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# مطالعه روی بهینه‌سازی تولید آنزیم ال-آسپاراژیناز ضد سرطان با استفاده از *باسیلوس سوبتیلیس* جداسازی‌شده از خاک استان آذربایجان شرقی

**غلامـــــی، نســـــرین:** کارشناس ارشد میکروبیولوژی، دانشکدهٔ علوم و فناوری‌های زیستی، دانشگاه شهید بهشتی، تهران، ایران، ngholami94@yahoo.com
**ابراهیمی پور، غلامحسین** [*]**:** دانشیار گروه میکروبیولوژی و زیست‌فناوری میکروبی، دانشکدهٔ علوم و فناوری زیستی، دانشگاه شهید بهشتی، تهران، ایران، g-ebrahimi@sbu.ac.ir
**یعقـوبی آوینـی، محمـد:** استادیار گروه میکروبیولوژی و زیست‌فناوری میکروبی، دانشکدهٔ علوم و فناوری زیستی، دانشگاه شهید بهشتی، تهران، ایران، m_yaghoubi@sbu.ac.ir

## چکیده

**مقدمه:** آنزیم ال-آسپاراژیناز به‌طور مؤثر برای درمان بیماران مبتلا به سرطان ازجمله لوسمی لنفوبلاستیک حاد، ملانوسارکوما، لنفوما استفاده می‌شود. منابع باکتریایی جزء منابع مهم و درخور توجه برای تولید انبوه آنزیم ال-آسپاراژیناز است.

**مواد و روش‌ها:** در این مطالعه، باکتری تولیدکنندهٔ ال-آسپاراژیناز از خاک‌های آذربایجان جداسازی شد. به‌طور کلی براساس روش اسپکتروفتومتری با معرف نسلر، ۴ جدایه با فعالیت آسپاراژینازی بالا مشاهده شد. جدایهٔ دارای فعالیت آسپاراژینازی و فاقد فعالیت گلوتامینازی، برای ادامهٔ مطالعه انتخاب شد. جدایهٔ مدنظر براساس آزمایشات میکروسکوپی و بیوشیمیایی شناسایی شد. بهینه‌سازی شرایط محیط کشت ازجمله فاکتورهای غذایی (منبع کربن و نیتروژن) و پارامترهای فیزیکی (pH و دما) برای تولید بالای آنزیم آسپاراژیناز سنجش شد.

**نتایج:** مطالعات بهینه‌سازی نشان می‌دهند بالاترین میزان آنزیم ال-آسپاراژیناز در pH ۷، دمای ۳۷ درجه سانتی‌گراد، در حضور ۱ درصد آب پنیر به‌عنوان منبع کربن و ۰/۲ درصد آسپاراژین به‌عنوان منبع نیتروژن دیده می‌شود.

**بحث و نتیجه‌گیری:** از این ارگانیسم می‌توان برای تولید صنعتی آنزیم ال-آسپاراژیناز و درنهایت، درمان سرطان استفاده کرد. در شرایط بهینهٔ به‌دست‌آمده، تولید ال-آسپاراژیناز تا ۲ برابر (۷/۸ واحد بر میلی‌لیتر) افزایش یافته است.

**واژه‌های کلیدی:** ال-آسپاراژیناز، ال-گلوتامیناز، درمان سرطان، بهینه‌سازی

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# A Study of the Optimization of Anti-Tumor L-Asparaginase Production Using *Bacillus subtilis* Isolated from the Soil of East Azerbaijan Province

**Nasrin Gholami**
Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran,ngholami94@yahoo.com
**Gholamhossein Ebrahimipour***
Department of Microbiology and amp; Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran,g-ebrahimi@sbu.ac.ir
**Mohammad Yaghoubi-Avini**
Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran, m_yaghoubi@sbu.ac.ir

## Abstract

**Introduction:**L-asparaginase can be effectively used for the treatment of cancers, such as acute lymphoblastic leukemia, melanosarcoma, and lymphosarcoma. Bacterial sources contribute to the mass production of L-asparaginase.

**Materials and methods:**In this study, a novel L-asparaginase-producing bacterium was isolated from the soil of East Azerbaijan Province. Totally, 4 isolates were shown to have high L-asparaginase activities by using Nessler's reagent through the spectrophotometric method. The strain with glutaminase-free L-asparaginase activity was chosen for further studies. The isolated strain was identified via microscopic and biochemical tests. The growth conditions, including nutritional factors (carbon and nitrogen sources) and physical parameters (pH and temperature), were assayed and optimized for maximum L-asparaginase growth rate and production.

**Results:***B. subtilis* as a potential microorganism was isolated, screened, and identified for the production of L-asparaginase. The optimization study revealed that maximum L-asparaginase could be produced at pH 7.0 and temperature of 37$^{o}$C in the presence of 1% whey as the most suitable carbon source and 0.2% asparagine as a nitrogen source.

**Discussion and Conclusion:**The mentioned organism could be exploited for industrial production of L-asparaginase to be eventually utilized in cancer therapy. Under the obtained optimal conditions, L-asparaginase production was enhanced up to 2-fold (7.8 U/ml).

**Key words:**L-asparaginase, L-glutaminase, cancer therapy, optimization

*Corresponding Author



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## Introduction

L-Asparaginase (L-asparagine amino hydrolase, E.C. 3.5.1.1, LA) is an important enzyme that hydrolyses L-asparagine into L-aspartic acid and ammonia (1). L-asparaginase is one of the most efficient factors for treating lymphoblastic leukemia, lymphosarcoma, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, and melanosarcoma chemotherapy (2). Apart from its clinical usage, L-asparaginase has a great potential for use in food processing and reducing acrylamide formation in foods rich in carbohydrates and L-asparagine when subjected to high temperatures. For the first time in 1953, L-asparaginase application for curing tumor cells was reported (3). There is a difference between cancer and normal cells expressing L-asparagine. Cancerous cells do not survive without L-asparagine as it urges tumor cells to be in need of a foreign source of amino acid for reproduction and growth (4). The anticancer mechanism of L-asparaginase is to inhibit tumor cell growth by depleting extracellular L-asparagine. Bio-degradability, non-toxicity, and easy administration at local sites can be mentioned as the principal properties of L-asparaginase, thus being preferred for cancer therapies (5). Also, this enzyme serves as an alternative to reducing acrylamide in food industry without changing the product texture and taste (6). Although being useful for treating cancer, L-asparaginase has different side effects, such as weight loss, fever, pancreatitis due to hepatocellular dysfunction, chills, vomiting, and death during treatment of Acute Lymphoblastic Leukemia (ALL) (7). This is due to its intrinsic toxicity in the presence of L-glutaminase activity. L-glutamine is significant for nitrogen transport in the blood, while prolonged depletion of amino acids during L-asparaginase treatment causes severe biochemical disorders in the body (8). Nowadays, most of the studies have focused on bacterium-based production of glutaminase-free L-asparaginase since glutaminase toxicity adversely affects the anticancer activity of L-asparaginase (2).

L-asparaginase is produced by animals, plants, bacteria, and fungi, but bacterial L-asparaginases have attracted more attention because of their anticarcinogenic activities and eco-friendly nature, besides being cost-effective (9). Due to its greater accumulation in the culture medium and simplified downstream process, extracellular L-asparaginase is preferred in industrial processes (2). L-asparaginase secretions by *Escherichia* (10), *Aerobacter* (11), *Erwinia* (1), *Serratia*(6), *Pseudomonas aeruginosa* (12), *Citrobacter sp*. (13), and *Bacillussp*. (14) have been already reported. *Bacillus* species is one of the most abundant species due to its resistance to extreme environments. Amen et al. reported that the production of L-glutaminase-free L-asparaginase makes *B. subtilis* premier to the commercially available asparaginases that cause diverse severe health problems in patients when associated with glutaminase activity during anti-cancer therapy (7). Process optimization plays a vital role in industrial production processes, in which even small improvements would be decisive for commercial success (6). Medium compositions (carbon and nitrogen sources) and culture conditions (pH and temperature) affect L-asparaginase production in the culture medium. Enhancement of anticancer enzyme productivity with a low cost is feasible either by improving the strains or by optimizing the growth conditions (2).

The goal of the present paper was to optimize the conditions for maximum production of L-glutaminase-free L-asparaginase and characterize the effects of

chemical and environmental parameters on the enzyme production.

## Materials and Methods

**Chemicals:** Nessler's reagent was purchased from Fluka (Buchs, Switzerland). Bromothymol blue and phenol red were purchased from Spectrochem (India). All the other chemicals and media components used in this study were of the highest purity obtained from Hi-media Laboratories (Mumbai, India).

**Bacterium and cultivation:** The soil samples were collected from various places in East Azerbaijan Province in Iran. They were taken from a certain depth of up to 10 cm. Then, they were placed in zip-locked plastic bags at 6$^{o}$C (5). Suspensions were provided by mixing each sample with sterile distilled water. After 6 h, the clear supernatants were serially diluted. Afterwards, 100 µl of $10^{-4}$ and $10^{-6}$ dilution tubes were spread on a modified M9 medium (pH 7.0) containing 1% Glucose, 0.05% $K_2HPO_4$, 0.02% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$, 0.001% $CaCl_2.2H_2O$, 0.1% L-Asparagine, 0.1% trace elements ($ZnCl_2$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $CuCl_2.\ 2H_2O$, and $NaMoO_4.2H_2O$), and 1.5% agar with 0.04-0.36 ml of phenol red (2.5%) as an indicator and incubated at 37$^{o}$C for 48 h. The purified colonies were obtained on nutrient agar by the streaking technique (6).

**Isolation of L-asparaginase-producing bacteria without L-glutaminase production:** Screening for L-glutaminase production was done by culturing on a modified M9 medium with 0.1% L-glutamine instead of 0.1% L-asparagine. After incubation, the L-asparginase-producing bacteria without L-glutaminase activity were chosen for subsequent studies (15).

**Identification of the isolated strain:**Identification of the isolated strain was done based on 16S rRNA gene sequences via phylogenetic analysis and morphological and physiological tests as described by Bergey's Manual of Systematic Bacteriology. PCR amplifications were performed in a Primus 25 PCR system (PEQLab, Germany). The universal 16S rRNA primers, *i.e.*, 27F (5-AGAGTTTGA TCCTGGCTCAG-3) and 1492R (5-GGTTACCTTG TTACGACTT-3), were utilized in this procedure. The amplification process of the purified DNA was divided into the following stages: 1) initialization step (heating the reaction mixture up to 94-96°C for 5 min); 2) denaturation step (30 cycles of heating the reaction mixture up to 94°C for 1 min); 3) annealing step (decreasing the reaction temperature to 53°C for 40 s); 4) extension step (heating the reaction mixture up to 72°C for 1 min); and 5) final extension step (heating the reaction mixture up to 72°C for 10 min). To check whether the PCR generated the anticipated DNA, 1% agarosegel electrophoresis was employed for size separation of the PCR products. Nucleotide sequencing analysis was performed by the dideoxy chain termination method (SEQLAB, Germany) (16). 16S rRNA gene sequence of the strain was compared with similar sequences of the reference organisms by using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Enzyme assay:** The isolated strain was cultured in a modified M9 broth medium at 37ºC for 24 h. The supernatant was harvested via centrifugation at 10000×g for 10 min. Enzyme activity of the cell-free supernatant obtained from cultures of the isolated bacteria was measured by the modified Nessler's method (17). To do this, 0.5 ml of the supernatant was combined with 3 ml of the reaction mixture containing 1 ml L-asparagine (10 µmol/l) in Tris-HCl buffer (pH 8.6, 50 µmol/l) and the mixture was incubated at 37ºC for 2 h. The reaction was terminated by adding 200 µl of 15% trichloroacetic acid. For separating

the precipitation, the mixture was centrifuged at 13000×g for 4 min. The released ammonia in the supernatant was distinguished by adding 200 µl of Nessler's reagent to the sample containing 1 ml of the supernatant and 3 ml of distilled water. The resulting mixture was incubated at room temperature for 15 min. Then, the Optical Density (OD) of the mixture was measured at 450 nm. Asparaginase activity was determined by measuring the amount of ammonia produced in the reaction mixture. Similarly, a control sample was prepared by adding TCA before adding the enzyme. One unit of LAse activity was equal to the amount of enzyme, which liberated 1 µmol/l of ammonia per minute under the experimental conditions. All the assays were performed in triplicate and the average of the 3 assays was taken to evaluate the activity units.

**Effect of incubation period on L-asparaginase production:** For investing an optimal incubation time for enzyme production, the bacterial culture was inoculated in the growth media and the enzyme activity was measured at different time intervals up to 108 h. The samples were withdrawn aseptically at every 6-h intervals and asparaginase activity was determined. All the experiments were performed in triplicates (2).

**Optimization of asparaginase production:** Optimization of asparaginase production was conducted using One-Factor-at-A-Time (OFAT) approach (18). L-asparaginase production basically depends on many factors like carbon and nitrogen sources, temperature, and pH. Thus, these parameters had to be optimized in order to achieve higher yields of L-asparaginase. To this goal, 250-ml conical flasks containing 100-ml M-9 were employed for asparaginase production. The components of M-9 medium (pH 7.0) were glucose (10 g/l), yeast extract (5 g/l), $K_2HPO_4$ (0.5 g/l), $MgSO_4$7H2O (0.2 g/l), $FeSO_4$.7H2O (0.01 g/l), $CaCl_2$.2H2O (0.01 g/l), and trace elements (1 ml). An overnight strain culture medium was centrifuged with a final OD of 0.2 at 600 nm to inoculate the fermentation media. During the optimization process, 2 ml of the medium in each flask was centrifuged (10000×g for 10 min) and used for assessing the enzyme activity. All the experiments were performed in triplicates (19).

**Optimization of nutritional factors for L-asparaginase production:**For optimizing carbon sources, the medium was supplemented with various carbon sources (glucose, sucrose, molasses, whey, sodium acetate, sodium citrate, and starch) at the concentration of 1% instead of glucose in the M9 medium. Also, the effects of various concentrations of the nitrogen source (asparagine) were evaluated (20).

**Optimization of physicochemical parameters for asparaginase production:**After optimizing the nutritional factors, the different physicochemical parameters, including temperature (21) (20, 25, 30, 35, 37, and 40ºC) and pH (5.0, 6.0, 7.0, 8.0, and 9.0), were optimized to maximize L-asparaginase production (22).

**Statistical analysis:**To compare the impacts of temperature, pH, and nitrogen and carbon sources on the bacterial growth rates and asparaginase production, One-way ANOVA was applied. Significant differences were determined using Tukey's test. All the analyses were done using SPSS 19 software.

## Results

**Isolation and screening of L-asparaginase-producing bacteria:** In this research, a total of 30 colonies were isolated from the soil, from among which 17 isolates altered the color of the culture media to pink due to pH changes as a result of asparagine production (Fig. 1). Among all the isolates, 4 isolates produced an intense pink zone around the colonies.

Among the isolated strains, 3 isolates showed L-glutaminase activity. Strain B was selected for subsequent studies due to lacking glutaminase activity (Fig. 3).

Fig. 1- Detection of L-asparaginase-producing isolates in the modified M9 medium

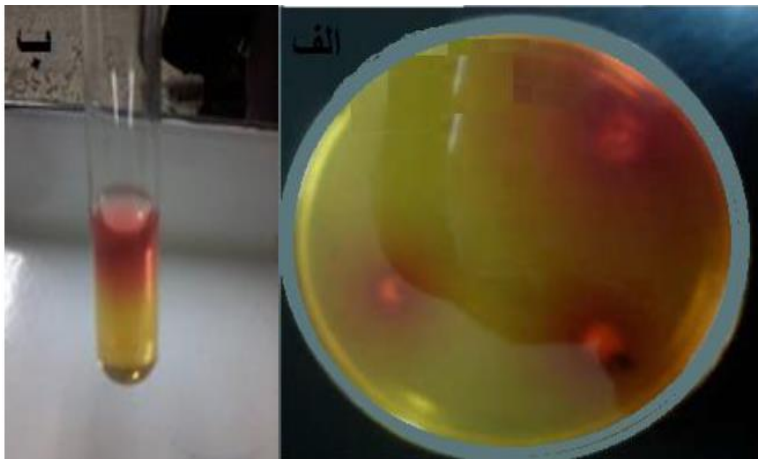


Fig. 2- Qualitative screening of L-asparaginase-producing bacteria in the modified M9 medium after 24 hours of incubation

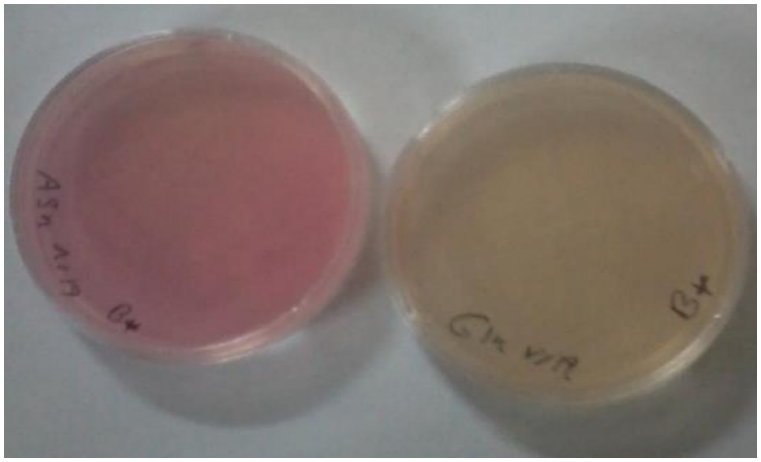

Fig. 3- Plate assay for detecting Strain B with L-glutaminase activity

**Characterization and identification of Strain B**: The PCR results are illustrated in Fig. 4. The sequence was reversed, aligned, and compared with similar database

sequences using Bioedit software (Ibis Biosciences, USA). The BLAST analysis demonstrated 99% similarities with *Bacillus subtilis*, *Bacillus gibsonii*, *Bacillus tequilensis sp.*,and *Bacillus vallismortis sp.* The results of morphological and biochemical properties of the isolated bacterium are summarized in Table 1, which indicates similarity of Strain B to *Bacillus subtilis*.

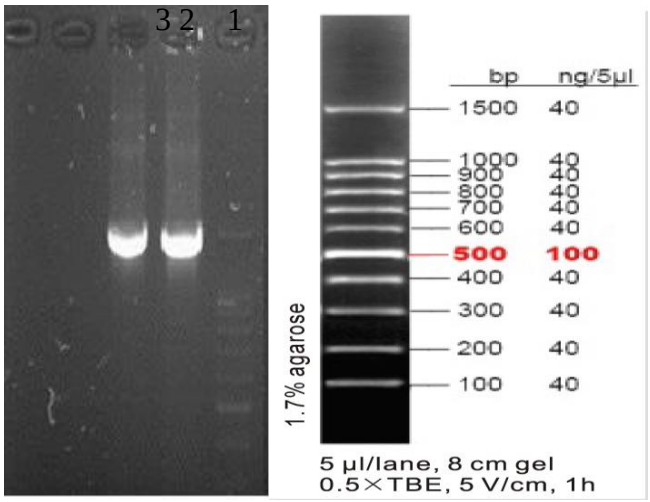


Fig. 4- A) Separation of the PCR product of 16S rDNA on 1% agarose gel (1: 100 bp DNA ladder, 2: Strain B, 3: negative control); B) 100-bp DNA ladder

Table 1- Biochemical tests

| Test | Strain B | *Bacillus subtilis* | *Bacillus tequilensis* |
|---|---|---|---|
| Gram reaction | Gram-positive Bacilli | +Ve | +Ve |
| KOH | - | - | - |
| Catalase | + | + | + |
| Oxidase | + | + | + |
| Spore forming | + | + | + |
| Growth in anaerobic condition | - | - | - |
| TSI (triple sugar-iron agar) | A/A | A/A | A/A |
| Indole | - | - | + |
| $H_2S$ production | - | - | - |
| Citrate utilization | + | + | + |
| Methyl red | - | - | - |
| Voges-proskauer | + | + | + |
| Motility | + | + | + |
| Urease | + | + | + |

A: Acid production; $H_2S$: Hydrogen sulfide

**Effect of incubation period on asparaginaseproduction:** Kinetics of the bacterial growth and asparaginase production was studied by inoculating Strain B in a mineral salt medium. As shown in Fig. 5, enzyme production started at the beginning of the stationary phase and reached its maximum rate after 60 h and then reduced with time.

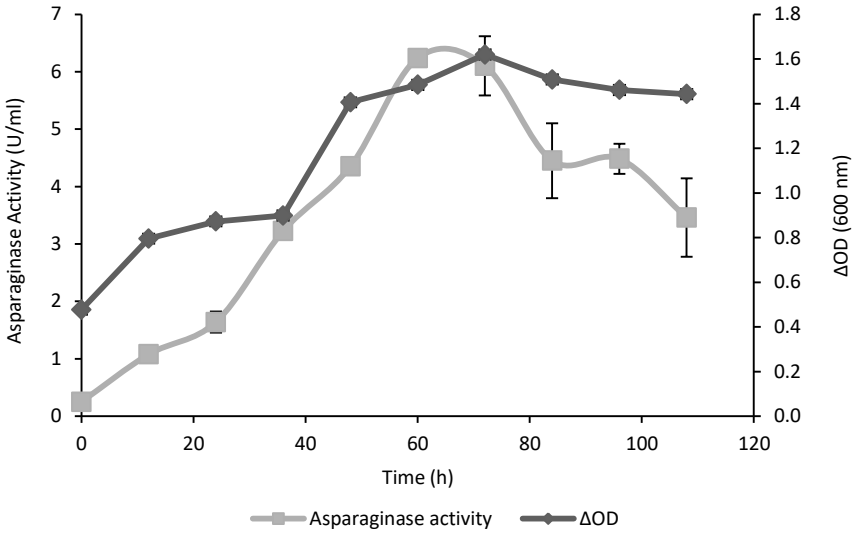


Fig. 5-Effect of incubation time on asparaginase production by Strain B (Each value represents means±SD, n=3)

**Optimization of culture conditions for L-asparaginase production**

**Effects of the different concentrations of asparagine on L-asparaginase production:** The results presented in Fig. 6 show achievement of high L-asparaginase activity (4.08 U/ml)at 0.2% asparagine. L-asparaginase activity decreased with an increase in asparagine concentration from 3.75 U/ml at 0.3% asparagine to 2.26 U/ml at 0.4% asparagine. The lowest enzyme production (2.03 U/ml) was obtained at 0.02% asparagine (Fig. 6).

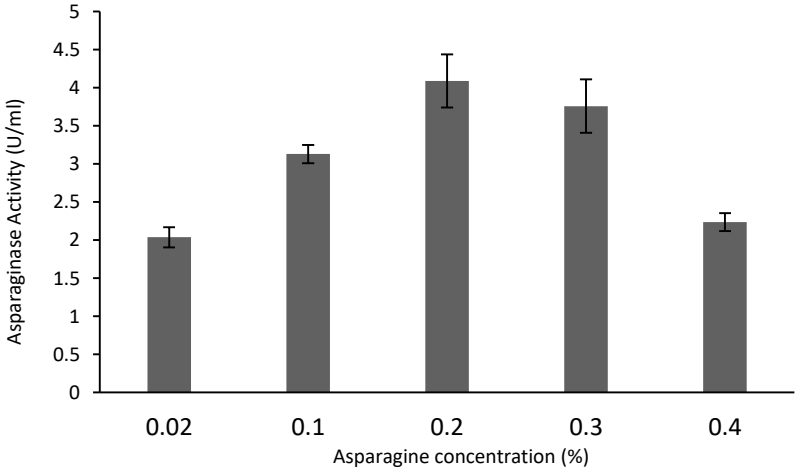


Fig. 6- L-asparaginase production at various asparagine concentrations

**Effects of carbon sources on L-asparaginase production:** Different carbon sources were utilized at 1% for L-asparaginase production. The data revealed that whey and starch were the best of all the substrates tested with L-asparaginaseactivities of 7.32 U/ml and 7.26 U/ml. Hence, whey was determined to be the best carbon source followed by starch and acetate, while molasses resulted in low L-asparaginase production, allowing an activity of 2.16 U/ml (Fig. 7).

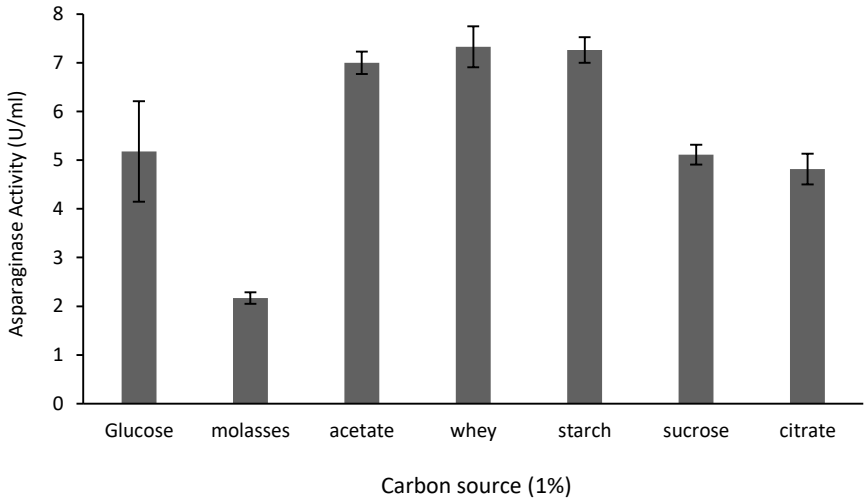


Fig. 7-L-asparaginase production with different carbon sources

**Effect of pH on L-asparaginase production:** The pH of the culture medium was another important factor to be studied. Therefore, buffers with the pH values of 5.0, 6.0, 7.0, 8.0, and 9.0 were taken into account. Fig. 8 displays asparaginaseactivity at various pH conditions. According to the results, the highest asparaginase activity of 7.62 U/ml was recorded at a pH value of 7.0. Minimum asparaginase activity (2.89 U/ml) was observed at acidic pH.

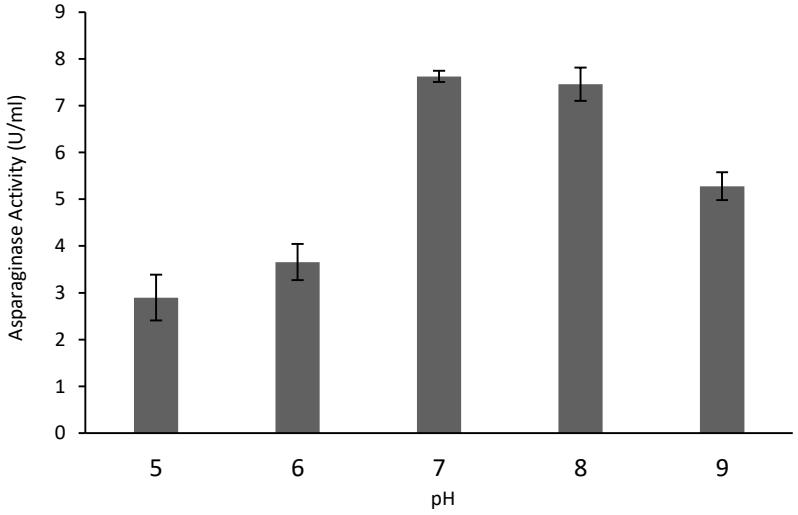


Fig. 8- L-asparaginase production at different pH conditions (Each value represents means±SD, n=3)

**Effect of temperature on L-asparaginase production:** One of the parameters that affect all cell functions is temperature. Temperature controls energy metabolism and enzyme production and activity in bacteria. To determine an optimal temperature for the enzyme production, Strain B was cultivated over the temperature range of 4-40°C. The highest asparaginase activity (7.85 U/ml) was observed at 37°C as depicted in Fig. 9. A further increase in temperature resulted in the enzyme activity reduction to 3.08 U/ml.

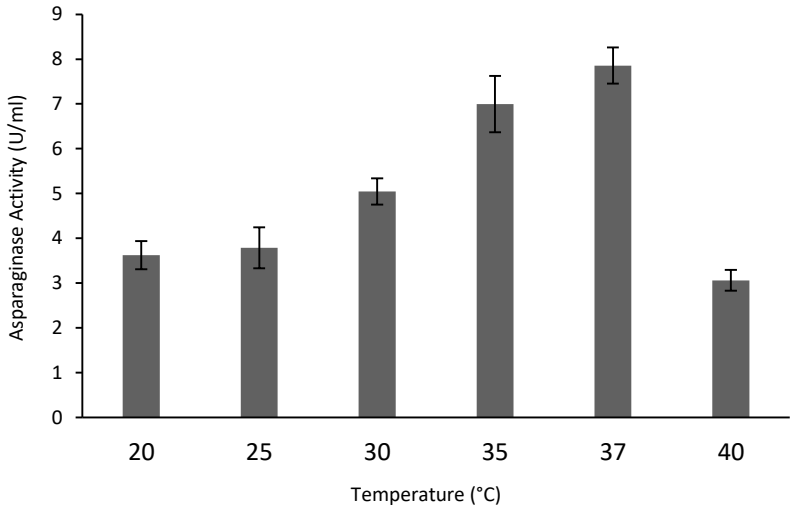


Fig. 9-L-asparaginase production at different temperatures (Each value represents means±SD, n=3)

## Discussion and Conclusion

The requirement for significant therapeutic enzymes has kept on increasing because of their vast variety of uses in oncology. Thus, scientists have been always looking for enzymes with desired properties. In this study, from among a total of 4 isolates with positive L-asparaginase activities, only Strain B showed no L-glutaminase production and was chosen for further studies. The main L-asparaginase producer displayed no glutaminase activity, which was a favorable characteristic for cancer therapy (23). There are few reports on glutaminase-free L-asparaginase productions by bacteria, such as *Pectobacteriumcarotovorurm*MTCC 1428 (24), *Bacillus velezensis*(23), and *Leucosporidiummuscorum* (19). The isolated strain was gram-positive, besides being aerobic, rod-shaped, and spore-forming. According to morphological, biochemical, physiological, and molecular features, Strain B was close to *Bacillus subtilis*. The genus of *Bacillus* includes strains that are capable of generating applicable enzymes, such as asparaginase, protease, amylase, lipase, and cellulose (25). Shukla and mandal showed that optimum production of L-asparaginase(18.4 U/ml) was obtained by using *Bacillus subtilis* after 48 h of incubation time (26).

About 40% of costs of industrial processes is being allocated to substrates for bacterial growth and enzyme production; hence, it is necessary to supply media with cost-effective ingredients (27). This research concerned with the optimization of bacterial growth and L-asparaginase production. The isolated strain indicated a typical bacterial growth curve. Production of extracellular L-asparaginase was started at the beginning of the stationary phase. Maximum amount of L-asparaginase was obtained after 60 h. Rajeswara's results showed that 30 h was the optimum time of incubation for asparaginase production by *Enterobacter sp.* (2).

In this research, among various carbon sources, whey and starch were capable of improving L-asparaginase production up to 7.32 U/ml and 7.26 U/ml, respectively. However, low asparaginase activity was displayed in the media supplemented with molasses. Deshpande et al. reported that glucose was the main carbon source for L-asparaginase production (28). Maximum activity of L-asparaginase was produced by *Nocardiopsisalba* NIOT-VKMA08 as

predicted to be 9.51 U/ml in the presence of maltose (20). Also, different concentrations of L-asparagine were assayed, while maximum L-asparaginase production was observed with 0.2% asparagine. Erva et al. showed that ammonium chloride (0.15%) proved to be the best nitrogen source as producing an activity of 6.43 U/ml induced by *E. aerogenes* MTCC111 (2). *Leucosporidiummuscorum* produced a greater amount of L-asparaginase (2.72 U/ml) when proline was applied as the nitrogen source (19).

According to the data, maximum enzyme production was achieved at pH 7.0. *Pseudomonas stutzeri*MB-405 produced a high amount of L-asparaginase in the pH range of 7.0-8.0 (29). Dhavegi and Poorani reported maximum L-asparaginase production at a pH between 8.0 and 8.5 (30). Likewise, Prakasham et al. reported that maximum L-asparaginase production was obtained at pH 7.5 using *Staphylococcus sp.* (-6A) (4).

In the current work, a high amount of L-asparaginase was produced at 37°C. Similar observations have been reported for asparaginase production from *B. subtilis*(Strain KDPS1) (31) and *Citrobacter*(AS-2) (13). Likewise, Ghosh et al. reported that maximum L-asparaginase production was achieved at the natural pH value of 7.0 and temperature of 37°C using *Serratiamarcescens* (NCIM2919) (32). The optimum production of L-asparaginase enzyme by *Bacillus subtilis* was observed at pH 7.0 and 37°C (26).

In the present investigation, we screened L-asparaginase activities of 17 isolates. Most isolates could utilize L-asparagine and L- glutamine. However, Strain B could interestingly produce glutaminase-free L-asparaginase. Beacause of L-glutaminase side effects, the glutaminase activity of L-asparaginase is not desired for anticancer treatments and thus, there has been an attempt for producing glutaminase-free L-asparaginase. The optimization studies demonstrated that maximum amount of L-asparaginase was produced at pH 7.0 and Temp. 37°C in the presence of 1 and 0.2% whey and asparagine as the most suitable carbon and nitrogen sources, respectively. Under the obtained optimal conditions, the traditional OFAT method provided us with the finest enzyme activity (7.8 U/ml), which revealed a 2-fold increase compared to the initial value. Hence, this organism may be exploited for the industrial production of L-asparaginase, which can be eventually applied in cancer therapy.

All the selected factors showed an impact on L-asparaginase enzyme production by the mentioned isolated strain either at the individual or interactive level. In conclusion, significant improvement (2.5-fold increase) in the enzyme production by this isolate was noted under an optimized environment.

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