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Original Research Paper

Chlorella vulgaris, a novel microalgal source for L-asparaginase production

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ABSTRACT

L-asparaginase is an amidohydrolase enzyme which is used for acute lymphoblastic leukemia chemotherapy. In this experiment we have collected 40 microalgal isolates from south of Iran. Intra- and extracellular L-asparaginase activity of these isolates were examined and the isolated with the highest activity selected for molecular identification by 18S rDNA sequencing. A partial sequence of ~600 bp was amplified and sequenced. The sequence similarity analysis is done using the BLAST program from the National Centre for Biotechnology Information (NCBI) and there was 100% similarity with other reports for 18S rDNA sequences of *Chlorella vulgaris*. Also, the GeneDoc software, version 2.6.002, was used for more 18S rDNA sequence investigation. We have found that *C. vulgaris* can produce an inducible intracellular L-asparaginase in the presence of L-asparagine, and L-asparagine can induce more growth of this microalga.

1. Introduction

L-asparagine is a non-essential amino acid which is synthetized in ordinary human cells by L-asparagine synthetase from aspartic acid. But neoplastic cells cannot synthesize L-asparagine due the absence of this enzyme; hence, L-asparagine is an essential amino acid for these cells and should be obtained from circulating sources. Therefore, this difference is used as a key in acute lymphoblastic leukemia chemotherapy. By reducing L-asparagine content of blood which obtained from dietary sources, the leukemic cells will turn into asparagine starvation phase along with a selective effect on leukemic cells. Nowadays this is done by intravenous (IV) injection of L-asparaginase from *Escherichia coli* or in some cases from *Erwinia* in combination with other chemotherapy agents in treatment of acute lymphoblastic leukemia. L-asparaginase is an amidohydrolase that catalyzes the hydrolysis of amino acid asparagine to aspartic acid and ammonia, consequently reduce the blood L-asparagine content (Peterson and Ciegler, 1969; Gulati et al., 1997; Ebrahiminezhad et al., 2011; Ghasemi et al., 2008a; Thenmozhi et al., 2011).

In 1953 Kidd found that guinea pig serum inhibited a number of transplantable lymphomas in mice and rats as well as certain spontaneous and radiation-induced leukemia in mice. Broome has presented some evidences that the antitumor principle in guinea pig serum is L-asparaginase (Ghasemi et al., 2008a; Roberts et al., 1966). Deamidation of L-asparagine by extracts of *E. coli* was first reported in 1957 (Cedar and Schwartz, 1968). Later, Mashburn and Wriston observed that L-asparaginase (L-asparagine amidohydrolase, Enzyme Commission 3.5.1.1) purified from cell extract of *E. coli* has an antitumor activity similar to that of guinea pig serum (Tosa et al., 1971). Production of this enzyme have reported in a lot of other microorganisms such as *Aerobacter*, *Bacillus*, *Erwinia*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Photobacterium* (Peterson and Ciegler, 1969), *Streptomyces* (DeJong, 1972), *Proteus* (Tosa et al., 1971), *Vibrio* (Kafkewitz and Goodman, 1974) and *Aspergillus* (Sarquis et al., 2004). But as mentioned, the asparaginase from *E. coli* and *Erwinia* had chance to be used in medicine, because of their biochemical properties such as half-life in serum and affinity to substrate.

Administration of foreign proteins always cases immunological problems and limits their usage. So there is a demand to new drug proteins with different immunological properties. That is why asparaginase from *Erwinia* came about in medicine. These enzymes are produced by biotechnological methods in recombinant strains of *E. coli* (Cornea et al., 2002). But drug production using bacterial strains which produce toxic compounds like endotoxins is not suitable and makes purification steps more difficult and critical. Recently there is a great interest to find and produce drugs from marine sources, especially microalgae (Anitha et al., 2012; Kim and Kim, 2012; Devi

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and Bhimba, 2012; Zbakh et al., 2012; Alghazeer et al., 2013). Microalgae do not need complicated carbon and nitrogen sources. They use sunlight as energy source, carbon dioxide as carbon source, ammonium salts and even in some cases atmospheric nitrogen as nitrogen source. Some marine microalgae are cultured in open ponds using marine water without the need to extreme and expensive proses of sterilization (Silva Benavides et al., 2013). So these organisms are so economic for drug production in large scales. Unlike other plant which produce greenhouse gases a pharmaceutical plant which works based on microalgae consumes CO_2 and produces O_2 as a green waste. In this experiment we have tried to find a microalga which can produce L-asparaginase in significant amounts.

2. Materials and methods

2.1. Microalgae isolation

Water and soil samples collected from Fars province in the south of Iran and cultivated on BG-11 agar medium contains $NaNO_3$, 1.5 g; K_2HPO_4, 0.04 g; $MgSO_4 \cdot 7H_2O$, 0.075 g; $CaCl_2 \cdot 2H_2O$, 0.036 g; citric acid, 0.006 g; ferric ammonium citrate, 0.006 g; EDTA (disodium salt), 0.001 g; Na_2CO_3, 0.02 g; trace metal mix, 1 mL and 1.7 g agar per litre. Trace metal mixture contains H_3BO_3, 2.86 g; $MnCL_2 \cdot 4H_2O$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $Na_2MoO_4 \cdot 2H_2O$, 0.39 g; $CuSO_2 \cdot 5H_2O$, 0.079 g and $Co(NO_3)_2 \cdot 6H_2O$, 0.0494 g per litre (Stanier et al., 1971). Plates were incubated for 14 days at 25 °C and illuminated continuously with fluorescent lamps at 60 $\mu E\ m^{-1}\ s^{-1}$ intensity (Ghasemi et al., 2008b). After colonization, pure cultures were prepared by subculturing with agar plate and then transferring to broth medium.

2.2. Enzyme production

Enzyme production was carried out in two media, BG-11 and modified BG-11. To promote the isolated microalga to produce more L-asparaginase, we have modified the BG-11 medium by replacing $NaNO_3$ with L-asparagine (10 g L^{-1}). Microalgal isolates were inoculated in 250 mL Erlenmeyer flasks with 50 mL broth medium and incubated at 25 °C by continuously illuminating with fluorescent lamps at 60 $\mu E\ m^{-1}\ s^{-1}$ intensity. After 14 days, cells were harvested by centrifuge at 4 °C, 5000 × *g* for 10 min. The supernatant was used to assay extracellular L-asparaginase activity. For determination of the intracellular L-asparaginase, cell pellets were freeze-dried and 0.1 g of dry cell weight was suspended in 80 μL sonication buffer (50 mM tris and 10 mM EDTA, pH 7.5) in a 1.5 mL thin wall micro tube. Cells were disrupted using probe-sonicator (Output 20, Duty 50, 10 min) on ice. Cell debris was brought down by centrifugation at 4 °C, 13,000 × *g* for 20 min. The supernatant was used as an enzyme solution for intracellular L-asparaginase activity assay (Ebrahiminezhad et al., 2011).

2.3. L-asparaginase assay

L-asparaginase activity was measured by Nessler's reaction. The assay procedure is based on direct nesslerization of ammonia. Enzyme solution (30 μl) was added to Tris–HCl (pH 8.5, 50 mM) in a final volume of 1.5 mL. The reaction was started with addition of 0.5 mL L-asparagine solution (10 mM, in 50 mM Tris–HCl, pH 8.5) and the reaction tubes were incubated in 37 °C water bath for 20 min. The reaction was terminated with addition of 0.5 mL trichloroacetic acid 15% (w/v) and the volume was adjusted to 4.5 mL with distilled water. Nessler's reagent (0.5 mL, 45.5 g HgI_2 and 35.0 g KI per litre distilled water containing 112 g of KOH) was added and the tubes were incubated at room temperature for 15 min. The absorbance was measured at 500 nm, using visible spectrophotometer. A standard curve was drawn with various concentrations of ammonia (Baran et al., 2002; Willis and Woolfolk, 1974). The microalgal isolate with the highest L-asparaginase activity was selected for molecular identification.

2.4. Molecular identification

Identification of selected strain was done by 18S ribosomal DNA (rDNA) sequencing, using universal 18S rDNA primers 5′-GTCAGAGGTGAAATTCTTGGATTTA-3′ as forward and 5′-AGGGCAGGGACGTAATCAACG-3′ as reverse, which amplify an about 600-bp region of the 18S rDNA. Template DNA was prepared by heat extraction method. For this purpose microalgae cultured in BG-11 broth medium. After centrifugation at 50,000*g* in 10 min and twice washing with distilled water, the pellets were selected for heat extraction at 95 °C for 5 min in TBE buffer. PCR was performed in a final volume of 50 μl containing PCR amplification buffer (1 ×), *Taq* DNA polymerase (2.5 U), dNTPs (4 mM), primers (0.4 μM) and template DNA (10 μl, 1 μg). Amplification conditions were as follow; initial denaturation at 94 °C for 5 min, 10 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min. 20 cycles at 92 °C for 30 s, 50 °C for 30 s and 72 °C for 2.5 min with a final extension of 72 °C for 5 min. *Taq* polymerase was added to the reaction after initial denaturation. The lower denaturation temperature (92 °C) during the 20 cycle step was used to avoid loss of enzyme activity. The samples were electrophoresed in a 1% (w/v) agarose gel, using TBE buffer containing ethidium bromide (1 $\mu g\ mL^{-1}$). A single ~600 bp DNA fragment was cut and extracted from the gel, using a Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company with the primers. The sequence similarity searches were conducted using the BLAST program that is available from the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and GeneDoc software, version 2.6.002. (Ebrahiminezhad et al., 2011; Ghasemi et al., 2008b).

3. Results

3.1. Microalgae isolation and enzyme production

At first 40 microalgal isolates were isolated from water and soil samples collected from south of Iran. Our initial observations showed that most of the isolates had better growth in BG-11 medium than in modified BG-11 (supplemented with L-asparagine). Five isolates failed to growth in modified BG-11, maybe due to their disability to produce sufficient L-asparaginase and utilize L-asparagine as nitrogen source. However, there were isolates which had more growth rate in modified BG-11.

3.2. L-asparaginase activity assay

Intra- and extra-cellular L-asparaginase activity of 35 microalgal isolates which can growth in modified BG-11, were examined in both BG-11 and modified BG-11 medium. No significant L-asparaginase production was seen in BG-11 medium. Also there was no adequate extracellular L-asparaginase in modified medium. The best activities were due to intracellular enzymes which produced by growth in modified medium. The most intracellular L-asparaginase activity was 10 IU g^{-1} dry cell weight. The isolate with the highest activity was selected for molecular identification. This isolate was one of the strains with better growth in modified BG-11.

3.3. Analysis of 18S rDNA sequence

Universal primers were used for amplify ~600 bp of 18S rDNA sequence. As one can see in Fig. 1, in addition to 18S rDNA these