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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 pI 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.

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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, DH5 α 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'-CCCAAGCTTTGCGCTTACAGCA CCGTCGATCAA-3') and reverse (5'-CGCGGATCCTTGAAAGAACATGTGTTACACCT-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* DH5 α 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'-**CCCAAGCT**TCTATGTTT-GCAAAACGATTCAA-3') whereas *Xho* I restriction site (indicated

in bold) was inserted in the reverse primer (5'-CCG**CTCGAG** CTCAATGGGAAAGAGAAC-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 μ g/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 μ g/ml kanamycin and 40 μ l 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 μ g/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.escript.ibcp.fr/EScript/EScript).

2.5. Alpha-amylase assay

The amyloytic 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

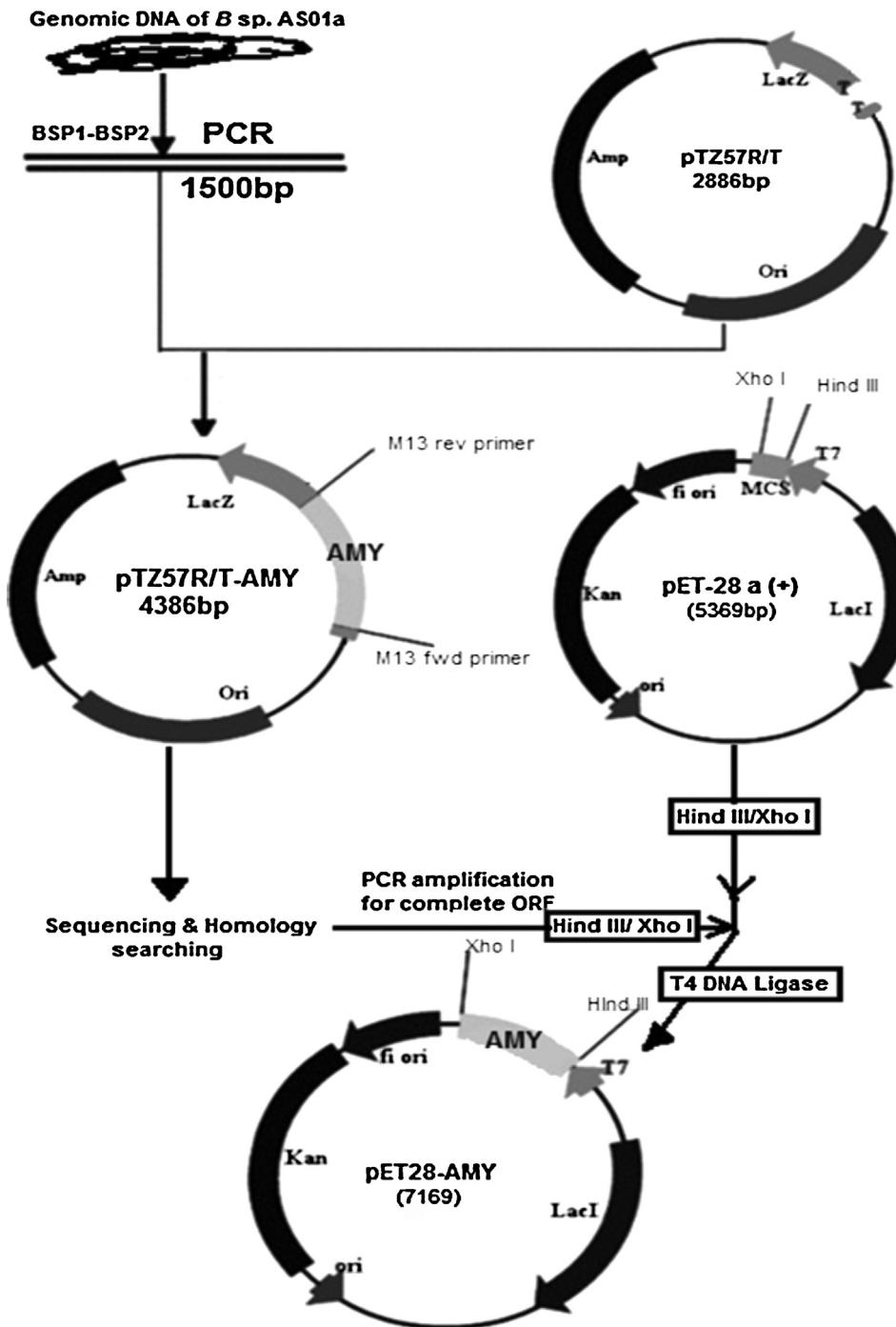


Fig. 1. Strategy used for the cloning of *amyBS-I* from *B. subtilis* strain AS01a into cloning and expression vectors.

by our previous illustrated method [12]. One unit of enzyme activity was defined as the liberation of reducing sugars equivalent to 1 μmol of D-glucose/min under the assay conditions [12].

2.6. Enhancement of extracellular expression of α -amylase into the expression media using response surface methodology

Response surface methodology [13] was applied to optimize the cultivation conditions for the over-expression of recombinant α -amylase in *E. coli*. The optimization was designed based on a rotatable central composite design (CCD) with 30 experimental trials involving 8 star points and 6 replicates at the central points.

The CCD results were analyzed using DESIGN EXPERT (State-Ease, USA). The following four parameters viz. concentrations of IPTG (C_1), post-induction time (C_2), temperature (C_3), and concentrations of EDTA (C_4), were optimized through RSM. The rationale for selection of the above parameters was based on studies showing their crucial role in the extracellular expression of recombinant proteins in *E. coli* [8,14,15]. Details of lower limit, central values, and the upper limit are shown in Table 1. For statistical calculations, the relation between the coded values and actual values is described by Eq. (1):

$$C_i = \frac{A_i - A_0}{\Delta A_i}, \quad i = 1, 2, 3, \dots, k \quad (1)$$

Table 1

Design matrix in both coded and actual (in bracket) values with their corresponding experimental and predicted activities of the recombinant AmyBS-I.

| Run order | IPTG (mM) (C_1) | Time of post Induction (h) (C_2) | Temperature (°C) (C_3) | EDTA (mM) (C_4) | Enzyme activity (U/ml) | Predicted values |
|-----------|---------------------|--------------------------------------|----------------------------|---------------------|------------------------|------------------|
| 1 | -1.0 (0.2) | -1.0 (12) | -1.0 (20) | -1.0 (17) | 101.67 | 95.28 |
| 2 | 1.0 (1) | -1.0 (12) | -1.0 (20) | -1.0 (17) | 116.79 | 111.61 |
| 3 | -1.0 (0.2) | 1.0 (24) | -1.0 (20) | -1.0 (17) | 186.83 | 172.45 |
| 4 | 1.0 (1) | 1.0 (24) | -1.0 (20) | -1.0 (17) | 136.51 | 131.6 |
| 5 | -1.0 (0.2) | -1.0 (12) | 1.0 (30) | -1.0 (17) | 123.56 | 124.08 |
| 6 | 1.0 (1) | -1.0 (12) | 1.0 (30) | -1.0 (17) | 159.73 | 154.31 |
| 7 | -1.0 (0.2) | 1.0 (24) | 1.0 (30) | -1.0 (17) | 159.35 | 151.37 |
| 8 | 1.0 (1) | 1.0 (24) | 1.0 (30) | -1.0 (17) | 124.98 | 124.41 |
| 9 | -1.0 (0.2) | -1.0 (12) | -1.0 (20) | 1.0 (34) | 129.00 | 117.25 |
| 10 | 1.0 (1) | -1.0 (12) | -1.0 (20) | 1.0 (34) | 120.30 | 121.11 |
| 11 | -1.0 (0.2) | 1.0 (24) | -1.0 (20) | 1.0 (34) | 217.34 | 215.59 |
| 12 | 1.0 (1) | 1.0 (24) | -1.0 (20) | 1.0 (34) | 175.11 | 162.26 |
| 13 | -1.0 (0.2) | -1.0 (12) | 1.0 (30) | 1.0 (34) | 94.50 | 92.24 |
| 14 | 1.0 (1) | -1.0 (12) | 1.0 (30) | 1.0 (34) | 107.93 | 109.99 |
| 15 | -1.0 (0.2) | 1.0 (24) | 1.0 (30) | 1.0 (34) | 147.84 | 140.69 |
| 16 | 1.0 (1) | 1.0 (24) | 1.0 (30) | 1.0 (34) | 102.04 | 101.26 |
| 17 | -2.0 (0) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 98.26 | 122.35 |
| 18 | 2.0 (1.4) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 144.82 | 150.74 |
| 19 | 0.0 (0.6) | -2.0 (6) | 0.0 (25) | 0.0 (25.5) | 105.42 | 109.46 |
| 20 | 0.0 (0.6) | 2.0 (30) | 0.0 (25) | 0.0 (25.5) | 162.48 | 177.90 |
| 21 | 0.0 (0.6) | 0.0 (18) | -2.0 (15) | 0.0 (25.5) | 95.74 | 114.18 |
| 22 | 0.0 (0.6) | 0.0 (18) | 2.0 (35) | 0.0 (25.5) | 80.95 | 81.98 |
| 23 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | -2.0 (8.5) | 115.52 | 127.92 |
| 24 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 2.0 (42.5) | 119.66 | 126.74 |
| 25 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 51.86 | 51.21 |
| 26 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 54.28 | 51.21 |
| 27 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 52.26 | 51.21 |
| 28 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 56.28 | 51.21 |
| 29 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 50.86 | 51.21 |
| 30 | 0.0 (0.6) | 0.0 (18) | 0.0 (25) | 0.0 (25.5) | 52.26 | 51.21 |

where C_i is a coded value of the variable, A_i is the actual value, A_0 is the actual value of A_i at the center point, and the star point was set with α of 2.0 from the coded center point. A mathematical model was developed using Design-Expert and the experiments were conducted according to design matrix (Table 1) arranged by the statistical software. The significance of difference among the same sets of experimental data was analyzed using the ANOVA test. A P -value (probability $> F$) of less than 0.05 indicated that the model terms were significant. Adequacy of the developed model was further validated using numerical method optimization option of the Design-Expert (version 7.0.Stat-Ease, Inc.) software.

2.7. Isolation and purification of α -amylase from recombinant *E. coli* strain

Solid ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ was gradually added to the cell-free culture supernatant (obtained after 24 h of incubation) at 4 °C to attain 1.0 M solution. The solution was then stirred slowly for additional 30 min at 4 °C. It was then applied to a FPLC (AKTApureifier, GE Healthcare, Uppsala, Sweden) coupled Phenyl-Sepharose column (5 mm × 20 mm) previously equilibrated with 50 mM Na-phosphate buffer, pH 7.4 containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with a linear gradient of 1.0–0.0 M $(\text{NH}_4)_2\text{SO}_4$ in the same buffer at a flow rate of 1 ml/min, and 2.0 ml fraction was collected in each tube. The active fractions were pooled and then dialyzed to remove the salt and subsequently it was concentrated by ultrafiltration unit (50-kDa cutoff membrane) (Amicon, Beverly, MA, USA). This was followed by fractionation of the concentrated solution through HiPrep 16/60 Sephadryl S-200 HR (GE Healthcare Bio-Sciences Corp., USA) gel filtration column coupled with a FPLC system. The column was then equilibrated with 50 mM Na-phosphate pH 7.4, and elution of protein(s) was carried out with the same buffer at a flow rate of 30 ml/h. One milliliter fraction was

collected in each tube, and each fraction was checked for α -amylase activity and protein content.

2.8. Determination of purity and molecular mass of recombinant protein

The homogeneity as well as molecular mass of the pooled gel-filtration fractions displaying α -amylase activity was checked by 10% SDS-PAGE analysis of the protein(s) [16]. Amylase zymography analysis was done as described by Roy et al. [2]. The purified enzyme so obtained was named as AmyBS-I, and was used for characterizing its biochemical properties and application in starch processing industry.

2.9. Biochemical characterization of purified AmyBS-I

The optimum pH and temperature for recombinant α -amylase (AmyBS-I) was determined by incubating 10 µg of enzyme with 1% (w/v) starch dissolved in different buffers (pH 4.0–12.0), or at different temperatures (30–90 °C) at optimum pH 6.0. In order to determine its thermostability, purified α -amylase (10 µg/ml) was incubated at different temperatures ranging from 40 to 100 °C for 30 min, in both presence and absence of 5 mM Ca^{2+} ions. This was followed by assaying its residual activity against control.

To investigate the effects of divalent metal ions, inhibitors and metal chelator on enzymatic activity of AmyBS-I, it was treated with divalent cations (5 mM) (Cu^{2+} , Fe^{2+} , Co^{2+} , Hg^{2+} , Ca^{2+} , Zn^{2+} , Ni^{2+} , Mn^{2+} , Mg^{2+}), phenyl methyl sulfonyl fluoride (2 mM) and EDTA (2 mM) for 30 min at room temperature [12]. The enzyme activity without any of the above-mentioned metal ions/inhibitors was considered as 100% activity and other values were compared with that [2,12]. The kinetic properties viz. K_m and V_{max} values of the purified AmyBS-I toward soluble starch were determined using Lineweaver–Burk double reciprocal plot [2].

2.10. Reaction end product determination by thin layer chromatography (TLC)

To determine the end product of starch hydrolysis, a reaction mixture containing 5.0 ml of 1% (w/v) soluble starch (Hi-media, India) dissolved in 50 mM Na-phosphate buffer (pH 6.0), was incubated with AmyBS-I at a concentration of 10 µg/ml of starch solution at 70 °C. An aliquot of reaction mixture was withdrawn at an interval of 1, 3, 6 and 12 h after the incubation, and starch hydrolysis products were analyzed by Slica gel G thin layer chromatography [17]. The product formation was quantitated by densitometry scanning of TLC plates followed by analysis with ImageJ software (www.rsbweb.nih.gov/ij).

2.11. Raw starch hydrolysis by the AmyBS-I

The efficiency of AmyBS-I to digest the raw starch obtained from various sources (rice, wheat, and potato) was determined by incubating 5.0 ml of 2% (w/v) starch dissolved in 50 mM Na-phosphate buffer (pH 6.0) with AmyBS-I at a concentration of 10 µg/ml at 60 °C for 6 h. The extent of starch hydrolysis was determined by estimating the amount of reducing sugars released from various starch by the action of AmyBS-I. The raw starch hydrolysis (R_h) potential of AmyBS-I was calculated using the formula: $R_h (\%) = (A_1/A_0) \times 0.9 \times 100$, where A_1 was the amount of sugar (mg/ml) in the supernatant after the reaction and A_0 was the amount of raw starch (mg/ml) before the reaction. The factor 0.9 (162/180) is the conversion factor due to the addition of water molecules to glycosyl moiety on hydrolysis [18]. After 6 h of hydrolysis, all starch grains were washed twice with pure ethanol and examined under scanning electron microscope at 15 kV (JEOL model JSM-6390 LV) as described previously [2].

2.12. Anti-staling effect of AmyBS-I on bread preparation

The application of AmyBS-I as anti-staling agent on bread making was evaluated by the procedure described by Sharma and Satyanarayana [19]. The dough mixture was supplemented with the enzyme preparation [AmyBS-I or commercial α-amylase (Hi-media, Mumbai)] in a ratio of 10 U/g of dough, followed by mechanical blending with 60% (w/v) water for 30 min to produce the dough. Control dough was also prepared where no enzyme was added. These were then kept for proofing followed by fermentation for 1 h at 33 °C and 85% relative humidity. The proved doughs were baked in electric oven at 220 °C for 60 min. Subsequently, bread loaves were cooled (60 min), packed in polythene bags, and stored for five days at room temperature.

To determine the reducing sugar content of the breads, 1 g of each bread sample was dissolved in 5 ml of 0.1 M Na-acetate buffer (pH 4.5), mixed well by vortexing, and centrifuged at 10,000 rpm for 15 min. Presence of reducing sugar in the supernatant was determined using DNS reagent [2]. Moisture content of the bread sample was estimated by drying the bread at 105 °C to constant weight. To determine the bread color, a breadcrumb was attached to HunterLab (Ultra-scan VIS, USA) then three readings were taken, and average value was recorded. The brownness index (BI) for the bread color was calculated [20] using the following equation:

$$BI = \frac{100(x - 0.31)}{0.17}, \quad \text{where } x = \frac{(a + 1.75L)}{(5.645L + a - 3.012b)} \quad (2)$$

For assessing the self-life, bread samples kept at room temperature were visually inspected on alternate days for texture, softness, and staling. The crumb texture of the loaves (hardness, springiness,

cohesiveness, and gumminess) was evaluated by texture profile analysis (TPA) method using a texture analyzer (TA-HD-plus, Stable Micro Systems, UK.) having a 35-mm flat-end aluminum compression disk (probe P/35). Slices of 2 cm thickness were compressed to 40% of their original height in a TPA double compression test, at 1 mm/s speed test, with a 20 s delay between the 1st and 2nd compression [21]. A semi-trained panel did sensory analysis for overall acceptability of the bread samples. All these experiments were performed in triplicates, and the average values are presented.

3. Results and discussion

3.1. Cloning and sequencing of the gene encoding α-amylase from *B. subtilis* AS01a

The molecular cloning of the α-amylase from *B. subtilis* strain AS01a would be helpful for hyper-production of this enzyme, thereby to meet industrial needs and for the development of novel properties with enzyme engineering such as pH profile and thermostability. Overexpression of recombinant protein would also be helpful to understand its structure-function relationships and development of a better enzyme by site-directed mutagenesis (enzyme engineering) [22]. Furthermore, the expression of this α-amylase in *E. coli* system would be of great value to reduce the energy consumption during the enzyme production process because the parent strain *B. subtilis* AS01a needs to be cultivated at 45 °C [2]. Approximately 2 kb fragment containing complete ORF of α-amylase gene (*amyBS-I*) was amplified by PCR using *B. subtilis* strain AS01a genomic DNA as the template (Supplementary Fig. S1A). The PCR amplified product was successfully cloned first into pTZ57R/T vector; then into pET-28a expression vector for sequencing and expression of α-amylase gene, respectively (Fig. 1). The *amyBS-I* sequence contained a complete ORF with a length of 1980 bp, which encoded a polypeptide of 659 amino acid residues (including the SPS) with a calculated molecular mass of 72.4 kDa (Fig. 2).

The nucleotide sequence of *amyBS-I* from *B. subtilis* AS01a reported here has been deposited into GenBank database under accession number (KC113313). The homology search revealed a significant similarity of *amyBS-I* with α-amylases from *B. subtilis* strains (Fig. 2). The deduced amino acid sequence of *amyBS-I* was found to contain a prokaryotic SPS (Fig. 2). The analysis of deduced amino acid sequence of mature protein (AmyBS-I) using conserved domain search of NCBI (www.ncbi.nlm.nih.gov) suggested that it belongs to GH 13 family of glycoside hydrolase (GH) [1]. The calculated pI/M_w of the AmyBS-I was estimated to be 5.8/72,387 and 5.5/68,875.6 for the native protein (containing SPS) and for the mature peptide, respectively.

3.2. Expression of *B. subtilis* AS01a α-amylase gene in *E. coli* BL21 (DE3)

A recombinant plasmid, pETAMY, containing *B. subtilis* AS01a α-amylase gene into pET-28a vector was constructed for the extracellular expression of the AmyBS-I. Since the SPS helps in the extracellular expression of a recombinant protein; therefore, complete ORF of α-amylase gene including its SPS from the *B. subtilis* strain AS01a was amplified and cloned into the expression vector. The formation of halo zones around the recombinant bacterial colonies on the starch agar plate after flooding with Gram's iodine re-confirmed the successful extracellular over-expression of AmyBS-I (Supplementary Fig. S1). The SDS-PAGE analysis of the cell extract as well as culture supernatant from *E. coli* BL21 harboring pETAMY showed a prominent protein band with an apparent

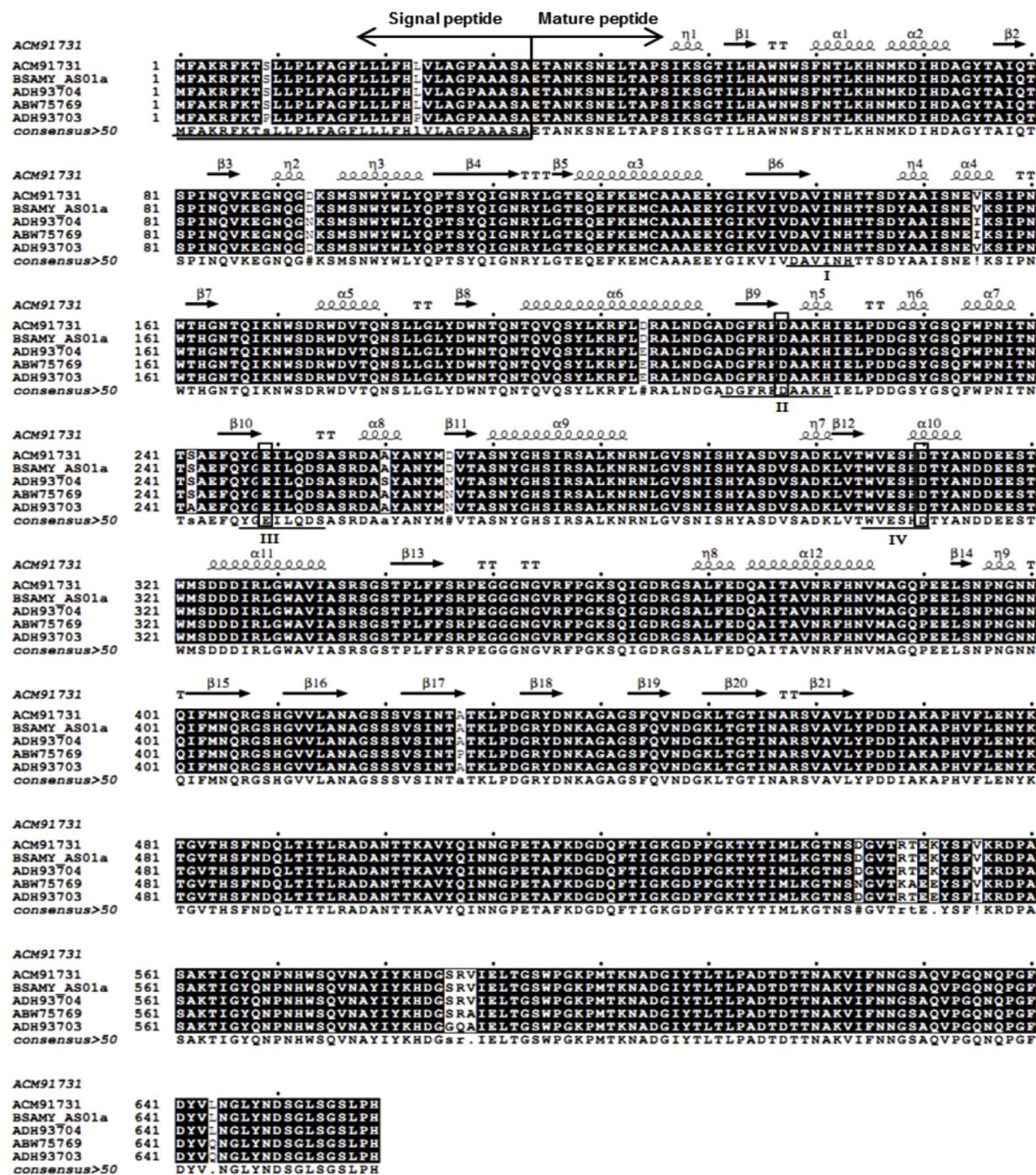


Fig. 2. Multiple sequence alignment of *amyBS-I* with the homologous α -amylase sequences reported in the databases. The *amyBS-I* showed 99% sequence homology with *B. subtilis* HSSK-3 (ADH93704), and *B. subtilis* strain OI1085 (ACM91731), whereas it showed 98% sequence similarity with *B. amyloliquefaciens* DL 341(ADH93703) and *B. subtilis* (ABW75769). The SPS is marked underlined, the four conserved regions of *amyBS-I* are marked in underline with numerals and, the catalytic sites residues are enclosed in rectangular boxes. The conserved sequences of α -amylases were displayed as the white letters on the black background. The secondary structure assignments of the AmyBS-I are indicated at the top of the alignment. The TTT and TT letters represent strict alpha and beta turns, respectively.

molecular mass of ~70 kDa (data not shown). The mass of this protein was found to be the same as estimated from the deduced amino acid sequence of the AmyBS-I suggesting extracellular expression of the recombinant enzyme. However, α -amylase assay as well as SDS-PAGE study suggested that majority of the target proteins were accumulated as inclusion bodies in *E. coli*. Therefore, in order to reduce the formation of inclusion bodies and efficient secretion of functionally active recombinant α -amylase into the culture medium, the *E. coli* culture parameters were optimized using RSM.

3.3. Optimization of culture conditions for the extracellular expression of AmyBS-I

It is well recognized that media components and cultivation parameters play an important role in extracellular expression of recombinant protein by *E. coli* [14,15]. Further, targeting recombinant protein into the periplasmic space or to the culture medium simplifies downstream processing, folding and *in vivo* stability; thus, enabling the cost-effective production of soluble and biologically active proteins [23]. Therefore, the four most significant

Table 2

Analysis of variance (ANOVA) and regression analysis for the expression of extracellular AmyBS-I.

| Source | Sum of squares | Degrees of freedom | Mean squares | F-value | Prob. (<i>P</i>) > <i>F</i> |
|------------------|----------------|--------------------|--------------|---------|-------------------------------|
| Model | 51,933.7 | 14 | 3709.55 | 25.14 | <0.0001 |
| Residual (error) | 2213.01 | 15 | 147.53 | | |
| Lack of fit | 2193.64 | 10 | 219.36 | 56.64 | 0.0002 |
| Pure error | 19.36 | 5 | 3.87 | | |
| Total | 54,146.71 | 29 | | | |

$R^2 = 0.96$, Adj. $R^2 = 0.92$, Pred $R^2 = 0.79$ and CV = 10.58%.

parameters viz. IPTG concentration, temperature, time of post-induction and EDTA concentration were optimized for the efficient extracellular expression of AmyBS-I. By processing these variables, a model was obtained as shown in Eq. (3). The model was evaluated by multiple regression analysis to predict the optimum cultivation conditions for optimizing the extracellular expression of AmyBS-I into the culture media. All the terms regardless of their significance are included in the following equation, where *Y* is the response, that is, the enzyme activity (U/ml) and $C_1 - C_4$ are the actual values of the test variables.

$$\begin{aligned} Y = & 390.89 - 135.51C_1 - 8.95C_2 - 10.5C_3 - 23.36C_4 + 173.57C_1^2 \\ & + 0.64C_2^2 + 0.46C_3^2 + 3.04C_4^2 - 5.95C_1C_2 + 1.74C_1C_3 - 3.12C_1C_4 \\ & - 0.42C_2C_3 + 0.35C_2C_4 - 1.08C_3C_4 \end{aligned} \quad (3)$$

The coefficient of the model including the significance of each coefficient was determined by *P*-values; the model terms with the *P*-value (probability > *F*) less than 0.05 are considered to be significant. Results showed that C_1 , C_2 , C_3 , C_1^2 , C_2^2 , C_3^2 , C_4^2 , C_1C_2 , C_2C_3 , and C_3C_4 are significant model terms. The analysis of variance (ANOVA) by Fisher's statistical test was conducted for the quadratic model and the results demonstrated that the computed *F*-value for the model is 25.14, which implies that the model is significant (Table 2). There is only a 0.01% chance that a "Model *F*-Value" could occur due to noise. The model's fitness accuracy can also be checked by determining the coefficient of correlation ($R^2 = 0.96$). It is obvious from the value of R^2 that the deduced model could not explain only 4% of the total variation (Table 2). The value of adjusted R^2 (0.92) also supported the significance of the model (Table 2).

Response surface and contour plots are graphical representations for the regression equation. They investigate the interaction amongst the variables and determine the optimum levels. Fig. 3A–F shows the response surface curves, and contour plots for variations in extracellular AmyBS-I activity as a function of two variables at a time while the other variables were maintained at their respective zero level. Figs. 3A and B demonstrate that lowering the IPTG concentration as well as temperature has a positive effect on high-level secretion of AmyBS-I into the culture medium. Ayadi et al. [14] reported a similar phenomenon for *Paenibacillus* CGTase production in *E. coli*. On the other hand, increasing the EDTA concentration as well as enhancing the incubation time resulted in better expression and secretion of AmyBS-I into the culture medium (Fig. 3F). This observation is in accordance with Lo et al. [15] showing enhanced production of *Bacillus* CGTase in *E. coli* with respect to time.

By solving the regression Eq. (3) using numerical method, the optimum value for each of the four test variables was found as: IPTG concentration = 0.24 mM, post induction time = 26.7 h, temperature of incubation = 19.7 °C and EDTA concentration = 46.7 mM. The predicted value for the extracellular AmyBS-I production at optimized conditions was calculated as 389.44 U/ml. This value was in close agreement with actual experimental value of 378.24 U/ml

of AmyBS-I thus justifying the suitability of RSM model applied in this study for enhancing the AmyBS-I yield. The model exhibited a significant improvement in the extracellular expression of AmyBS-I (approx. 7-fold) under optimized conditions as compared to its production under non-optimized conditions. Our study showed that the culture conditions such as low IPTG concentration, low temperature, and increase in post induction time play a critical role in the heterologous extracellular expression of AmyBS-I. All these factors keep the *E. coli* under low induction level, give sufficient time to properly fold and export the AmyBS-I through the cell wall membrane into the culture medium. Further, the expression and secretion of AmyBS-I in *E. coli* at a low temperature (~20 °C) are unique findings of this study and hardly few reports are available showing extracellular expression of recombinant protein in *E. coli* at such a low temperature without the aid of co-expression of chaperone [24]. The role of EDTA in extracellular secretion of AmyBS-I may be assumed by permeabilization of the outer membrane of *E. coli* [25].

3.4. Purification and biochemical characterization of AmyBS-I

The AmyBS-I was eluted from the Phenyl-sepharose column with 0% $(\text{NH}_4)_2\text{SO}_4$ salt concentration (Supplementary Fig. S2A). Further fractionation of this active fraction through Sephadryl S-200 resulted in elution of active enzyme in a single peak (Supplementary Fig. S2B), and proteins of this peak was found to be homogenous by SDS-PAGE (Fig. 4). Under both denatured and native conditions, the enzyme yields a single band of approximately 69.0 kDa in SDS-PAGE. This suggests that the α -amylase under study is monomeric in nature, which is in accordance with the predicted size of the amino acids sequence (Fig. 4). The purity of preparation was further re-confirmed by amylase-zymographic study where the enzyme showed a single clear zone of starch hydrolysis (Fig. 4). A summary of purification of the recombinant enzyme AmyBS-I is shown in Supplementary Table S1. The AmyBS-I was purified up to 3.9-fold with 19.5% of recovery of the total enzyme activity (Supplementary Table S1). In general, molecular mass of α -amylases from bacteria particularly from *Bacillus* sp. is reported in between 48 and 60 kDa [26] and there is a dearth of report on such a high molecular mass α -amylase from bacteria.

Before assessing the biotechnological potential of any enzyme, characterization of its biochemical properties relevant to industrial application is most important and advantageous [27]. AmyBS-I retained its activity over a broad range of pH (5.0–9.0) and temperature (40–80 °C) (Supplementary Fig. S3); however, the enzyme displayed optimum activity at pH 6.0 and at 70 °C, suggesting its application in starch saccharification industry. The AmyBS-I also showed higher temperature optima than many of the α -amylases purified from *Bacillus* sp. KSM 1378 [4], *Bacillus* sp. GM 8901 [4], and *Bacillus* sp. YX1 [7].

The thermo-stability study demonstrated that heating of AmyBS-I for 30 min at 70 °C had no effect on its enzyme activity. However, heating beyond this temperature resulted in a gradual

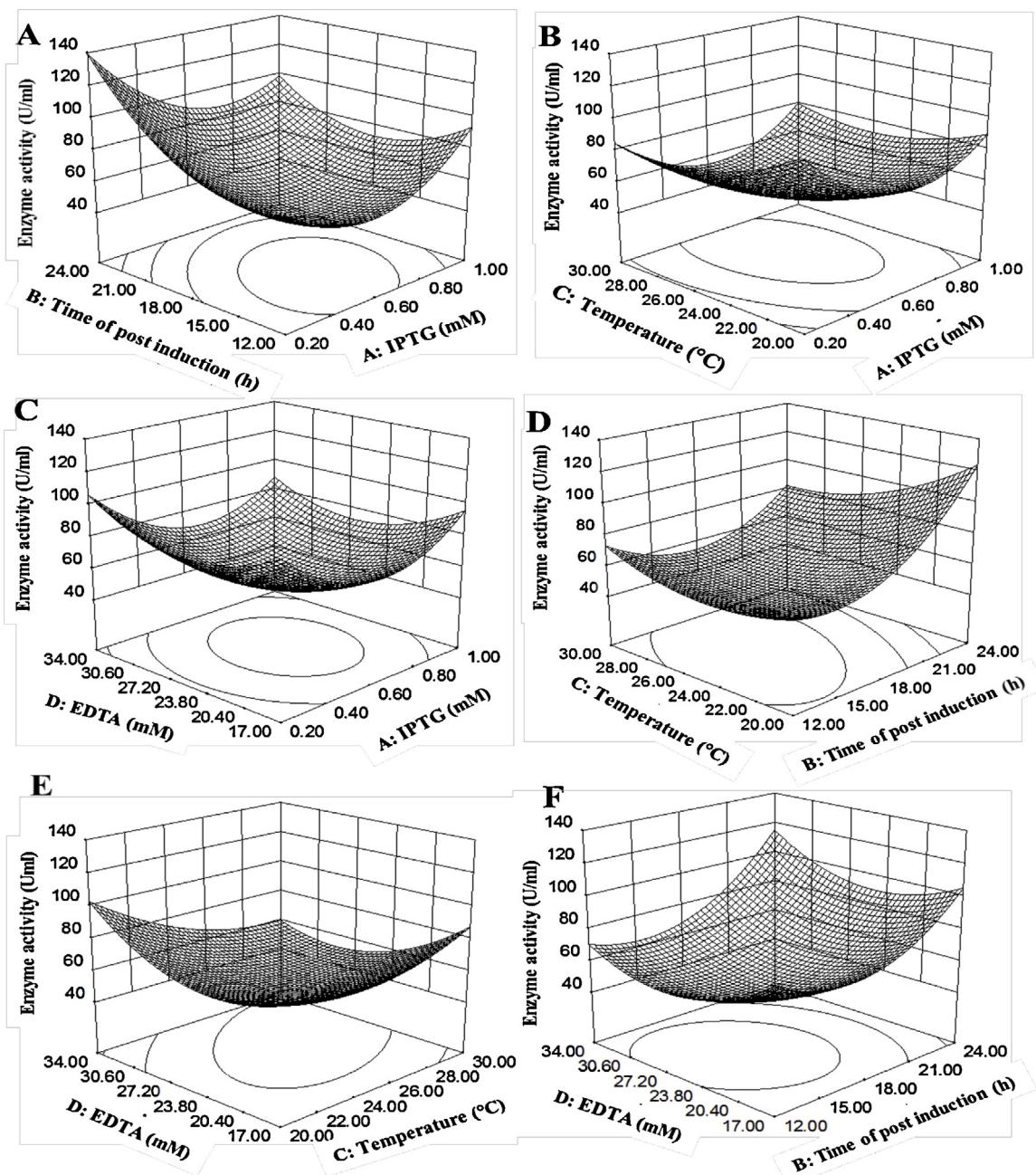


Fig. 3. Response surface plots and contour plots showing interaction effect of test variables on extracellular over expression of AmyBS-I in *E. coli* BL21 (DE3) strain. (A) IPTG concentration (mM) versus time of incubation after induction (h), (B) IPTG concentration (mM) versus temperature (°C), (C) IPTG concentration (mM) versus EDTA concentration (mM), (D) time of incubation after induction (h) versus temperature (°C), (E) temperature (°C) versus EDTA concentration (mM) and (F) time of incubation after induction (h) versus EDTA concentration (mM).

loss of enzyme activity (Supplementary Fig. S4). The thermostability of the AmyBS-I was not influenced by the presence of Ca^{2+} ion (Supplementary Fig. S4) which is an added advantage for application of AmyBS-I in starch saccharification process at a higher temperature because it eliminates the requirement of Ca^{2+} ion for improving the thermo-stability of α -amylases [2]. Therefore, application of AmyBS-I in starch processing industry may be considered as a cost-effective process.

The activity of various enzymes is influenced by the presence of divalent cations and additive molecules. In the present study, α -amylase activity of AmyBS-I in presence of Fe^{2+} , Cu^{2+} , Zn^{2+} , and Hg^{2+} were inhibited to 70.5%, 79.2%, 84.6%, and 94.5%, respectively of its original activity. The other tested divalent metal ions and

EDTA did not show adverse effect on the enzyme activity of AmyBS-I. In the presence of PMSF, AmyBS-I lost around 22% of its original activity suggesting the presence of serine in the active site, which was also confirmed by AmyBS-I sequence analysis. The K_m and V_{max} values of AmyBS-I toward soluble starch were found to be 2.7 mg/ml and 454.0 $\mu\text{mol}/\text{min}/\text{mg}$, respectively. The K_m value of AmyBS-I for starch hydrolysis is higher than the K_m value of alkaline α -amylase (1.9 mg/ml) purified from the same strain of *Bacillus* [2]. However, this K_m value is comparable with the K_m value of α -amylases from *B. subtilis* (3.85 mg/ml) [26] and *Bacillus* sp. TM1 (4 mg/ml) [28].

The end-products of starch hydrolysis by AmyBS-I by TLC analysis demonstrated the formation of oligosaccharide mixtures after

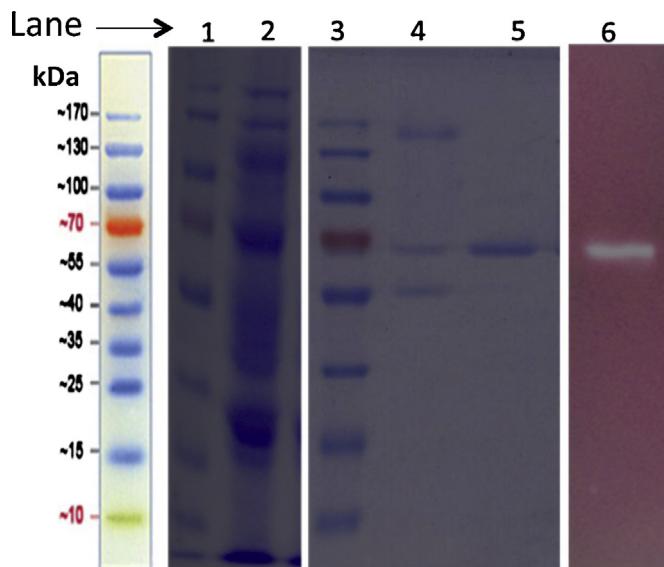


Fig. 4. SDS-PAGE analysis of AmyBS-I. Lanes 1 and 3, pre-stained protein molecular weight markers; lane 2, crude culture supernatant containing recombinant protein (30 µg); lane 4, phenyl-sepharose pooled fraction (25 µg); lane 5, purified gel filtration fraction (25 µg); and lane 6, zymogram of AmyBS-I (20 µg).

1 h of treatment; however, formations of maltose and glucose were observed after 3 h of treatment (Supplementary Fig. S5). After 12 h of treatment of soluble starch with AmyBS-I, formation of glucose (82.5%) surpassed maltose production (17.5%) (Supplementary Table S2). This result proves that the enzyme AmyBS-I is an endo-acting type α -amylase enzyme, which may be useful for the food and starch industry [2]. The biochemical properties as well as molecular mass of AmyBS-I were found to be different from the previously reported alkaline α -amylase from the same parent strain (Supplementary Table S3). These data provide convincing evidences that the recombinant enzyme (AmyBS-I) from *B. subtilis* strain AS01a is a different α -amylase than previously reported alkaline α -amylase from the same strain [2]. A bacterium can produce several isoenzymes and other metabolites to cater its requirement as well as to sustain its growth in a particular environment from where it was isolated and therefore, production of isoforms of the same enzyme is not an uncommon phenomenon.

3.5. Raw potato starch hydrolysis by AmyBS-I

The α -amylases capable of digesting various raw starches are attractive from the industrial perspective since they reduce the energy consumption as well as starches from different sources can directly be used for the hydrolysis by the same enzyme [7,18]. Further, conventional starch processing involves two enzymatic steps after starch slurry gelatinization by heating at 100–105 °C [5,18]. The first enzymatic step is the liquefaction by α -amylase at 90–95 °C and second step of starch saccharification is carried out at 60–65 °C using glucoamylase [5], thus the process involves repeated steps of cooling and heating which is costly and high energy consuming process. Since AmyBS-I can digest various raw-starches at 60 °C, therefore, application of AmyBS-I in starch saccharification process may eliminate the unnecessary repeated cooling and heating steps.

The raw starch digestibility of AmyBS-I showed that it could hydrolyze the wheat, potato and rice raw starches to 61%, 58%, and 44%, respectively after six hours of incubation at 60 °C. Moreover, the raw potato starch degradation ability of AmyBS-I suggests its potential application in potato starch hydrolysis since only a few

bacterial α -amylases are reported to digest this most commonly available starch to such an extent [29]. Further, the SEM analysis of starch degradation showed that AmyBS-I, like alkaline α -amylase from *B. subtilis* AS01a [2], also formed pit and deep holes on the surface of all the tested raw starches (Fig. 5). Results of this study suggest that AmyBS-I very efficiently digests raw starch from various sources (wheat, potato, and rice) which makes this enzyme as a better candidate for its industrial application in food and fermentation industries [30]. It is noteworthy to mention that there are very few examples of α -amylases which show potency to digest raw starches from different sources [5].

3.6. Anti-staling effects of AmyBS-I on bread

Acidic and moderate thermostable amylases, which are able to produce fermentable sugars and dextrin for further use by the yeast, are applied in baking industry to improve self-life of breads [19]. Supplementation of AmyBS-I and commercial α -amylases to dough resulted in increase in self-life of bread; however, AmyBS-I supplementation resulted in a considerable higher anti-staling effect as compared to supplementation of commercial α -amylases in bread. It was also found that the AmyBS-I supplemented bread has a better loaf volume and crumb color as compared to the commercial α -amylase supplemented and control (no supplementation) breads (Fig. 6). Further, the moisture content of the fresh bread has a direct correlation with the softness of the baked products and its ideal level should be around 35–40% [19]. Lower moisture content is a desirable property to prevent the microbial growth; however, the moisture content of the bread cannot be kept less than 30% because bread having lower moisture content than this value cannot be refreshed even after heating [31]. The moisture content of AmyBS-I supplemented bread was found within the range of this ideal level and least among the tested samples which suggests adequate softness of the bread and least susceptible to the microbial attack after storage for some period of time (Table 3). Sharma and Satyanarayana [19] have also opined that the addition of amylase increases the self-life of the bread due to their anti-staling effect on bread. In addition, the AmyBS-I supplemented bread has a higher sugar content as compared to other test samples (Table 3). The higher sugar content results in improvement of the taste, crust color, and toasting quality of bread [19]. The brownness index (BI) of bread is an important parameter to determine its acceptability. The BI of AmyBS-I, and commercial amylase supplemented bread was found to be 38.2 and 37.6, respectively. For the control bread, this value was determined as 29.4 (Table 3).

The TPA evaluation of bread before and after its storage may directly be correlated with the staling rate of bread by physical examination of baked products [21]. In fact, these mechanical characteristics of TPA evaluation consisting of hardness, gumminess, cohesiveness, and springiness can be correlated well with the sensory perception of bread and can define bread quality [21]. In general, on storage of bread, its hardness and gumminess are increased whereas cohesiveness and springiness are found to decrease with time; therefore, the rate of these changes determines the quality of bread [32]. Table 4, shows that significant changes observed in all the tested parameters of bread stored for five days; except the commercial α -amylase (Himedia) supplementation to the bread did not result in a significant change ($P > 0.05$) in gumminess with respect to control. It was also observed that the bread supplemented with AmyBS-I had significant resistance to hardness and gumminess changes over the time as compared to control as well as commercial α -amylase (Himedia) supplemented bread. This result indicates that breads supplemented with AmyBS-I have adequate softness and low staling rate over time as compared to other tested samples (Table 4). Gambaro et al. [32] also reported that the

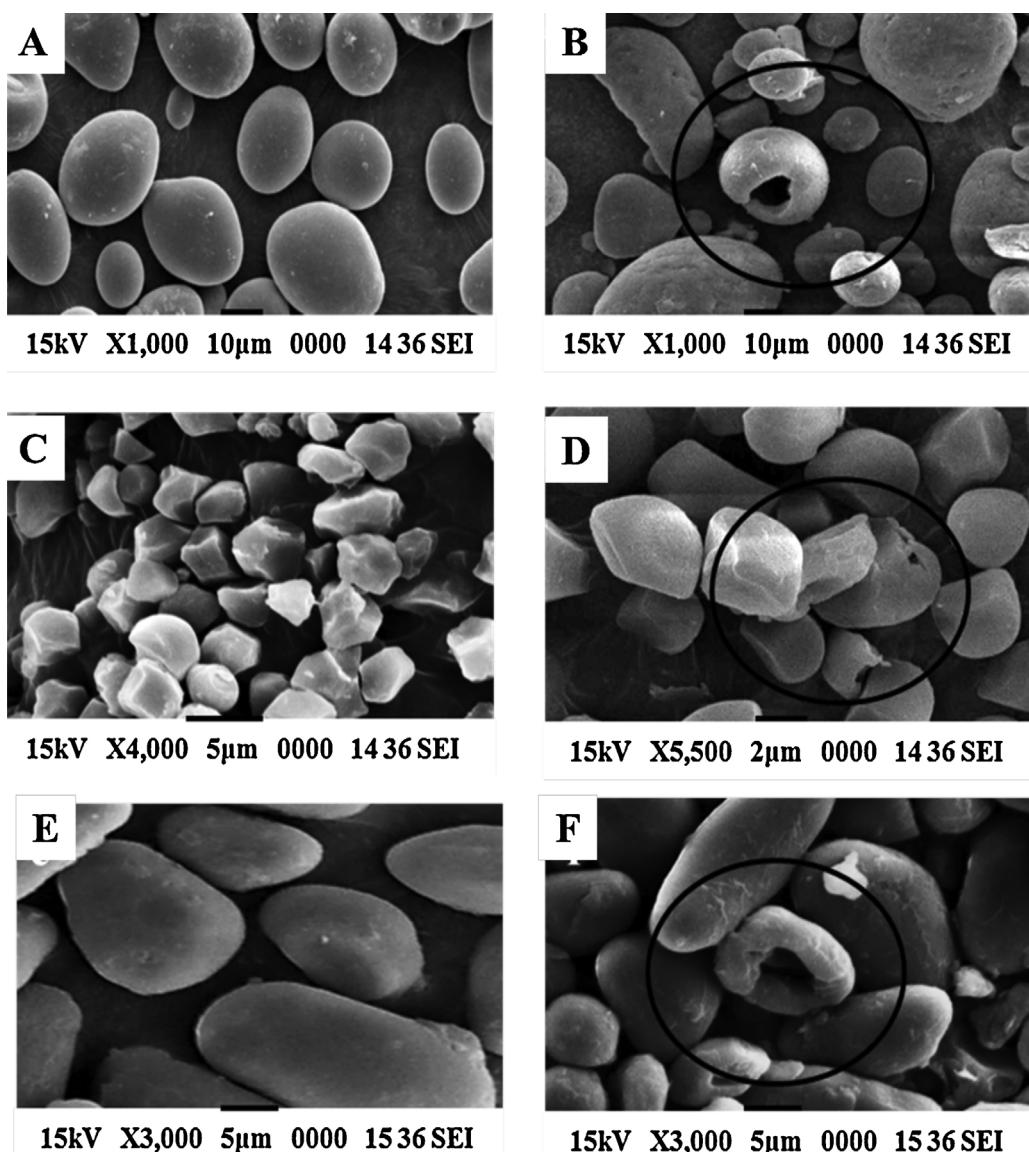


Fig. 5. SEM images of raw starch granules before and after hydrolysis with AmyBS-I for 6 h. (A) Untreated potato starch, (B) AmyBS-I treated potato starch, (C) untreated rice starch, (D) AmyBS-I treated rice starch, (E) untreated wheat starch, and (F) AmyBS-I treated wheat starch.

Table 3

A comparisons of properties of bread supplemented with commercial α -amylase and AmyBS-I. Control bread was made without supplementation of α -amylase to the dough.

| Parameter | Control | Commercial α -amylase | AmyBS-I |
|--|------------|------------------------------|---------------------------|
| Weight of dough (g) ^a | 100 ± 5.0 | 100 ± 5.0 | 100 ± 5.0 |
| Weight of bread (g) ^a | 87.5 ± 4.4 | 76.5 ^b ± 3.8 | 77 ^c ± 3.8 |
| Dough rise (cm) ^a | 2.2 ± 0.1 | 2.9 ^b ± 0.2 | 3.4 ^{c,d} ± 0.2 |
| Self-life (days) | 4 | 5 | 5 |
| Bread moisture (%) ^a | 44.2 ± 2.2 | 39.1 ^b ± 1.9 | 35.6 ^{c,d} ± 1.7 |
| Reducing sugars (mg/g of bread) ^a | 37.6 ± 1.8 | 42.4 ^b ± 2.1 | 44.0 ^c ± 2.2 |
| Overall acceptability ^e | ++ | +++ | ++++ |
| Bread color ^f | | | |
| L-value | 49.3 | 48.1 | 50.9 |
| a-Value | 1.2 | 2.5 | 2.9 |
| b-Value | 12.7 | 14.0 | 14.9 |

^a Each value is expressed as mean ± standard dev. ($n = 3$).

^b $P < 0.05$ between the values of control and commercial α -amylase treated bread within the same row.

^c $P < 0.05$ between the values of control and AmyBS-I treated bread within the same row.

^d $P < 0.05$ between the values of commercial α -amylase and AmyBS-I treated bread within the same row.

^e Sensory evaluation (+ = average, ++ = good, +++ = very good, ++++ = the best).

^f L = lightness index (0–100 = black – white), a = redness and greenness [(+100) – (-80) = red – green] while, b = yellowness and blueness [(+70) – (-80) = yellow – blue].

Table 4

Difference in TPA (between 0th day and 5th day) on storage of various bread samples supplemented with AmyBS-I and a commercial α -amylase. Values are mean \pm standard deviation of triplicates data.

| | Control | Bread supplemented with | |
|---------------------------|------------------|-------------------------------|---------------------------------|
| | | Commercial α -amylase | AmyBS-I |
| Increase in hardness (N) | 15.83 \pm 0.79 | 4.05 ^a \pm 0.2 | 2.96 ^{b,c} \pm 0.14 |
| Increase in gumminess (N) | 1.7 \pm 0.08 | 0.58 ^a \pm 0.029 | 0.45 ^{b,c} \pm 0.02 |
| Decrease in springiness | 0.12 \pm 0.006 | 0.12 \pm 0.006 | 0.15 ^{b,c} \pm 0.007 |
| Decrease in cohesiveness | 0.18 \pm 0.009 | 0.12 ^a \pm 0.006 | 0.14 ^{b,c} \pm 0.007 |

^a P<0.05 between the values of control and commercial amylase within the same row.

^b P<0.05 between the values of control and AmyBS-I supplementation within the same row.

^c P<0.05 between the values of commercial amylase and AmyBS-I within same row.



Fig. 6. Crumb structure of the loaf supplemented with AmyBS-I/commercial α -amylase/control (without enzyme).

addition of amylase retarded starch retrograding, which maintains the bread freshness for a relatively longer period.

4. Conclusions

The SPS facilitated the efficient extracellular over expression of AmyBS-I gene in *E. coli* cells. Further, the response surface optimization of culture parameters was found to be highly effective for enhancing the extracellular expression of catalytically active

recombinant AmyBS-I. This suggests that this technique may be applied for the efficient extracellular expression of other recombinant proteins in *E. coli*. The biochemical properties of purified AmyBS-I suggested its candidature suitable for application in starch industry. Further, being capable of digesting raw starches from various sources, the application of AmyBS-I may reduce the production cost of starch-based foodstuffs. Additionally, due to amelioration of crumb structure and self-life of AmyBS-I supplemented bread; they may be stored for a longer period of time that is an added advantage to the baking industry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molcatb.2013.07.019>.

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