Gene amplification and overexpression of *Bacillus subtilis* L-asparaginase

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Background and objectives
The aim of this paper was to focus on improving production level of l-asparaginase by developing recombinant strains.
Materials and methods
Asp gene was cloned into the shuttle vector pNW33N, and the recombinant plasmid was used to transform *Bacillus subtilis* protoplast. *Asp* gene was expressed into both *Escherichia coli* and *B. subtilis.* Enzyme activity of the recombinant strains was measured as compared with wild-type strains.
Results
Asp gene was successfully subcloned into the recombinant plasmid named S-ASP-NRC-27. The gene was expressed efficiently in both host strains: *E. coli* and *B. subtilis*. The enzyme activity of the transformants was increased up to threefold under control of *Lac Z* promoter.
Conclusion
From the previous results, the shuttle vector pNW33N seemed to be a very useful plasmid as a cloning vector in a wide variety of the genus *Bacillus*. Both of *Asp* gene amplification and the control of *Lac Z* promoter had direct effects on producing the super *Asp*-expression strains.

Keywords:
antitumor, *Bacillus subtilis*, cloning, l-asparaginase

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Introduction

Asparaginase catalyzes the hydrolysis of l-asparagine to l-aspartic acid and ammonia. Bacterial l-asparaginases are classified into subtypes I and II, which are defined by their intracellular or extracellular localization [1]. The application of asparaginase I from *Bacillus subtilis* was extensively used in food processing industry, whereas type II l-asparaginases, in particular, had tumor inhibitory activity [2–4]. Asparaginase gene from different bacterial sources, such as *Helicobacter pylori* [5], *Erwinia chrysanthemi* [6], and *B. subtilis* [7], have been cloned and expressed into different bacterial hosts.

There are several methods to introduce plasmid DNA into *Bacillus* spp., such as transformation of competent cells, polyethylene glycol (PEG)-mediated transformation of protoplasts, electroporation, transduction, and conjugation [8]. Preliminary studies have reported that plasmid transformation procedures effective with *Escherichia coli* are unsatisfactory for transformation in *B. subtilis*. Plasmid transformation of protoplasts appeared to be an attractive approach as it does not require cell competence for DNA uptake [9]. Many reports indicated that protoplast transformation is a useful method for gene cloning of *B. subtilis* [10].

The objective of our study was to improve l-asparaginase production of indigenous *B. subtilis* strain through increasing the corresponding gene copies.

Materials and methods

Bacterial strains, plasmids, and medium

Bacterial strains and plasmids used in the present study are presented in Table 1. *B. subtilis* Alazhar, local isolate, and *E. coli* JM 107 were used as the cloning and expression hosts for l-asparaginase gene in transformation trials.

ASP-NRC-1 is a recombinant plasmid harboring *B. subtilis* l-asparaginase gene [11]. It was used to subclone the l-asparaginase gene.

The cloning plasmid pNW33N is a shuttle vector that shows stable replication in *B. subtilis*, *Geobacillus stearothermophilus*, and *E. coli*. It contains a large multiple cloning site and encodes a chloramphenicol

Table 1 Bacterial strains and plasmids used in the present study

Bacterial strain/plasmid	Characterization	Source/references
Bacillus subtilis Alazhar	Wild type indigenous strain	Microbial Genetics Department, National Research Centre, Egypt
Escherichia coli JM 107	General host for plasmids	Sambrock *et al*. [12]
ASP-NRC-1 plasmid	l- asparaginase recombinant plasmid	Hegazy *et al.* [11]
pNW33N (BGSC No. ECE 136)	Shuttle vector for *Escherichia coli* and *Bacillus subtilis*. Contains Cm^r	BGSC
S-ASP-NRC-27 plasmid	l- asparaginase recombinant ECE136 plasmid	This study
Escherichia coli JM 107 (S-ASP-NRC-27)	*ASP*, Cm^r transformant	This study
Bacillus subtilis Alazhar (S-ASP-NRC-27)-7	*ASP*, Cm^r transformant	This study

ASP, l- asparaginase gene; BGSC, *Bacillus* Genetic Stock Center.

acetyltransferase resistant gene (Cm^r). It was used for cloning *Asp* gene and transformation of both *E. coli* and *B. subtilis* Alazhar strains.

All molecular biology manipulations were performed according to standard protocols [12] and kit supplier's instructions, unless specified. DNA bands were purified from the gel using MEGAquick-spin T.

Media

Luria-Bertani (LB) medium [13] was used for bacterial growth. Regeneration medium was used for *B. subtilis* protoplast transformation. It contains 5 g glucose, 1.0 g NH_4PO_4, 3.5 g K_2HPO_4, 1.5 g KH_2PO_4, 0.33 mol/l sodium succinate, 5 g gelatin, 4.07 g $MgCl_2$. $6H_2O$, 5 g casamino acids, and 20 g agar/l [14].

E. coli and *B. subtilis* transformants were selected on media supplemented with the antibiotic chloramphenicol (Cm) (25 μg/ml and 5 μg/ml, respectively).

Construction of recombinant plasmid

The two plasmids, ASP-NRC-1 and pNW33N, were isolated, purified, and digested with *Bam* HI and *Xba* I (the two restriction enzymes flanking the l-asparaginase gene in ASP-NRC-1 plasmid). Both digested fragments were purified from agarose gel electrophoreses and ligated. The resulting recombinant plasmid designated as S-ASP-NRC-27 was transformed to both *B. subtilis* protoplast and *E. coli h*ost strains.

Escherichia coli transformation

The genetic transformation procedure of competent cells and the selection of recombinants were performed according to Sambrock *et al.* [12].

A volume of 5 μl of recombinant DNA (S-ASP-NRC-27, harboring *Asp* gene) was used to transform *E. coli* JM 107 competent cells using heat shock technique. Transformants (cm^r) were selected on LB agar medium containing chloramphenicol (25 μg/ml).

The recombinant plasmid from *E. coli* JM 107 transformants was isolated using DNA-spin plasmid DNA purification Kit (INtRON Biotechnology, Korea) and characterized by its digestion with different restriction enzymes.

Bacillus subtilis protoplast preparation

B. subtilis protoplast induction was performed according to Akamatsu and Sekiguchi [14] method with some modifications.

B. subtilis Alazhar was grown in LB medium with shaking (120 rpm) up to OD_{570}=0.4, and then 15 ml was centrifuged (6000 rpm for 15 min) and suspended in 2 ml of SMM buffer (0.5 mol/l sucrose, 0.02 mol/l maleic acid, 0.02 mol $MgCl_2$, pH 6.5). A volume of 2 mg of lysozyme (1 mg/ml) was added and incubated at 37°C for 1 h. After centrifugation (4000 rpm, 10 min), they were suspended in 2 ml of SMM buffer.

Protoplast transformation of *Bacillus subtilis* by constructed plasmid DNA

A volume of 1 ml of *B. subtilis* protoplasts was mixed with 100 μl of recombinant S-ASP-27 plasmid DNA and 3 ml of 40% PEG 4000 in SMM buffer. The mixture was kept at 0°C for 2 min and then incubated at 30°C for 2 h. The protoplast mixture was then centrifuged at 4000 rpm for 10 min and suspended in 2 ml SMM. Overall, 100 μl was added to overlay tube of regeneration medium (0.7% agar) and poured into regeneration medium supplemented with 5 μl/ml chloramphenicol and incubated at 30°C up to 3 days. The transformants (cm^r) were selected, and their plasmid content was isolated.

L-asparaginase assay

l-asparaginase activity was assayed according to Wriston [15]. The reaction mixture contained 0.1 ml culture supernatant and 0.9 ml of 0.01 mol/l

l-asparagine prepared in 0.05 mol/l Tris-HCl buffer, pH 8.6, and incubated for 30 min at 37°C. The reaction mixture was centrifuged at 6000*g* for 10 min, and the ammonia released in the supernatant was determined by Nesslerization reaction. In brief, to 0.5 ml of supernatant, 1.75 ml distilled H_2O, 0.25 ml of Nessler reagent was mixed. After 10 min, absorbance at 480 nm was read with appropriate control. One enzyme unit (U) is defined as the amount of enzyme that liberates 1 µmol of ammonia per min at 37°C. Standard curve of ammonium sulfate was used for calculating ammonia concentrations.

Results

Construction of recombinant plasmid

Asp gene in ASP-NRC-1plasmid is located on *Bam* HI/*Xba* I restriction fragment; the purified *Bam* HI/*Xba* I fragment, which contains *Asp* gene, was successfully ligated to *Bam* HI/*Xba* I-digested pNW33N plasmid. The two ligated DNA fragments formed a recombinant plasmid named S-ASP-NRC-27 (Fig. 1). It was used to transform each of *E. coli* JM 107 and *B. subtilis* strains.

Bacterial transformation

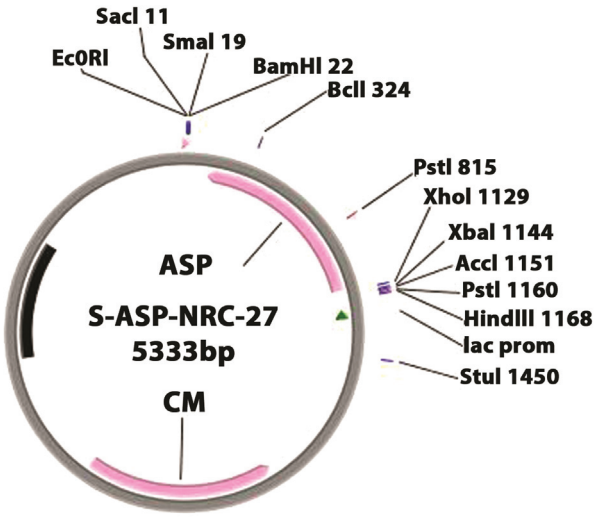
Following transformation trial of *E. coli* and *B. subtilis* using pS-ASP-NRC-27 plasmid, *E. coli* transformants were selected on LB supplemented with 25 µg/ml chloramphenicol and *B. subtilis* transformants on regeneration medium supplemented with 5 µl/ml chloramphenicol.

Plasmids were isolated from (cm^r) transformants, and agarose gel electrophoresis pattern of some obtained plasmids has been shown in Fig. 2. The restriction pattern of the digested plasmid with *Bam* HI+*Xba* I (lane 1), *Bam* HI+*Pest* I (lane 2), and *Xba* I+*Pest* I (lane 3) is illustrated in Fig. 3 to characterize its physical mapping. The data obtained confirmed the same expected DNA fragment lengths of Fig. 1 and that the *Asp* gene is under control of *lac* Z promoter of the recombinant plasmid.

Expression of *Asp* gene in *Bacillus subtilis* and *Escherichia coli* transformants

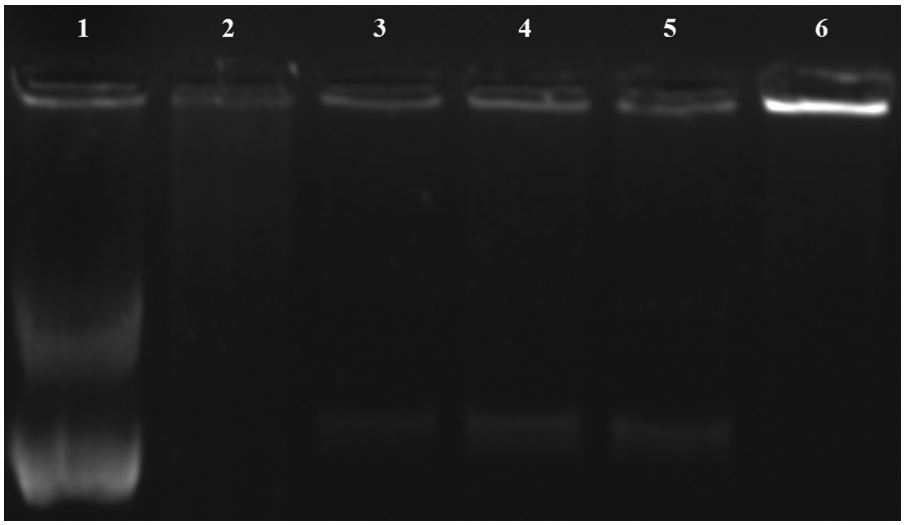
L-asparaginase activity of both recombinant strains, *E. coli* (S-ASP-NRC-27) and *B. subtilis* (S-ASP-NRC-

Figure 1



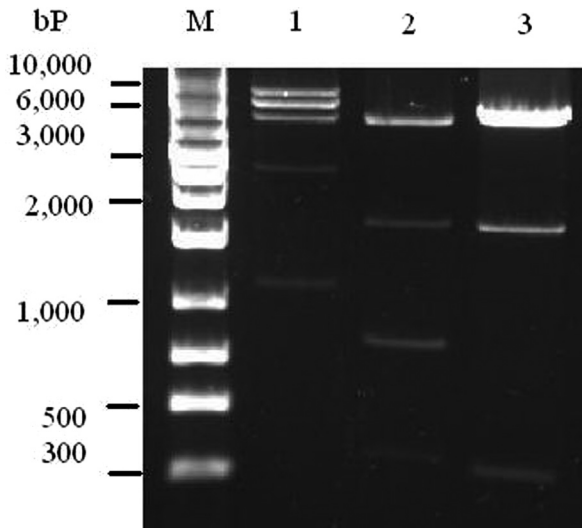
S-ASP-27 plasmid.

Figure 2



Agarose gel electrophoresis of isolated S-ASP-NRC-27 plasmids from *Escherichia coli* and *Bacillus. Subtilis* transformants. Lane 1: *Escherichia coli* (pS-ASP-NRC-27); lane 2: *Escherichia coli*; lanes 3,4,5: *Bacillus subtilis* (pS-ASP-NRC-27) 2, 3 and 7, respectively, lane 6: *Bacillus subtilis* Alazhar strain.

Figure 3



Agarose gel electrophoresis of S-ASP-NRC-27 plasmid cut with different DNA restriction enzymes. Lane M: DNA marker – Axygen DNA marker (300–10 000 bp); lane 1: S-ASP-NRC-27+BamHI+XbaI; lane 2: S-ASP-NRC-27+BamHI+PestI; lane 3: S-ASP-NRC-27+XbaI +PestI.

Table 2 L-asparaginase activity of *Escherichia coli*

Strain code	Enzyme activity (Ul/ml)	
	Extracellular	Intracellular
Escherichia coli JM 107	1.7	9.0
Escherichia coli JM 107 (S-ASP-NRC-27)	2.4	29.6
Bacillus subtilis Alazhar	3.0	16.0
Bacillus subtilis (S-ASP-NRC-27)-7	6.0	49.0

ASP, l- asparaginase gene.

27), was determined compared with their host strain: *E. coli* JM107 and *B. subtilis*. The data in Table 2 revealed that cloned *Asp* gene from *B. subtilis* was successfully expressed in *B. subtilis* as well as in *E. coli* JM107.; *E. coli* transformant harboring recombinant plasmid produced threefold increase of asparaginase yield, whereas *B. subtilis* transformant produced 2.9-fold increase as compared with parent strain.

Discussion

Cloning of *Asp* gene from *B. subtilis* strain and its nucleotide sequence was described by our team. The gene expressed efficiently in *E. coli* JM107, and the expression level is controlled by one of the two promoters T7and *lac*UV5 [11]. In the present study, we try to find an efficient strategy to transfer and express *Asp* gene into *B. subtilis* strain as well as *E. coli* JM107. For this purpose, two-step approach has been studied; protoplast transformation in *Bacillus* spp. and the usefulness of plasmid pNW33N DNA as a cloning vector in *Bacillus* spp. Many research studies described conditions of the plasmid transformation [10]. Transformation by plasmid DNA which is an essential step in most cloning experiments was improved by the use of protoplasts in PEG solution. However, protoplast transformation of *Bacillus* spp. has been reported [12].

Gene expression is dependent mainly on its promoter efficiency. In the present study, l-asparaginase-specific activity of *E. coli* JM 107 (pS-ASP-NRC-27) the gene was under control of *lac* Z promoter. These results reflect the superior efficiency of the *lac* Z promoter than both promoters studied before [14].

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Conflicts of interest
There are no conflicts of interest.

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