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RESEARCH ARTICLE

Cloning and Expression of Thermostable β-Amylase Gene of *Thermoanaerobacterium thermosulfurogenes* in *Escherichia coli* and *Bacillus subtilis* BR151 [1]

Bahri Devrim ÖZCAN * Numan ÖZCAN *

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* Osmaniye Korkut Ata University, Faculty of Arts and Sciences, Department of Biology, TR-80000 Osmaniye - TURKEY
** Çukurova University, Faculty of Agriculture, Department of Animal Science, TR-01330 Adana - TURKEY

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Summary

DNA fragment encoding thermostable β-amylase gene from *Thermoanaerobacterium thermosulfurogenes* was amplified by PCR and then cloned into pBluescript II KS/SK, pBT10, pNW33N and pUB110 plasmids. Recombinant plasmids were designated as pBluescriptβ, pBT10β, pNW33Nβ and pUB110β respectively. pBluescriptβ, pBT10β and pNW33Nβ recombinant plasmids were transferred into *Escherichia coli,* and pUB110β was electrotransformed into *Bacillus subtilis* BR151. Insert and PCR analysis of recombinant plasmids from *E. coli* and *B. subtilis* confirmed the 1935 bp β-amylase gene fragment on agarose gel electrophoresis. On LB-starch-agar plates, all recombinant *E. coli* colonies showed positive zones with I2 staining. Although thermostable β-amylase gene was cloned in *B. subtilis* BR151, the enzyme activity was not detected on LB-starch-agar plate. But after renaturation of extracellular proteins from *B. subtilis* on SDS-Starch-PAGE, β-amylase enzyme regained enzymatic activity by zymogram technique and thereby confirmed that enzyme was not folding properly in *B. subtilis* host.

Keywords: *Thermoanaerobacterium thermosulfurogenes, Escherichia coli, Bacillus, β-amylase, Cloning*

Thermoanaerobacterium thermosulfurogenes'e Ait Sıcaklığa Dirençli β-Amilaz Geninin *Escherichia coli* ve *Bacillus subtilis* BR151'de Klonlanması ve Ekspresyonu

Özet

Thermoanaerobacterium thermosulfurogenes DNA'sından sıcaklığa dirençli β-amilaz genini kodlayan DNA parçası PCR ile amplifiye edilerek pBluescript II KS/SK, pBT10, pNW33N ve pUB110 plazmidlerine klonlanmıştır. Rekombinant plazmidler sırasıyla pBluescriptβ, pBT10β, pNW33Nβ ve pUB110β olarak isimlendirilmişlerdir. pBluescriptβ, pBT10β ve pNW33Nβ rekombinant plazmidleri *Escherichia coli,* pUB110β plazmidi ise *Bacillus subtilis* BR151 bakterisine transfer edilmişlerdir. *E. coli* ve *B. subtilis* bakterilerinden izole edilen rekombinant plazmidlerin insört ve PCR analizleri, agaroz jel elektroforezde 1935 bç'lik β-amilaz genini doğrulamıştır. LB-nişasta-agar plaklarında, tüm rekombinant *E. coli* kolonileri I2 boyaması ile pozitif zon vermişlerdir. Sıcaklığa dirençli β-amilaz geni *B. subtilis* BR151 bakterisinde klonlanmasına karşın, LB-nişasta-agar plağında enzimatik aktivite gözlenememiştir. *B. subtilis* kökenli hücre dışı proteinlerin renatürasyonundan sonra, SDS-Nişasta-PAGE'de β-amilaz enzimatik aktivitesini tekrar kazanmış ve bu sonuç *B. subtilis* konukçusunda enzimin uygun katlanmayı yapamadığı görüşünü desteklemiştir.

Anahtar sözcükler: *Thermoanaerobacterium thermosulfurogenes, Escherichia coli, Bacillus, β-amilaz, klonlama*

İletişim (Correspondence)
+90 328 8251818/2539
devrimozcan@osmaniye.edu.tr

INTRODUCTION

Amylases are hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units [1-4]. Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths [5-8]. Exoamylases act from the non-reducing end successively resulting in short end products [1,4,6-8].

β-Amylases (EC 3.2.1.2) are exo-hydrolyses which release β-maltose from non- reducing ends of α-1,4-linked poly- and oligoglucans until the first α-1,6-branching point along the substrate molecule is encountered [9,10]. β-Amylases are produced from higher plants such as barley, sweet potato, soybean, wheat and also from certain thermophilic and mesophilic bacteria such as *Thermoanaerobacterium thermosulfurogenes* [11], *Bacillus cereus* [12,13], *B. polymyxa* [14,15] and *B. megaterium* [16]. β-Amylase produce maltose in combination with thermostable α-amylase from starch [6,7].

To construct of new recombinant *E. coli* and *Bacillus* strains producing thermostable β-amylase for easy purification of the enzyme by heat treatment, we aimed to clone β-amylase gene of *T. thermosulfurogenes* into these microorganisms by using pBluescript II KS/SK, pBT10 and pNW33N for *E. coli*, and pUB110 for *Bacillus subtilis* as vectors.

MATERIAL and METHODS

Bacterial Strains, Plasmids, Media, and Culture Conditions

T. thermosufurogenes ATCC 33743 was supplied from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), *B. subtilis* BR151 ATCC 33677 (β-amylase$^-$, α-amylase$^-$) was supplied from *Bacillus* Genetic Stock Center (The Ohio State University, Colombus) and *E. coli* XL1 Blue MRF' was supplied from Stratagene. The cloning vectors pBluescript II KS/SK, pUB110 and pNW33N were supplied from Strategene, Sigma and *Bacillus* Genetic Stock Center respectively. The *E. coli*/*Streptococcus* shuttle cloning vector pBT10 was modified from pL2 (pBluescript + *Gene e* + replication origine of pTRW10) [17] by removing of T4 bacteriophage lysozyme gene (*Gene e*). LB-broth (10 g bacto tryptone (Merck), 5 g yeast extract (Merck) and 10 g NaCl (Merck) per l, pH 7.5) and LB-agar (1.5% wt/v agar) were used to grow *E. coli* and *Bacillus* strains [18]. *T. thermosulfurogenes* was grown in selective media (5.5 g dextrose, 5 g yeast extract, 2.5 g NaCl, 1.5 g casitone, 0.5 g thioglycolic acid, 0.5 g L (-) cystin, 1 ml 0.1% resazurin, 0.75 g agar per l, pH 7.7) at 60°C under anaerobic conditions. Transformants were selected on LB-agar plates supplemented with appropriate antibiotics.

Genomic and Plasmid DNA Isolation

Extraction of *T. thermosulfurogenes* chromosomal DNA was performed as described by [19]. pUB110 plasmid DNA was prepared as described by [20] from recombinant *B. subtilis* overnight culture supplemented with 20 µg/ml kanamycin. pBluescript II KS/SK, pBT10 and pNW33N plasmids were prepared as described by [21] from recombinant *E. coli* overnight cultures. Growth media supplemented with 50 µg/ml ampicillin for pBluescript II KS/SK and pBT10, and 25 µg/ml chloramphenicol for pNW33N.

Amplification of β-Amylase Gene and Ligation Procedures

The following oligonucleotide primers were used for amplification of β-amylase gene from *T. thermosulfurogenes*: forward primer 5'-GAAAGGATCCTAT AATAGCTGGTGCTGTAAAG-3' and reverse primer 5'-GTTT GGATCCCACCGGAATATATTCCAGTGT-3'. *Bam* HI restriction site was added to 5' ends of the primers. PCR reaction was performed with a DNA thermal cycler (Techne) with each primer (20 pmol) plus genomic DNA (2.2 µg) from *T. thermosulfurogenes* (94°C for 1 min, 58°C for 2 min, and 72°C for 3 min for 30 cycles). The reaction mixture contained 200 µM deoxynucleotide triphosphates, 1.25 U of Pfu DNA polymerase (to produce blunt ended products-Fermentas) and reaction buffer in a reaction volume of 50 µl. PCR product was purified from agarose gel with a PCR product purification kit (Fermentas). The cloning vector pBluescript II KS/SK was cleaved with *Sma* I to produce blunt ended products and the other vectors pNW33N, pBT10 and pUB110 were cleaved with *Bam* HI. Approximately 0.55 µg of pBluescript II KS/SK were ligated to amplified but undigested 0.2 µg β-amylase gene insert. 5% wt/v PEG was added to the reaction mixture. Approximately 0.5 µg of other vectors (pNW33N, pBT10 and pUB110) were ligated to *Bam* HI cleaved DNA fragment harbouring β-amylase gene extracted from pBluescriptβ vector.

Transformation and Electroporation Procedures

6 µl of each ligation mixtures of pBluescript II KS/SK, pNW33N and pBT10 were transformed into competent *E. coli* XL1 Blue MRF' as previously described by [22].

Competent cells from *B. subtilis* BR151 were prepared

as described by [23]. For electroporation 60 µl of the competent cells were mixed with 5 µl of ligation mixture of pUB110 and then transferred to an ice-cold electroporation cuvette (1 mm gap). After incubation for 2-3 min, the cells were exposed to a single electrical pulse using a Gene-Pulser (Invitrogen) set at 25 µF, 200 Ω, 1650 V and 25 mA resulting in time constant of 5 ms. Immediately following the electrical discharge, 1 ml of electroporation medium (LB containing 0.5 M sorbitol and 0.38 M mannitol) was added to the cells. After incubation at 37°C for 3 h the cells were plated on LB-agar containing 20 µg/ml kanamycin and incubated at 37°C for overnight. Transformants of *E. coli* and *B. subtilis* carrying recombinant plasmids were selected from agar plates containing appropriate antibiotics.

Restriction and PCR Analysis of Recombinant Plasmids

Recombinant plasmids pBluescriptβ, pNW33Nβ, pBT10β and pUB110β were isolated from transformants. To detect the β-amylase gene, all plasmids were cleaved with *Bam* HI restriction endonuclease and analysed by agarose gel (0.8% wt/v) electrophoresis. Furthermore, PCR reactions performed with all recombinant plasmids analysed by agarose gel (0.8% wt/v) electrophoresis.

Electrophoretic Analysis of Extracellular and Intracellular Proteins

To obtain the extracellular proteins of recombinant strains from LB-broth, the cells were pelleted by centrifuge. The extracellular extracts (supernatants) were mixed with 1:1 volume of 20% wt/v TCA. After the incubation at room temperature for overnight, protein patterns were obtained by centrifuge. Air-dried proteins were dissolved in 0.1 M Tris-HCL buffer (pH 8.0). To obtain the intracellular proteins of recombinant *E. coli* strain from LB-broth, the cells were pelleted by centrifuge and then the pellets were dissolved in equal volume of water. After the sonication process (50-60 kHz) the samples were centrifuged. The protein patterns were obtained from the supernatant using 20% wt/v TCA as described previously.

SDS-PAGE and SDS-Starch-PAGE (0.2% starch) were done as described by [24] with slab gels (12% wt/v acrylamide). After the electrophoresis, the gel was stained for 1 h with Coomassie blue R 250 dye in methanol-acetic acit–water solution (4:1:5, by volume) and destained in the same solution without dye [25,26]. For activity staining (zymogram analysis), SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 20% for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. Renaturation of enzyme proteins was carried out by keeping the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C. Gel was then transferred onto a glass plate, sealed with film, and incubated at 55-65°C for 4 h. Gel was stained in a solution of iodine (Iodine 5 g/l, KI 50 g/l), for 30 min, clear bands indicate the presence of amylase activity [27,28].

RESULTS

To construct pBluescriptβ plasmid vector, 1935 bp amplified blunt-ended β-amylase gene from *T. thermosulfurogenes* was transferred into *Sma* I cleaved pBluescript vector *(Fig. 1).* The β-amylase gene was re-extracted from pBluescriptβ by *Bam* HI and then ligated to *Bam* HI cleaved pBT10, pNW33N and pUB110 plasmids respectively. The recombinant plasmids pBluescriptβ, pBT10β and pNW33Nβ were transferred into *E. coli* but pUB110β was electrotransformed into *B. subtilis* BR151. pBluescriptβ and pBT10β carrying *E. coli* colonies were selected on LB-agar plates supplemented with ampicillin (50 g/L) and X-Gal (40 g/L), while *E. coli*/pNW33Nβ colonies were selected on LB-agar plates supplemented with chloramphenicol (25 g/L). On the other hand, pUB110β carrying *B. subtilis* colonies were selected on LB-agar plates supplemented with kanamycin (20 g/L).

To select recombinant *E. coli* cells bearing pBluescriptβ, white colonies grown on solid growth media were collected and transferred onto new LB-starch-agar plates containing ampicillin. Seven recombinant *E. coli* colonies carrying pBluescriptβ detected on LB-starch-agar plates by I_2 staining *(Fig. 2).*

Plasmid DNA from each recombinant clone were isolated. Digestion of recombinant plasmids by *Bam*

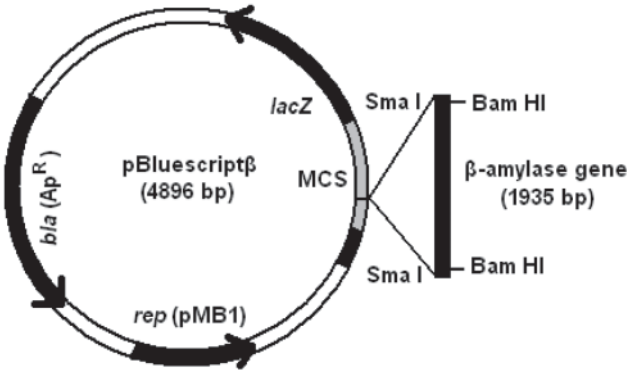

Fig 1. Structure of pBluescriptβ [pBluescript (2961 bp) plus β-amylase gene (1935 bp)]

Şekil 1. pBluescriptβ'nın yapısı [β-amilaz geni (1935 bç) taşıyan pBluescript (2961 bç)]

HI was showed the 1935 bp β-amylase gene insert and 2961 bp pBluescript plasmid DNA *(Fig. 3a)*.

Bam HI digested β-amylase gene insert was purified from 0.8% wt/v agarose gel and ligated to *Bam* HI cleaved pBT10, pNW33N and pUB110 plasmids. The ligation mixtures containing pBT10β and pNW33Nβ were transferred into *E. coli* XL1 Blue MRF' competent cells. Recombinant *E. coli*/pBT10β and *E. coli*/pNW33Nβ colonies showed clear zone by I_2 staining (data not shown). The ligation mixture containing pUB110β was transferred into *B. subtilis* BR151. Recombinant *B. subtilis* BR151β cells grew on LB-starch-agar plates supplemented with kanamycin but they did not produce β-amylase activity by I_2 staining. But insert and PCR analysis of pUB110β produced both 4548 bp pUB110 and 1935 bp β-amylase gene fragments *(Fig. 3b)*. The recombinant plasmids pBT10β and pNW33Nβ were isolated from *E. coli*. Both recombinant plasmids were also subjected to insert and PCR analysis on 0.8% wt/v agarose gel electrophoresis. Digestion of recombinant plasmids by *Bam* HI were showed the 1935 bp β-amylase gene inserts. β-Amylase gene fragments from PCR amplification were determined on agarose gel electrophoresis too (data not shown).

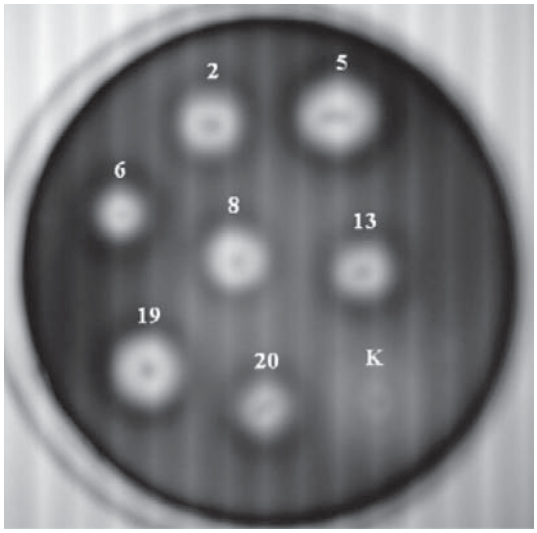

Fig 2 .β-Amylase positive (numbered) and negative control (K) *E. coli* colonies by I_2 staining

Şekil 2. I_2 boyaması ile β-amilaz pozitif (numaralandırılmış) ve negatif kontrol (K) *E. coli* kolonileri

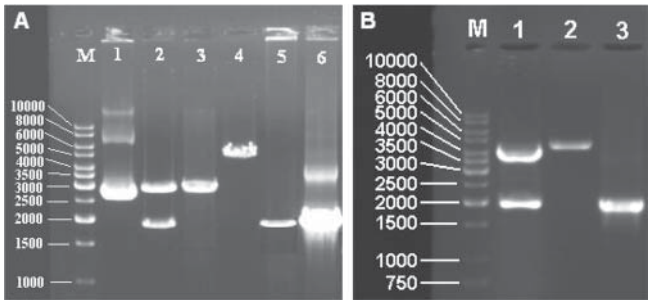

Fig 3. A) Insert and PCR analysis of pBluescriptβ on agarose gel (M: marker, 1: pBluescriptβ, 2: pBluescriptβ/*Bam* HI, 3: pBluescript/*Bam* HI, 4: pBluescriptβ/*Eco* RI, 5-6: PCR fragments of β-amylase gene (5: from *T. thermosulfurogenes*, 6: from pBluescriptβ). B) Restriction and PCR analysis of pUB110β from *B. subtilis* BR151β (M: Marker, 1: pUB110β/*Bam* HI, 2: pUB110/*Bam* HI, 3: PCR fragment of β-amylase gene from pUB110β)

Şekil 3. A) pBluescriptβ'nın agaroz jelde insört ve PCR analizleri (M: markır, 1: pBluescriptβ, 2: pBluescriptβ/*Bam* HI, 3: pBluescript/*Bam* HI, 4: pBluescriptβ/*Eco* RI, 5-6: β-Amilaz genine ait PCR fragmentleri (5: *T. thermosulfurogenes*'den, 6: pBluescriptβ'dan). B) *B. subtilis* BR151β'dan izole edilen pUB110β'nın restriksiyon ve PCR analizleri (M: Markır, 1: pUB110β/*Bam* HI, 2: pUB110/*Bam* HI, 3: pUB110β'dan izole edilen β-amilaz genine ait PCR fragmenti)

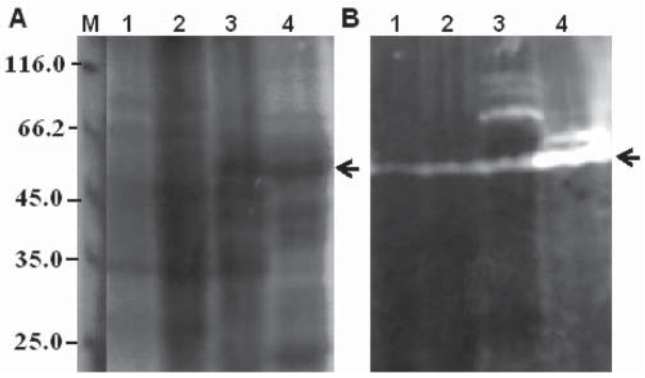

Fig 4. Comparison of total proteins by Coomassie blue staining of SDS-PAGE (A) and β-amylase bands by I_2 staining of SDS-Starch-PAGE (B) (M: Marker, 1: Extracellular proteins of *E. coli*/pBluescriptβ, 2: Intracellular proteins of *E. coli*/pBluescriptβ, 3-4: Extracellular proteins of *B. subtilis* BR151β and *T. thermosulfurogenes* respectively)

Şekil 4. Toplam proteinlerin Coomassie blue boyaması ile SDS-PAGE'de (A) ve β-amilaz bantlarının I_2 boyaması ile SDS-Nişasta-PAGE'de karşılaştırılması. (M: Markır, 1: *E. coli*/pBluescriptβ hücre dışı proteinleri, 2: *E. coli*/pBluescriptβ hücre içi proteinleri, 3-4: *B. subtilis* BR151β ve *T. thermosulfurogenes* hücre dışı proteinleri)

Extracellular proteins of *B. subtilis* BR151β and *T. thermosulfurogenes*, and extracellular and intracellular proteins of *E. coli*/pBluescriptβ were compared on SDS-PAGE including 2% starch by Coomassie blue staining *(Fig. 4a)*. On zymogram analysis, 57 kDa size β-amylase band of *T. thermosulfurogenes* was showed a clear zone with counterparts from all other recombinant *E. coli* and *B. subtilis* strains *(Fig. 4b)*.

DISCUSSION

Thermostable β-amylase gene of *T. thermosulfurogenes* was cloned, expressed and sequenced in *B. subtilis* previously [29]. In this study, we have cloned this gene using PCR technique instead of shut-gun cloning for expression. *Bam* HI endonuclease sequences located end of the primers to cleave the gene from vectors

enzymatically. Pfu DNA polymerase was used because of its proofreading activity and to produce blunt ended products for direct ligation. A 1935 bp PCR product carrying β-amylase gene from *T. thermoslufurogenes* chromosome was ligated to blunt ended pBluescript II KS/SK. The *Bam* HI digested fragments coding β-amylase gene from pBluescriptβ re-ligated to *Bam* HI cleaved pBT10, pNW33N and pUB110 respectively. The new recombinant vectors harbouring β-amylase gene were designated as pBT10β, pNW33Nβ and pUB110β. pBT10β and pNW33Nβ were introduced into *E. coli* by transformation, and pUB110β was introduced only into *B. subtilis* BR151 by electroporation.

B. polymyxa and *B. cereus* β-amylase genes were cloned in *E. coli* previously [13-15]. But *T. thermosulfurogenes* β-amylase gene was cloned in *E. coli* for the first time with this study. On the other hand, *T. thermosulfurogenes* β-amylase gene was cloned in *B. subtilis* 1A289 strain previously [29]. We also cloned the same gene in *B. subtilis* BR151 strain with this study too. The main aims of this study were enhanced production of thermostable β-amylase by multi-copying plasmids, and also to produce the thermostable enzyme in mesophilic *B. subtilis* BR151 strain therefore purify the enzyme by heat treatment easily.

Reddy et al.[30] have reported that various surfactants stimulated the enzyme production by *T. thermosulfurogenes* SV2. Among these surfactants, Triton X-100, CHAPS, Tween-80 and sodium taurocholate were increased 140, 34, 88 and 28% more β-amylase than the control (lacking surfactants) respectively. Nanmori et al.[31] have isolated a mutant *B. cereus* strain excreting about 40-fold more β-amylase than does the wild-type strain, after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It was reported by the same researchers that, the amylase production of this strain was observed to increase in the presence of 0.5% glucose or 1% maltose and, more markedly, in the presence of 2% soluble starch in the culture medium. In another study, Nipkow et al.[32] have reported that β-amylase concentration reached 90 U/ml at a dilution rate of 0.07/ h^{-1} in a 3% starch medium by *T. thermosulfurogenes* mutant strain. We also intented to construct an industrial strain producing thermostable β-amylase in *B. subtilis* BR151. Although restriction and PCR analysis showed the β-amylase gene was cloned into *B. subtilis* BR151, no amylolytic activity was observed on LB-starch-agar plates with I_2 staining. Several reasons were thought that; firstly the promotor sequences of the gene could not idendified by *Bacillus*, secondly produced enzyme might be denaturated by host proteolytic activity or a folding problem could be occured in host *Bacillus* cells.

It was reported previously that the enzymes expressed by cloned genes could be exposed to proteolytic activity by proteases presented at cell wall during folding stage [33-35]. But revealing of β-amylase activity bands on zymogram analysis (SDS-Starch-PAGE) by I_2 staining after renaturation of proteins by removing of SDS and other denaturing agents was seemed that enzymes were expressed in host, but could not fold properly. Because three dimensional structure by appropriate folding is required for enzyme function [36,37]. Cloning and experssion of thermostable β-amylase gene of *T. thermosulfurogenes* in *B. subtilis* 1A289 which has low proteolytic activity in previous study [29,38] considered us that *B. subtilis* BR151 strain used in this study may be unsuitable host because of both folding and proteolytic activity features. In future studies, it is thought to clone the β-amylase gene into lactic acid bacteria by pBT10β for starch industry and *B. stearothermophilus* to be produced both thermostable α- and β-amylases by pNW33Nβ.

The results of our investigation showed that DNA fragment encoding thermostable β-amylase gene from *Thermoanaerobacterium thermosulfurogenes* was amplified by PCR and then cloned into pBluescript II KS/SK, pBT10, pNW33N and pUB110 plasmids. pBluescriptβ, pBT10β and pNW33Nβ recombinant plasmids were transferred into *Escherichia coli*, and pUB110β was electrotransformed into *Bacillus subtilis* BR151. On LB-starch-agar plates, all recombinant *E. coli* colonies showed positive zones with I_2 staining. Although thermostable β-amylase gene was cloned in *B. subtilis* BR151, the enzyme activity was not detected on LB-starch-agar plate. But activity band on SDS-Starch-PAGE showed that β-amylase enzyme was not folding properly in *B. subtilis* host and then did not show positive zones on LB-starch-agar plates.

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