Biological Journal of Microorganism
4th Year, Vol. 4, No. 16, Winter 2016
Received: August 31, 2015/ **Accepted:** October 28, 2015. **Page:** 11- 20

Gene sequencing, cloning, and expression of the recombinant L- Asparaginase of *Pseudomonas aeruginosa* SN4 strain in *Escherichia coli*

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Abstract

Introduction: L- asparaginase is in an excessive demand in medical applications and in food treating industries, the request for this therapeutic enzyme is growing several folds every year.

Materials and methods: In this study, a L- asparaginase gene from *Pseudomonas aeruginosa* strain SN4 was sequenced and cloned in *E. coli.* Primers were designed based on L- asparaginase from *P. aeruginosa* DSM 50071, which show high similarity to SN4 strain, according to *16S rRNA* sequence. The L- asparaginase gene was exposed to restriction digestion with NdeI and XhoI enzymes and then ligated into pET21a plasmid. The ligated sample was transformed into competent *E. coli* (DE3) pLysS DH5a cells, according to $CaCl_2$ method. The transformed *E. coli* cells were grown into LB agar plate containing 100 µg/ml ampicillin, IPTG (1 mM).

Results: Recombinant L- asparaginase from *E. coli* BL21 induced after 9 h of incubation and showed high L- asparaginase activity about 93.4 IU/ml. Recombinant L- asparaginase sequencing and alignments showed that the presumed amino acid sequence composed of 350 amino acid residues showed high similarity with *P. aeruginosa* L- asparaginases about 99%. The results also indicated that SN4 L- asparaginase has the catalytic residues and conserve region similar to other L- asparaginases.

Discussion and conclusion: This is the first report on cloning and expression of *P. aeruginosa* L- asparaginases in *Escherichia coli*. These results indicated a potent source of L- asparaginase for *in vitro* and *in vivio* anticancer consideration.

Key words: L- asparaginase, Cloning, *Pseudomonas aeruginosa*, Sequence alignments

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Introduction

Bacterial L- asparaginase has been broadly used as an actual therapeutic agent against lymphosarcoma and acute lymphoblastic leukemia (1- 3). Numerous cases of current research are accessible regarding the use of L- asparaginase in melanoma therapy (4- 6). The use of anti-tumor therapy is based on its capacity to hydrolase L- asparagine to L- aspartic acid and ammonia in serum and cerebro- spinal fluid. Since tumor cells are unable to make endogenous L- asparagine, starvation for this amino acid hints to death of these cells (7). L- asparaginase received enlarged care in current years as food handling helps to diminish the development of acrylamide in carbohydrate-based foods that were fried, baked or roasted (8 & 9). L- asparaginase has also been considered for presentation in the L- asparagine biosensor for leukemia (10). Meanwhile, the statement that *E. coli* L- asparaginase has an anticancer activity similar to that of guinea pig serum, there has been extensive attention in L- asparaginase from numerous sources particularly microorganisms. Many plants, mammalian and bacterial species can produce L- asparaginases. Although other bacteria, such as *Thermococcus gammatolerans* (11), *Pectobacterium carotovorum* MTCC 1428 (12 & 13), *Cladosporium* sp. (14), *Streptomyces* (15), *Pseudomonas aeruginosa* (16), and *Actinomycetes* (17), *Escherichia coli* (18) have a latent for L- asparaginase making, but the enzymes from *Erwinia* sp. and *E. coli* (1, 7 & 19) has been used as an anti-cancer agent.

Up to now, only L- asparaginase from *Erwinia* and *E. coli* has been used in medical application. Because of various side effects of these enzymes, finding other bacterial L- asparaginase received more attention. So, isolation of L- asparaginase gene from *Pseudomonas aeruginosa* strain SN4, cloning, expression in *E. coli* is the aim of this study.

Materials and methods

Chemical materials: L- asparagine and trichloroacetic acid (TCA) were obtained from Sigma Chemicals Co. L- asparaginase- specific primers used in this study were synthesized by Bioneer Co. (Soft Korea). Taq polymerase and dNTPs were procured from Sinaclone (Iran).

Cloning of PaA gene: *Pseudomonas aeruginosa* strain SN4 was previously isolated and identified in our lab (20). A BLAST search of *P. aeruginosa* genome sequence using as a quire, the gene encoding for L- asparaginase from *P. aeruginosa* yielded a single 1041- bp ORF that was hypothesized to code for L- asparaginase. This strain was grown- up at 30°C in a medium containing 0.5% yeast extract, 1% peptone and 1% NaCl. After 16 h, cells were harvested by centrifugation (10,000× g 10 min, at 4°C) and genomic DNA was isolated according to a standard method (21). Genomic DNA isolated from *P. aeroginosa* strain SN4 was used as the template for amplification of L- asparaginase gene using the primers: pCFP (5- GGAATTC*CATATG*ATGGCCCTGATGCTC- 3) and pCRP (5- CCG*CTCGAG*GTATTCCCAGAAGAT- 3), where, the italic sequences are the

restriction sites of Nde1 and Xho1 enzymes, respectively. The DNA amplification was approved in a total volume of 25 µl, containing 2.5 µl PCR Buffer, 1 µl of DNA templet, 0.5 µl lµl dNTP mix, 1 µl each of both primers, 0.2 µl Taq polymerase, and 18.7 µl Milli q water. The PCR method included 33 cycles of 45 s at 94°C, 60 s at 55°C and 45 s at 72°C. A final extension time at 72°C for 10 min was done after 33 cycles (22 & 23). The PCR product was sequenced and the L-asparaginase gene was exposed to restriction digestion with NdeI and XhoI enzymes and then ligated into pET21a plasmid (Novagen, Inc.) which was previously having the restriction sites (Fig. 1).

The ligated sample was transformed into competent *E. coli* (DE3) pLysS DH5a cells, according to $CaCl_2$ method. The transformed *E. coli* cells were grown into LB agar plate containing 100 µg/ml ampicillin, IPTG (1mM) and X- gal (1mM). The recombinant clones were grown up in 50 ml LB medium containing 100 µg/ml ampicillin at 37°C. The plasmid was isolated from *E. coli* DH5a and was used to transform competent *E. coli* BL21 cells, which were screened with blue/white selection. Colony PCR, double digestion and sequencing were performed to further approve the presence of L- asparaginase gene and also to check the orientation of integration in the plasmid.

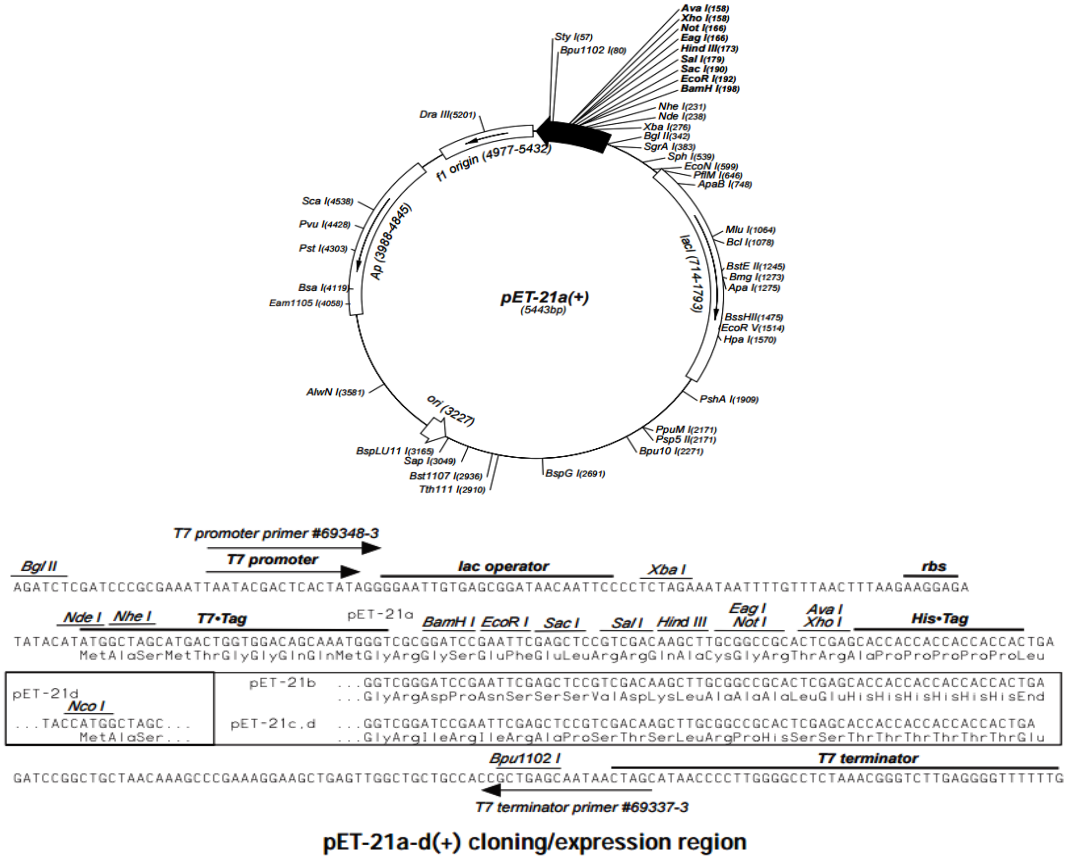

Fig. 1- Map of pET21a vector

L- asparaginase production and purification: The *E. coli* BL21 cells were grown into LB agar plate containing ampicillin (100 µg/ml). 2 ml of an overnight cultures inoculated into 20 ml of LB medium in Erlenmeyer flasks (100 ml) and grown for 24 h at 37°C in a 180 rpm shaker incubator. L- asparaginase synthesis was induced by the addition of 1mM IPTG (isopropyl- β- D-thiogalactopyranoside) after 9 h of growth. 9 hours after induction, cells were pelleted by centrifugation at 5000 g and 4°C for 15 min, resuspended in sodium phosphate buffer (10 mM, pH 7.5), sonicated and centrifuged at 10,000×g for 15 min. The supernatant was gathered and used for purification.

The lysate of *E. coli* BL21 containing pET21aASN was applied to a Q-Sepharose column, an anion exchanger, pre- equilibrated with 45 mM Tris- HCl buffer, pH 8.5. The proteins were eluted (1 ml/min) with NaCl gradient (0.1- 0.5 M) and 45 mM Tris- HCl buffer (pH 8.5). The active fractions were concentrated with ammonium sulphate (80%) and dialyzed. The purity of the enzyme was valued by SDS- PAGE. SDS- PAGE was done according to the method of Laemmli (24) on a slab gel comprising 10.0% (w/v) polyacrylamide (running gel) and 5.0% (w/v) (stacking gel). The molecular size of the purified L- asparaginase was estimated using molecular markers and the proteins were stained with Coomassi Brilliant Blue R-250. The purified L- asparaginase was dialyzed to remove salt.

L- asparaginase assay: The L-asparaginase activity was investigated by the technique of Imada, utilizing the Nessler reaction (25). In this method, the ammonia liberated from L- asparagine in the enzyme reaction measured by the Nessler reaction. The reaction was started by adding 100 µl cell suspension into the 900 µl of 100 mM L- asparagine prepared in 50 mM Tris/HCl buffer, pH 8.0 and incubated for 20 min at 37°C. The reaction was stopped by the addition of 100 µl 1.5 M TCA. The reaction mixture was pelleted by centrifugation at room temperature (10,000 g for 10 min) to remove the precipitate and the ammonia released into the supernatant was determined colorimetrically (A_{480}) by adding 0.2 ml Nessler reagent into tubes containing 0.3 ml supernatant and 1.5 ml H_2O. The content in the tubes was vortexed and incubated at room temperature for 10 min, and the A_{480} values were measured against the blank that received TCA before the addition of cell suspension. One L-asparaginase unit (U) is defined as the amount of enzyme that liberates 1 µmole of ammonia per min at 37°C. Ammonium sulphate was used as the standard for enzyme activity calculations. For rapid colorimetric assay the recombinant bacteria were screened using the modified M9 medium (1 l: 3.0 g KH_2PO_4; 6.0 g $Na_2HPO_4 \cdot 2H_2O$; 0.5 g NaCl; 5.0 g L-asparagine; 0.5 g $MgSO_4.7H_2O$; 0.014 g $CaCl_2.2H_2O$; 2.0% (w/v) glucose, and 15.0 g agar) combined with a pH indicator (phenol red). Development of pink color was considered as a positive result for L-asparaginase production (20).

Results

Cloning PaA: A BLAST search of *P. aeruginosa* SN4 genome sequence in NCBI database (http://www.ncbi.nlm.nih.gov/) using the amino acid sequence of L-asparaginase from *P. aeruginosa* DSM 50071 (accession number: CP012001) as a query sequence, generated a high-probability match to a single 1041- bp ORF, encoding a predicted protein of 346 amino acids. To define whether the supposed *P. aeruginosa* protein was actually an active L- asparaginase, we cloned and expressed the full- length gene in *E. coli* and confirmed for its capacity to hydrolysis L- asparagine.

PCR was used to amplify the full- length gene from genomic DNA of *P. aeruginosa* SN4 (Fig. 2). The PCR product was cloned into the pET21a vector. This plasmid was used to transform the *E. coli* BL21 (DE3) pLysS as an expression host. L- asparaginase from *Erwinia carotovora* has been cloned in *E. coli* BL21 (26).

L- asparaginase expression: *E. coli* BL21 containing pET21aASN was grown up in LB medium supplemented with 1 mM IPTG. After 9 h of induction, 2 ml of this culture was added to 2 ml of phosphate buffer (20 mM, pH 8.0) containing L-asparagine (10 mM) and phenol red (0.001%). After 10 min incubation at room temperature, a tube containing *E. coli* BL21 with 9h induction, showed a dense pink color against the other (Fig. 3). These results indicated high activity of recombinant L- asparaginase about 93.4 IU/ml.

After induction with 1 mM IPTG, sample was picked up at different time incubation (3, 6 and 9 h). Results showed that the maximum L-asparaginase production was echived after 6 h of incubation (Fig 4b).

Purification results showed that active fraction was obtained in 15% of NaCl concentration. After dialysis, active fraction was loaded on SDS–PAGE gel and then the gel stained with Coomassie Blue. Results showed the attendance of only one band with the molecular weight of 35.0 kDa (Fig. 4a).

Gene sequence analysis: The sequence of recombinant L- asparaginase gene consisted of 1041 bp showed more than 99% identity with similar sequences of different *P. aeruginosa* strains (Fig. 5 and Fig. 6). Results in Fig. 5, showed the multiple sequence alignment of *H. pylori* CCUG 17874 (Hepy; EIE30409.1), *B. subtilis* W168 (Basu; AAA22243.1), *E. coli* (Esco; NP 417432.1), and *Er. chrysanthemi* 3937 (Erch; AAS67028.1), *B. licheniformis* (BaLi, WP_044789473), *K. pneumoniae* JM45 (Klpn, AGT24790), *P. aeruginosa* SN004 (Pasn) and *P. aeruginosa* (Paea, WP_023099587).

Fig. 3- L- asparaginase activity in medium supplemented with phenol red and L- asparagine. Lane 1; *E. coli* BL21 containing pET21aASN without induction and Lane 2; extract of *E. coli* BL21 containing pET21aASN after 9 h of incubation induction with 1mM IPTG.

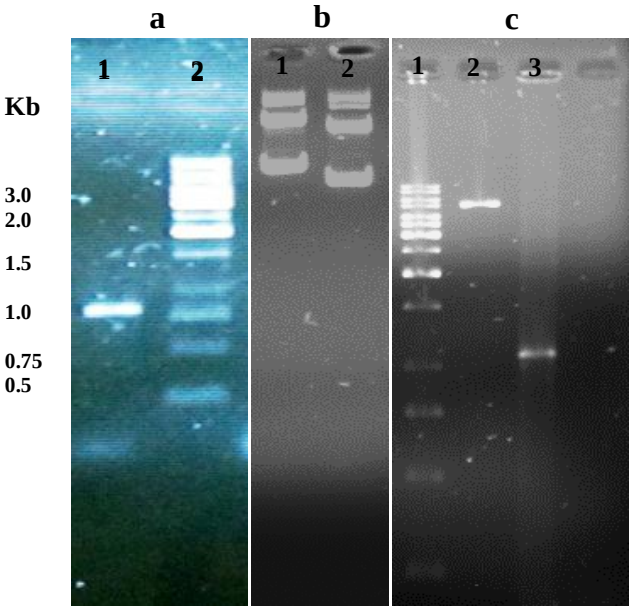

Fig. 2- Agarose gel in the cloning process. a) Electrophoresis in 1% agarose gel of PCR product (1) and DNA ladder (2). b) Recombinant construct (1; 6.5 Kb), (2) pET21a vector without insert. c) Restriction endonuclease digestion of polymerase chain reaction product (3; 1.0 Kb), pET21a digested with NdeI and Xho1 (2; 5.4 Kb) and DNA ladder (1).

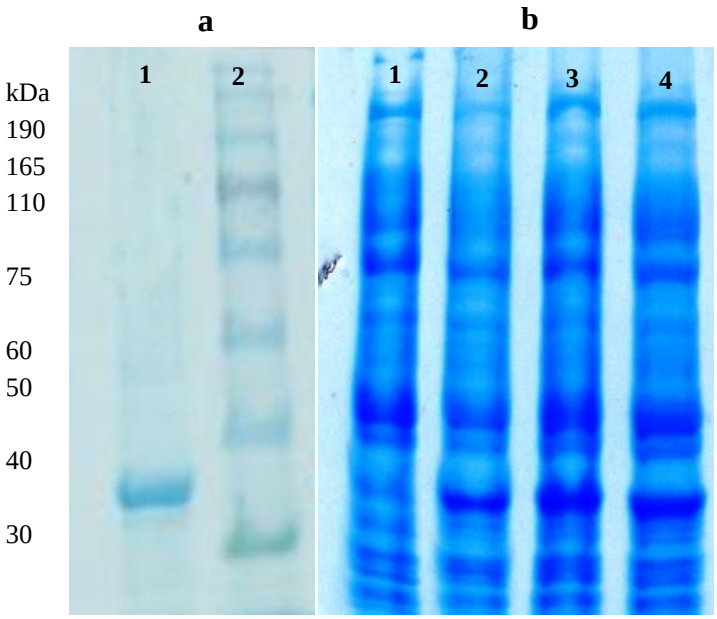

Fig. 4- SDS- polyacrylamide gel electrophoresis of L- asparaginase induction and purification. Protein bands were stained with Coomassie Brilliant Blue R- 250. **a**) L- asparaginase purification, Lane 1 (molecular weight markers), Lane 2 (purified L- asparaginase). **b**) *E. coli* crude extract without induction (lane 1) and after 3h (lane 2), 6h (lane 3) and 9 h (lane 4) of incubation induction with 1mM IPTG.

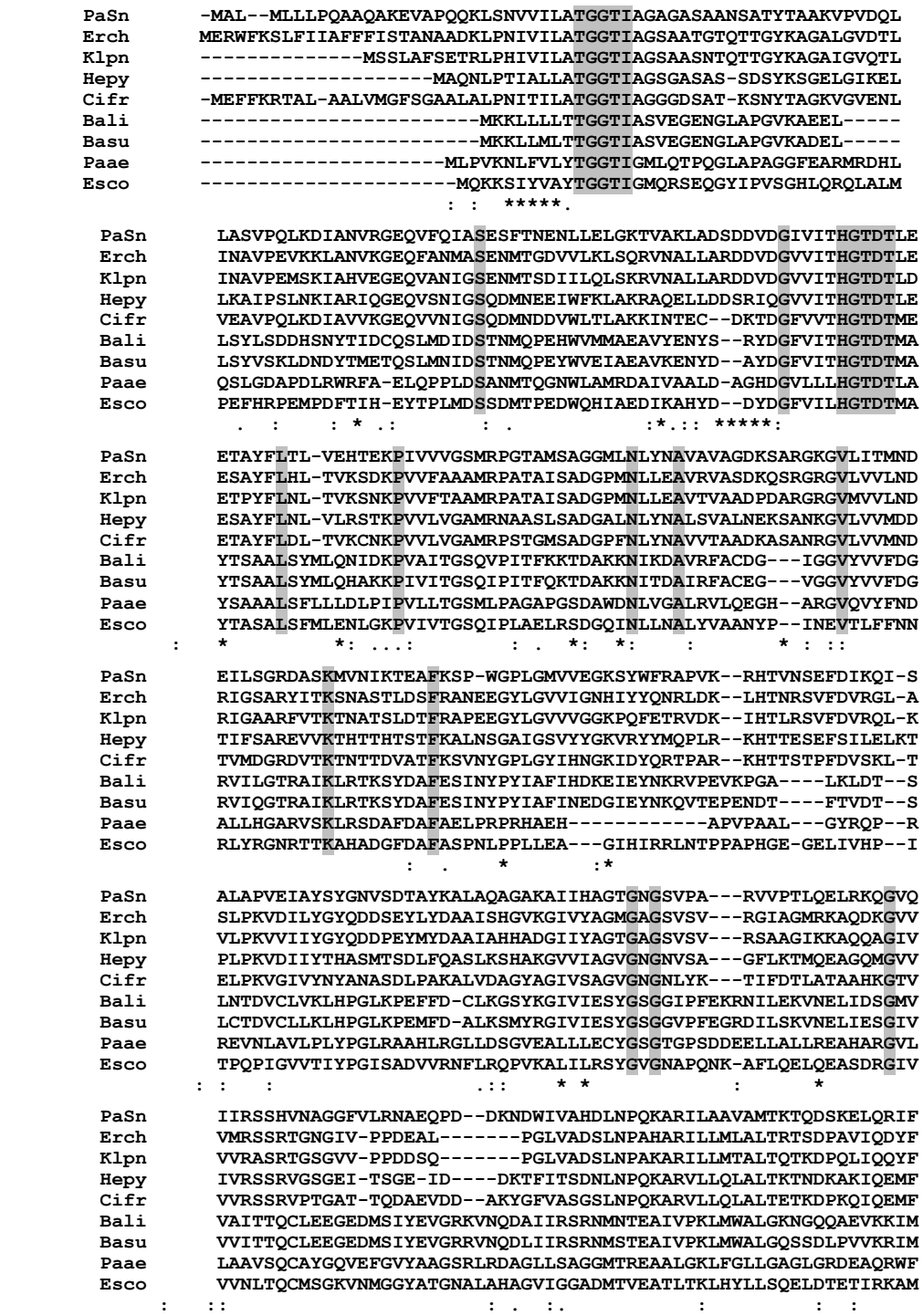

Fig. 5- Sequence alignments of microbial l-asparaginases. The alignment was performed using Clustal Omega program. Amino acid residues that are identical in all of the displayed sequences are marked by black. The origins of l-asparaginase and their GenBank accession numbers are as follows: *P. aeruginosa* SN004 (Pasn), *H. pylori* CCUG 17874 (Hepy; EIE30409.1), *B. subtilis* W168 (Basu; AAA22243.1), *E. coli* (Esco; NP 417432.1), and *Er. chrysanthemi* 3937 (Erch; AAS67028.1), *B. licheniformis* (BaLi, WP_044789473), *K. pneumoniae* JM45 (Klpn, AGT24790), *P. aeruginosa* (Paea, WP_023099587).

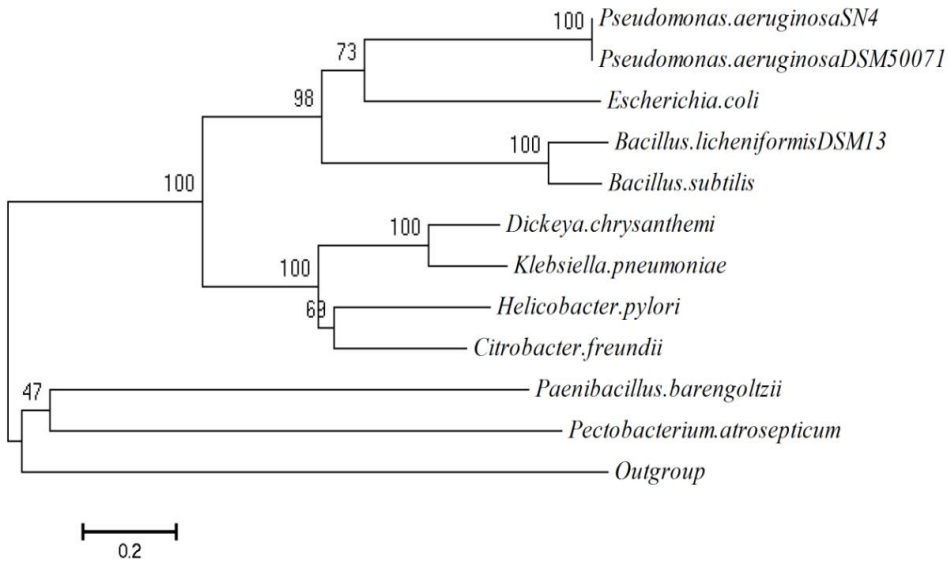

Fig. 6- Phylogenetic analysis of the L- asparaginase protein of *Pseudomonas aeruginosa* strain SN4. Bootstrap values and scale bar depicting substitution rate per site are indicated. The phylogenetic tree constructed by the neighbor- joining method showing the position of L- asparaginase of of *Pseudomonas aeruginosa* strain SN4. Protease from *Escherichia coli* was used as outgroup.

Discussion and conclusion

Because of various side effects of *Erwinia* L- asparaginase, finding other bacterial L- asparaginase received more attention. So, production of recombinant L- asparaginase has academic and practical importance. Up to now, there is only one report about cloning of L- asparaginase gene of *P. fluorescens* into *E. coli* BL21 (27) and there is no report about cloning of *P. aeruginosa* L- asparaginase. Multiple sequence alignment of L- asparaginases showed that *P. aeruginosa* L- asparaginase SN4 has 43% similarity with other L- asparaginases from *E. coli* (WP_047081447) and *Bacillus subtilis* (AID16240). The results also indicated that SN4 L- asparaginase has the catalytic residues and two conserve regions similar to other L- asparaginases from *E. coli*, *B. subtilis*, *H. pylori*, *Er. chrysanthemi* 3937, *B. licheniformis*, *K. pneumoniae* JM45 and *P. aeruginosa*. In this study, phenol red used as a pH indicator to screen recombinant L- asparaginase. Molecular weight of *P. aeruginosa* L- asparaginase strain SN4 was about 35 kDa. In this respect, L- asparaginases purified from *Pseudomonas stutzeir* MB- 405, *Thermus thermophilus* and *Escherichia coli* were with smaller Mr values, ranging from 33- 34 kDa, (28- 30). Purified L- asparaginase from *Bacillus* sp., *Streptomyces gulbargensis*, *Streptomyces albidoflavus* and S. PDK2 exhibited a molecular weight of 45, 85, 112 and 140 kDa, respectively (31- 34). Recombinant L- asparaginase showed high activity with about 93.4 IU/ml. Gilbert et al., Liu et al. and Oza et al. have cloned and expressed *E. coli*, *E. chrysanthemi* and *Withania somnifera* L- asparaginase gene into *E. coli*. The enzyme activities of these recombinant strains were 49, 106 and 17.3 IU/ml, respectively. High

activity of recombinant SN4 L-asparaginase indicated a potent source of L-asparaginase for anticancer examination *in vitro* and *in vivio*.

Acknowledgement

The authors express their gratitude to Research Council of Shahid Bahonar University of Kerman for financial support during the course of this project.

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تعیین توالی ژن، کلونینگ و بیان آل- آسپاراژیناز نوترکیب از *سودوموناس آیروجینوزا* سویه SN4 در *اشریشیاکلی*

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چکیده

مقدمه: ال- آسپاراژیناز جایگاه با ارزشی از جنبه کاربردهای پزشکی و صنایع غذایی دارد. تقاضا برای این آنزیم دارویی هرساله چندین برابر می‌شود.

مواد و روش‌ها: در این پژوهش، یک آسپاراژیناز از *سودوموناس ایروجینوزا* سویه SN4، تعیین توالی و در *اشریشیاکلی* کلون شد. پرایمرها بر اساس آل- آسپاراژیناز از *سودوموناس ایروجینوزا* سویه DSM50071 طراحی شدند که بر اساس توالی ژن *16S rRNA* شباهت بالایی را نسبت به سویه SN4 نشان می‌داد. ژن *آسپاراژیناز* در معرض هضم آنزیمی توسط آنزیم‌های برشی *NdeI* و *XhoI* قرار گرفت و سپس، به داخل پلاسمید pET21a الحاق شد. محصول الحاق به درون سلول‌های مستعد *E. coli* (DE3) pLysS DH5a با روش CaCl2 ترانسفورم شد. سلول‌های *E. coli* ترانسفورم شده در محیط LB آگار حاوی ۱۰۰ میکروگرم/ میلی‌لیتر آمپیسیلین و IPTG (1mM) رشد داده شدند.

نتایج: ال-آسپاراژیناز نوترکیب در باکتری *E. coli* BL21 پس از ۹ ساعت انکوباسیون، به میزان بالایی القا شد و فعالیت آسپاراژینازی بالایی به میزان ۹۳/۴ واحد در میلی‌لیتر را نشان داد. ال- آسپاراژیناز نوترکیب تعیین توالی شد و نتایج همردیفی نشان داد که توالی پیشنهادی اسید آمینه‌ایی آن از ۳۵۰ اسید آمینه تشکیل شده که شباهت بالایی با ال- آسپاراژینازهای سایر *سودوموناس ایروجینوز*ها در حدود ۹۹ درصد را نشان می‌دهد. نتایج نشان می‌دهد که آل- آسپاراژیناز SN4، باقی‌مانده‌های کاتالیزی و نواحی حفظ شده مشابه با سایر آسپاراژینازها را داراست.

بحث و نتیجه‌گیری: پژوهش حاضر نخستین گزارش در مورد کلونینگ و بیان آل- آسپاراژیناز نوترکیب از *سودوموناس آایروجینوزا* در *اشریشیاکلی* است که منبع بالقوه‌ایی از ال- آسپاراژیناز را برای بررسی اثر ضدسرطانی آن هم در شرایط آزمایشگاه و هم در موجود زنده فراهم می‌کند.

واژه‌های کلیدی: ال- آسپاراژیناز، کلونینگ، *سودوموناس آایروجینوزا*، همردیفی توالی

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* نویسنده مسؤول مکاتبات

تاریخ دریافت: ۱۳۹۴/۰۶/۰۹- تاریخ پذیرش: ۱۳۹۴/۰۸/۰۶