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Cloning and extracellular expression of a raw starch digesting α-amylase (Blamy-I) and its application in bioethanol production from a non-conventional source of starch

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The aim of this study was to clone and efficiently express a raw starch-digesting α-amylase enzyme in the culture media and also to investigate the potential application of this recombinant enzyme in the digestion of non-conventional raw starch for bioethanol production. A raw starch digesting α-amylase gene isolated from *Bacillus licheniformis* strain AS08E was cloned and extracellularly expressed in *E. coli* cells using the native signal peptide. The mature recombinant α-amylase (Blamy-I) consisting of 483 amino acid residues was found to be homogenous with a mass of 55.3 kDa (by SDS-PAGE analysis) and a predicted pI of 6.05. Structural and functional analysis of Blamy-I revealed the presence of an extra Ca^{2+}-binding region between the A and C domains responsible for higher thermostability of this enzyme. The statistical optimization of *E. coli* culture conditions resulted in an approximately eightfold increase in extracellular expression of Blamy-I as compared to its production under non-optimized conditions. Blamy-I demonstrated optimum enzyme activity at 80 °C and pH 10.0, and efficiently hydrolyzed raw starch isolated from a non-conventional, underutilized jack fruit seeds. Further utilization of this starch for bioethanol production using Blamy-I and *Saccharomyces cerevisiae* also proved to be highly promising.

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Starch is the second most abundant biosynthesis product of plants after cellulose [1], which has a great industrial demand because it is the most inexpensive starting material for the production of sugar syrups to be used in the food industries [2]. Besides, starch has also been considered as an excellent raw material for the production of biofuel such as bioethanol [3], which accounts for more than 90% of the total use for biofuel globally [4]. Furthermore, in recent years, production of bioethanol replacing the gasoline has gained a considerable interest in the global market [5] and is expected to touch 100 billion liters of global production in 2015 [6]. However, the major hurdle in its large-scale expansion has been its dependency on grain-based raw materials, which escalates the cost of production. Therefore, low-cost agricultural wastes as well as non-conventional sources of starch are now being increasingly used and utilized as better alternatives for bioethanol production. It has been estimated that with the use of these resources, worldwide bioethanol production may increase to 16-fold as compared to the present level [5].

A typical method for conversion of starch into smaller oligosaccharides consists of two steps; starch liquefaction and saccharification. These processes are energy intensive, which ultimately escalate the price of starch-based final products [7]. Raw starch degrading enzymes

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such as α-amylases (EC 3.2.1.1: 1, 4-α-D-glucan-glucanohydrolase) can act directly on raw starch granules below the starch gelatinization temperature and thus can significantly reduce the energy consumption up to 10–20% [7, 8]. Therefore, application of raw starch digesting enzymes in energy reduction process in starch industry can prove more favorable than the conventional starch conversion process [7].

The constant increase in demand for commercial crops in order to feed the ever-increasing population has limited the use of such crops for starch production. Therefore, search from lesser-known and underutilized crops, which may have many potential uses, has become the focus of research in the recent years [9]. Jackfruit (*Artocarpus heterophyllus*), which is native to South-East Asia including the eastern and northeastern regions of India, have always been used as delicious food however, its seeds are underutilized. According to Sidhu [10], the annual production of jackfruit in India is around 1,436,000 tons and if the seed content is 10% of the fruit [9, 11] then approximately 143,600 tons of seeds despite being a very good source of carbohydrates and proteins goes wasted [12]. On an average, dried jackfruit seeds contain 77.76% starch (after removal of the spermoderm), which is in fact higher than the starch extracted from maize (~70%), a predominantly commercial source of starch [13]. Consequently, jackfruit seeds may be considered as an alternative source of low cost starch for cost-effective bioethanol production. In addition, jackfruit seed starch may find many other applications in starch industry. Therefore, considering the quantity (tons) of jackfruit seeds generated per year, identification of jackfruit-seed starch degrading α-amylase enzyme may be a stepping-stone towards the industrial application of this underutilized starch for the food and energy sectors coupled with waste management.

Recently, we reported on the purification and characterization of an α-amylase from a *B. licheniformis* strain AS08E [14]. The above enzyme holds a great promise for its industrial application in raw starch digestion process [14]. Inspired by the above findings and due to good solvent stability of this enzyme [14], in the present study an effort has been made to clone and express the gene of this enzyme in a heterologous expression system such as *Escherichia coli*, to increase its production level, to understand its structure-function relationships and to explore the potential application of this recombinant enzyme in the digestion of raw starch from jackfruit seeds. An attempt has also been made to utilize the jackfruit seed starch for bioethanol production.

E. coli is known to be a "protein factory" for heterologous protein expression because of its ease of genetic manipulation, simple fermentation, faster growth, and cost-effectiveness as compared to expression of recombinant protein in other hosts [15]. Notably, some gram-positive *Bacillus* expression systems are available for heterologous protein expression albeit they are reported to secrete a large number of hydrolytic enzymes including proteases which complicates the downstream processing and may effect the product stability [16]. Therefore, it is advantageous to overexpress even *Bacillus* proteins in *E. coli* expression system. However, *E. coli* expression system also has some disadvantages. For example, it may form protein aggregates known as "inclusion bodies" (enzymatically in-active in case of enzyme) but this problem can be sorted out easily by targeting the recombinant enzyme into the culture medium or periplasm [15, 17]. Therefore in the present study, the recombinant α-amylase from *B. licheniformis* strain AS08E was successfully targeted into the *E. coli* culture media (extracellular expression) using a native signal peptide sequence and the expression of recombinant enzyme was significantly enhanced by optimization of the culture conditions.

Materials and methods

Bacterial strains, chemicals, and reagents

The genomic DNA of *B. licheniformis* strain AS08E was isolated using the genomic DNA isolation kit (Fermentas, USA). DH5α and BL21 (DE3) (Novagen, Inc., CA, USA) strains of *E. coli* were used for transformation and overexpression studies, respectively. The pJET1.2 PCR cloning vector, (Fermentas, USA) was used for direct cloning of PCR amplified products and pET28a vectors (Invitrogen, CA, USA) was used for expression of the α-amylase gene. The Miniprep kit and gel extraction kits, restriction enzymes, T4 DNA ligase and DNA polymerase were also procured from Fermentas (USA). All other molecular biology grade chemicals used in the present study was procured either from Merck (USA) or HiMedia (India).

Cloning of α-amylase gene in pJET1.2 and pET28a vectors

A set of forward and reverse primers (BLF1 & BLR2) were designed (Table 1) on the basis of closest match of *B. licheniformis* DSM13 (Genbank no: AE017333) α-amylase gene sequence, which we arrived by homology searching of N-terminal sequence of α-amylase isolated from *B. licheniformis* strain AS08E in NCBI database [14]. The α-amylase gene was amplified from *B. licheniformis* strain AS08E using the above set of primers. The amplified product (~1.5 kb) was then

Table 1. Primers used in cloning of α-amylase gene from *B. licheniformis* strain AS08E.

Primer name	Primer sequence	Restriction sites
BLF 1	5'-CCC**AAGCTT**CTGCAAATCTTAAAGGGACGCTG-3'	*Hind* III
BLR 2	5'-CCG**CTCGAG**CCTATCTTTGAACATAAATTGAAAC-3'	*Xho* I
BLF 3	5'-CCC**AAGCTT**CTATGAAACAACAAAAACGG-3'	*Hind* III
BLR 4	5'-CCG**CTCGAG**CCTATCTTT GAACATAAATTG-3'	*Xho* I

ligated into pJET1.2 PCR cloning vector using CloneJET PCR Cloning Kit (Fermentas, USA) and subsequently the ligated product was transformed into competent *E. coli* DH5α cells. After sequencing the recombinant plasmid, the cloned α-amylase gene was found to be incomplete because it lacked the signal sequence required to export the recombinant protein outside the bacterial cell membrane. Therefore, to cover the complete ORF, sequences showing highest percentage similarity with the amplified (Blamy-I) gene in NCBI database were further selected to design the new set of primers.

The ***Hind*** III and ***Xho*** I restriction sites (indicated in bold) were introduced in the forward primer (BLF3) and reverse primers (BLR4), respectively (Table 1), which were then used to amplify the complete ORF of the α-amylase genes as isolated from *B. licheniformis* strain AS08E. The PCR amplified products were then double digested with the ***Hind*** III and ***Xho*** I restriction enzymes and inserted into the ***Hind*** III and ***Xho*** I sites of the pET-28a (+) vector. The recombinant plasmids (pETBLA2) were then transformed into competent *E. coli* BL21 (DE3) cells and subsequently, the cells were plated onto a LBA plates containing kanamycin (30 μg/ml). The recombinant clones were then examined for the extracellular secretion of α-amylase on LBA plates as well as in broth culture using the methods described in our earlier report [17].

Primary structure determination

The recombinant plasmids so obtained from the recombinant colony were sequenced using automated DNA sequencing units (3130 Genetic Analyzer, Applied Biosystems, USA). The deduced amino acid sequence obtained from nucleotide sequence was subjected to homology searching in NCBI database using the BLASTp programme (http://www.ncbi.nlm.nih.gov). Expasy online primary structure analysis tools (www.expasy.org) was used to determine primary structure of the recombinant α-amylase and the signal peptide was predicted using SignalP 4.0 server [18]. The multiple amino acid sequence alignment was accomplished using CLUSTAL W2 program [19] of EMBL–EBI online software (www.ebi.ac.uk) and conserved residues were determined using NCBI-conserved domain program (www.ncbi.nlm.nih.gov). Subsequently, the secondary structures of the recombinant protein were predicted and were superimposed using the ESPript online programme (www.espript.ibcp.fr/ESPript/ESPript).

Prediction of Blamy-I tertiary structure by computer aided modeling

An automated online protein homology-modeling server (SWISS-MODEL) was used to generate the three-dimensional structure of Blamy-I by comparing the X-ray crystallography structure of an α-amylase (PDB: 1ob0A) from PDB server as the template. The recombinant α-amylase (BLA.NH1) from *B. licheniformis* NH1 was used for the comparative study [20]. Both the models (Blamy-I and BLA.NH1) were superimposed, compared, and illustrated using the Molsoft ICM-Browser software (http://www.molsoft.com/icm_browser.html).

Enzyme (α-amylase) assay and protein estimation

The enzymatic activity of the recombinant α-amylase was assayed by estimating the amount of reducing sugars released by the action of enzyme from 1% (w/v) soluble starch in 50 mM K-phosphate buffer, pH-7.0 by DNS method [21]. One unit of enzyme activity was defined as the liberation of reducing sugars equivalent to 1μmol of D-glucose liberated per min under the assay condition [21]. The protein content of the unknown sample was assayed using Folin–Lowry's method with bovine serum albumin as the standard [22].

Optimization of extracellular expression of recombinant proteins

With the help of signal peptide, although *E. coli* cells were able to express recombinant α-amylase outsides the cell membrane; however, a large fraction of it remained inside the cell. Furthermore, the influence of media components and cultivation parameters for the extracellular expression of recombinant proteins by *E. coli* are well recognized [23, 24]. Therefore, the four identified parameters viz, IPTG concentration, post induction time, incubation temperature and concentration of EDTA (Supporting Information Table S1), which were supposed to influence the extracellular expression of recombinant

protein in *E. coli*, were optimized using our previous optimization techniques (Response Surface Methodology) for maximum extracellular expression of recombinant enzyme in culture media [17].

Purification of recombinant α-amylase from the recombinant *E. coli* cells

For the production and purification of recombinant α-amylase, a single transformed colony harboring recombinant plasmid (pETBLA2) was grown in 5 ml of LB media supplemented with 30 μg/ml Kanamycin and incubated overnight at 37 °C in a shaking incubator. On the next day, the culture was transferred into 100 ml of Terrific Broth (TB) supplemented with the 30 μg/ml Kanamycin and bacteria was allowed to grow until they reached mid-logarithmic phase (~0.6 O.D. at 600 nm). Finally, for the extracellular expression of recombinant α-amylase into the culture media, *E. coli* cells were then induced under the optimized culture conditions as shown in Table 2. Subsequently, the cell free supernatant (CFS) was obtained for purification of recombinant α-amylase by HIC and gel-filtration chromatography, using our previously illustrated procedures [17].

Determination of purity and molecular mass of recombinant α-amylase

The homogeneity as well as molecular mass of the pooled gel-filtration fractions displaying α-amylase activity was determined by 10% SDS-PAGE analysis of reduced (treated with β-mercaptoethanol) and non-reduced proteins [25]. For amylase zymography study, 1% (w/v) soluble starch was incorporated into the native PAGE (without reducing agent and SDS) prior to the addition of ammonium persulphate solution in pre-casting gel mixture. Successively, the purified recombinant α-amylase was run at constant voltage and then the gel was stained with iodine solution until a clear band appeared [26].

A comparison of biochemical properties of native and recombinant α-amylases

To compare and determine the optimum pH and temperature of the purified recombinant enzyme (Blamy-I) from *E. coli* with that of purified α-amylase from wild-type strain [14], 10 μg of purified α-amylase was incubated with 1% (w/v) starch dissolved in different buffers (pH ranging from 4.0 to 12.0), and at different temperatures (ranging from 30 to 90 °C) for 30 min. The α-amylase activity at each condition was assayed against appropriate control (substrate without enzyme). In order to determine the heat-denaturation of catalytic activity, Blamy-I solutions (10 μg/ml) were incubated at different temperatures ranging from 40 to 100 °C for 30 min, either in the presence or in absence of 5 mM Ca^{2+} ion, followed by enzyme assay. Residual α-amylase activity was determined against the control (unheated) considering its activity as baseline (100% activity). The kinetic properties (K_m and V_{max} values) of the recombinant α-amylase towards soluble starch were determined from the Lineweaver–Burk double-reciprocal plot.

Isolation of raw starch from jackfruit seeds and its digestion by Blamy-I

Raw starch from jackfruit seed cotyledons was isolated by following the method of Dutta *et al*. [12] with slight modifications. The dried jackfruit seed cotyledons was wet ground in a blender with distilled water and the crushed mass was sieved. The filtrate was then suspended in 0.5% (w/v) sodium thiosulfate solution for 24 h at room temperature with stirring at regular intervals to remove any protein fractions adhering to the starch granules. The precipitate was then recovered by centrifugation at 5000 rpm for 5 min. Thereafter, it was neutralized with 0.1 M HCl and washed several times with deionized water followed by 50% ethanol to remove any ions. The material so obtained was eventually oven dried and stored for further study.

Raw starch digesting capability of Blamy-I was evaluated by incubating 100 U of the enzyme with 2% (w/v) of jackfruit seed raw starch for 6 h at 30 °C [17]. The degree of raw starch hydrolysis was determined using a mathematical equation suggested by Mehta and Satyanarayana [27]

$$\mathrm{Rh}(\%) = \left(\frac{A_i}{A_0}\right) \times 100 \times 0.9 \quad (1)$$

where A_i is the amount of reducing sugar (mg/ml) in the supernatant after enzymatic reaction, A_0 is the amount of raw starch (mg/ml) before the reaction, and 0.9 (162/180) is the conversion factor due to the addition of

Table 2. Fold increase in extracellular expression of recombinant Blamy-I from *E. coli* BL21 cells using native signal peptide and optimized culture conditions.

IPTG (mM/ml)	Time of post Induction (h)	Temp. (°C)	EDTA (mM/ml)	Pred. values (EU)	Exp. values (EU)	Fold increase in extracellular expression
0.1	24	20	49.8	427.62	409.5	~8.0

water molecules to glycosyl moiety on hydrolysis [27]. The different end products so obtained after raw starch hydrolysis were analyzed by Silica gel G thin layer chromatography as described previously [21]. The residual product of raw starch hydrolysis was analyzed by scanning electron microscopy [17].

Simultaneous saccharification and fermentation (SSF) of jackfruit seeds starch

SSF was carried out by the method described by Shanavas *et al*. [4] with little modifications. Briefly, *Saccharomyces cerevisiae* strain ARDMC1 (GenBank: KF414969) isolated from traditional starter culture of rice beer of Assam, India was cultured aerobically in YPD (yeast extract 5, peptone 5, dextrose 20 g/L) medium at 30 °C for 72 h, thereafter, cells were harvested by centrifugation at 5000 rpm for 5 min and washed twice with phosphate buffer saline. For SSF of raw jackfruit seeds starch (RJSS) directly into ethanol, 15% of RJSS was suspended in 50 ml reaction media containing 0.3% (w/v) of urea, 0.1% (w/v) washed yeast cells and Blamy-I enzyme (100 U/g of raw starch) in 100 ml fermentation bottles equipped with a bubbling CO_2 outlet. The reaction mixture was then incubated at 30 °C for 7 days with mild agitations (100 rpm) in between. A control reaction was run under identical conditions but without Blamy-I enzyme. The ethanol content in the fermentation broth was analyzed on Porapack Q column (2 mm internal diameter, 80–100 mesh, 2 m length) by gas chromatograph analyzer (Nucon-5765, India) equipped with a thermal conductivity detector, at oven temperature of 60 °C. The injector and detector temperature was set at 80 and 110 °C, respectively with argon gas as a carrier at a flow rate of 30 ml/min.

The fermentation efficiency was calculated using the formula described by *Shanavas et al*. [4], which is illustrated in the following equation:

$$\text{Fermentation Efficiency } (\%) = \frac{\text{Total ethanol produced (g/kg of starch)}}{\text{Theoretical yield (g/kg starch)}} \times 100 \quad (2)$$

As the theoretical yield of ethanol (g) from per kg of glucose is 511 g, while the amount of glucose (kg) formed per kg of starch is 1.11 kg, therefore, the theoretical yield of ethanol per kg of starch is computed as 567 g [4].

Results

Isolation and cloning of gene encoding α-amylase from *B. licheniformis* strain AS08E in *E. coli*

By PCR amplification, ~1.5 kb fragment from the genomic DNA of *B. licheniformis* strain AS08E was amplified (Supporting Information Fig. S1), which was first cloned into pJET1.2 cloning vector and then ligated into pET-28a expression vector for sequencing and expression of amylase gene in *E. coli*. Sequencing of pETBLA2 insert gene (*blamy*-I) revealed that it contained complete ORF with a fragment length of 1539 bp, which encodes for 512 amino acid residues (including its signal peptide) with a calculated molecular mass of 58.56 kDa. The nucleotide sequence of the insert α-amylase (Blamy-I) gene has been deposited into GenBank database under accession number of KC802019.

The homology search of nucleotide and deduced amino acid sequences of Blamy-I showed marked similarity with the sequences of previously reported α-amylases from *B. licheniformis* (Fig. 1). The deduced Blamy-I amino acid sequence showed 99% similarity with the α-amylase isolated from *B. licheniformis* strain CICC 10181 (Genbank no: ACN88150); 98% sequence similarity with *B. licheniformis* strain RH 101 (Genbank no: ABF61440); and 96% sequence identity with a hyper-thermostable α-amylase from *B. licheniformis* (Genbank no: AAO26743) as well as with *B. licheniformis* NH1 (Genbank no: ABL75259). The deduced amino acid sequence of the insert showed that it contained prokaryotic signal peptide at its N-terminal end (Fig. 1). The isoelectric point (pI) and molecular weight (Mw) of mature Blamy-I was predicted as 6.05 and 55.27 kDa, respectively.

Structural investigation of Blamy-I using homology modeling

In order to understand the structure and function of the purified Blamy-I, its homology modeling was carried out by SWISS modeling server using the crystal structure of *B. licheniformis* α-amylase (PDB code: 1oB0), as a template. Blamy-I showed up to 99% homology with other *B. licheniformis* α-amylase amino acid sequence (Fig. 1); however, the biochemical properties of most of the cloned α-amylases were either undetermined or not reported, thus restricting the comparison of Blamy-I with most of the other related α-amylases sequences. The BLA.NH1 (Genbank code: ABL75259) showing 96% similarity with Blamy-I was finally selected for the comparative analysis (Fig. 1). The QMEAN Z-scores, which determine the acceptability and stability of the models [28], were found within the acceptable range for both the models. The QMEAN Z-score for Blamy-I model was calculated as 1.046 while for BLA.NH1, it was determined as 1.005. The Ramachandran plot, which

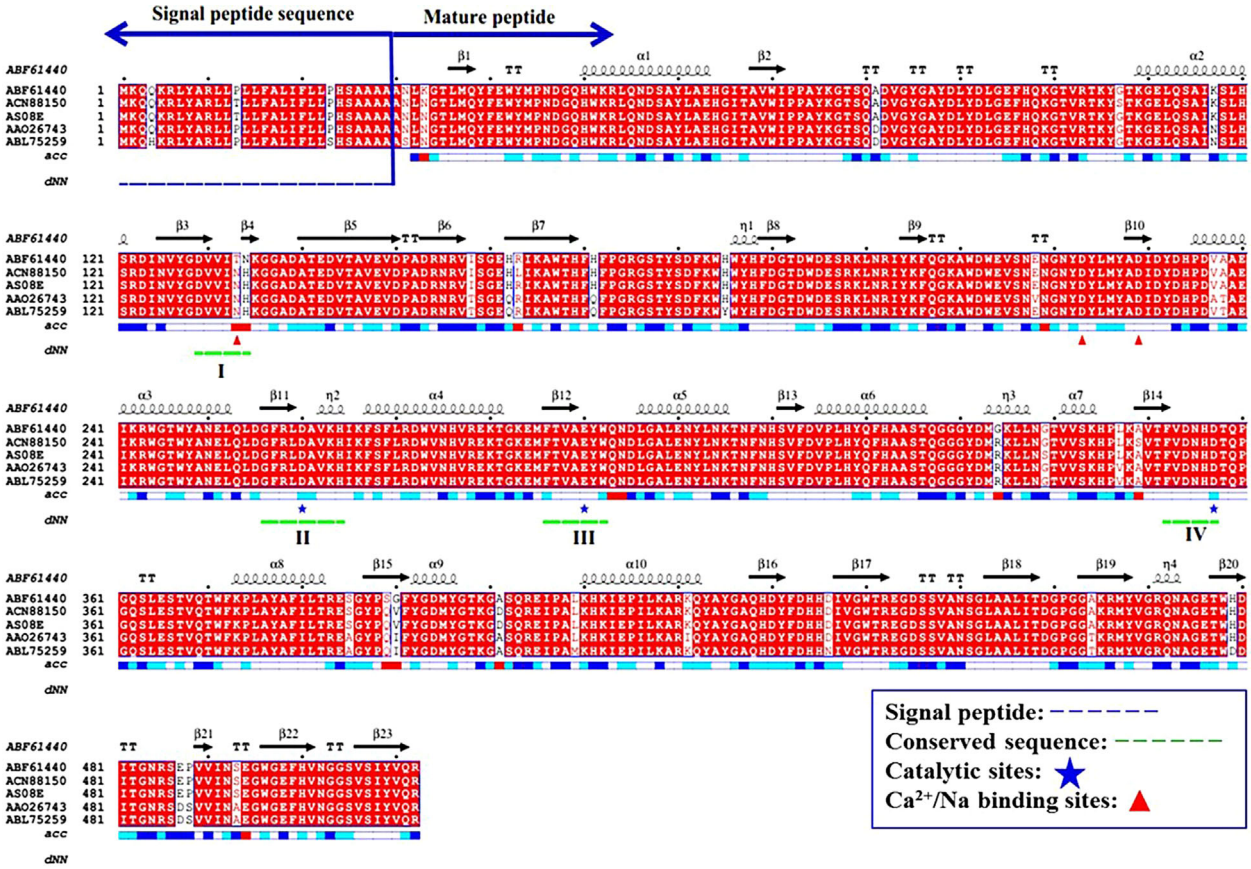

Figure 1. Multiple sequence alignment of Blamy-I with other homologous bacterial α-amylase sequences. The secondary structure assignments of the Blamy-I is indicated at the top of the alignment. The TTT and TT letters represent strict alpha and beta turns, respectively. The four conserved regions of Blamy-I are marked in underline (green color) with numerals and, the catalytic sites residues are marked with blue star sign and Ca^{2+}/Na^{+} binding sites are marked with red triangle. The conserved sequences of α-amylases are displayed as the white letters on the red background and the relative accessibility (*acc*) are shown in different colours (blue = accessible, cyan = intermediate, white = buried, and red = not predicted).

expresses how well the backbone conformations of all residues correspond to the known allowed areas in the plot [29], was determined in each case of Blamy-I and BLA.NH1 and it was found that in both the cases all the residues were in favored (98%) and allowed (>99.8%) regions. The structural superimposition of Blamy-I and BLA.NH1 did not show much difference in structure; however, Blamy-I possesses an additional Ca^{2+} binding site between the A and C domains (Fig. 2).

Extracellular expression of recombinant α-amylase in *E. coli*

The extracellular expression of recombinant α-amylase (Supporting Information Fig. S2) was confirmed by the formation of halo zones in starch-agar plates around *E. coli* colonies containing pETBLA2. By SDS-PAGE analysis of the cell extract as well as culture supernatant from *E. coli* BL21 cells harboring pETBLA2, a protein band of ~55 kDa equivalent to the size of Blamy-I was observed (data not shown). However, majority of the target proteins were accumulated as inclusion bodies in *E. coli* cells.

With the help of native signal peptide sequence of α-amylase from *B. licheniformis* strain AS08E, the amylase activity in culture supernatant of *E. coli* was found to be 51.2 ± 2.5 U/ml. Nevertheless, by statistical media optimization techniques and by solving so obtained modeled equation presented in Supporting Information Table S2, approximately eight-fold increase in extracellular expression of recombinant active enzyme (409.5 ± 20 U/ml) was achieved compared to its production under non-optimized conditions (Table 2).

Biochemical properties of the native and recombinant enzyme (Blamy-I)

Blamy-I was purified to homogeneity by a combination of hydrophobic interaction column chromatography

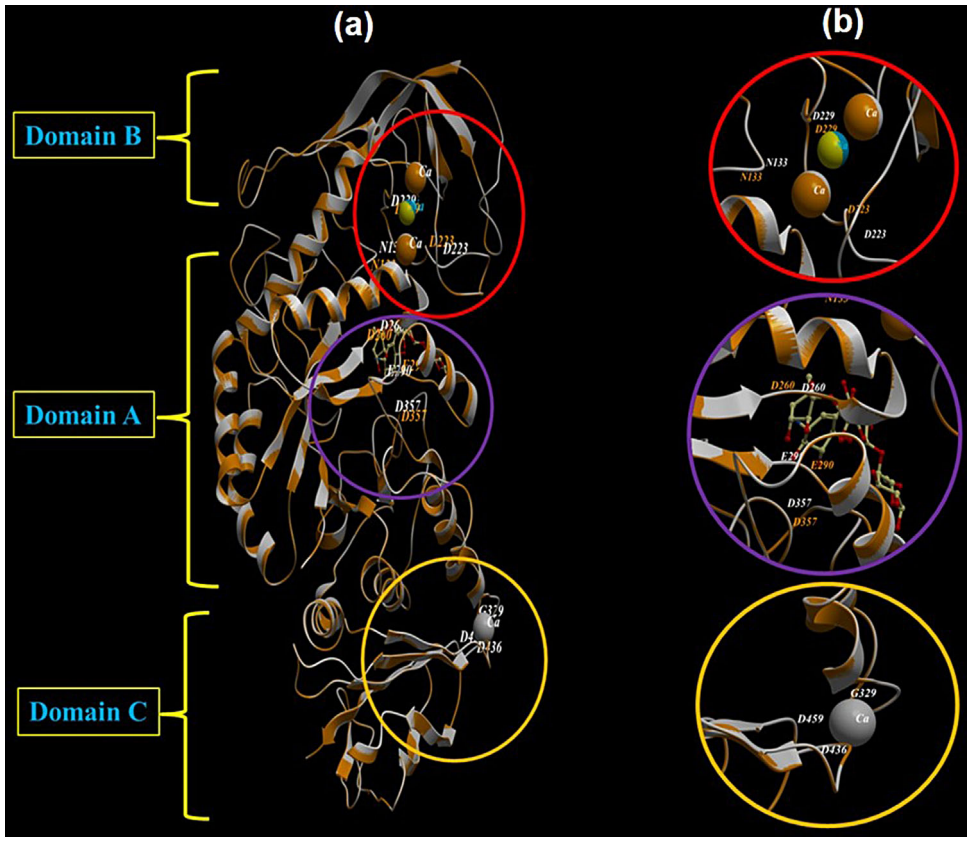

Figure 2. Superimposed model structure of Blamy-I and α-amylase from *B. licheniformis* NH1. (a) Superimposed backbone structure of Blamy-I (brown color) and BLA.NH1 (grayish white color) model showing the three domains (A, B, and C) and the conserved catalytic sites containing substrate analog (acarbose) along with the metal ions binding sites. (b) Amplified views of catalytic active residues and metal binding sites in the superimposed model.

(Supporting Information Fig. S3) followed by gel-filtration (Supporting Information Fig. S4) chromatography. The SDS-PAGE analysis of the purified Blamy-I showed a single band of approx. 55 kDa under both denatured and native conditions (Fig. 3). Zymographic analysis of the purified Blamy-I also indicated the appearance of a single clear zone of starch hydrolysis on the native PAGE (Fig. 3) suggesting that Blamy-I was catalytically active due to its expression in correctly folded form. A summary of the purification of this recombinant α-amylase (Blamy-I) is presented in Table 3. Blamy-I was purified to 14.2-fold with 19% recovery of the total α-amylase activity (Table 3). Additionally, both the purified native and recombinant enzymes showed the same specific activity towards starch hydrolysis. However, the percent yield of Blamy-I was much higher (19%) in comparison with the yield of the same enzyme (6.9%) isolated from the culture supernatant of *B. licheniformis* strain AS08E.

Biochemical characterization of Blamy-I was essentially carried out to eliminate the possibility of any mutation in the gene of this enzyme during PCR

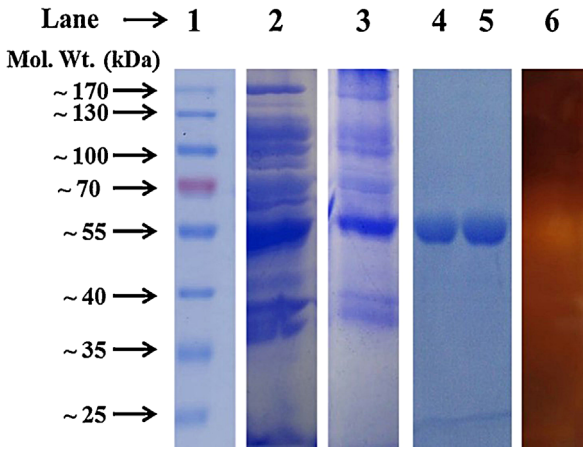

Figure 3. SDS-PAGE analysis of purified Blamy-I from *E. coli* BL21 (DE3) strain; lane 1: pre-stained protein marker; lanes 2: crude culture supernatant (15 μg); lane 3: phenyl-sepharose pooled fraction (15 μg); lane 4: purified gel-filtration fraction in reduced condition (10 μg); lane 5: purified gel filtration fraction in non-reduced condition (10 μg); and lane 6: zymograph of purified Blamy-I.

Table 3. Summary of purification of Blamy-I from the culture supernatant of recombinant *E. coli* BL21 (DE3) cells. The data represents a typical experiment.

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Enzyme recovery (%)	Purification fold
Culture supernatant	525.0	39150.0	74.57	100	1.0
Phenyl-sepharose	50.0	12000.0	240.0	30.6	3.2
Sephacryl S-200	7.0	7440.0	1062.85	19.0	14.2

amplification, which otherwise might have led to significant changes in the physico-chemical properties of recombinant enzyme compared to the native enzyme purified from wild-type [14]. A comparative analysis of native (AmyBL-I) and recombinant (Blamy-I) enzymes showed their identical biochemical properties (Table 4). This result further validates that the cloned enzyme (Blamy-I) in *E. coli* was indeed the same enzyme (AmyBL-I) purified from *B. licheniformis* strain AS08E [14]. Further, biochemical comparison of Blamy-I with that of structural and sequence similar BLA.NH1 enzyme [20] showed that despite possessing significant similarity (96%) in their primary structures, they displayed a significant difference in their biochemical properties (Table 4).

Raw starch digestion by Blamy-I

Our previous study showed that the α-amylase purified from wild-type *B. licheniformis* strain AS08E could efficiently hydrolyze raw starches obtained from various sources [14]. In the present study, we explored the jackfruit seed raw starch digestion potency of Blamy-I for its industrial application in bioethanol production. The hydrolysis of jackfruit seeds-starch by Blamy-I revealed that this recombinant enzyme can hydrolyze raw jackfruit seed starch up to 52% after 6 h treatment at 30 °C. The SEM analysis of the digested raw starch from jackfruit seed showed that Blamy-I could erode and form pits and holes on the smooth surfaces of the raw starch granules (Fig. 4). This confirm that like the α-amylase from parent *B. licheniformis* strain AS08E [14], Blamy-I is equally efficient in hydrolyzing the raw starch granules. Analysis of jackfruit seed starch hydrolysis product by TLC revealed that Blamy-I forms a range of oligosaccharides along with the trisaccharides and disaccharides from starch after 6 h of treatment under the experimental conditions (Supporting Information Fig. S5). It was also observed that Blamy-I hydrolyzed starch to produce maltose as one of the end products (Supporting Information Fig. S5), suggesting Blamy-I is a maltose-liberating enzyme [14].

Simultaneous saccharification and fermentation of jackfruit seeds starch

Simultaneous saccharification and fermentation (SSF) of non-conventional jackfruit seeds as a source of starch for

Table 4. Biochemical properties of the purified wild type (AmyBL-I) and recombinant (Blamy-I) α-amylase from *B. licheniformis* strain AS08E and its comparative analysis with structural and sequence similar α-amylase from *B. licheniformis* strain NH1.

Biochemical properties	Wild type α-amylase (AmyBL-I)	Recombinant α-Amylase (Blamy-I)	Recombinant α-Amylase (BLA.NH1)
Molecular wt. of the protein (kDa)	~55.0	~55.0	~58.0
Optimum pH	10.0	10.0	6.5
Optimum Temp. (°C)	80.0	80.0	90.0
pH stability	6.0–12.0	6.0–12.0	5.0–12.0
Thermostability (°C) in presence of Ca^{2+} ions	Up to 80.0 (upto 60–70°C in absence of Ca^{2+} ions)	Up to 80.0 (upto 60–70°C in absence of Ca^{2+} ions)	Up to 70.0 (absence/presence of Ca^{2+} ions)
K_m	5.0 mg/ml	5.56 mg/ml	Not defined
Ca^{2+} requirement	Ca^{2+}-dependent	Ca^{2+}-dependent	Ca^{2+} independent
Inhibitors	EDTA, 4-BPB, Hg^{2+}	EDTA, 4-BPB, Hg^{2+}	EDTA, Hg^{2+}
End product of starch hydrolysis	Maltose and high molecular oligosaccharides	Maltose, maltotriose and high molecular oligosaccharides	Maltose, maltotriose and maltopentaose

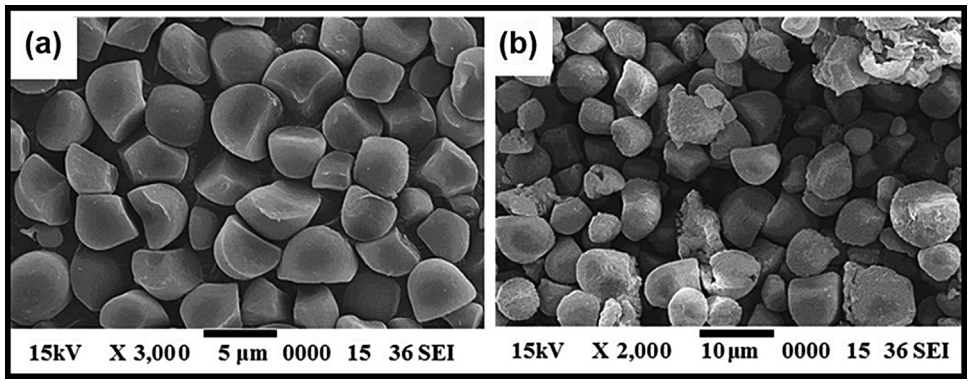

Figure 4. SEM images of untreated (control) and Blamy-I treated raw starch granules after 6 h of treatment at 30 °C. (A) Untreated jackfruit seed starch, and (B) treated jackfruit seed starch.

the production of bioethanol with the aid of Blamy-I and yeast is found to be an efficient feedstock, as the fermentation yielded approximately 5.3% (v/v) ethanol (Supporting Information Fig. S6). The final ethanol yield was calculated as 353.33 g/kg of starch while the fermentation efficiency [4] was calculated as 62%, suggesting that only 62% of raw jackfruit seeds starch was fermentable. However, there are reports which suggest that up to 98% fermentation efficiency could be achieved by conventional methods which requires the intensive input of energy [4]. Conversely, present work demonstrates a simple approach to scale up the bioethanol production using RJSS for efficient bulk industrial production.

Discussion

In order to study the structure-function relationship and to ensure overproduction of the Blamy-I enzyme, the α-amylase gene isolated from highly alkalophilic *B. licheniformis* strain AS08E was cloned and extracellularly expressed in *E. coli* BL21 cells. The homology search of nucleotide and deduced amino acid sequences of Blamy-I showed marked similarity with sequences of previously reported α-amylases from *B. licheniformis*. Furthermore, the deduced amino acid sequence found to contain prokaryotic signal peptide at its N-terminus. The analysis of amino acid sequence of Blamy-I also suggests that Blamy-I belongs to the glycosyl hydrolase family 13 of the α-amylase family [30]. The structural superimposition of Blamy-I and BLA.NH1 did not show much difference in structure; however, Blamy-I possesses an additional Ca^{2+} binding site between the A and C domains which is believed to play a key role in the observed thermo-stabilization of Blamy-I [31].

The superimposed structure of Blamy-I also revealed that it retained the key features of α-amylase enzyme, for example, it had three domains (A, B, and C) among which, domain A [$(\alpha/\beta)_8$ TIM barrel] formed the core of the molecules containing the three catalytic site residues. Among the three catalytic residues, Asp230 and Glu260 are believed to play the main catalytic role while the third residue Asp357 normally assists in the catalysis by hydrogen bonding to the substrate [32]. Domain A is also found to contain a Ca—Na—Ca metal triad, which is believed to play a key role in maintaining the structural stability of the α-amylases [31]. Domain B is composed of β-sheets, which normally vary substantially in numbers among the various members of α-amylase family; however, the main functions remain associated with this domain are substrate specificity and stability [32]. Domain C consists of C-terminus of the molecules and forms a Greek key motif structure. However, the function of this domain is unknown, but the third Ca^{2+}-binding site bridging domains A and C, found exclusively in bacterial α-amylases, are responsible for their enhanced thermostability [31].

Further, with the help of native signal peptide sequence of α-amylase from *B. licheniformis* strain AS08E, the recombinant protein was successfully expressed in *E. coli* culture media. However, majority of the target proteins remain accumulated as inclusion bodies in *E. coli* cells. Therefore, to reduce the formation of inclusion bodies and to overproduce the biologically active recombinant α-amylase into the culture media, the culture conditions of the recombinant *E. coli* BL21 cells were further optimized following our previously optimization techniques [17]. Optimization of culture conditions yielded approximately eight-fold increase in extracellular expression of recombinant active enzyme

(Blamy-I) as compared to its production under non-optimized conditions. Similar observations were made by various researchers [17, 23, 24].

The purification of recombinant Blamy-I showed a single band of approx. 55.0 kDa on SDS-PAGE which was identical to the native α-amylase purified from parent *B. licheniformis* strain AS08E [14] suggesting that α-amylase gene cloning was successful in *E. coli*. The percent yield of Blamy-I from *E. coli* culture supernatant was 19%, which also advocate that the majority of the secreted protein in cell-free supernatant was the recombinant enzyme and it was less contaminated with other proteins of *E. coli*. Further, structural and biochemical investigation of both the enzymes revealed that Blamy-I was much more thermostable than BLA.NH1, which may be due to the presence of extra Ca^{2+} binding site in the former enzyme. However, BLA.NH1 demonstrated higher temperature optima as compared to the same property shown by Blamy-I [20]. Thus, identifying and engineering these residue(s) in Blamy-I might lead to the development of a better enzyme molecule capable of enduring harsh industrial processes.

The starch hydrolysis profile of Blamy-I showed that it can also form maltose as one of the end products, suggesting that it is a maltose-forming enzyme [14] and such enzymes have a great market demand for bioethanol production as well as in the baking industry [33]. In our previous investigation it was found that wild-type α-amylase purified from *B. licheniformis* strain AS08E could easily digest the raw starch from various sources [14] and thus in the present study we investigated the digestibility of raw jack fruit seeds starch which is considered as underutilized agro waste. Recently, Dutta *et al*. [12] have also conducted acid hydrolysis of jackfruit seed starch and found that this technique had certain limitations. Nevertheless, the enzymatic hydrolysis of the same starch by Blamy-I using our simple and cost-effective method shows superiority over the chemical hydrolysis of starch molecules. Moreover, application of α-amylase enzyme, a green chemical, for the hydrolysis of jackfruit seed starch may be considered as an eco-friendly process to mitigate the environmental pollution problems by acid hydrolysis. Besides, Blamy-I also has an added advantages as they can form porous starch with jackfruit seed starch which can be used as adsorbent for volatile compounds and as fat substitute [34].

Simultaneous saccharification and fermentation (SSF) has several advantages over traditional methods of bioethanol production such as it maintains the concentration of available sugar at lower level which facilitates enzymatic reaction in forward direction and prevents bacterial contaminations [4]. The use of raw starch digesting enzyme in SSF for bioethanol production also eliminates the energy intensive enzymatic hydrolysis step [8]. Furthermore, exploration of non-conventional source of starch for bioethanol production may also reduce the production cost as well as dependency on commercial crops which are used for human consumption. The present study suggests that the use of non-conventional jackfruit seeds as a source of starch may serve as an efficient feedstock for the production of bioethanol, as the fermentation yielded approximately 5.3% (v/v) bioethanol in the culture supernatant. With further optimization of fermentation parameters, the ethanol yield could be enhanced substantially.

Conclusion

The present study shows that using signal peptide sequence of α-amylase gene from parent *B. licheniformis* strain AS08E and statistical optimization of culture parameters of recombinant *E. coli* cells, resulted in the efficient secretion of catalytically active recombinant enzyme (Blamy-I) into culture media. The percent yield of Blamy-I was significantly higher compared to the yield of the same enzyme from parent *B. licheniformis* strain AS08E. This justifies the production of recombinant α-amylase in *E. coli* system rather than its isolation and purification from wild-type bacteria for industrial applications. Furthermore, Blamy-I efficiently hydrolyzed the raw starch from jackfruit seed, a good source of unutilized, non-conventional starch. Taken together, the future application of Blamy-I as an eco-friendly green catalyst in the hydrolysis of raw starch from non-conventional sources, such as jackfruit seed, for the production of bioethanol and sugar syrup is highly promising.

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Conflict of interest

The authors declare that they have no competing interests.

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