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Research Article

Cloning and Expression Assessment of Uricase Gene from *Bacillus subtilis* in Some *Escherichia coli* Strains

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Abstract

Objective: The purpose of this study was cloning and expression of uricase gene from *Bacillus subtilis* in BL21, DH5a and TOP10 *Escherichia coli* (*E. coli*) strains.

Methods: Following the amplification, the PCR product was digested by NCOI and XhoI and cloned into pET28a vector. The ligation product was transformed into DH5α, TOP10 and BL21 *E. coli* strains using chemical methods. Cloning of uricase in pET28a was checked using colony PCR, digestion check, and sequencing. After confirmation of cloning, the expression of the urate oxidase encoding gene was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG).

Results: The uricase gene was successfully cloned into the pET28a and transformed into BL21, while did not transform into the DH5α successfully, and impaired into the TOP10, losing 500 nucleotide of the gene. The gene had been cloned downstream of T7 promotor. However, no expression occurred even after successful cloning into Bl21, and the western-blotting result was negative.

Conclusion: The uricase gene from *B. subtilis* was successfully cloned in BL21 strain, but two other strains of TOP10 and DH5α were not suitable for this purpose. Furthermore, the expression of the gene was not successful after cloning. It was demonstrated that uricase gene could not be expressed by the BL21 host.

Keywords: *Uricase Gene; Cloning; Hyper Uremia; Gout; Bacillus subtilis*

Introduction

The uricase enzyme found in several bacterial and multi-cellular organisms causes the dissection of urea and formation of alantoin. The purine nucleotides metabolism makes uric acid, thus in organisms without uricase enzyme the uric acid is the final product. In human and primates, the uricase gene is silent and is not expressed due to a mutation, thus there is the possibility of hyper uricemia among them [1]. When the uric acid of blood exceeds the normal level, the hyper uricemia will occur. Many diseases have been reported to be in relation to the hyper uricemia, such as gout, tumor lysis syndrome, metabolic syndrome, Lesch-Nyhan Syndrome, type 2 diabetes and many degenerative nervous system and cardio-vascular diseases [2,3]. The most important complication of increased blood uric acid is gout, occurring because of low solubility of uric acid and its precipitation in joints and soft tissues and formation of crystals [4,5]. Treatment of hyper

acid uremia is performed by prescription of some drugs such as allopurinol which inhibits the xantin oxidase enzyme and thus decreases the production level of uric acid and probecid which increases the excretion of uric acid from kidneys [6]. However, these compounds are not able to efficiently decrease the uric acid level. The uricase enzyme was firstly used as a non-recombinant drug from *Aspergillus spp* cultures in 1992, but it caused sensitivity and showed allergic effects because of some impurities [7]. After years in 2009, it was prepared via cloning of the encoding gene from *Aspergillus* to *Saccharomyces cerevisiae*, and recombinant production of the enzyme. Several other organisms produce uricase enzyme, however the stability and activity under physiological conditions vary among them [8,9]. By cloning of the gene and making recombinant bacteria, there may be the possibility of producing oral probiotics metabolizing uremic toxins such as uric acid. Moreover, evaluation of proper host bacteria for this purpose seems to be a critical aspect.

Aim of the Study

The aim of the present study was cloning and expression assessment of the uricase gene from *B. subtilis* into some *E. coli* host standard strains.

Materials and Methods

Bacterial strains and culture conditions

In this study, three standard bacterial strains including TOP10, DH5α and BL21 and *B. subtilis* were used. Culture media including Luria Bertani (LB, pH = 7.4, with and without 100 mg/ml Kanamycin) broth and LB agar (containing 100 mg/ml kanamycin) and SOC media were prepared for bacterial culture from Pasteur institute, Tehran Iran.

Culture of *B. subtilis* and genomic DNA extraction

B. subtilis was cultured in 15 ml LB broth with shaking and incubated for 16h. After reaching to stationary phase, 4 ml of culture was taken and centrifuged for 3 minutes at 6000g and precipitated. The DNA was isolated as previously described. Briefly, bacterial precipitate was collected in a micro tube. Two-hundred and fifty microliter of CTAB solution was added to it and incubated at 55°C for 12h. two-fold of present volume, iso-amyl alcohol-chloroform was added and mixed completely and centrifuged for 5 minutes at 13000g. The supernatant was transferred to a new tube and cold isopropanol 100% equal to 2/3 of volume and placed at -20°C for 1h. The supernatant was discarded and the precipitate was washed with 70% ethanol and placed at 50°C in order to dry the ethanol. The dried precipitate was dissolved in 50 - 100 µl distilled water and incubated at 50°C for 15 minutes.

PCR reaction

PCR was performed in order to amplify and cloning of uricase gene (with 1500bp size) using specific primers including F: 5-CGTCCATGGTGACAATGGATGACCTGAACCAAATG-3 and R: 5-TCGCTCGAGGGCCTTCAGTGCATCCGCTAATG-3. The digestion sites in F and R primers were designed for NCOI and XhoI, respectively. The uricase gene was extracted form gel for the purification of specific size band.

Plasmid extraction (mini-prep)

Plasmid was extracted by using mini-prep kit (Bionear) and following the manufacturer's instructions. Bacterial strains that contained pET28a were cultured in LB broth with antibiotic for 16h in a shaking incubator adjusted at 37°C. Four milliliter of culture was centrifuged at 5000g and precipitated. Then, 250 ul re suspension buffer (RB) was added to the precipitate and dissolved completely by quick severe shaking and 250 ul lysis buffer (LB) was added to the tube and was gently shaken for 5 minutes. Next, 400 ul of Neutralization Buffer (NB) was added and was shaken gently and placed at room temperature for 3 minutes, then centrifuged at 14000g for 10 minutes. The supernatant was transferred to silica column and centrifuged at 10000g for 1 minute. The silica column was washed by Washing Buffer (WB) and the centrifugation repeated. The tube was centrifuged again and placed in 50°C for 10 minutes in order to remove the excess ethanol. The column was transferred to a new tube and 40 ul Elution Buffer (EB) was added, and incubated at 50°C for 2 minutes and centrifuge at 14000g, then the extracted plasmid was collected. Two microliter of plasmid DNA was electrophoresed in 1% agarose gel. The map of PET28a was assessed using the Snape gene software (www.snapegene.com).

Enzymatic digestion of pET28a

The pET28a plasmid was digested using the fast NCOI and XhoI restriction enzymes (Cinagen, Iran) as previously have been reported. Briefly, 30 ul of PCR product, 2 ul of each NCOI and XhoI restriction enzymes and 6 ul universal digestion were added in a final volume of 60 ul. The mixture was placed at 37°C for 30 minutes. the product (5kbp size) of digestion was extracted using Cleanup PCR Product kit.

The ligation reaction and transfer to bacteria

The T4 ligase (Cinagen, Iran) was used for the ligation of uricase gene PCR product to the pET28a and recombinant plasmid formation. For this purpose, 15 ul of digested plasmid and 30 ul of the uricase gene were added to a tube and 5 ul ligation buffer and 2 ul T4 ligase were added and the mixture was placed at 37°C for 2h. Then, the ligation product was transferred to the competent bacteria (BL21, TOP10 and DH5α). For recombination confirmation, the enzymatic digestion and PCR amplification were used.

Protein expression and purification

The expression of protein was performed in BL21 *E. coli* strain as the suitable host for this purpose and by utilizing 100 ul IPTG (100 ug/ml) inducer at 29°C. The medium was incubated at 30°C for 12h by 180rpm shaking. Next, the bacteria were lyzed and the protein was purified by the affinity chromatography method. Because of histidine label on the downstream of multiple cloning site (MCS) of pET28a, the expressed proteins will be more easily isolated in the Nickel-sepharose vertical column.

SDS-PAGE of protein

The SDS-PAGE of protein was conducted as described before. Buffers and solutions included: stacking and separating gels, ammonium per sulfate, buffer tank and sample, Thermo Scientific Pierce Tetra methyl ethylene di-amine (TEMED) solution, de-coloration solution, acrylamide and 30% bis-acrylamide and 10% SDS.

Protein assessment by the Bradford method

The Bradford is a simple method for protein assessment. The Coomassie brilliant green G-250 will attach to the protein (wavelength of 465 - 595 nm) in the Bradford acidic solution and changes the color from brown to blue color in. the bovine serum albumin (BSA 1mg/ml) was used as the standard sample.

Results

Amplification and cloning of the uricase gene

The PCR product (1500bp) of uricase gene has been shown in figure 1. The recombinant Pet28a with 6kbp has also been depicted in figure 1. The growth of bacteria was observed on the LB medium surface. Following the ligation of product and plasmid, the recombinant product was transferred to the DH5α, TOP10 and BL21 host strains.

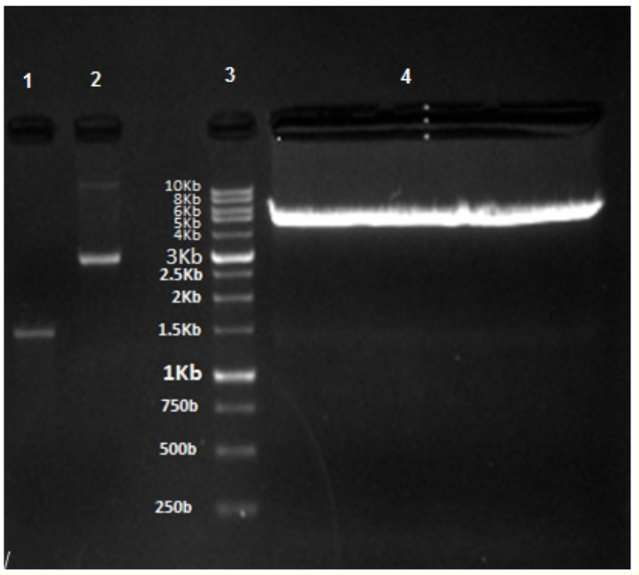

Figure 1: *Electrophoresis of PCR product of uricase gene (line1, digested by XhoI and NCOI enzymes), pET28a mini-prep plasmid (line 2) and pET28a digested by the both enzymes (line 4).*

The strains were cultured on kanamycin containing media in order to grow those containing the recombinant plasmid. After plasmid extraction and digestion of the recombinant plasmid by NCOI and XhoI enzymes, the product of 1500bp was also obtained on 1% agarose gel. The PCR amplification of uricase gene on the extracted plasmid was also exhibited the 1500bp band. Moreover, the product cloned completely in BL21 was sent for the sequencing analysis and the results were confirmed. The recombinant plasmid was partially cloned to TOP10, but not cloned in the DH5α strain.

Protein expression and SDS-PAGE

After protein expression assays and purification by the affinity chromatography, the product was not detected on the SDS-PAGE (Figure 2). In fact the results showed that uricase gene could not be expressed by BL21 even after successful cloning in the BL21 host (Figure 2).

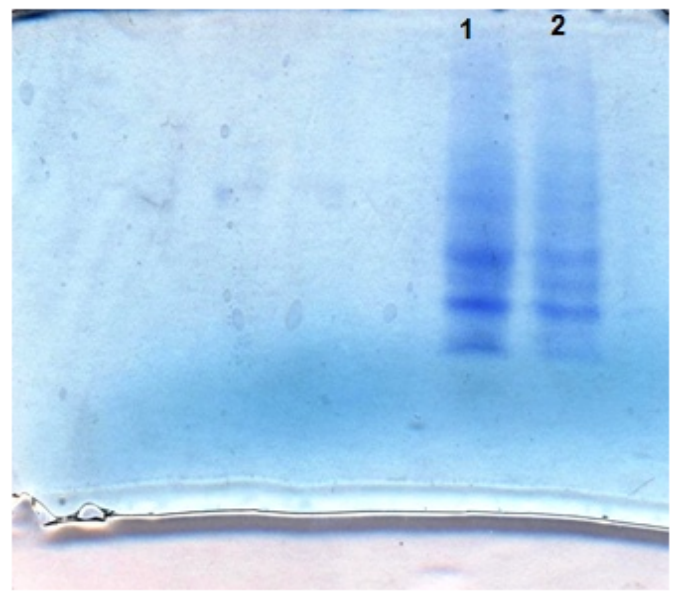

Figure 2: *The SDS-PAGE of the uricase enzyme product. It was exhibited that the uricase gene had not been expressed and was not observed in the SDS-PAGE.*

Discussion

There is a great interest of using bacterial enzymes in the field of industry, medicine, and analytic purposes. The uricase exceed in blood leads to some disorders, most of which is goat resulting from the precipitation of uric acid in tissues. Thus using of uricase enzyme can be a resort to remedy or prevent the disease. The uricase gene has been reported from several bacterial species in addition to *B. subtilis*, including *Candida utilis*, *Pseudomonas aeruginosa* [10] and *Aspergillus flavus* [11]. Although there are many sources of bacterial uricase enzyme, several limitations have made it difficult to reach the desired purpose including low levels of the gene expression, lower stability and drawbacks in purification for commercial implications [12]. In this study, the uricase gene was obtained from a strain of *B. subtilis* producing uricase enzyme and cloned into three *E. coli* strains including BL21, TOP10 and DH5α. The result of cloning and expression of the uricase gene from *B. subtilis* into BL21 host strain was successful but in case of TOP10 strain, partially cloning was occurred, whereas it was not cloned into DH5α strain suggesting a limitation for the gene expression into these hosts. The gene was not expressed even by

the Bl21 host. A study by Shaaban depicted that the uricase gene of *P. aeruginosa* was successfully expressed in *E. coli* using pRSET-B expression vector, but they did not assessed that from *B. subtilis* [10]. Several studies have shown the production of uricase by *A. niger* and *Proteus vulgaris* in the presence of uric acid in media [13-15]. In a previous study, the uricase gene from *B. subtilis* was successfully cloned and purified 58% using a Ni-NTA column [16]. In a recent study, two mutants of the uricase encoding gene known as 6E9 and 8E279 were designed which showed 2.99 and 3.43 times higher catalytic efficiency, respectively. in addition, three amino acids including D44V, Q268R and K285Q were recognized as the three most beneficial amino acid substitutions [17]. Kotb demonstrated that *Bacillus subtilis*, the strain RNZ-79 had a high level expression of the uricase enzyme cultured in culture with shrimp shell (highest V_{max} (0.42 μM $mg^{-1}min^{-1}$) and lowest value of Km (56 μM), and Maximum productivity at pH 7.6 and 45°C with an inducer concentration of 0.4% (w/v)) especially using optimized conditions [18]. In this study, we could note the reason why TOP10 and DH5α were not suitable hosts for cloning and expression/production of uricase gene from *B. subtilis*. Thus more studies using *E. coli* or other bacterial or fungal strains as hosts are required to optimize better conditions for a higher level of uricase enzyme production.

Conclusion

In this study, the uricase gene from *B. subtilis* was successfully cloned into the BL21 strain, but two other strains of TOP10 and DH5α were not suitable for this purpose. However, we observed that the uricase gene could not be expressed by BL21 host.

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