Biotechnological approach for the production of L-asparaginase from locally *Bacillus subtilis* isolate

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Background and objectives
L-asparaginase is a therapeutic enzyme used for the treatment of hematopoietic diseases, for example, acute lymphoblastic leukemia. It has many applications other than as an anticancer agent, which includes in the treatment of autoimmune diseases, infectious diseases, antimicrobial property, canine and feline cancers. The aim of this study is to increase the production level of L-asparaginase by the cloning and expression of *Bacillus subtilis* L-asparaginase (*Asp*) gene in *Escherichia coli*.

Materials and methods
PCR was used for the amplification of Asp gene of *B. subtilis*. *Asp* gene was cloned with the blunt vector pJET1.2 under the control of *T7* or *lacUV5* promoters. Transformation of both plasmids was done in *E. coli* JM 107. L-asparaginase was determined in *E. coli* JM 107 recombinant strains.

Results
Bacillus Asp gene was amplified using PCR and the primers were deduced from the published *ansA* sequence. PCR program was optimized. The PCR product (1112 bp DNA fragment containing the *Asp* gene) was detected, purified, and sequenced. The DNA sequence including the complete Asp CDS sequence was deposited in GenBank (GenBank accession number KJ642620.1). The amplicon was cloned using the blunt vector pJET1.2 under the control of *T7* or *lacUV5* promoters. The recombinant plasmids containing the Asp gene were transformed and expressed into *E. coli* JM 107. The expression level of *Asp* in the recombinant stains was increased up to 22 U/ml, which is 2.5-fold higher than that of *E. coli* JM 107 wild type. The vector promoters had regulatory effect on L-asparaginase production, where the activity under the control of *T7* promoter was increased by 47% compared with that of *lacUV5* promoter.

Conclusion
L-asparaginase production could be improved by cloning and by the expression of its corresponding gene in *E. coli*. The *T7* promoter had a higher regulatory effect on L-asparaginase production level than *lacUV5* promoter.

Keywords:
antitumor, cloning, GenBank accession number: KJ642620.1, L-asparaginase, PCR

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Introduction

L-asparaginase is an important enzyme; which is used for the treatment of hematopoietic diseases [1]. It destroys asparagine external to the cell by hydrolysis of asparagine to aspartate and ammonia [2]. All asparagine needed by normal cells are made internally; at the same time, tumor cells which require huge amounts of asparagine become depleted rapidly and die.

L-asparaginase has many applications other than as an anticancer agent, which includes autoimmune diseases, treatment of infectious diseases, antimicrobial property, and treatment of canine and feline cancers. Its significance is also established in the food sector to reduce acrylamide concentration [3]. Because of its wide range of applications, there has been a huge market demand for L-asparaginase. A search for better L-asparaginase-producing sources, including high yields and low immunogenicity, is the aim of this industry.

Asparaginase is known to be produced by many animal tissues [4], bacteria [5], yeast [6], fungi [7], and plants [8]. The major sources of L-asparaginase was reviewed and a wide range applications of this important enzyme have been discussed [9,10].

L-asparaginases produced by bacteria are classified into two subtypes according to their intracellular or extracellular localization [11]: the cytosolic (type I) and the periplasmic (type II) type. They also differ in their affinity for L-asparagine. The first type has a lower affinity, while the second one has a higher affinity. L-asparaginases produced by plants differ structurally than bacterial L-asparaginases [12] and have a different evolutionary origin.

L-asparaginase is isolated from bacteria, among them are *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) [13]. These therapeutic agents may cause severe immunological reactions. The toxic side effects including hypersensitivity reactions is partially attributable to the glutaminase activity of these enzymes [14]. *Bacillus stratosphericus* asparaginase shows decreased glutaminase activity, so it is believed to have fewer side effects in leukemia therapy [15]. The side effects of L-asparaginase therapy remain to be elucidated.

Cloning and expression of L-asparaginase (*Asp*) genes has been performed from different organisms including *E. chrysanthemi* [16]. These cloned varieties showed different enzymatic characteristics and side effects when used as an antileukemic drug. Searching for new bacterial L-asparaginase can lead to an enzyme with less adverse effects. The expression of *Asp* genes from such sources into suitable bacterial strain may allow access to improve products for used in chemotherapy of leukemia. The main objective of this study is to improve enzyme production through the recombinant technique. The high-yield strains with low immunogenicity could be also used in food industry and medical applications.

Materials and methods

Bacterial strains, vector, and culture condition

Bacillus subtilis Al Azhar, local isolate, Microbial Genetics Department, National Research Centre, Egypt, was used as a source of DNA donor for *Asp* gene. It was grown on Lauria-Bertani (LB) plate at 37°C. *E. coli JM 107* was used in transformation trials.

LB medium [17] was used for bacterial growth.

The pJET1.2 blunt vector (Fermentas Life Sciences, Vilnius, Lithuania) with the two promoters, *lacUV5* promoter and *T7* promoter was used for cloning and expression of the *Asp* gene. This vector has a positive selection of successful cloning (i.e. a lethal gene is disrupted by ligation of a DNA into the cloning site of the vector).

DNA manipulation

All molecular biology manipulations were performed according to the standard protocol of Sambrock *et al.* [18] and as per the kit supplier's instructions unless specified.

The genomic DNA from crude lysate cells was isolated as described by Ostuki *et al.* [19].

PCR amplification of L-asparaginase gene

According to the published sequence of *Asp* gene (*ansA*) of *B. subtilis* in the NCBI Nucleotide Sequence Database (NC_000964.3), the two DNA primers were designed and synthesized to allow PCR amplification of the entire *Asp* gene. To incorporate the suitable restriction enzyme sites into designed primers, the sequence was analyzed for its restriction endonuclease cutting by using the WebCutter 2.0 software (Yale, USA).

Primers were designed using the Primer3 software (Cambridge, Massachusetts, USA); the reverse primer was 5′-CCCAAGGAAGTCTT TTTCCA-3′ and the forward primer was 5′-AGTGAAGAGGTGCATGGTATGA –3′.They were chosen as they were flanking the entire *Asp* CDS. By using these primers, the expecting amplicon is 1100 bp. Two restriction enzymes sites were added to facilitate gene manipulation. Bam HI sequence was added to the reverse primer and the XhoI sequence site was added to the forward primer, so the expecting amplicon is 1112 bp.

B. subtilis was cultivated on LB plates at 37°C for 18 h. The DNA from crude lysate cells was isolated as described by Dong *et al.* [20] and subjected to PCR amplification using Asp forward and reverse primers.

To optimize the reaction mixture of the PCR, different parameters were tested. A final volume of 50 μl reaction mixtures was used by mixing DreamTaq Green PCR Master Mix (Fermentas Life Sciences) with 3 or 6 μl of cell lysate and using 10 or 20 pmol of each primer. The thermal cycler (Nyx Technik, San Diego, CA, USA) was used. The PCR program consisted of a denaturation cycle at 94°C (3 min), followed by 35 cycles of 1 min at 94°C, 2 min at 48°C, and 3 min at 72°C. Then an additional cycle at 71°C was performed for a final chain elongation.

The PCR product was analyzed using 1% agarose gel electrophoresis. The 1112 bp DNA fragment, containing the Asp gene, was purified using Ron's PCR-Pure Kit (BIORON, Römerberg, Germany).

This DNA was sequenced by Macrogen Co. (Seoul, South Korea), and was used for cloning experiments.

Cloning and subcloning of L-asparaginase gene

The PCR product was blunt ended and ligated with the pJET1.2 blunt vector using CloneJET PCR Cloning Kit (Fermentas Life Sciences).

The plasmid ASP-NRC-1 (this study) was subcloned by its digestion with Bam HI and XhoI, blunt ended and then ligated with pJET1.2 blunt vector. Recombinant plasmids were transformed into *E. coli* JM 107. The occurrence of *Asp* gene in the random selected clones was confirmed by PCR amplification, using both pJET vector primers and asparaginase primers. The expected amplicon size was 1112 bp when using *Asp* primes and was 1230 bp when using the pJET primers.

Bioinformatics and primer design

Different internet sites have been used through these studies, they include: The National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), WebCutter 2.0 software (http://rna.lundberg.gu.se/cutter2/), primer design (Primer3, http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and Plasmid Mapping [20] http://wishart.biology.ualberta.ca/PlasMapper/).

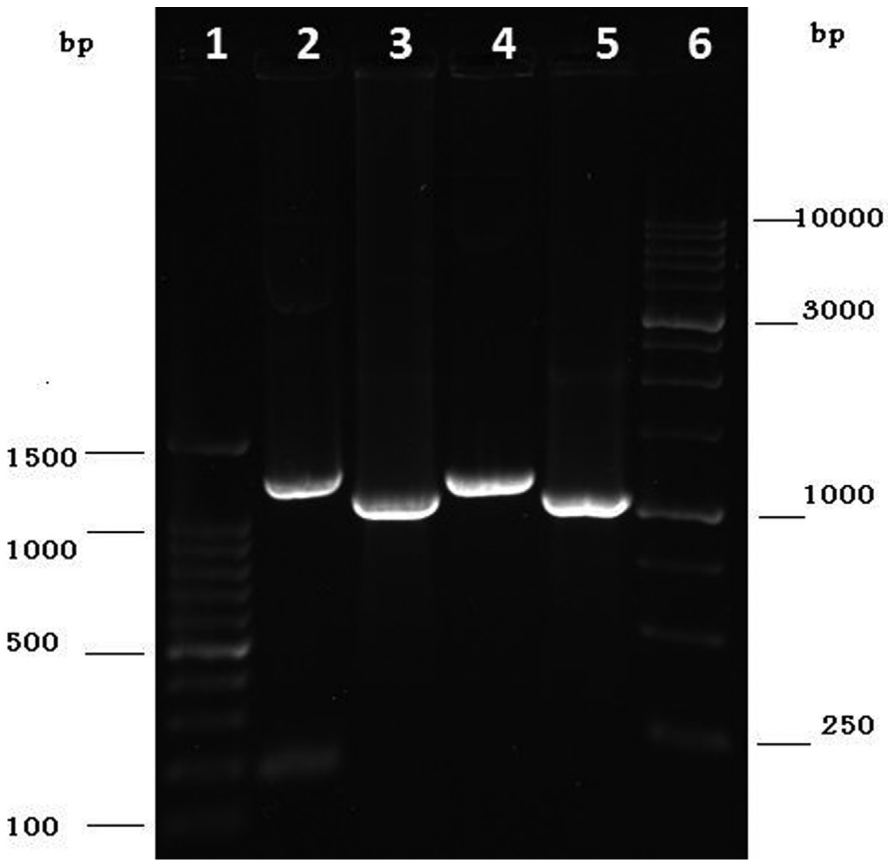
L-asparaginase assay

The L-asparaginase activity was measured in the whole cells or cell-free extracts of a 24 h bacterial culture according to Wriston [21]. The ammonia released was determined by Nesslerization reaction of the supernatant produced after centrifugation at 6000*g* for 10 min. One enzyme unit (U) is defined as the amount of enzyme that liberates 1 μmol of ammonia per min at 37°C. The ammonia concentrations were calculated using a standard curve of ammonium sulfate.

Results

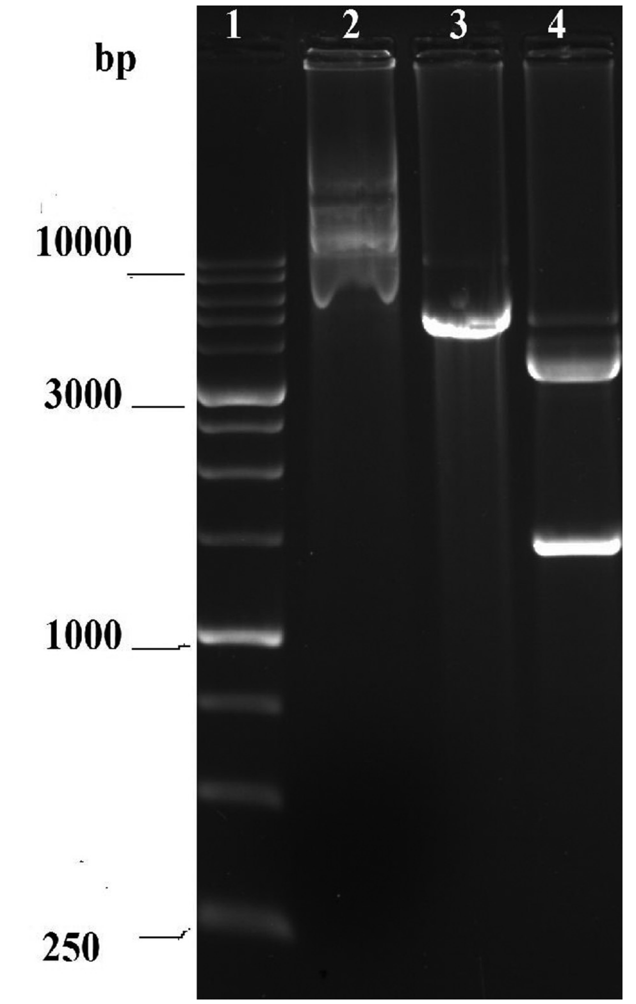
The efficiency of different indigenous bacterial strains was screened for asparaginase activity, among them the local strain *B. subtilis* Al Azhar was selected as the most promising asparaginase-producing strain and its chromosomal DNA was used as a source of *Asp* gene.

Figure 1



Agarose gel electrophoreses of PCR amplicons of two clones. Lane 1: 100 bp DNA ladder, lane 2: ASP-NRC-4 with pJET vector primers, lane 3: ASP-NRC-4 with asparaginase primers, lane 4: ASP-NRC-1with pJET vector primers, lane 5: ASP-NRC-1 with asparaginase primers, and lane 6: 1 kb DNA ladder.

Figure 2



Agarose gel electrophoreses of plasmid ASP-NRC-1 digested with different enzymes. Lane 1: 1 kb DNA ladder, lane 2: ASP-NRC-1, lane 3: ASP-NRC-1+Hind III, and lane 4: ASP-NRC-1+Hind III+Bam HI.

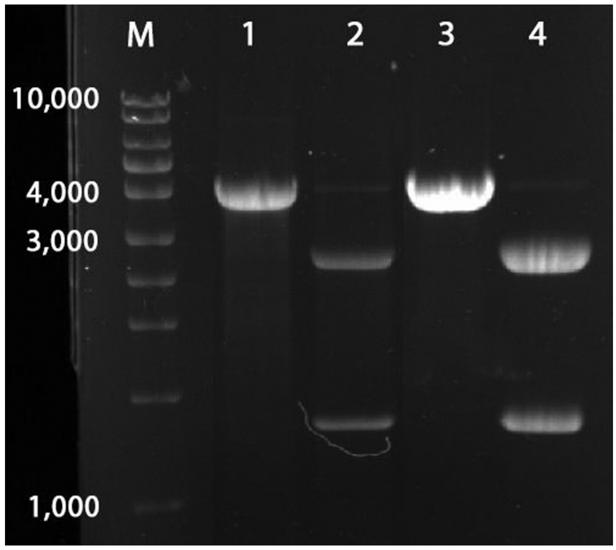
PCR amplification of L-asparaginase gene

The DNA sequence including the complete *Asp* CDS was deposited into GenBank with accession number (KJ642620.1).

Cloning and expression of L-asparaginase gene

The purified PCR product was blunt ended and ligated to pJET1.2 blunt vector; the recombinant plasmid was inserted into *E. coli* JM 107. Different *E. coli* transformants were screened for *Asp* gene. The presence of the gene in the recombinant strain was detected using two types of primers; pJET vector primers and asparaginase primers in PCR amplification. The results are summarized in Fig. 1, confirmed the existence of *Asp* gene in both tested clones; ASP-NRC-1 and ASP-NRC-4, where the size of DNA fragments size was as expected, that is, 1112 bp when using *Asp* primers and 1230 bp when using pJET primers.

Figure 3



Agarose gel electrophoreses of plasmids ASP-NRC-4 and ASP-NRC-2-II digested with different enzymes. M: DNA ladder, 1: ASP-NRC-4+ XhoI, 2: ASP-NRC-4+ XhoI+ Hind III, 3: ASP-NRC-2-II+ XohI, and 4: ASP-NRC-2-II + XhoI+ Hind III.

Position of the L-asparaginase gene

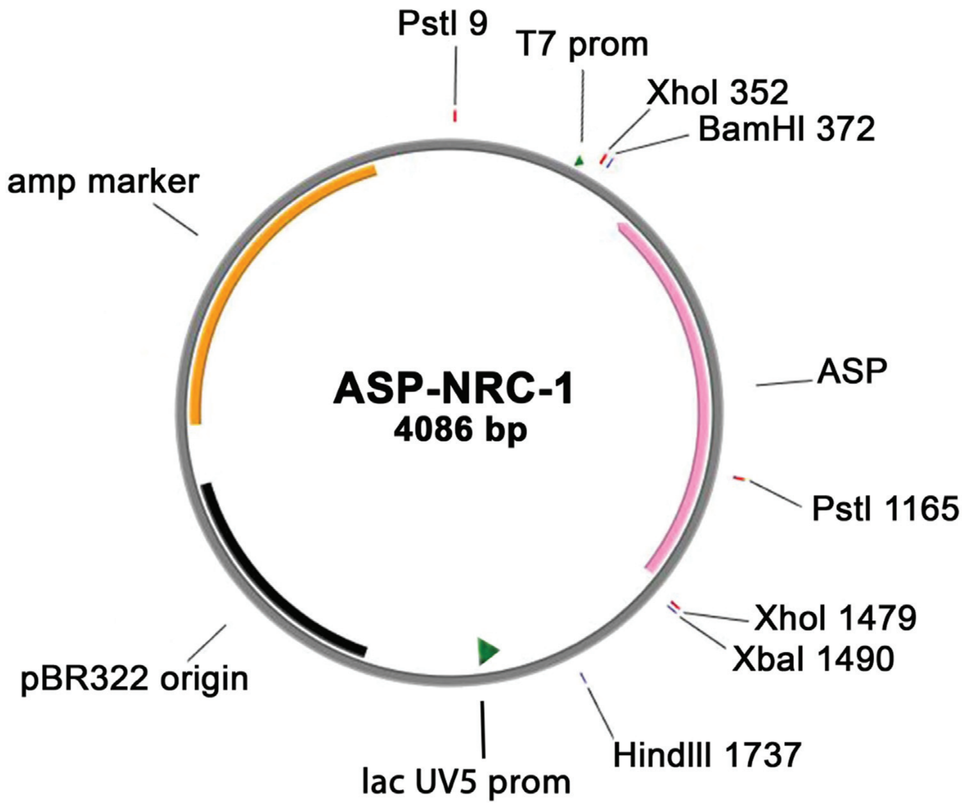
Asp gene direction was investigated in two strains: ASP-NRC-1 and ASP-NRC-4 by agarose gel electrophoreses after their digestion with restriction enzymes as illustrated in Figs 2 and 3. After digestion of ASP-NRC-1 with *Hind* III and *Bam* HI (Fig. 2), two DNA fragments (2721 and 1365 bp) were obtained., since the smallest DNA fragment is more than the ligated amplicon (1112 bp); this indicated that *Bam* HI site is far away from *Hind* III site and hence the *Asp* gene is under control of *lac*UV5 promoter (Fig. 4).

After digestion of ASP-NRC-4 plasmid with *Xho*I alone or with *Hind* III, the *Xho*I fragment of more than 4050 bp was obtained, while the double digestion with both enzymes produced two DNA fragments. The smallest one is more than amplicon (1112 bp); this indicated that the *Asp* gene is under control of *T7* promoter (Fig. 3).

L-asparaginase gene expression

Three *E. coli* transformants harboring recombinant plasmids, that is, *E. coli* (ASP-NRC-1), *E. coli* (ASP-NRC-4), and *E. coli* (ASP-NRC-7), were selected. Extracellular and intracellular L-asparaginase activities of the strains were measured. From the data represented in Table 1, it is clear that the enzyme activity is expressed mainly as intercellular. The highest activity was obtained by *E. coli* (ASP-NRC-4) reaching 16.2 U/ml, while *E. coli* (ASP-NRC-1) and *E. coli* (ASP-NRC-7) were of identical intercellular enzyme activity recording 11 U/ml. Nevertheless, minor activity was detected in the

Figure 4



Plasmid ASP-NRC-1.

Table 1 L-asparaginase activity of *Escherichia coli* transformants

Escherichia coli strain	Promotor	Enzyme activity (U/ml)	
		Extracellular	Intracellular
E. coli JM 107 (ASP-NRC-1)	*lac*UV5 promoter	3.0	11.0
E. coli JM 107 (ASP-NRC-4)	T7 promoter	2.9	16.2
E. coli JM 107 (ASP-NRC-7)	*lac*UV5 promoter	2.8	11.0
E. coli JM 107 (ASP-NRC-2-I)	*lac*UV5 promoter	2.0	18.6
E. coli JM 107 (ASP-NRC-2-II)	T7 promoter	4.0	22.0
E. coli JM 107	–	1.7	9.0

supernatant of all culture filtrates not exceeding one-fifth of the total enzyme activity.

It was also observed that L-asparaginase production depends mainly on different promoter's effect, where the asparaginase activity under the control of *T7* promoter in ASP-NRC-4 was increased by 47% compared with the activity under the control of *lacUV5* promoter in ASP-NRC-1 clone (Table 1).

Subcloning and orientation of L-asparaginase gene

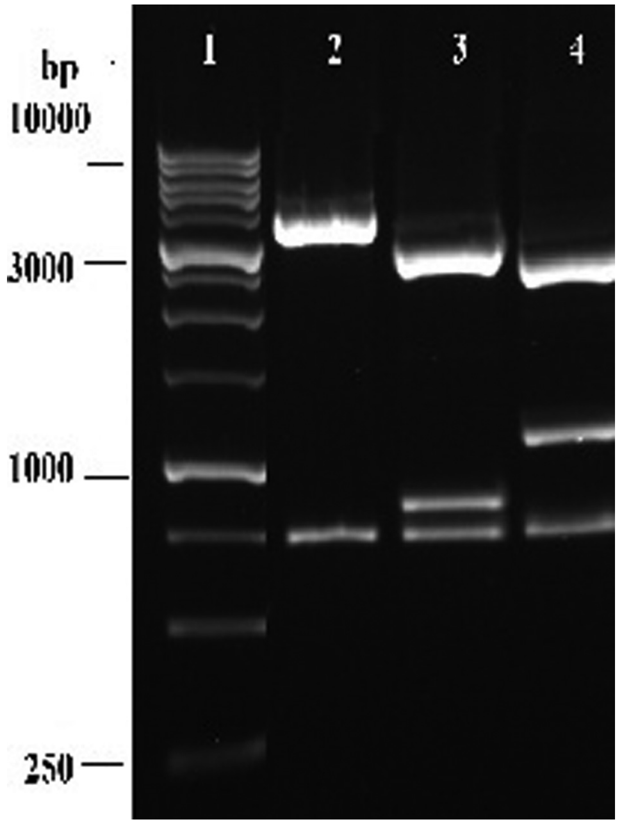
To confirm the effect of the two promoters on *Asp* gene expression, the recombinant plasmid ASP-NRC-1 was subcloned as described above. Several ampicillin-resistant transformants were obtained and randomly selected for further studies. Plasmids were isolated from each and digested with restricted enzymes to identify the *Asp* gene orientation. Among the tested transformants, ASP-NRC-2-I and ASP-NRC-2-II were selected, where they represent the two *Asp* orientations. In the clone ASP-NRC-2-I, the orientation of the *Asp* gene was anticlockwise and the gene was under the control of *lac*UV5 promoter (Figs 5 and 6). While in the clone ASP-NRC-2-II the orientation of *Asp* gene was clockwise and the gene was under the control of *T7* promoter (Figs 3 and 6).

L-asparaginase activity was investigated in both *E. coli* (ASP-NRC-2-I) and *E. coli* (ASP-NRC-2-II) (Table 1) indicating that, the two promoters *T7* and *lacUV5* enhanced the yield of bacterial L-asparaginase. Intracellular enzyme activity of *E. coli* (ASP-NRC-2-I) was 18.6 U/ml, which represents about double the activity of the control strain (9 U/ml).

Moreover, *E. coli* (ASP-NRC-2-II) yielded the highest enzyme activity reaching 22 U/ml, that is, near twice and half the control untreated *E. coli* strain. However, the two genetically modified *E. coli* strains also keep a low extracellular activity in the range of 10–15%; these results confirmed the previous evidence obtained in this study that *T7* promoter induces higher expression of L-asparaginase than the *lacUV5* promoter.

The current study has shown that one could succeed to increase the expression of the intracellular enzyme by using genetic techniques.

Figure 5



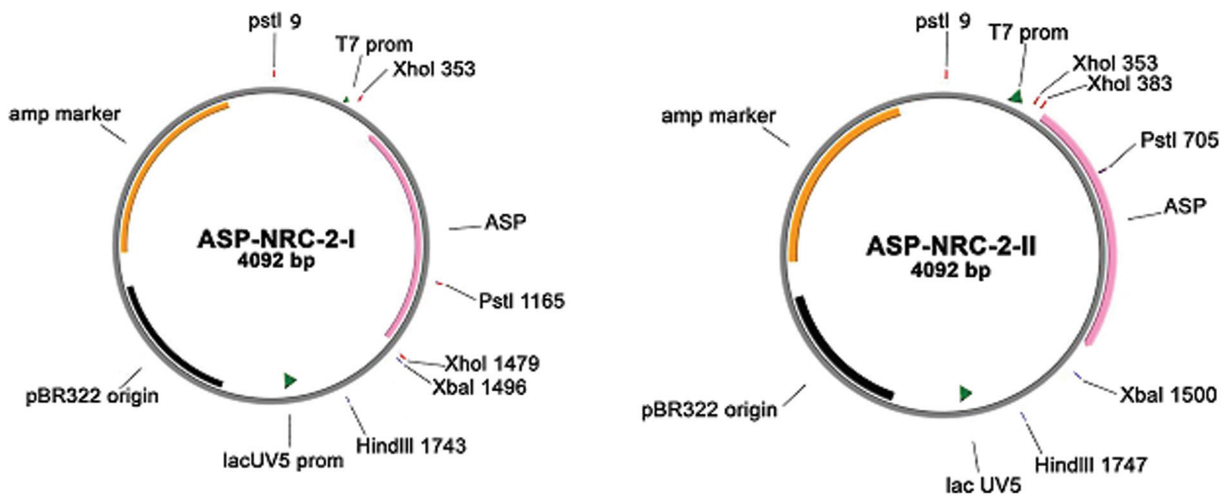
Agarose gel electrophoreses of the subclone Asp-NRC-2-I digested with different enzymes. Lane 1: 1 kb DNA ladder (GeneRuler 1 kb DNA Ladder, Fermentas Life Sciences), plasmid Asp-NRC-2-I digested with Pst I (lane 2) with Pst I + Xba I (lane 3) and with Pst I + Hind III (lane 4).

Discussion

E. coli and *E. chrysanthemi* asparaginases are used for the treatment of acute lymphoblastic leukemia for over 30 years. However, serious side effects have been observed. An effort to discover novel L-asparaginases with potential chemotherapeutic utility in acute lymphoblastic leukemia treatment was carried out [22]. *B. subtilis* could be used as an alternative source of L-asparaginase with less adverse side effects. The main purpose of this study is to search for new bacterial strain producing large amounts of the enzyme used for clinical studies. To this goal a number of bacteria were screened for their enzyme activity; among them, *Asp* gene from *B. subtilis* Al Azhar was isolated, analyzed, and studied.

Gene coding for *B. subtilis* L-asparaginase was cloned and expressed into *E. coli* JM 107 under control of two vector promoters; that is, *T7* and *lacUV5*, in the pJET1.2 blunt vector. The level and expression of *Asp* gene under control of the two vector promoters was demonstrated. The *Asp* gene position and orientation was confirmed, and their effects on asparaginase expression were studied. *E. coli* harboring recombinant plasmid produces different levels of asparaginases according to the

Figure 6



Constructed plasmids with asparaginase gene under the control of lacUV5 promoter (ASP-NRC-2-I) and the control of T7 promoter (ASP-NRC-2-II).

orientation of *Asp* gene in the plasmid. The expression level under control of the *T7* promoter was much higher than those under control of *lacUV5*. The higher yield of asparaginases from the *E. coli* recombinant strain compared with *E. coli* wild type may be due to that the vector promoters were more powerful in inducing asparaginases than the natural asparaginase promoter in *E. coli*. This observation was confirmed by the previous finding which stated that the recombinant plasmid containing the *Asp* gene when introduced into *Erwinia carotovora* caused increased synthesis of the enzyme two to four-fold higher than the production strain [23]. All strains under investigation produce minor asparaginase activity in their cell-free extract (extracellular); a similar result was detected by Jain *et al.* [24] who stated that only 10 isolates of *E. coli* showed extracellular production while 25 isolates did not. At the same time, 27 *E. coli* isolates showed intracellular L-asparaginase. They added that maximum L-asparaginase activity was found in the intracellular extract of VRY-15 showing 19.56 μmol/mg of specific activity.

Conclusion

From the previous results, it is clear that the expression of *Asp* gene could be remarkably increased up to 2.5-fold. This study also revealed that *T7* promoter induced a higher production level of L-asparaginase than *lacUV5* promoter.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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