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Original Article

بهینه‌سازی تولید ال-آسپارژیناز از سویه بومی باسیلوس جداشده از خاک و ارزیابی فعالیت ضدسرطانی آن

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چکیده

سابقه و هدف: باکتری‌ها منابع مهم آنزیم عامل ضدسرطان ال-آسپارژیناز (ASNase) هستند که برای درمان لوسمی لنفوبلاستیک حاد در سراسر جهان استفاده می‌شود. این مطالعه با هدف تعیین ویژگی‌های ASNase از باکتری‌های جدا شده از خاک شمال ایران انجام شد.

مواد و روش‌ها: در مطالعه حاضر، تولید ASNase توسط سویه‌های باکتریایی جدا شده از نمونه‌های خاک جنگلی استان گیلان، شمال ایران مورد بررسی قرار گرفت. شرایط بهینه تولید آنزیم، سینتیک، اثر فعال‌کننده‌ها و مهارکننده‌ها و فعالیت ضدسرطانی ال-آسپارژیناز نیمه خالص‌شده در برابر رده سلولی MCF-7 مورد مطالعه قرار گرفت.

یافته‌ها: یک جدایه با قابلیت مطلوب تولید ASNase به روش تعیین توالی ژن ۱۶S rRNA به‌عنوان باسیلوس شناسایی شد. فعالیت گلوتامینازی آنزیم ۵/۹ برابر کمتر از فعالیت آسپاراژینازی آن بود و آنزیم با Km و Vmax به‌ترتیب ۰/۵۵۰ مولار و ۳۵/۷۱ میکرومولار بر میلی‌لیتر در دقیقه، دارای میل ترکیبی با ال-آسپاراژین بود. آنزیم ASNase مورد مطالعه در محدوده pH بین ۶/۵ تا ۸/۵ و تا دمای ۵۵ درجه سلسیوس پایدار بود. فعالیت ASNase تحت تاثیر حضور یون‌های فلزی Na^{+}، K^{+} قرار نگرفت و یون Mg^{2+} به طور قابل توجهی سبب افزایش فعالیت آنزیم شد در حالی که Ca^{2+} فعالیت آنزیم را کاهش داد. فعالیت ضدسرطانی ال-آسپاراژیناز در برابر رده‌های سلولی MCF-7 با IC50 معادل ۲۱میکروگرم بر میلی‌لیتر شناسایی شد.

نتیجه‌گیری: گونه باسیلوس جداشده از خاک گیلان به‌عنوان گزینه‌ای برای تولید ال-آسپارژیناز شناسایی شد و می‌تواند برای استفاده در صنایع دارویی و غذایی مورد مطالعه قرار گیرد.

کلمات کلیدی: الـ آسپاراژیناز، بهینه‌سازی، خاک، باسیلوس.

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Original Article

Optimization of L-asparaginase production using native soil-isolated Bacillus sp. and evaluation of its anticancer activity

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Abstract

Background and Objectives: Bacteria are one of the most important sources of L-asparaginase (ASNase) production which is used as an anticancer agent in the treatment of acute lymphoblastic leukemia worldwide. This study aimed to optimize the ASNase production by bacteria isolated from the soil in northern Iran, and to determine its anti-cancer activity.

Materials and Methods: ASNase production by bacterial strains isolated from forest soil samples in Guilan Province, northern Iran was investigated. The optimized condition of enzyme production, kinetics, effect of activators and inhibitors and anticancer activity of the partially purified L-asparaginase against MCF-7 cell lines were studied.

Results: A promising ASNase producing isolate, was identified by 16S rRNA gene sequencing as Bacillus sp. The glutaminase activity of the enzyme was found to be 5.9 times lower than its asparaginase activity and the enzyme showed affinity for L-asparagine with a Km value and Vmax of 0.055M and 35.71 µM/mL/min, respectively. The current ASNase enzyme was stable from pH 6.5 to 8.5 and stable up to 55°C. ASNase activity was not significantly affected by the presence of two metal ions Na+, K+; Mg2+ showed enhancement in enzyme activity, while Ca2+ decreased it. Anticancer activity of the purified L-asparaginase was detected against MCF-7 cell lines with IC50 of 21µg/ml.

Conclusion: The soil isolate Bacillus sp. was identified as a candidate for L-asparaginase production. The future prospect of this enzyme recommends its utility in pharmaceutical and food industry.

Keywords: Fatty acids, *Candida glabrata*, Yeast, Microbial species, GC-MS.
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Introduction

L-asparagine, a major requirement of the cells for the production of protein, is a nonessential amino acid which can be synthesized from central metabolic pathway intermediates within the cell using an enzyme named L-asparagine synthetase in humans and is not required from external source (1).

L-Asparagine aminohydrolase (EC 3.5.1.1) is an important enzyme which hydrolyzes L-asparagine to aspartic acid and ammonia. In contrast with healthy cells, lymphocytic leukemia cells don't have L-asparagine

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synthetase and are not capable of synthesizing L-asparagine, so rely on the exogenous pool of this amino acid for their growth and survival. L -asparaginase exploits this dependency of the leukemia cells on L-asparagine to kill them (2). Thus, L-asparaginase (ASNase) inhibits protein synthesis in T-cells and this catalytic reaction is essentially irreversible under physiological conditions (3). L-asparaginase is also used in food industry to counter the formation of acrylamide in food processing at extreme temperatures, retaining their nutritional properties (4).

L-asparaginase is present in mammals, birds, plants, yeasts and an extensive range of bacteria but not in humans. This enzyme is produced by a large number of microorganisms such as *Enterobacter, Aerobacter, Bacillus, Erwinia, Pseudomonas, Serratia, Xanthomonas, Photobacterium, Vibrio* and *Proteus* (1,5). However, because of high substrate affinity, only the purified enzymes from *Escherichia coli* and *Dickeya dadantii* (formerly known as *Erwinia chrysanthemi*) are currently used as efficient drugs in lymphocytic leukemia (2,4).

The therapeutic use of L-asparaginase from the above two sources was limited due to immunological responses. L-asparaginase of bacterial origin has several issues like hypersensitivity due to long-term use, which may lead to allergic reactions and anaphylaxis, as well as resistance to asparaginase, leukemoginicity, and glutaminase activity (6). Therefore, there is a need for newer asparaginases with new immunological properties (such as lower allergic reactions). The aim of this study is to isolate L-asparaginase producing bacteria from the soil in Guilan province, northern Iran, optimize the enzyme production process and evaluate its kinetics and anticancer activity.

Materials and Methods

1. Isolation of microorganisms and screening for L-asparaginase production: The forest soil samples were collected at a depth of 10 cm from different sites in Guilan province, northern Iran.

Bacteria have been isolated from one gram of each soil sample using standard dilution plate procedure on M9 media (6.0g $Na_2HPO_4.2H_2O$; 3.0g KH_2PO_4; 0.5g NaCL; 2.0g L-asparagine, 0.5g $MgSO_4.7H_2O$; 0.014g $CaCl_2.2H_2O$; 2.0 glucose (w/v); and 20g agar, pH=7.0) supplemented with phenol red (few drops) as indicator. Two control plates were also prepared using modified M9 media; one without the dye and the other one without asparagine. The plates were incubated at room temperature for 24 hours. Extracellular L-asparaginase production was identified by formation of a pink zone around colonies and change of color from yellow to pink in medium due to the ammonia release which increased the pH of the medium. The isolates obtained from the plates were further screened for L-asparaginase production. The inoculated agar plates were incubated at 37 °C. Pink zone radius and colony diameter were measured in positive isolates after incubation for 24 hours (5). For identification of L-asparaginase producing bacteria, different morphological and biochemical characteristics of the isolates were studied and compared with the standard description of Bergey's Manual of Determinative Bacteriology (2).

2. DNA extraction and 16S rDNA gene amplification: Total genomic DNA was extracted from the isolated strain employing the GenElute Bacterial Genomic Kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol. A 1500bp of *16S rRNA* gene fragment was amplified using universal Primers (2). The amplified fragment

was analyzed using 1% agarose gel and subsequently sequenced.

3. Determination of L-asparaginase activity: The bacterial isolates were cultivated in M9 broth and incubated at 37 °C in three culturing periods; 24, 36 and 48 hours. L-asparaginase activity was measured by Nessler's reaction to determine the rate of hydrolysis of L-asparagine and release of ammonia. A mixture consisting of 0.8 ml of 50mM Tris-HCl buffer pH 8.6 and 0.2 ml of 40 mM L -asparagine was prepared. The reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 0.5 ml of 1.5M trichloroacetic acid. After centrifugation at 10000 rpm, 0.5 ml of the supernatant was diluted to reach 7 ml using distilled water and treated with 1 ml of Nessler's reagent.

Precipitated proteins were removed by centrifugation and the liberated ammonia was measured spectrophotometrically at 410 nm by nesslerization. Enzyme activity was determined with reference to ammonia liberated using ammonium sulphate standard graph. One unit (U) of L-asparaginase is defined as the amount of enzyme that liberates 1 mol of ammonia/min/ml under the above assay conditions. Specific activity was considered as units of enzyme per mg protein (2,7).

3. Enzyme purification: The partial purification of enzyme was done by adding chilled trichloroacetic acid (saturation of 70%) to crude enzyme and keeping it for overnight incubation at 4°C. The precipitates were collected by centrifugation at 10000 xg for 20 min, dissolved in 5 ml of 50 mM tris-HCl buffer, pH 7.5 and subsequently dialyzed overnight against the same buffer. For the present study, dialysis membrane-110 (HiMedia) was used. The dialysis was carried out in a pre-treated dialysis tube. The precipitate was dissolved in 1M tris buffer, pH 8.0, until the dialysate was negative when tested for ammonia. The precipitate formed during dialysis was removed by centrifugation and was discarded (8,9).

4. Characterization of partially purified enzyme: Effect of pH, temperature and substrate concentration on L-asparaginase activity. The one-factor-at-a-time optimization was used to determine the effect of pH, temperature and substrate concentration on L-asparaginase activity. The activity of the partially purified enzyme was studied under the pH range of 3.5 to 10.5. Acetate buffer (0.1 M, pH range: 3.5 -5.5), phosphate buffer (0.1M, pH range: 6.0-8.0) and Tris HCl buffer (0.1M, pH range: 8.5-10.5) were used for this purpose. In addition, the stability of the enzyme was determined by incubating the enzyme at the same pH range in the absence of substrate for 30 min and then the residual activity of enzyme was measured.

The optimum temperature for the enzyme activity was determined by incubating the assay mixture at temperatures of 25 to 55ºC. For stability check, the enzyme was incubated at the same temperatures in the absence of substrate for 30 min and then the residual activity of enzyme was measured. Moreover, the effect of different L-asparagine concentrations ranging from 0.01 M to 0.1 M on L-asparaginase production was investigated (10,11).

5. Determination of L-Glutaminase activity: The enzyme was checked for its glutaminase activity. The activity was determined using the L-asparaginase assay, and using 40 mM glutamine instead of 40 mM asparagine as substrate. The specific activity of the enzyme for glutamine was calculated and compared to that of asparagine (2).

6. Effect of Nitrogen sources on enzyme production: Different nitrogen sources such as L-asparagine, yeast extract, peptone, tryptone, glycine and glutamine were added to the glucose asparagine broth at a concentration of 0.1% (w/v). The optimal concentration of the best nitrogen source (0.02-0.15 % w/v) for L-asparaginase production was determined (2).

7. Effect of Carbon sources on enzyme production: To investigate the effect of different carbon sources on production of L-asparaginase by the strain, the glucose asparagine broth with optimal amount of superior nitrogen source was supplemented with different carbon sources such as lactose, glucose, galactose, mannose, sucrose and glycerol at a concentration of 1% (w/v). The best carbon source was checked at different concentrations (0.5-2.5 % w/v) for production of L-asparaginase by the strain (2).

Effect of EDTA and metal ions

The effect of metal chelator EDTA and metal ions (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}) on L-asparaginase activity was tested at a concentration of 5mM. The assay was performed by pre-incubating 0.25 ml of the partially purified enzyme with 0.75 ml of the EDTA and respective metal ions at 37 °C for 30 min. After pre-incubation, enzymatic assay was performed under optimal conditions, and enzyme activity was expressed as the percentage of the activity without addition of EDTA and metal ions (12,13).

8. Determination of kinetic properties (Km, Vmax): The kinetic parameters, Michaelis–Menten constant (Km) and maximal velocity (Vmax) of the partially purified L-asparaginase were determined with different concentrations of L-asparagine (0.0 to 0.1M) as substrate. The Michaelis-Menten parameters were determined based on Lineweaver-Burk plots using the equation derived from linear-regression analysis of the curve (14).

9. Anticancer activity of L-asparaginase: In order to study the anticancer activity of L-asparaginase produced by *Bacillus sp.* against MCF-7 (human mammary gland adenocarcinoma, ATCC Number HTB-22) cell line, the cells were purchased from cell bank of Pasteur Institute of Iran and grown in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/mL). The cells were maintained at 37°C with 5% CO_2 in a humidified CO_2 incubator. Then, MCF-7 cell suspensions (1×10^4 cells/wells) were seeded onto 96-well tissue culture plates and cells were treated with different concentrations (0–100 µg/mL) of the partially purified enzyme and incubated for 24 h at 37°C, 5% CO_2, and relative humidity. Anti-proliferative effect of L-asparaginase was measured by the colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to an insoluble, colored (dark purple) formazan product, which is solubilized in organic solvent and the absorbance was measured by spectrophotometric microplate reader at a wavelength of 570 nm (15).

10. Statistical analysis: The data obtained from One-factor-at-a-time on L-asparginase production were subjected to One-way ANOVA analysis. $P \leq 0.05$ was considered as significant.

Results

1. Bacterial isolate: Out of 30 bacterial isolates with L-asparaginase activity which were determined by pink color zones on M9 medium supplemented with L-asparagine and phenol red, one isolate with the highest

L-asparaginase production was identified as *Bacillus sp.* on the basis of biochemical tests. Subsequently, sequence analysis of *16S rRNA* was performed and phylogenetic trees for this strain were inferred using neighbor joining method with the help of MEGA 6.0 software package, as shown in Figure 1.

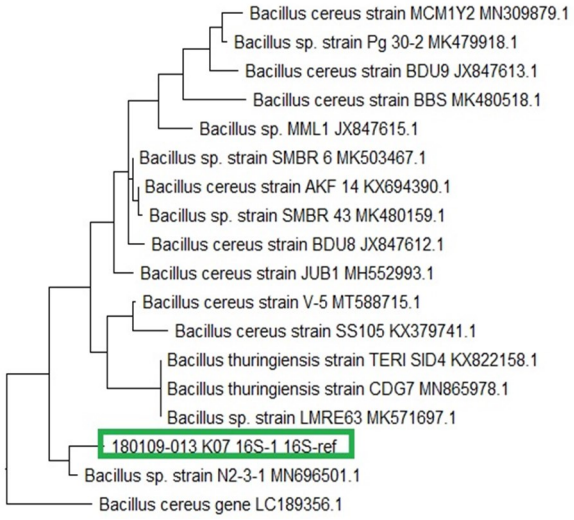

Figure 1: Phylogenetic relationship between the isolated *Bacillus sp.* and other 16S rRNA gene sequences.

2. Effect of time and substrate concentration on L-asparaginase activity: Maximum enzyme production was archived after 48-hour incubation of bacterial culture. In order to characterize the produced L-asparaginase, the partially purified enzyme was incubated at a time interval of 30 min with different concentrations of asparagine in the range of 0.02-0.14 % w/v. It was observed that at 0. 1 % w/v, L-asparagine showed the maximum velocity and further increase in L-asparagine concentration did not enhance the enzyme activity significantly (Figure 2).

3. Glutaminase activity: The glutaminase activity of L-asparaginase produced by test isolate was 12.11 IU/ml, which was 5.9 times lower than specific activity for asparagine.

4. Effect of temperature on L-asparaginase activity: As demonstrated in Figure 3, the maximum activity of L-asparaginase was noticed at 37 °C for isolated strain. The enzyme showed high stability in a wide range of temperature up to 55 °C. Enzyme activity decreased at higher temperature (Figure 4).

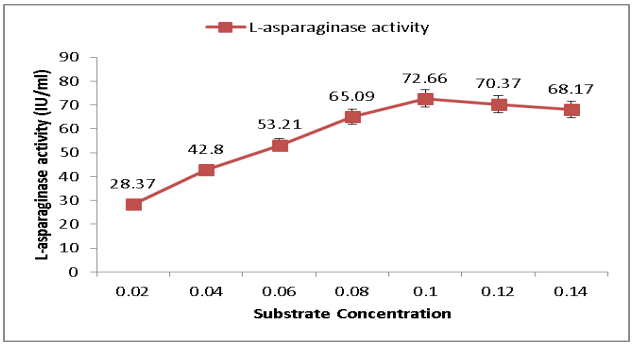

Figure 2: Effect of substrate concentration on L-asparaginase activity.

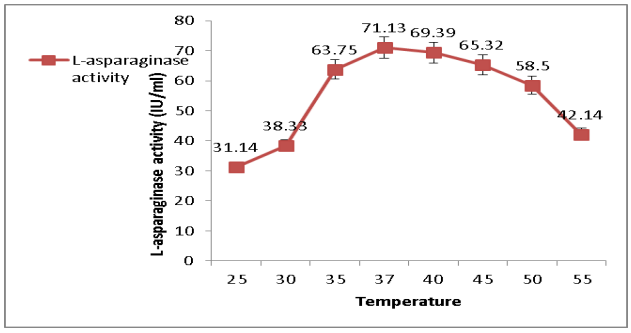

Figure 3: Effect of varying temperature on l-asparaginase activity.

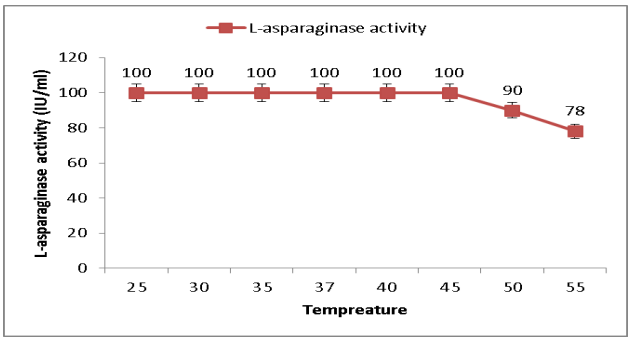

Figure 4: Stability of L-asparaginase in varying temperature.

5. Effect of pH on L-asparaginase activity: The activity of L-asparaginase was evaluated at different pH values. The maximum L-asparaginase activity was observed at pH 7.0 (Figure 5). The enzyme was relatively stable at pH 6.5-8.5 (Figure 6).

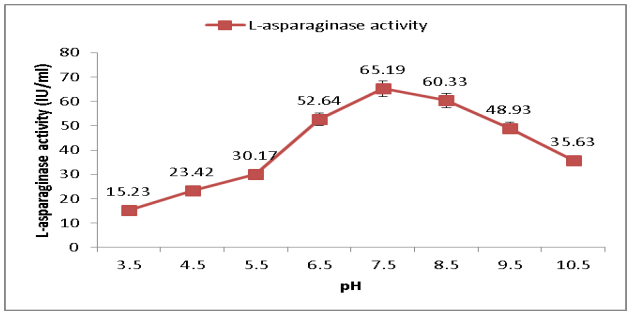

Figure 5: Effect of varying pH on l-asparaginase production.

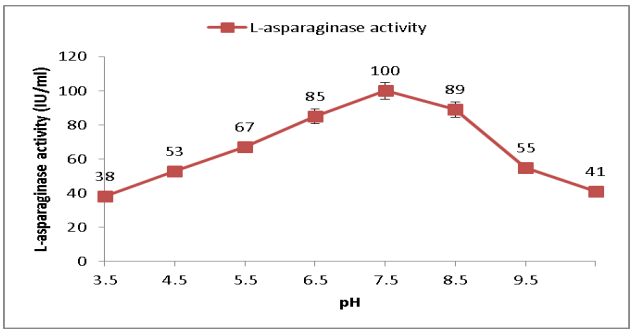

Figure 6: Stability of L-asparaginase in varying pH.

6. Effect of Carbon and Nitrogen sources on L-asparaginase production: Different nitrogen sources like yeast extract, peptone, tryptone, glycine and glutamine were amended in glucose asparagine broth to determine their impact on L-asparagine production. Of all nitrogen sources, L-asparagine showed the highest level of enzyme production (Figure 7). Among different concentrations of L-asparagine, culture medium amended with 0.1% L-asparagine favored maximum enzyme production for *Bacillus sp.*

Furthermore, among different carbon sources, including lactose, sucrose, glucose, galactose, trehalose and glycerol, the highest enzyme production was determined in the presence of glucose (Figure 8). Among different concentrations of glucose, culture medium amended with 2% glucose favored maximum enzyme production. The supplementation of optimized nitrogen and carbon sources yielded to the production of 73.23 IU/ml L-asparaginase at 37 °C and pH 7.0.

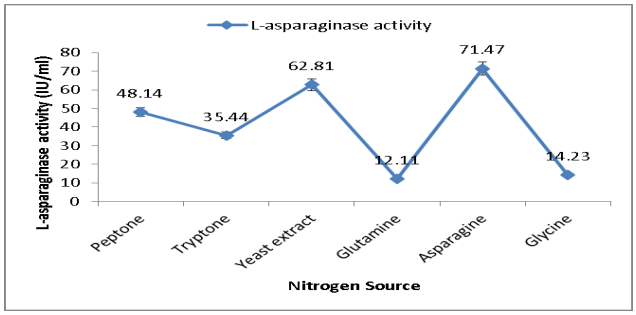

Figure 7: Effect of varying Nitrogen sources on L-asparaginase production.

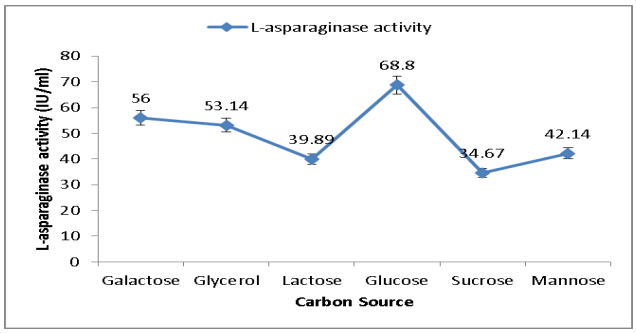

Figure 8: Effect of varying carbon sources on L-asparaginase production.

7. Effect of EDTA and metal ions on L-asparaginase activity: Effect of EDTA and some metal ions on L-asparaginase activity is represented in Table 8. L-asparaginase from this *Bacillus* strain was not significantly affected by the presence of metal ions Na+, K+, and Mg^{2+}, which showed enhancement in enzyme activity, while Ca^{2+} decreased L-asparaginase activity by about 20%. The presence of EDTA acted as inhibitor of L-asparaginase activity, reducing its activity by 37.7% (Figure 9).

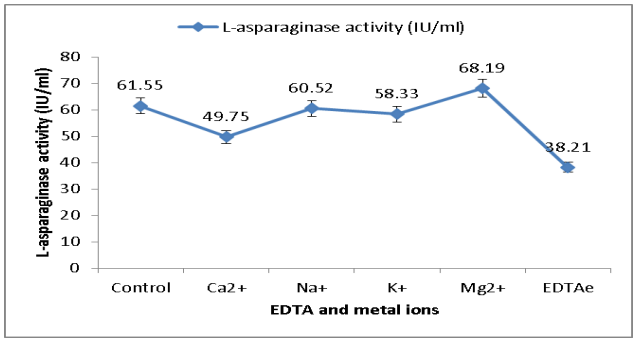

Figure 9: Effect of EDTA and metal ions on L-asparginase activity.

8. Determination of kinetic properties (Km, Vmax): The Km and Vmax values of L-asparaginase were calculated based on the reciprocal plots of substrate concentration versus reaction velocity. The enzymes from *Bacillus sp.* revealed Michaelis-Menten kinetics when L-asparagine was used as a substrate. The calculated values of Km and Vmax were 0.055M and 35.71 µM/mL/min, respectively.

9. Anticancer activity of L-asparaginase: The results of MTT assay was calculated as viability percentage of the cells cultured with six serial concentrations (3.12, 6.52, 12.5, 25, 50, 100 µg/ml) of L-asparaginase from *Bacillus sp.* for 24 h. The obtained results concluded that the inhibition of cell viability with L-asparaginase was dose dependent and gradual increases in the dose of enzyme resulted in a gradual inhibition of cell growth (Figure 10). The inhibitory concentration (IC_{50}) of enzyme was detected about 21µg/ml.

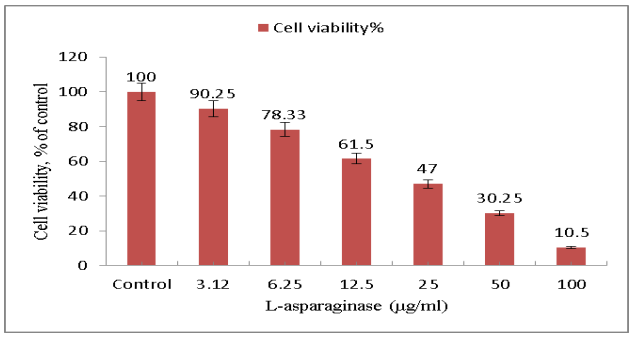

Figure 10: Survival rate of MCF-7 cells in different concentrations of L-asparaginase.

Discussion

L-Asparaginase has been well documented to possess different therapeutic applications, including anti-lymphoma, anti-leukemic and antineoplastic activities (16). The current commercially available L-Asparaginase produced by bacteria can lead to hypersensitivity and toxicity during therapy. Therefore, researchers are trying to find the new sources of this enzyme (14). In the present study, different L-asparaginase producing bacteria have been isolated from various sources and the L-asparaginase producing bacterium identified as *Bacillus sp.* has been isolated from forest soil samples in northern Iran. This strain showed the highest L-asparaginase activity among 30 isolates on M9 media and was optimized for maximum enzyme production. The classical method one-factor-at-a-time was employed for optimization of carbon and nitrogen sources for L-asparaginase production. According to this assay, 2% glucose and 0.1% asparagine were detected as potent carbon and nitrogen sources for enzyme production. In addition, highest enzyme activity of this strain has been shown at pH 7.0 and 37 °C. The supplementation of optimized nitrogen and carbon sources yielded to the production of 73.23 IU/ml L-asparaginase at 37 °C and pH 7.0. Earlier studies revealed that L-asparaginase production varies from species to species and is strongly influenced by the composition of fermentation media and the culture conditions of each organism (11,14). The optimized conditions of L-asparaginase production by *Bacillus sp.* identified in this study were in agreement with the results of Sindhwad and Desai in 2015 using *Bacillus pumilus* (2). Also, enhancement of L-asparaginase production by asparagine in mangrove derived from *Bacillus cereus* has already been reported (18). The yield of L-asparaginase production obtained under optimized conditions in the present study was also comparable with the results of other researches on *Bacillus spp.* It was higher than the L-asparaginase produced by *Bacillus polymyxa*, *Bacillus firmus*, *Bacillus velezensis* and *Bacillus licheniformis* which showed optimum enzyme production of 7.037 U/mL, 5.368 U/mL, 14.03 U/mL and 36.08 U/mL,

respectively (14, 19, 20). On the other hand, maximum L-asparaginase production by *Bacillus pumilus* was approximately 157.03 U/mL and (2).

In the present study, the enzyme was also examined for its glutaminase activity which was 5.9 times lower than that for asparagine. In a previously reported data, the glutaminase activity of L-asparaginase from *Bacillus pumilus* was 3.5 times lower than that of asparagine (2). This low L-glutaminase activity has several medical advantages. Because this activity of enzyme can cause severe symptoms in the liver and pancreas, leucopenia, neurological crisis, and coagulation abnormalities that lead to intracranial thrombosis (14, 20).

The optimal enzyme production evaluated in this study was obtained at 37 °C and pH 7.5. This maximum enzyme productivity in physiological condition previously isolated, which is a major requirement for future therapeutic uses, is similar to L-asparaginase from *Bacillus subtilis* (21), *Bacillus licheniformis* (14), *Bacillus* PG03 and *Bacillus* PG04 (22). The enzyme produced by present *Bacillus cereus* strain in this study has a broad temperature range of activity and was relatively thermostable, and was also active over a wide range of pH i.e. from 6.5 to 8.5. These findings are similar to those of Alrumman et al., 2019 and Mahajan et al., 2014, which reported a broad range of pH stability for L-asparaginase from *B. licheniformis* (14,15).

As described previously, the culturing period plays an essential role in enzyme productivity by bacterial strains (14). The maximal production of the L-asparaginase investigated in this study was observed at 48 hours which is higher than the optimal enzyme production as 36 hours for *Bacillus subtilis* and 24 hours for *Bacillus sp.* R36 reported in earlier studies (21,23).

Also in this study, EDTA acted as inhibitor of L-asparaginase activity and reducing its activity by 37.7%. As described previously, reduction of L-asparaginase activity in the presence of chelating agent EDTA may be indicating that it was a metalloprotein (24).

In this study, the kinetic constants Km and Vmax of L-asparaginase were determined as 0.055M and 35.71 µM/mL/min, respectively. Many factors affect the kinetic parameters of enzymes, such as the source, type, and form of an enzyme, as well as changes in enzyme conditions and assay procedures (25). Alrumman et al. reported Km and Vmax values for L-asparaginase from *B. licheniformis* to be 0.0499 and 45.45 µmol/ml/min, respectively, while Km and Vmax values of the purified enzyme from the same bacterium were measured to be 1.4×10^{-5}M and 4.03 IU (15), and 0.42 mM and 2778.9 µmol/min (26), respectively. Although, L-asparaginase is found in various microorganisms including yeast, fungi, and bacteria, the bacterial enzyme has gained more attention because of the higher substrate specificity and longer half-life (27).

The viability of MCF-7 cell line was used as an indicator of cell toxicity of ASNase produced by B. cereus. In agreement with our results, the antiproliferative activity of L-asparaginase on MCF7 cells was reported in different studies (14,28,29). Mahajan et al. (2014) and Arjun et al. (2016) reported that ASNases from marine Bacillus sp (29,19) and *B. licheniformis* strains exhibited high cytotoxic effect against the cancer cell line MCF-7 with IC50 value of 0.78 IU. In addition, Shafei et al. (2012) described an ASNase that inhibited the breast cancer cell lines with IC50 value of 12.5 µg/mL (30).

Previous findings showed that tumor cells with excessive requirement for L-asparagine do not

have an L-asparagine synthetase gene and cannot produce L-asparagine. Thus, decrease in the amount of this amino acid is likely to prevent the growth of tumor cells and disrupt the cell cycle (14).

Conclusion

The soil isolate *Bacillus cereus* was identified as a candidate for L-asparaginase production. The future prospect of this enzyme recommends its utility in pharmaceutical and food industry.

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Conflict of interests

The authors declare that there are no conflicts of interest in this study.

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