Simultaneous production of alpha and beta amylase enzymes using separate gene bearing recombinant vectors in the same Escherichia coli cells

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Abstract: The present study describes the simultaneous expression of thermostable industrial alpha (α) and beta (β) amylase enzymes that have been used widely in starch industry. Genomic DNA of Bacillus stearothermophilus DSM 22 strain for α amylase and, Thermoanaerobacterium (Clostridium) thermosulfurogenes DSM 2229 strain for β amylase were used as gene sources. Both genes were ligated into pETDuet-1 expression vector separately and resulting recombinant vectors were transformed into Escherichia coli BL21 competent cells by electroporation. The cells were first transformed by pETDuet-1/ αAmy recombinant plasmid, then the competent cells carrying this plasmid were prepared for the transformation of pETDuet-1/ βAmy plasmid. Enzymatic activities of bacterial colonies were detected on LB agar staining with iodide. Both enzymes were more produced by IPTG induction in BL21 cells and were purified using Ni-NTA agarose column. SDS-PAGE and western blot analyses showed that the molecular weight of purified α and β amylase to be approximately 60 kDa and 55kDa, respectively. The concentration of the purified α and β amylase were calculated as 4.59 μg/mL and 3.17 μg/mL with IPTG as an inducer in LB medium. The present study proposes a novel and efficient method for the production of thermostable α and β amylases at the same E coli cells containing separate engineered plasmid vectors.

Key words: Alpha amylase, beta amylase, dual gene expression, purification of recombinant enzyme

1. Introduction
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low activity at temperatures below the growth conditions of the organisms. Due to their overall natural features, the thermal denaturation of host cell culture ensures separation from mesophilic enzymes resulting high level and stable enzyme that suitable for industrial processes. Moreover, these enzymes are also resistant to proteolysis of the host cell (Bertoldo and Antranikian, 2002).

Alpha amylase enzyme, is the first enzyme used commercially (Radley, 1976). Alpha amylase, an endoenzyme, splits starch polymers (chain) into macromolecular dextrins and maltose by hydrolyzing α-1,4 bonds at random points. With its effect on amylose, viscosity decreases quickly, and starch's property of rendering blue color with iodine disappears. The effect of this enzyme on the amylopectin molecule is similar to amylase, however, in this molecule, fragmentation does not take place in the α-1,6 bonds with branching (Reed, 1966). The field of application of thermostable Bacillus α amylase has been greatly extended and diversified. It has been used in paper industry to liquefy the starch, to produce glucose and fructose syrups and glue and in the fermentation of alcohol (Kiran et al., 2005).
Betamylase, an exoenzyme, enables the formation of maltose units with two glucose molecules starting from the nonactive lead of chain by watering α-1,4 link of starch molecule. Enzyme does not hydrolyze amylase and amylopectin at random points but it follows a regular order (Ertugay, 2010). The most important feature of β amylase enzyme is that it is not activated at high temperatures. It was determined that Clostridium thermosulfurogenes produce a thermo-amylase enzyme β. Beta amylase enzymes are used in maltose production and food and beverage industry. The resulting high purity maltose syrup is used in production of jam, confectionery, bread and beer, also as a sweetener in food (Tatar, 2007).

Like other polymers, starch molecules need combination of amylase enzymes to be completely hydrolyzed, and when α and β amylase enzymes are used together, they split starch better compared to their separate use (Haki and Rakshit, 2003; Ertugay, 2010). Simultaneous expression of two or more enzymes in one host has the advantages of avoiding repeated fermentations, reduces the extracting and purifying works, and improves the cost-effectiveness of the processes (He et al., 2014). Therefore, α and β amylase enzymes are used together in food, textile, detergent, and adhesive industries (Liu et al., 2003). In this study, molecular cloning and expression of α and β amylase genes were investigated with a special emphasis on recovery and partial molecular characterization of purified α and β amylases.

2. Materials and methods
2.1. Materials
The genomic DNA of Bacillus stearothermophilus (Geobacillus stearothermophilus) DSM 22 strain and Thermoanaerobacterium thermosulfurogenes (Clostridium thermosulfurogenes) DSM 2229 strain were commercially obtained from Leibniz Institute DSMZ firm (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) for α amylase (α Amy) and β amylase (β Amy) enzymes and genes, respectively.

2.2. Primer design
The PCR amplification of full-length α Amy and β Amy genes, primers (A-SacI-F-5'-AAATGCATTCCGCTGCTCAGTTC-3', A-HindIII-R-5'--GGAATTCACCCCGTGGCTG-3'), (B-BamHI-I-F-5'-'GAGCTCAGATTTGTCTCAG-3', B-XhoI-R-5'--GAGCTCAGATTTGTCTCAG-3') were designed using published sequence from the genomic database of B. stearothermophilus and T. thermosulfurogenes. Access number for α Amy gene: M57457 and for β Amy gene: M22471). The primers were compatible with the endonuclease restriction sites (SacI, HindIII, BamHI, and XhoI) of pETDuet-1 expression vector (Novagen, Merck KGaA, Darmstadt, Germany). To each primer, four additional unrelated nucleotides (shown in italics) were added at their 5’end. For the amplification of both genes, a final volume of 50 µL PCR mixture contained: 2 µL of DNA, 5 µL of 10× reaction buffer (200mM Tris–HCl pH: 8.4, 500mM KCl), 3 µL of MgCl₂ (25 mM), 1 µL of dNTPs (10mM each), 1 µL of each primer (100 pmol), 0.4 µL of GoTag G2 Hot Start DNA polymerase and 36.6 µL of DNase free sterile water. The complete α Amy gene was amplified by PCR with the following thermal cycling scheme: 2 min at 95 °C, 30 cycles of 1 min at 94 °C, 1.5 min at 55 °C, and 2 min at 72 °C followed by a final extension at 72 °C for 10 min. The complete β Amy gene was generated by the following thermal cycling scheme: 2 min at 94 °C, 30 cycles of 20 sec at 94 °C, 20 sec at 55 °C, and 2 min at 72 °C followed by a final extension at 72 °C for 3 min. The PCR products were separated on 1% agarose gel and recovered by gel extraction kit (ThermoGeneJET Gel Extraction Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Construction of plasmids for the production and purification of recombinant His-tagged α and β amylase
The amylase gene sequences were cloned into the SacI and HindIII site (for α Amy gene) and BamHI and XhoI site (for β Amy gene) of pETDuet-1 vector containing 6xHis-Tag coding sequence according to the protocol recommended by the manufacturer. The recombinant vectors were then transformed into competent cells of E. coli BL21(DE3)pLysS cells by Gene Pulser® apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA), respectively. Then, they were plated on a LB agar containing ampicillin (100mg/mL). Positive clones were identified by colony-PCR, sequence analysis or by restriction endonucleases. Positively identified clones were inoculated into 10 mL of LB broth containing ampicillin and were grown to OD₆₀₀ 0.6. Ihe bacterial growth was induced by adding isopropyl β-D thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM followed by an incubation with constant shaking at 37 °C for 18h. The total broth media were centrifuged and bacterial cells were pelleted and resuspended with water containing Complete™ Mini EDTA-free tablet to inhibit proteolytic activity of a broad range of proteases (Roche Diagnostics International AG, Rotkreuz, ZG, Switzerland). After adding 0.5 mL of TrisHCl (pH7.5), 1 mL of NP40 (% 10), 25 µL of MgCl₂ (1M), 7 µL of 2-mercaptoethanol and 20 µL of DNase I (10 U/µL), the suspension was sonicated four times (3–5 s) in ice, then, the mixture was incubated at 4 °C for 45 min on a shaker. After adding 0.3g of NaCl (final concentration of 0.5 M) the protein extract was ultracentrifuged at 30.000 rpm for 30 min (at 4 °C). Recombinant His-tagged α Amy and β Amy proteins were purified by chromatography on a Ni²⁺-NTA agarose resin column. After washing the column with TL buffer (2.5 mL 1M Tris-HCl pH 7.5, 1.46g
NaCl, 5 mL %10 NP40, 35 µL 2-mercaptoethanol, 1mL 1M imidazole pH 8.0) the proteins was eluted with TE buffer (50 % of imidazole and 50 % of TL buffer). The protein concentrations were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.4. Preparation of competent cells and transformation

A single bacterial colony was picked up from the plate incubated at 37 °C for overnight and transferred into 10 mL of LB medium overnight at 37 °C with shaking. The culture than added into 1000 mL of LB medium for 4 to 5 h at 37 °C with shaking at 250 rpm until reach the OD₆₀₀ 0.6. The culture centrifuged two times with ice-cold centrifuge tubes. The pellet was resuspended with 4 mL of chilled 0.8 % glycerine and aliquoted 100 µl of each tube. Recombinant pETDuet-1/αAmy plasmid was transferred to competent cells. Bacterial cells bearing pETDuet-1/αAmy plasmids were then served for the preparation of competent cell for transformation of β Amy containing recombinant plasmids. Transformation was made by electroporation using a Gene Pulser® apparatus (Bio-Rad Laboratories, Inc.).

2.5. SDS-PAGE and western blot analysis and starch–iodine assay for extracellular amylase production

Levels of protein expression of recombinant BL21/αAmy and BL21/βAmy were analyzed by 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) along with molecular weight markers. The purified enzymes were loaded on PAGE gel than stained by coomassie blue as described by Laemmli (1970). Western blot analysis were carried out essentially according to Sambrook et al. (1989) using semidry blotting system. Purified proteins were transferred to PVDF-plus membrane by electro-blotting. Monoclonal antibody recognizing the His-tag residue was used to capture α and β amylases. Molecular weight of enzymes were estimated from a calibration curve of Log₁₀ molecular weight of standard proteins. The amylolytic activity of transformed bacteria was determined by the method described by Cowan (1991), using SW-10 medium supplemented with 0.5% (w/v) soluble starch. The plates were than filled with 0.3 % I₂ and 0.6 % KI solution and incubated at 37 °C for one week to observe a clear zone around the bacterial growth.

3. Results

3.1. Cloning and expression of thermostable α Amy and β Amy genes

A full length α Amy and β Amy gene fragments of 1699 bp and 1675 bp were amplified from genomic DNA of B. stearothermophilus DSM 22 strain and T. thermosulfurogenes DSM 2229 strain, respectively, using gene specific primers. The cloning of α Amy and β Amy genes into pETDuet-1 expression vector resulted the secretion of His-tagged recombinant enzymes into the periplasmic space and subsequently into culture medium. After induction with IPTG, the both recombinant enzymes were efficiently secreted. Under the standard assay conditions, the concentration of α and β amylase enzymes were measured as 4.59 µg/mL and 3.17 µg/mL, respectively. As seen in Figures 1A and 1B, the extracellular location of the enzymes are clearly evident form transformed bacteria that secrete α and β amylases (zigzags). The enzymes hydrolyzed the starch, creating a clear zone in which the iodine causes blue coloration of starchy medium. Iodine then degraded by secreted α and β amylase enzymes.

3.2. Analysis of α and β amylases expressed in BL21/αAmy bacteria by SDS-PAGE

The α Amy and β Amy genes were under control of the T7 promoter in pETDuet-1 expression vector. For the induction of high expression, isoprpyl β-D-1-thiogalacpyranoside (IPTG) was used. The induced bacterial cells were used for affinity purification on Ni-NTA agarose. Levels of protein expression of recombinant BL21/αAmy and BL21/βAmy bacteria were analyzed by 10% SDS-PAGE (Figure 2). Recombinant α and β amylases showed a molecular mass of ca. 60 kDa and 55kDa on SDS-PAGE, respectively.

3.3. Western blot analysis

His-tag specific monoclonal antibodies captured α and β amylases resulting signals in western blot analyses. Signals were observed at a molecular weight of 60 and 55kDa confirming the presence of His-tagged recombinant proteins of α and β amylases, respectively (Figure 3).

4. Discussion

As an expression host, E. coli has been widely used for the production of numerous industrial enzymes (Lin and Hsu, 1997; Lo et al., 2001; Shiina et al., 2007; Yamabhai et al., 2008). Generally, the enzymes are recovered from periplasmic space or cultivation medium (Shiina et al., 2007; Yamabhai et al., 2008). In the present study, transformants containing multiple copies of α and β amylase genes were obtained by electrotransformation of β amylase gene carrying plasmids to the α amylase gene bearing cells. The bacterial cells were then screened on plates for high-level expression of α and β amylase enzymes. In general, both enzymes have similar biochemical properties. Therefore, we firstly expressed a amylase in E. coli cells than these cells were served to prepare competent cells for the transformation of β amylase gene carrying plasmids in order to coexpress the two enzymes. Thus, the industrially advantageous of coexpression of both enzymes has been shown in the same host. The simultaneous expression of two or more enzymes in the same host has been reported by several researchers (Su et al., 1993; Li et al., 2011; He et al., 2014). He et al. (2014) demonstrated a significant improvement in amylase activity through coexpressing RpGla with RpAmy in Pichia pastoris.
Figure 1. Testing of recombinant BL21/αAmy (A) and BL21/βAmy (B) bacteria with iodine staining developed in environment containing starch. Clear zones along zigzags indicate starch hydrolysis.

Figure 2. 10% SDS-PAGE analysis of purified α (A) and β (B) amylase. Panel (A): Lane 1, 2, and 3 IPTG induced bacterial cells, Lane 4, 5, and 6 uninduced transformed cells, Lane 7 and 8 untransformed control cells. Panel (B): Lane 1 and 2 induced cells, Lane 3 untransformed control cells. M: Prestained protein molecular weight markers.
The correlation between starch hydrolysis zone and the relative enzyme expression rates has been shown in Figure 1. According to the hydrolysis result, starch digestion assay indicated that α and β amylases rapidly hydrolyzed the starch and created a clear zone on starch containing medium. The larger clear zones may be due to the high copy number of α amylase bearing plasmids and high level expression of amylases in E. coli. This finding coincides with the literature reported by Su et al. (1993). In the similar experiment carried out in Zymomonas mobilis, the larger zones of clearing produced by an enzyme indicate a high level of enzyme production.

Based on western blot analysis, the purified recombinant α and β amylases showed two single band corresponding to MW 60 kDa and 55 kDa, respectively. The analysis confirmed that the both fusion proteins were correctly expressed. Molecular analysis showed that both proteins have molecular weights consistent with their monomeric native structure. In previous studies carried out by Gandhi et al. (2015) and Özcan et al. (2001), the molecular mass of the recombinant α amylase from Geobacillus stearothermophilus was calculated as 59 kDa and 65 kDa from Bacillus subtilis, respectively. The molecular mass of the recombinant β amylase from barley calculated as 60 kDa by Ziegler (1999). There has been a high variation in molecular mass of α and β amylases which varies in the range of 10–210 kDa (Gupta et al. 2003).

We purified two different recombinant amylases which cosecreted from same bacterial cells in varying amounts.
However, α amylase molecule having higher molecular weight was seen predominant over the β amylase. The concentration of purified α amylase was approximately 20% higher than β amylase. This may be due to low copy numbers of β amylase bearing plasmids in *E. coli* cells. In a previous study, Castro et al. (1992) reported a simultaneous production of bacterial α and β amylases with a continuous culture technique in separate hosts. However, novel approaches are needed to obtain high level of expression in the same host from the cloned genes. In the present study, we developed an efficient expression system for the heterologous expression of recombinant α and β amylase genes using T7 promoter-based pET vector at the same cells. *E. coli* BL21(DE3)pLysS cells carrying α and β amylase bearing plasmids simultaneously produces recombinant α and β amylase when growing in LB culture medium. The enzyme concentrations were comparable with those reported enzymes produced alone. The present study offers a promising method to prepare a high yield of α and β amylase enzymes. To our knowledge, the present study is the first report on the simultaneous expressing of α and β amylase enzymes using separate expression vectors in the same host.

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**References**

Bertoldo C, Antranikian G (2002). Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Current Opinion in Chemical Biology 6: 151-160.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Castro GR, Ferrero MA, Abate CM, Méndez S, Sifferiz F (1992). Simultaneous production of alpha and beta amylases by *Bacillus subtilis* MIR-5 in batch and continuous culture. Biotechnology Letters 14: 49-54.

Cowan DA (1991). Industrial enzymes. In: Moses V, Cape RE (editors). Biotechnology, the Science and the Business. Amsterdam, Netherlands: Harwood Academic Publishers, pp. 311-340.

Çukurova University, Adana, Turkey.

Castro GR, Ferrero MA, Abate CM, Méndez S, Sifferiz F (1992). Simultaneous production of alpha and beta amylases by *Bacillus subtilis* MIR-5 in batch and continuous culture. Biotechnology Letters 14: 49-54.

Cowan DA (1991). Industrial enzymes. In: Moses V, Cape RE (editors). Biotechnology, the Science and the Business. Amsterdam, Netherlands: Harwood Academic Publishers, pp. 311-340.

Çukurova University, Adana, Turkey.

Ertugay Z (2010). Buğdayda amilolitik aktivite ve unların alfa amilaz koncentrasyonu. Atatürk Üniversitesi Ziraat Fakültesi Dergisi: 173-180 (in Turkish). Available online at https://dergipark.org.tr/tr/pub/ataunizfd/issue/2994/41533

Gandhi S, Salleh AB, Rahman R, Leow TC, Oslan SN (2015). Expression and characterization of *Geobacillus stearothermophilus* SR74 recombinant α-amylase in *Pichia pastoris*. BioMed Research International 2015: 1-9.

Gözükara F (2009). Isolation of thermophilic *Bacillus* sp., production, characterization and determination of technological application of lichenase (β-1,3 and 1,4 glucanase). MSc, Çukurova University, Adana, Turkey.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-amylases: a biotechnological perspective. Process Biochemistry 38: 1599–1616.

Haki GD, Rakshit SK (2003). Developments in industrially important thermostable enzymes: A Review. Bioresource Technology 89: 17-34.

He Z, Zhang L, Mao Y, Gu J, Pan Q et al. (2014). Cloning of a novel thermostable glucoamylase from thermophilic fungus *Rhizomucor pusillus* and high-level co-expression with α-amylase in *Pichia pastoris*. BMC Biotechnology 14 (1): 114.

Kiran Ö, Cömlekçıoğlu U, Arıkan B (2005). Effects of carbon sources and various chemicals on the production of a novel amylase from a thermophilic *Bacillus* sp. K-12. Turkish Journal of Biology 29: 99-103.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680-685.

Li S, Zuo ZR, Niu D, Singh S, Permaul K et al. (2011). Gene cloning, heterologous expression, and characterization of a high maltose-producing alpha-amylase of Rhizopus oryzae. Applied Biochemistry and Biotechnology 164: 581-592.

Lin LL, Hsu WH (1997). Lactose-induced expression of *Bacillus* sp. TS-23 amylase gene in *Escherichia coli* regulated by a T7 promoter. Letters in Applied Microbiology 24: 365-368.

Liu HL, Chen WJ, Chou SN (2003). Mechanisms of aggregation of α and β amylases in aqueous dispersions. Colloids and Surfaces B: Biointerfaces 28 (2-3): 215-225.

Lo HF, Lin LL, Li CC, Hsu WH, Chang CT (2001). The N-terminal signal sequence and the last 98 amino acids are not essential for the secretion of *Bacillus* sp. TS-23 amylase in *Escherichia coli*. Current Microbiology 43: 170-175.

Özcan N, Altunalan A, Ekinci MS (2001). Molecular cloning of an α-amylase gene from *Bacillus subtilis* RSKK246 and its expression in *Escherichia coli* and in *Bacillus subtilis*. Turkish Journal of Veterinary and Animal Sciences 25: 197-201.

Radley JA (1976). Production of microbial amylolytic enzymes: Starch Production Technology. In: Underkofler LA (editor). England: Applied Science Publisher Ltd., pp. 295-309.

Rajagopalan G, Krishnan C (2008). α-Amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresource Technology 99: 3044-3050.

Reddy NS, Nimmagadda A, Sambasiva Rao KRS (2003). An overview of the microbial α-amylase family. African Journal of Biotechnology 2: 645-648.
Reed G (1966). Enzymes in Food Processing. Amsterdam, Netherlands: Elsevier.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press, pp. 633-1636.

Shiina S, Ohshima T, Sato M (2007). Extracellular production of amylase during fed-batch cultivation of recombinant Escherichia coli using pulsed electric field. Journal of Electrostatics 65: 30-36.

Su P, Liu CQ, Lucas RJ, Delaney SF, Dunn NW (1993). Simultaneous expression of genes encoding endoglucanase and beta-glucosidase in Zymomonas mobilis. Biotechnology Letters 15: 979-984.

Tatar S (2007). Amylase enzyme production and investigation of possibilities in industrial usage from thermophile moderately halophilic Bacillus sp. MSc, Çukurova University, Adana, Turkey.

Yamabhai M, Emrat S, Sukasem S et al. (2008). Secretion of recombinant Bacillus hydrolytic enzymes using Escherichia coli expression systems. The Journal of Biotechnology 133: 50-57.

Zafar A, Aftab MN, Iqbal I et al. (2019). Pilot-scale production of a highly thermostable α-amylase enzyme from Thermotoga petrophila cloned into E. coli and its application as a desizer in textile industry. RSC Advances 9: 984-992.