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Cloning and overexpression of raw starch digesting α-amylase gene from *Bacillus subtilis* strain AS01a in *Escherichia coli* and application of the purified recombinant α-amylase (AmyBS-I) in raw starch digestion and baking industry

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ABSTRACT

Considering the economic and industrial relevance of α-amylases used in food and starch industries, a raw starch digesting α-amylase gene (*amyBS-I*) from *Bacillus subtilis* strain AS01a was cloned and expressed in *Escherichia coli* BL21 cells. The gene also includes its signal peptide sequence (SPS) for facilitating the efficient extracellular expression of recombinant α-amylase (AmyBS-I) in correctly folded (enzymatically active) form. The native AmyBS-I consists of 659 amino acids with a molecular mass and p*I* of 72,387 Da and 5.8, respectively. The extracellular secretion of AmyBS-I after response surface optimization of culture conditions was found to be 7-fold higher as compared to its production under non-optimized conditions. Purified AmyBS-I demonstrated optimum activity at 70 °C and pH 6.0. It shows K_m and V_{max} values toward soluble starch as 2.7 mg/ml and 454 U/ml, respectively. Further, it does not require Ca^{2+} ion for its α-amylase activity/thermo-stability, which is an added advantage for its use in the starch industry. The AmyBS-I also hydrolyzed a wide variety of raw starches and produced maltose and glucose as main hydrolyzed products. The bread dough supplemented with AmyBS-I showed better amelioration of the bread quality as compared to the bread supplemented with commercial α-amylase.

1. Introduction

Alpha-amylases (1,4-α-D-glucan glucanohydrolase, [E.C. 3.2.1.1]) are extracellular starch hydrolytic enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. This results in the formation of soluble maltodextrins, maltose, and glucose as end products of starch hydrolysis. Alpha-amylases comprise 30% of world's enzyme market [1,2] and are applied in many industrial processes such as starch liquefaction, textile, paper, brewing, baking, detergent, distilling industries, preparation of digestive aids, production of cakes, fruit juices, starch syrups, and pharmaceuticals [3]. Amylases, which show optimum activity in acidic pH, are primarily used in glucose syrup and baking industries, whereas those showing activities at alkaline pH have found applications in laundry detergent formulations [4].

Since starch is the second most abundant source of carbon and energy, therefore, a worldwide interest has been engrossed to use this economic carbon source in food processing industry to produce valuable products, like glucose, fructose and maltose syrups [5]. In addition, starch may also be converted to bio-ethanol [5]. However, the conventional way of starch processing requires a high input of energy, which in turn escalates the price of starch-based products [6]. Therefore, considerable efforts have been made to produce raw starch-digesting amylases capable of acting at acidic pH and at a moderate temperature much below the gelatinization temperature, which would be economical for the starch processing industries [6,7].

Recently we have reported the purification, characterization, and industrial application of an alkaline α-amylase from a high titer α-amylase producing *Bacillus subtilis* strain AS01a isolated from the soil sample of Assam, India [2]. In the present study, an attempt has been made to clone an α-amylase gene from the above strain and its expression in a mesophilic host (*Escherichia coli*). Interestingly, this recombinant enzyme (AmyBS-I) was found to have distinct properties from the previously reported alkaline α-amylase from the same bacterium [2]. However, the expression of heterologous proteins in

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E. coli has some limitations [8]. For example, most of the recombinant proteins synthesized in the cytoplasm of *E. coli* are not secreted out of the cell because of the complex arrangement of cell wall [8]. Moreover, it occasionally accumulates as 'inclusion bodies' in the cytoplasm which does not show any enzymatic or biological activity and become lethal to the cell [9]. From an industrial perspective, extracellular secretion of significant amount of recombinant protein in its enzymatically active form is highly appreciable for ease of downstream processing as well as application of this enzyme [8].

Recovery of biological active form of recombinant protein from inclusion bodies is a complicated and costly process. Therefore, various strategies have been developed for the periplasmic and extracellular production of recombinant proteins in *E. coli* [10]. Several prokaryotic SPS, including PelB, OmpA, PhoA, endoxylanase, and StII [10] have been used for the efficient, extracellular production of heterologous recombinant proteins in *E. coli*. The present study shows the efficient, extracellular expression of an α-amylase gene that contains its SPS from *B. subtilis* AS01a in *E. coli* [2]. Furthermore, the secretion of the recombinant enzyme was enhanced through response surface optimization of production conditions. Here we also put forth the purification, biochemical characterization and raw starch digestion potential of this recombinant enzyme (AmyBS-I) as well as its application in baking industry.

2. Materials and methods

2.1. Bacterial strains, chemicals and reagents

The genomic DNA of *B. subtilis* strain AS01a was isolated and purified by using GeneJET genomic DNA purification kit procured from Fermentas (USA). *E. coli* strains, DH5α was used for transformation studies while BL21 (DE3) (Novagen, Inc., CA, USA) was used for the over expression studies. The TA cloning vector (Fermentas, USA) and pET28a (Invitrogen, CA, USA) were used for cloning and expression of the amylase gene, respectively. The gel extraction kits, restriction enzymes, T4 DNA ligase, and DNA polymerase were procured from Fermentas (USA). All other molecular biology grade chemicals used in the present study were procured from either Merck (USA) or Hi-media (India).

2.2. Cloning of α-amylase gene in pTZ57R/T and pET 28a vectors

Cloning of the amylase gene from *B. subtilis* strain AS01a was carried out as illustrated in Fig. 1. Briefly, based on the available data of α-amylase gene sequence from *B. subtilis* in NCBI database (www.ncbi.nlm.nih.gov), the α-amylase gene sequence of *B. subtilis* BF7658 (Genbank accession No. FJ463162) was retrieved randomly from NCBI database for designing primers. Using the above retrieved sequence and freely available CloneAssistant 1.0 software (www.bis.zju.edu.cn/clone), the forward (5′-CCCAAGCTTTTGCGCTTACAGCA CCGTCGATCAA-3′) and reverse (5′-CGCGGATCCTTGAAAGAATGTGTTACACCT-3′) primers were designed to amplify α-amylase gene sequence (*amyBS-I*) from the genomic DNA of *B. subtilis* strain AS01a [2]. The amplified product (1.5 kb) so obtained was then inserted into a pTZ57R/T vector using InsTAclone PCR Cloning Kit (Fermentas, USA), following the instruction of the manufacturer. The recombinant vector was then transformed into *E. coli* DH5α competent cells. However, after sequencing of recombinant vector, the α-amylase gene was found to be incomplete. Therefore, the entire open reading frame (ORF) including SPS of the α-amylase of highest similar strain was taken to design the new set of primers.

A unique *Hind* III restriction site (indicated in bold) was introduced in the forward primer (5′-CCC**AAGCTT**CTATGTTT-GCAAAACGATTCAA-3′) whereas *Xho* I restriction site (indicated in bold) was inserted in the reverse primer (5′-CCG**CTCGAG** CTCAATGGGGAAGAGAACC-3′). These primers were used to amplify the complete ORF of the α-amylase gene from *B. subtilis* strain AS01a. The PCR amplified product (∼2 kb) was then double digested with the *Hind* III and *Xho* I and inserted into the *Hind* III and *Xho* I restriction sites of the pET-28a (+) vector. Subsequently, the recombinant plasmid (pETAMY) was transformed into *E. coli* BL21 (DE3) competent cells; it was then plated on LBA plates containing kanamycin (30 μg/ml). The recombinant clones so obtained were further examined for extracellular secretion of α-amylase by culturing the individual clone on LBA plate supplemented with 0.5% (w/v) soluble starch, 30 μg/ml kanamycin and 40 μl of IPTG (isopropyl β-D-1-thiogalactopyranoside) (100 mM). After incubating the plates for 18 h at 37 °C, they were stained with the iodine solution to visualize the zones of starch hydrolysis (indicator of α-amylase production) surrounding the colonies.

2.3. Induction and overexpression of B. subtilis AS01a α-amylase gene in E. coli BL21 (DE3)

The *E. coli* BL21 (DE3) transformant harboring the pETAMY was grown in LB medium containing kanamycin (30 μg/ml) at 37 °C, 200 rpm until the culture reached the mid-logarithmic phase (∼0.6 absorbance at 600 nm). The expression of recombinant protein was then induced by the addition of 1.0 mM IPTG. After the different period of induction, cells were harvested by centrifugation (6000 rpm for 10 min at 4 °C) and the cell-free culture supernatant was used for the measurement of extracellular α-amylase production and SDS-PAGE analysis. The cell-free culture supernatant from native (non-recombinant) *E. coli* cells was used as negative control. For the determination of intracellular α-amylase activity, if any, the cell pellets were re-suspended in Tris–HCl buffer (pH 8.0) and the cells were disrupted by lysozyme treatment followed by sonication. The cell lysate was then subjected to centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant of clear lysate was used for the measurement of intracellular α-amylase activity.

2.4. Amino acid analysis and structure determination of recombinant enzyme

The recombinant plasmid containing α-amylase gene was isolated from the *E. coli* cells and then sequenced using automated DNA sequencer (3130 Genetic Analyzer, Applied Biosystem, Switzerland). From the gene sequence, primary structure of the recombinant α-amylase was deduced using the Gene Runner software (www.generunner.net). The nucleotides and the deduced amino acid sequence homology searches were performed using BLAST program of NCBI database (http://www.ncbi.nlm.nih.gov). The signal peptide sequence was predicted using SignalP 4.0 server online program (http://www.cbs.dtu.dk/services/SignalP). The multiple amino acid sequence alignment was accomplished using CLUSTAL W2 program [11] of EMBL-EBI online software (www.ebi.ac.uk). The resulted aligned sequences were then investigated for the conserved domain searched in NCBI database (www.ncbi.nlm.nih.gov). Subsequently, the secondary structure of the recombinant protein was then predicted and superimposed using the ESPript online programme (www.espript.ibcp.fr/ESPript/ESPript).

2.5. Alpha-amylase assay

The amylolytic activity was assayed by measuring the amounts of reducing sugar released by the action of enzyme from 1% (w/v) of soluble starch dissolved in 50 mM K-phosphate buffer (pH – 6.0) at 60 °C. The amounts of reducing sugar released were estimated