced by each dialysis strain was purified and the amount of enzyme in each sample was measured. Amount of enzyme produced at PH 7 and 8 are specified in Table 2. Increased enzyme production was observed at PH 8 and the results show that PH plays an important role in the production of L-asparaginase.

Enzyme molecular weight

The protein-purified enzyme was profiled by sodium dodecyl sulphate–polyacrylamide gel electrophoresis to determine the molecular weight The resulting enzyme thus produced a protein band of a molecular weight of approximately 43 kDa (Fig. 6).

The anti-cancer activity of the crude enzyme

Te viability (%) of MCF-7, HepG-2 and was used as an indicator of cytotoxicity, It was treated with different concentrations of ASNase produced by three isolates for 72 h. The enzyme-induced toxicity of MCF-7 and HepG-2 was dose-dependent because a gradual increase in ASNase dose led to a seal of cell growth (Figure 12). Purified ASNase enzyme was shown to selectively inhibit cancer and the effects of the enzyme extracted from C^+ isolate were greater than those of other isolates. The cytotoxic effect ASNase of C^+ isolate showed significant toxic activity toward HepG-2 cells (IC50 = 11.66 µg/ml), that was more than the effect observed against MCF-7 cells (IC50 values of 14.55 µg/ml). Isolated ASP^+ and B^+ also showed cytotoxic effects on cell line HepG-2 and MCF-7 cells. IC50 was obtained for ASP^+ isolate these two cell lines 8.6 and 8.1 µg/ml , and IC50 for ASNase extracted from isolate B^+, 7.8 and 8.1 µg/ml respectively.

16S rRNA Gene Sequence Analysis

In the present research, 64 isolates were isolated from 250 soil samples, with three isolates showing the best enzyme production. After performing PCR and sending samples for sequencing and sequence analysis Three isolate were placed on *Streptomyces* genus using molecular methods (Figure 3). ASP+ showed the highest similarity of phylogenetic sequence to *Streptomyces flavoviridis* and isolates B and C were very similar to *Streptomyces griseus* and *Streptomyces hypolyticus* (Fig. 4).

Discussion

L-a has been reported to have anti-neoplastic activity. Organisms such as *Escherichia coli*, *S. marcescens*, *E. carotovora* and *P. acidivoras* L-asparaginase produce the L-asparaginase compound. Enzyme activity was consistent in our study as previously reported. In this research study, the enzyme activity recorded at pH 8 which were 112.572, 99.706, and 138.572 (IU/ml) for the strains ASP, B, and C, respectively. The final preparation was examined using SDS-PAGE (with and without β-mercaptoethanol), which revealed that its molecular mass was 43 kDa. Enzymes with different molecular weights are isolated from different types of bacteria. From 5 different soil samples, 61 bacteria producing asparagine were isolated. This is probably because soil is a source of enzyme-producing microorganisms. ASP + and B + identified the effects of cytotoxicity on HepG-2 and MCF-7 cells. IC50 was obtained for ASP^+ isolate these two cell lines 8.6 and 8.1 µg/ml, and IC50 for ASNase extracted from isolated B^+,

TABLE 2. Amount (µg/ml) of enzyme produced at pH 7 and 8

Strain code	Protein concentration (pH 7)	Protein concentration (pH 8)
ASP^+	425.4	562.86
B^+	258.6	489.53
C^+	512.7	692.86

TABLE 3. Enzyme activity of L-asparaginase

Strain	L-asparaginase activity (IU/ml)
ASP^+	112.572
B^+	99.706
C^+	138.572

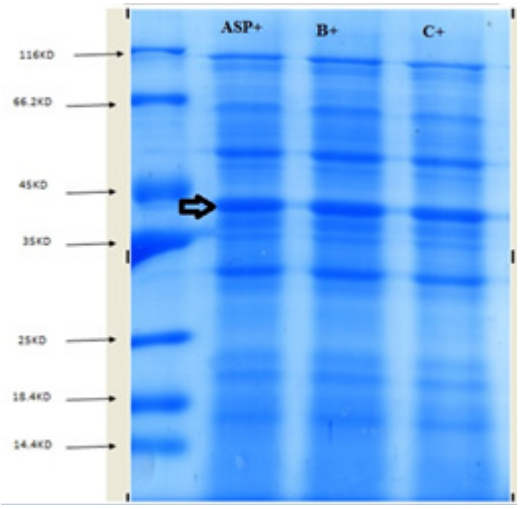

Fig. 2. SDS PAGE of the purified asparaginase

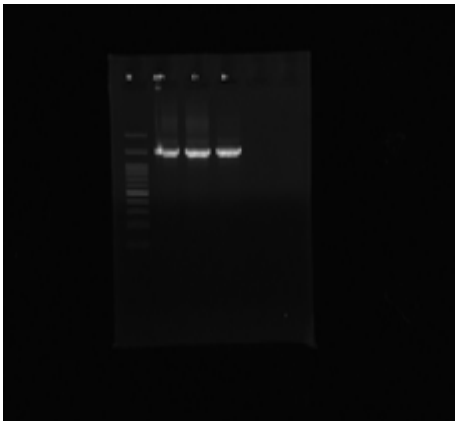

Fig. 3. Electrophoresis of 16S rRNA PCR products using 1492R and 27F primers

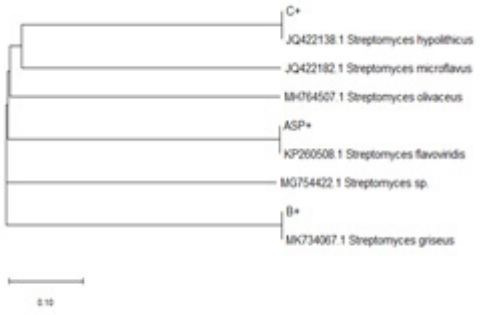

Fig. 4. 16S rRNA phylogenetic tree using the neighbor-joining method.

7.8 and 8.1 µg/ml respectively. There are many studies in the world to isolate the bacteria that produce this enzyme, so each study has isolated a specific isolate and examined the amount of enzyme production and its effects, and this shows that researchers are still looking to introduce bacteria with more enzyme production capacity. In 2019, Fatima et al. Isolated this enzyme from *Pseudomonas aeruginosa*, which showed good effects on the HeLa cell line. In 2010, Moorthy et al. Extracted the enzyme L-a from the genus Bacillus, which had a molecular weight of 45 kDa, which was almost identical to our study. Mahajan et al. Isolated this enzyme from Bacillus lichene formis, which showed a good effect on clone E6-1, MCF-7 and K-562 cell lines. In general, these studies show the importance of the subject in introducing productive bacterial species in order to industrialize enzyme production.

Conclusion

It has been shown that L-a can be used to treat cancer. The results showed that L + asparaginase isolated from C + can effectively inhibit the growth of cells of cancer and can be used for therapeutic purposes.

Authors' contributions

All authors were involved in study design, data collection, and article approval.

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Conflict of interest

None.

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