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Cloning and expression of L-asparaginase from *E. coli* in eukaryotic expression system

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ABSTRACT

L-Asparaginase is an anti-cancer agent which prevents the proliferation of cancerous cells by decreasing the level of asparagine in the blood. L-asparaginase from *Escherichia coli* which is encoded by *ansB* gene is widely used because of its substrate specificity and less glutaminase activity. Here, we are reporting the expression studies in yeast which has many added advantages like protein folding and processing. The expression studies were carried out in a new protein expression system based on the yeast *Pichia pastoris* called PichiaPink™. *ansB* gene isolated from *E. coli* which is coded for asparaginase was cloned into pPink HC-α plasmid and transformed into protease knock out pichia pink strain by electroporation. The recombinant enzyme was extracellular and showing the activity of 2.5 IU/ml. It was then purified using Ni-NTA column since the enzyme contains His-tag at the C-terminal end. The new way of expression would be efficient in making the enzyme humanized by glycosylation patterns which is similar to mammals.

1. Introduction

Asparaginase is known to us from 1967 and used widely as an anti-neoplastic agent against childhood acute lymphoblastic leukemia and Hodgkin's lymphoma [1]. Out of the many available sources, bacterial asparaginases gained more importance in therapeutic industry since it was showing high substrate specificity and long half life. Asparaginase from *Escherichia coli* and *Erwinia carotovora* [2] is mostly used. Bacterial asparaginases are of two types, one which occurring in cytoplasm called asparaginase I and asparaginase II, which occurs in periplasm. Since asparaginase II is more specific to its substrate asparagine, it is used more for therapeutic purposes [3]. The basis for its oncolytic nature is it depletes the asparaginase present in the blood, thus, make cancer cells deprived of asparagine. At the same time normal cells escapes this starvation by making use of asparagine synthetase enzyme which is deficient in tumor cells. Lack of this non-essential amino acid asparagine will affect the protein machinery and thus, the growth of the cell [4].

So far asparaginase was expressed in the prokaryotic systems like *E. coli* [5,6], *E. carotovora* [7,8,2], *Bacillus subtilis* [9], *Bacillus licheniformis* [10] and yeasts such as *Saccharomyces cerevisiae* [11]. Even though sources of the asparaginases in the nature are high, it is essential to modify the existing ones to improve its clinical efficiency. Methods are mainly focused on the site directed mutagenesis to increase thermo stability [12] and also recent techniques like layer by layer technique [13], nanoparticle coating [14] which mainly masks the enzyme from antibodies, thus, reducing immunogenicity. The pichia pink expression system that we are using here has many added advantages like high transformation efficiency, screening by ADE2 complementation, i.e., adenine auxotrophy which eliminates the use of antibiotics, thus, obtain correct transformants, the strains are protease knock out, thus, avoids degradation of secreting recombinant protein.

The main aim of this present study is to express asparaginase II of *E. coli* in pichia pink expression system. *E. coli* asparaginase was isolated and ligated into the plasmid pPink α-HC and then transformed into pPink host. The expression of the recombinant protein was studied; purified and optimum temperature was also calculated.

2. Materials and methods

2.1. Bacterial source

E. coli MTCC 739 was used for isolating asparaginase II in this work [5].

2.2. Pichiapink™ expression system

Pichia pink expression system was purchased from Invitrogen. It includes pPink expression strain kit, vector kit, media kit and

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secretion signal kit provided with an expression system manual. Out of the four pPink strain, the strain used in this work was pPink 4 in which both pep4 and prb1 proteases are knock out, and the vector used was pPink α-HC, which is a high copy number plasmid.

2.3. Cloning of ansB gene

Genomic DNA isolated from *E. coli* MTCC 739 was used as the template for PCR amplification of *ansB* gene using the primers:

- pPinkF (5′-GCG**AGGCCT**GTTACCCAATATCACCA-3′) and
- pPink R (5′- GGCG**TTAA**GTACTGATTGAAGA-3′),

where, the highlighted sequence is the restriction sites of *StuI* and *FseI* enzymes, respectively. PCR conditions used was initial denaturation at 95 °C for 4 min, denaturation for 95 °C at 40 s, annealing at 55.8 °C for 35 s, extension for 72 °C at 10 min and final extension for 4 °C α.

Amplicon size is 1.3 kb. After amplification, the *ansB* gene was subjected to restriction digestion with *StuI* and *FseI* enzyme and then ligated into pPink α-HC plasmid which was already having the restriction sites. The alpha mating factor (AMF) of *S. cerevisiae* for secretion of recombinant protein and His tag which enables the easy purification was also cloned in frame with the gene, but the former is in the N-terminal end the latter in the C-terminal end. The recombinant plasmid was then transformed into competent DH5α cells.

2.4. Clone confirmation

Clones were analyzed by PCR amplification using both vector specific and gene specific primers. The primer sequences of vector specific primers are alpha mating factor forward primer as 5′-ATGAGATTTCTTTCAATTTTTAC-3′ and CYC termination primer reverse as 5′-GGAAAAGGGGCCTGTA-3′. The *ansB* gene was then single digested with *StuI* enzyme to find out the shift, and thus, the integration of gene and also sequencing was done to further confirm the presence of gene and also to check the orientation of integration in the plasmid.

2.5. Isolation and linearization of pPink α-HC

Recombinant plasmid was isolated from DH5α using kit method (QIAGEN midi prep kit) and it was then linearized with *SpeI* (ACTAGT) restriction enzyme which makes cut in TRPI locus. It is necessary that the concentration of the plasmid should be 5–10 μg according to kit protocol and it is essential to increase transformation efficiency.

2.6. Electroporation of recombinant plasmid in the host pPink 4

Linearized plasmid was then transformed into pPink 4 strain by electroporation. Electrocompetent pPink cells were prepared by 1 M sorbitol. Electroporation was carried out in Gene Pulser Xcell™ electroporator system with 0.2 cm cuvette (BIORAD). After electroporation, YPDS media was added to it and incubated for 4 h at 37 °C. The culture was then spread onto PAD (pichia adenine dropout) agar plate and incubated for 7–10 days at 24–30 °C.

2.6.1. Conditions used

Better transformation efficiency was found at the OD_{600} of 1 at the primary inoculum and OD_{600} of 1.3–1.5 in the secondary inoculum. Electroporation was carried out at the voltage of 1.5 kV cm^{-1}, capacitance of 25 lF and a resistance of 200 X.

2.7. Expression and purification

The colonies were picked and yeast DNA was isolated (BMC, Biotechnology-2014) and expressed with inducer as methanol according to manufacturer's protocol. SDS-PAGE and silver staining was also used for the confirmation of expression. His tag containing expressed protein was then purified with Ni-NTA column (QIAGEN, Germany) using affinity chromatography. Protein was eluted with 250 mM of imidazole containing elution buffer. It was then dialyzed with MilliQ water and samples were collected and used for characterization.

2.8. Enzyme assay

Activity of the expressed enzyme was checked using asparaginase assay in which Nesslerization is its main principle [15]. The ammonia released due to the hydrolysis of asparagine was detected by nessler's reagent. Culture supernatant and purified samples were mixed with 40 mM of asparagine as substrate and 50 mM of Tris-HCl of pH 7. It was then incubated at 37 °C for 1 h and then the reaction was stopped by adding 1.5 M TCA and supernatant was mixed with nessler's reagent and readings were taken at 450 nm. Ammonium sulphate was used as the standard for enzyme activity calculations and it is expressed in IU/ml. 1 IU/ml corresponds to amount of enzyme required for the conversion of 1 μM of ammonia in 1 min.

2.9. Optimum temperature

Optimum temperature for the highest activity was determined at the temperatures of 37 °C, 42 °C, 50 °C and 60 °C.

3. Results and discussions

3.1. Cloning of ansB gene

ansB Gene was isolated from *E. coli* and included alpha mating factor for extracellular secretion in the N-terminal end and and His-tag in the C-terminal end was cloned into pPinkα-HC downstream of AOX1 promoter using restriction sites *StuI* and *FseI*. The recombinant plasmid containing the gene was then transformed into chemically competent DH5α cells. Fig. 1 shows the linearized diagram of recombinant pPinkα-HC–*ansB* vector.

3.2. Clone confirmation

Clones obtained after transformation were checked for the presence of gene and the orientation of integration. PCR was used as one way of confirmation. PCR amplification was obtained with both pPinkα-HC specific primers (vector specific) and also *ansB* gene specific primers in the corresponding size of 2.5 kb and 1.3 kb, respectively. The isolated plasmid was then subjected to single restriction digestion to analyze the shift and observed the upward shift at 8.9 kb compared to the 7.9 kb of control plasmid having no gene as insert. It was again confirmed by sequencing and showing 99% similarity to asparaginase II when checked with NCBI-BLAST.

3.3. Isolation and linearization of pPink α-HC

Since the clone got confirmed, the isolated plasmid was linearized by single digestion with *SpeI* restriction enzyme and got the concentration of 7 μg/30 μl.