Research Article

Khurram Jahangir Toor[#], Nasir Ahmad[#], Majida Atta Muhammad, Naeem Rashid*

TK-PUL, a pullulan hydrolase type III from *Thermococcus kodakarensis*, a potential candidate for simultaneous liquefaction and saccharification of starch

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Abstract: TK-PUL, a novel thermo-acidophilic pullulanase from *Thermococcus kodakarensis* and a unique member of glycoside hydrolase family GH13 was successfully produced in *Escherichia coli* grown by fed batch culture in a fermenter and partially purified by simple heat treatment. Specific activity of partially purified TK-PUL was 28 U/mg. Corn starch was successfully liquefied and saccharified using this single enzyme at pH 4.2. Simultaneous liquefaction and saccharification of corn starch by TK-PUL was comparable to Termamyl, a commercially available starch-hydrolyzing industrial enzyme. Both enzymes efficiently hydrolysed corn starch into sugar syrups having major proportions of maltose. TK-PUL performs efficiently at the natural pH of starch (~4.5) in the absence of any metal ions, hence is a potential candidate for starch industry.

Keywords: pullulan hydrolase; liquefaction; saccharification; thermostable; expression; *Thermococcus kodakarensis*.

Abbreviations

AMG 300L, amyloglucosidase from *Aspergillus niger*; CBM, carbohydrate-binding modules; CD, cyclodextrin; DE, dextrose equivalent; DNS, dinitrosalicylic acid; GH, glycoside hydrolase; HPLC, high-performance liquid chromatography; IPTG, isopropyl β -D-thiogalactopyranoside; TK-PUL, pullulan hydrolase type III from *Thermococcus kodakarensis*; TLC, thin-layer chromatography.

1 Introduction

Industrial synthesis of sweetener syrups and solid sweeteners from starch is a multi-billion business [1, <https://www.ers.usda.gov/data-products/sugar-and-sweeteners-yearbook-tables/>]. They have worldwide applications in foods and pharmaceuticals. The sweeteners from corn starch are increasingly replacing the traditional cane sugar all over the world [2, <https://www.ers.usda.gov/data-products/sugar-and-sweeteners-yearbook-tables/>]. Glucose and maltose syrups obtained from starch serve as feed stock in several biotechnological transformations and are being employed in the synthesis of organic acids, sorbitol, ethanol and amino acids [3,4]. United States of America, world's largest producer of fuel ethanol, obtains more than 80% of fuel ethanol from fermentation of glucose syrup [5]. High fructose syrup is synthesized from glucose syrup with the help of glucose isomerase [6].

Starch is the starting material for these sweeteners [7] and conversion of starch into glucose is achieved in two steps. The first step is liquefaction, which involves starch hydrolysis with the help of bacterial amylases in the presence of calcium (40 ppm) at 95–105 °C and a pH of ~6.0. During the second step (saccharification), the liquefied starch is further hydrolysed with the help of

*Corresponding author: Naeem Rashid, School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan, E-mail: naeem.ff.sbs@pu.edu.pk; naeemrashid37@hotmail.com

Khurram Jahangir Toor, Nasir Ahmad, Majida Atta Muhammad, School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan

[#] K. J. Toor and N. Ahmad contributed equally to this work and, in their opinion, should be considered as joint first author

Table 1: Comparison of the properties of type III pullulan hydrolases [3].^a

Property	<i>Thermococcus kodakarensis</i> KOD1	<i>Thermococcus aggregans</i>
Molecular mass (kDa)	84.4	80
Catalytic subunits	Monomer	Not determined
Optimum temp (°C)	95-100	95
Activity at 120 °C (%)	>60	>35
pH range	3.0-8.5	3.5-8.5
Activity at pH 3.5 (%)	100	50
Optimum pH for activity	3.5	6.5
Ca ²⁺ requirement	No	No
Half-life at 100 °C (min)	45	90
K_m (mg/mL) for pullulan	2	2.38
V_{max} (U/mg) for pullulan	109.17	16.6
Complete hydrolysis of pullulan	10 min	16 h
End products of pullulan hydrolysis	G3, panose, G2, isomaltose and G1	G3, panose, G2 and G1
Hydrolysis of glycogen	Yes	No
Hydrolysis of CD	All types	Only β -CD and γ -CD
Smallest oligosaccharide hydrolysed	G3	G4

^a G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; β -CD, β -cyclodextrin; γ -CD, γ -cyclodextrin.

bacterial pullulanase and fungal amyloglucosidase at 60 °C and pH 4.5. Currently available amylases (TERMAMYL[®] from *Bacillus licheniformis* and THERMOLASE[™] from *Bacillus stearothermophilus*) are unable to work efficiently in the absence of calcium and at low pH, therefore pH of starch slurry has to be increased from its natural pH (~4.5) to pH 6.0 [7]. After completion of liquefaction, the pH of the hydrolysates had to be brought back to ~4.5 that is optimal pH of fungal amyloglucosidase subsequently used in saccharification. Addition of calcium during liquefaction step, required for thermostability of bacterial amylases, is a nuisance because it inhibits the activity of glucose isomerase [6]. Another disadvantage of calcium is the synthesis of a by-product calcium oxalate that deposits in plate heat exchangers and pipelines, choking them and increasing the process cost. Despite the above-mentioned problems, thermostable bacterial amylases were the only option for starch processing industry because nearly 100 °C is required for gelatinization.

In order to improve yields of maltose and glucose syrups, pullulanase is employed as a debranching enzyme, which hydrolyses α -1,6-glucosidic linkages at the branch points of limit dextrins. Both α - and β -amylases are unable to hydrolyze α -1,6 bonds [8]. On the basis of sequence similarities pullulanases are grouped into

glycoside hydrolase (GH) families GH13 and GH57 [9,10] and, eventually, also in the family GH49 (<http://www.cazy.org/>). Similarly, depending upon reactions catalysed and substrate specificities, pullulanases are grouped into five types – pullulanases I, II and pullulan hydrolases I, II and III. In contrast to other four types, pullulan hydrolase type III has the unique property of hydrolysing both α -1,4- and α -1,6-glycosidic linkages in pullulan. Till now, only two members of this class have been characterized. The first report was from *Thermococcus aggregans* [11], while second one was from *Thermococcus kodakarensis* [3]. A comparison of catalytic properties of both these pullulan hydrolases is given in Table 1.

The pullulan hydrolase type III from *T. kodakarensis*, TK-PUL, is highly thermostable and optimally acts at pH ~4.5 [3]. It has the ability to completely hydrolyse starch without the help of any other enzyme. Moreover, it does not require any additional metal ion for its activity or stability [3]. Three-dimensional structure of TK-PUL has already been resolved [8]. Like other members of GH13, TK-PUL is a multi-domain enzyme consisting of an N-terminal domain (residues 185-280), a central catalytic domain (residues 281-694) in the form of typical TIM-barrel structure and a C-terminal domain (residues 694 onwards). The tertiary structure of TK-PUL was significantly different from

homologous structures at the active-site and at the last parts of the N- and C-terminal domains [8].

Here we report the production of recombinant TK-PUL through fed batch fermentation and production of maltose-rich syrups by simultaneous liquefaction and saccharification of starch using partially purified TK-PUL.

2 Materials and methods

2.1 Chemicals, strains and plasmids

The chemicals used in the study were of high grade and purchased from Sigma-Aldrich or Fluka, if not mentioned otherwise. *Escherichia coli* BL21-CodonPlus (DE3)-RIL strain was used as expression host, while recombinant plasmid Pul-pET was used for production of recombinant TK-PUL [3]. Industrially employed liquefying amylase, TERMAMYL® 120L, and saccharifying amyloglucosidase, AMG 300L, were from Novozymes.

2.2 Production of recombinant TK-PUL through fed batch fermentation

Recombinant TK-PUL was produced in *E. coli* BL21-CodonPlus (DE3)-RIL cells and purified as described earlier [3] but on a large scale. Laboratory scale fermenter (Bio Flo 110, New Brunswick Scientific) was used for large-scale cultivations. Inoculation of the host cells containing Pul-pET plasmid was made in the fermenter vessel containing 1,800 mL of sterilized LB medium (1% trypton, 0.5% yeast extract, 0.5% NaCl) supplemented with 0.5% glycerol, 0.05% glucose, 2 mM magnesium sulphate and ampicillin (100 µg/mL). Cells were grown at 37 °C while keeping agitation speed 200 rpm and constant aeration (1 vvm). Dissolved oxygen level was maintained at 30% of air saturation either by regulation of agitation speed or supplementation of air with pure oxygen during high cell density cultivation. The pH of the culture was kept constant at 7.2 ± 0.1 with the addition of NH_4OH or HCl. Antifoam Y-30 Emulsion (Sigma Cat. # A6457) was intermittently added, at controlled rate, to avoid foaming. Feedings were given at appropriate intervals. Gene expression was induced with 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) and cultivation was continued for further four hours. Cells were harvested by centrifugation at 6,000 rpm for 20 min and supernatant was discarded. Cell pellet was washed and re-suspended in 50 mM Tris-Cl buffer of pH 8.0.

2.3 Partial purification of recombinant TK-PUL

E. coli cells (180 g) containing recombinant TK-PUL were suspended in 200 mL of 50 mM Tris-Cl buffer (pH 8.0) and disrupted by sonication in ice water. The cell lysate was centrifuged at 6,000 rpm and 4 °C for 20 min to separate soluble and insoluble protein fractions. The soluble fraction was heated at 80 °C for 30 min in water bath and incubated on ice for 30 min. Heat coagulated host cell proteins were removed by centrifugation at 12,000 rpm and 4 °C for 20 min.

2.4 Dextrinization assay

Starch-iodine assay was performed to examine the dextrinization of starch. Reaction mixture was prepared by mixing 130 µL of 2% starch and 320 µL of 50 mM sodium citrate buffer of pH 4.2. The mixture was pre-heated at 90 °C for 10 min and then 50 µL of properly diluted TK-PUL was added. Commercially available liquefying amylase, Termamyl® from Novozymes, was used as positive control. For Termamyl assay, the reaction mixture containing 130 µL of 2% starch, 300 µL 50 mM sodium acetate buffer of pH 6.9 and 20 µL of 50 mM calcium chloride was pre-heated at 90 °C for 10 min. Then 50 µL of properly diluted Termamyl was added. Further incubations of the reaction mixtures were performed at 90 °C for 5-10 min. A substrate control was prepared in the same way but without starch. A protein control was included without the addition of protein. After incubation, the reaction mixtures were quenched on ice for 10 min. Stock solution of potassium iodide/iodine solution (35 g potassium iodide and 5 g iodine was dissolved in 500 mL of distilled H_2O) was diluted 4-times and 250 µL was added in the reaction. The reaction volume was raised upto 1 mL with distilled H_2O . In order to determine the extent of dextrinization, optical density (OD) of the mixture was measured spectrophotometrically at 580 nm by properly diluting the mixture [12]. One Kilo Novo α -amylase (TERMAMYL®) unit (KNU) was defined as the amount of enzyme, which dextrinizes 5,260 mg dry starch/hour under standard conditions (at 90 °C and pH 6.9). An equivalent Kilo Novo pullulanase (TK-PUL) unit (KNU) was defined as the amount of enzyme, which dextrinizes 5,260 mg dry starch /hour under standard conditions (at 90 °C and pH 4.2).

2.5 Activity assay

Enzyme activity was measured in terms of reducing sugars released upon incubation of TK-PUL with soluble starch, which were determined by dinitrosalicylic acid (DNS) method [13]. Maltose was used as standard. The assay mixture containing 100 μ L of 2% starch solution and 50 mM sodium citrate buffer of pH 4.2 was pre-incubated at 90 °C for 10 min. Properly diluted TK-PUL was added and the reaction mixture was further incubated at the same temperature for 5 min. Reaction was stopped by quenching on ice for 10 min. DNS reagent was added in it and placed in boiling water bath for 5 min. The mixture was diluted and absorbance was measured at 540 nm. One unit was defined as the amount of enzyme required to release one μ mol maltose in 1 min under standard assay conditions.

2.6 Conversion rate of TK-PUL during starch hydrolysis

In order to evaluate the conversion rate, recombinant TK-PUL (0.5 KNU/g substrate on dry basis) was incubated with 1% (w/v) corn starch under optimal conditions (pH 4.2, 90 °C). At regular intervals samples were taken and saccharide profile was analysed by high-performance liquid chromatography (HPLC) as described previously [3].

2.7 Saccharide profiling by thin-layer chromatography (TLC)

TLC was performed by slightly modifying the method described by Hay et al. [14]. Mobile phase was prepared by mixing solution A (*n*-butanol : diethyl ether : acetic acid : water in 9:3:3:1 ratio) and B (isopropanol : acetone : water in 2:2:1 ratio) in 1:1 ratio. Mixture of glucose, maltose and maltotriose (1% each mixed in 1:1:1) was used as a standard. The sugar spots after TLC were visualized by spraying with 5% (v/v) sulphuric acid (in methanol) followed by baking at 110 °C for 10 min.

2.8 Measurement of dextrose equivalent (DE)

Reducing sugars were measured according to the Association of Official Analytical Chemists method No. 31-035 [15]. Glucose was used as a standard. Reducing sugar content of the properly diluted starch hydrolysates was determined by titration against Fehling's solution. DE

was determined by the following formula: $DE = (250 \times a \times 10) / (b \times g \times d)$, where “a” is the factor from table [15]; “b” is volume (mL) of test solution used for titration; “g” is the mass (in grams) of sample dissolved in 250 mL distilled water and “d” is percent dry solids in the sample.

2.9 Simultaneous liquefaction and saccharification of 10% (w/v) corn starch

Corn starch (60 g) was mixed with 600 mL of 50 mM sodium citrate buffer of pH 4.2 and heated in boiling water bath for 5 min to gelatinize it. Partially purified TK-PUL (3.2 mg) was added, mixed properly and placed in water bath at 90 °C for 2 h. The liquefied starch slurry was then shifted to dry heat oven at 95 °C and incubated for overnight. In order to examine the extent of liquefaction at various intervals, samples were taken and analysed through colorimetric iodine assay.

2.10 Production of glucose syrup

Saccharification by TK-PUL was compared with the commercially available amyloglucosidase from *Aspergillus niger* (AMG 300L). For this purpose, corn starch liquefied by TK-PUL was divided into two halves (300 mL each). AMG 300L was added in one of the halves and incubated at 60 °C, while the other half, containing TK-PUL since liquefaction step, was incubated again at 95 °C. At the completion of experiment, the saccharide profiles were analysed by TLC and HPLC [3].

2.11 Simultaneous liquefaction and saccharification of 30% (w/v) corn starch

Various concentrations of partially purified TK-PUL and Termamyl 120L were mixed individually with 17 mL of 30% (w/v) corn starch under respective optimum reaction conditions. The samples were placed in boiling water bath (100 °C) for 2 h and then shifted to dry heat oven at 105 °C. Samples were taken out at regular intervals and analysed through colorimetric iodine and DNS methods. DEs were also calculated and saccharide profiles were analysed by TLC.

3 Results and discussion

3.1 TK-PUL and sequence-based classification

Sequence-based classification system of GHs, introduced by Henrissat in 1991 [16], categorizes pullulanases into families GH13 and GH57 [9,10], and – according to recent update of the Carbohydrate-Active enZyme (CAZy) database (<http://www.cazy.org/>) [17] – also in the family GH49. Among them, GH13 is the largest family, currently containing 104,223 sequences, from which 825 have been characterized [17]. Members of the family GH13 share following common features: (i) they act on the α -glucosidic linkages and catalyse either hydrolysis or transglycosylation or both (hydrolysis and transglycosylation) to produce α -anomeric mono- or oligosaccharides (containing α -1,4- or 1,6-glycosidic linkages); (ii) they contain a $(\beta/\alpha)_8$ -barrel (i.e. TIM-barrel) type catalytic domain; (iii) their primary structures contain the four highly conserved sequence regions; and (iv) their catalytic triad is constituted by the three acidic residues (two aspartic acids and one glutamic acid). Biochemical characterization and three-dimensional structure analysis of TK-PUL revealed that it possessed all the features of the family GH13 [3,8].

Despite sharing several structural features, the members of the family GH13 display a wide variety of substrate specificities, bond cleavage patterns and potential to catalyse transglycosylation and/or cyclization reactions [18]. Around 30 different catalytic activities have been reported for GH13 members; therefore, this superfamily of GHs has been subdivided into 42 subfamilies [17,19].

TK-PUL along with pullulan hydrolase type III from *T. aggregans* [11] has been assigned to the subfamily GH13_20 [20]. Other members of subfamily GH13_20 include neopullulanase, cyclomaltodextrinase and maltogenic amylase [20,21]. All of these enzyme possess hydrolytic activity towards cyclodextrins (CDs) [22]. TK-PUL also displayed cyclodextrinase activity and γ -CD was the second most preferred substrate after pullulan [3].

Carbohydrate-binding modules (CBMs) refer to the structural motifs responsible for holding or orienting the carbohydrate ligands to the active sites of carbohydrate-active enzymes. Individual CBMs do not play any role in catalysis, they rather facilitate the enzyme to better interact with substrate. CBMs were first identified in cellulases and amylases. In amylases, such domains were previously known as starch-binding domains. CAZy system classifies

them into CBM families [23]. Bacterial members of GH13_20 normally contain starch-binding domain at the N-terminus (absent in classical amylases), which is classified as the family CBM34 [20,22]. This N-domain of bacterial enzymes help them to adopt a homodimer configuration and generation of the active site cleft [24]. Besides N-domain, archaeal counterparts possess an additional N'-domain, which is classified as CBM48 family [22]. Because of this novel domain, monomers of archaeal enzymes remain successful to adopt a unique active-site configuration for cyclodextrinase activity [24,25]. Tertiary structure of TK-PUL [8] was found similar with the structures of other archaeal members of the subfamily GH13_20, which are maltogenic amylase from *Staphylothermus marinus* [24] and α -amylase/cyclomaltodextrinase from *Pyrococcus furiosus* [25]. TK-PUL lacks significant homology with these enzyme at the N-terminal region and probably due to this reason TK-PUL, in spite of high cyclodextrinase activity [3], is not a member of either CBM34 or CBM48 (<http://www.cazy.org/>).

Based on the amino acid sequence comparison, TK-PUL was annotated as pullulanase type II, the GH13 family member in the genome sequence of *T. kodakarensis* (TK0977; GenBank Accession No. AP006878.1). Han et al. [26] also characterized it as pullulanase type II, but with strong evidences we proved that TK-PUL was actually a pullulan hydrolase type III [3].

Type II pullulanases are also known as amylopullulanases. They are considered potential candidates for starch processing industry because of their dual activity as α -amylase (α -1,4-cleavage) as well as pullulanase (α -1,6-cleavage). Amylopullulanases may possess either a single active site for this dual activity or they have two different active sites [27]. Amylopullulanases from *Bacillus circulans* F-2 [28], *Bacillus* sp. strain KSM-1378 [29] and *Pyrococcus woesei* [30] have been reported to possess two different active sites on a single polypeptide chain responsible for dual catalytic activities. Amylopullulanases from *Desulfurococcus mucosus* [31], *Thermoanaerobium* strain Tok6-B1 [32], *Clostridium thermohydrosulfuricum* [33], *Thermoanaerobacterium saccharolyticum* B6A-RI [34], *Thermoanaerobacter ethanolicus* 39E [35], *Bacillus* sp. strain XAL601 [36] and *Bacillus* sp. DSM 405 [37] were shown to possess only one active site for cleavage of both α -1,4- and α -1,6-glycosidic linkages. Like most of the amylopullulanases, TK-PUL also possesses a single active site for dual catalytic activity [3].

3.2 Production of TK-PUL by fed batch method

In order to produce TK-PUL by fed batch culture, the host cells containing PUL-pET were grown in bench top fermenter using fed batch method. Aeration in the culture was maintained at 30% (v/v) oxygen saturation. pH was maintained at 7.2 ± 0.1 with the addition of NH_4OH or HCl . Feedings were given at appropriate intervals. Gene expression was induced with 0.1 mM IPTG when OD_{600} and wet cell mass were 31 and 79 mg/mL, respectively (Fig. 1). Synthesis of recombinant TK-PUL in *E. coli* started immediately after the IPTG induction and reached to about 40% of the total cell proteins in the second hour of post-induction period. Almost similar level of protein synthesis (~40% of the total cell proteins) was maintained in the next three hours (Fig. 2). Since it is well established during our shake flask experiments [3] and by other researchers that high production of recombinant proteins is attained in four to five hours after the IPTG induction [38,39], cells were therefore harvested after five hours post induction. Finally, 180 g cells were obtained from 3 L culture.

3.3 Partial purification of recombinant TK-PUL

Cells, after harvesting, were lysed by sonication. Soluble and insoluble fractions were separated. The soluble fraction containing TK-PUL was heated at 80 °C for 30 min in a water bath. The heat labile proteins were separated by centrifugation. Both the supernatant and the pellet were analysed by SDS-PAGE (Fig. 3). Partially purified TK-PUL was obtained in the supernatant, while most of the host cell proteins got precipitated during heating and were removed in the insoluble fraction. Partially purified TK-PUL displayed a specific activity of 28 U mg^{-1} at 90 °C and pH 4.2.

3.4 Comparison of TK-PUL and commercially available Termamyl

During two-step hydrolysis of starch into glucose and maltose rich syrups, pullulanase is normally added in the second step (saccharification) to hydrolyze the α -1,6-linkages at branch points that remain un-attacked by liquefying α -amylases [40]. For efficient enzymatic hydrolysis, complete solubility of starch slurry is required that is only attainable above 100 °C, while the natural pH of starch slurry is ~4.5. Therefore, the use of

Figure 1: Growth and activity curves representing production of TK-PUL in *E. coli* during fed batch cultivation. Wet cell mass (●), OD at 600 nm (○) and enzyme activity (◇).

Figure 2: Coomassie brilliant blue stained 12% SDS-PAGE showing production of TK-PUL in *E. coli* in fed batch culture. Lane 1, before induction; lane 2, 1 h after induction; lane 3, 2 h after induction; lane 4, 3 h after induction; lane 5, 4 h after induction; lane 6, 5 h after induction.

a thermoacidophilic enzyme would directly benefit the starch industry [7]. Owing to high thermostability and low pH (4.2) optima, TK-PUL possesses the promising features to meet industrial requirements. It hydrolyses both α -1,4- and α -1,6-glucosidic linkages and does not require calcium or any other metal ion for its activity or thermostability [3].

TK-PUL and Termamyl were used for starch hydrolysis for comparative studies. Termamyl (0.5 μg) completely dextrinized the starch in 5 min, while TK-PUL (10 μg) dextrinized the starch to same level in 40 min. One KNU is defined as the amount of enzyme which, under standard conditions (for TK-PUL, 90 °C and pH 4.2; while

for Termamyl, 90 °C and pH 6.9), dextrinizes 5,260 mg/h dry starch. One KNU of TK-PUL was equivalent to 13.33 mg enzyme, which dextrinized 5,260 mg of starch under standard assay conditions, while one KNU of Termamyl was equivalent to 83 µg.

3.5 Conversion rate of TK-PUL during starch hydrolysis

When tested against 1% (w/v) corn starch, recombinant TK-PUL (0.5 KNU/g substrate on dry basis) completely hydrolysed starch during initial six hours incubation at optimal conditions (pH 4.2, 90 °C). Initially generated maltooligosaccharides (maltoheptaose to maltotetraose) were rapidly converted to maltose and maltotriose, both of which reached to near maximum at around tenth hour. Afterwards these were synthesized approximately at a steady rate (Fig. 4). After 24 hours maltose concentration reached more than 50% of the reaction mixture and continued to increase but at slower rate (Fig. 4).

3.6 Simultaneous liquefaction and saccharification of corn starch (10% w/v) using partially purified TK-PUL

Corn starch (10% w/v) was prepared in 50 mM sodium citrate buffer (pH 4.2) in a total volume of 600 mL and gelatinized by heating in boiling water bath for 5-10 min, then 3.2 mg of partially purified TK-PUL were added. The reaction mixture was incubated at 95 °C for overnight. A sample of 100 µL was taken and diluted 10-times. Dextrinization of starch was checked by starch-iodine method. Starch was completely dextrinized and the blue colour of starch-iodine complex was disappeared as shown in Figure 5.

3.7 Production of glucose syrup

Corn starch, dextrinized by TK-PUL, was divided into two equal portions. Commercially available amyloglucosidase from *A. niger* was added (1.2 U) in one of the halves and placed at 60 °C, while the other half was placed at 95 °C in dry heat oven for overnight. The saccharide profiles, analysed by HPLC, revealed that the corn starch dextrinized by the sole action of TK-PUL contained a mixture of dextrans (48%) and saccharides smaller than maltohexaose (52%). Among smaller saccharides, maltose

Figure 3: Coomassie brilliant blue stained 12% SDS-PAGE demonstrating soluble and insoluble fractions after heat-treatment at 80 °C for 30 min. Lane 1, insoluble fraction; lane 2, soluble fraction.

Figure 4: Conversion rate of TK-PUL during corn starch hydrolysis. Maltose (■), maltotriose (○), oligosaccharides from maltotetraose to maltoheptaose (●), and higher saccharides including substrate (□).

had the predominant concentration (29%) (Table 2; Fig. 6A). The combined action of TK-PUL and amyloglucosidase resulted in complete conversion of larger saccharides into glucose (93.1%) with a few traces of higher saccharides (Fig. 6B). These results indicated that TK-PUL successfully liquefied and saccharified 10% (w/v) corn starch into maltose-rich syrup, while amyloglucosidase further hydrolysed these oligosaccharides into glucose.

Table 2: Saccharide profile of glucose and maltose syrups produced from 10% corn starch.^a

Enzyme	Product concentration (%)						
	DPn	G6	G5	G4	G3	G2	G1
None	100						
TK-PUL	47.7	2.2	2.8	6.6	3.9	29.0	7.8
TK-PUL + AMG			0.9		0.8	5.2	93.1

^a DP, degree of polymerization; AMG, amyloglucosidase, G6 to G1, maltohexaose to glucose, respectively.

Figure 5: Starch-iodine assay for confirmation of dextrinization of starch. 1, negative control without starch; 2, control without TK-PUL; 3, 10% starch dextrinized by TK-PUL.

3.8 Simultaneous liquefaction and saccharification of 30% (w/v) corn starch

Various concentrations of TK-PUL (6, 12, 25 mg), respectively, were used for simultaneous liquefaction and saccharification of 30% (w/v) corn starch. For comparison, different dosages of Termamyl (0.01, 0.1, 0.2 mg), respectively, were also used in the similar way as a positive control. Samples were taken after 1h, 2h and overnight incubations under respective conditions. Samples were diluted 30-times and assayed for dextrinization of starch using starch-iodine method. The extent of starch dextrinization gradually increased over the time and with increasing concentrations of TK-PUL or Termamyl (Table 3).

3.9 Determination of reducing sugars in glucose syrup

The reducing sugar content of the glucose syrups, obtained through simultaneous liquefaction and saccharification

Figure 6: Saccharide profile of syrups produced from 10% corn starch by the action of TK-PUL (A) and by the combined action of TK-PUL and AMG (B). DP denotes degree of polymerization, while G4 to G1 are representing maltotetraose to glucose, respectively.

of 30% w/v corn starch, was estimated by DNS method [13]. Reducing sugars in terms of maltose equivalents gradually increased over the time with the increase in concentrations of TK-PUL or Termamyl (Table 4).

3.10 Determination of dextrose equivalents in glucose syrup

Dextrose is the purified crystalline glucose obtained after complete hydrolysis of starch. The term glucose syrup refers to a concentrated solution of starch hydrolysates, which contains a mixture of glucose, maltose, maltotriose and higher oligosaccharides but DE higher than 20 [2]. Higher value of DE corresponds to higher degree of hydrolysis and vice versa. Starch has nearly zero dextrose equivalent, whereas 100 represents purified glucose/dextrose [7].

DEs of the glucose syrups in this study were calculated for the samples obtained after simultaneous liquefaction and saccharification of 30% (w/v) corn starch using

Table 3: Comparison of time course dextrinization of 30% corn starch with TK-PUL and Termamyl.

Time (h)	OD (580 nm)	
	TK-PUL (6 mg)	Termamyl (0.2 mg)
0	57477	57477
1	6171	833
2	4386	659
17	3819	586

Table 4: Comparative release of reducing sugars from corn starch treated with TK-PUL and Termamyl.

Time (h)	Maltose equivalents (μmol)	
	TK-PUL (6 mg)	Termamyl (0.2 mg)
1	1657	8435
2	2126	9067
17	5411	9983

Table 5: Comparison of the dextrose equivalents of the maltose syrups produced by using TK-PUL and Termamyl.

Enzyme	TK-PUL (mg)			Termamyl (mg)		
Dosage	6	12	25	0.01	0.1	0.2
DE	24	30	37	21	26	35

Figure 7: TLC demonstrating saccharide profiles of glucose syrups obtained from hydrolysis of 30% corn starch using different dosages of TK-PUL or Termamyl. Lane 1, standard mixture containing glucose, maltose and maltotriose; lane 2, hydrolysis products by 0.01 mg Termamyl; lane 3, hydrolysis products by 0.1 mg Termamyl; lane 4, hydrolysis products by 0.2 mg Termamyl; lane 5, maltose as standard; lane 6, maltotriose as standard; lane 7, hydrolysis products by 6 mg partially purified TK-PUL; lane 8, hydrolysis products by 12 mg partially purified TK-PUL; lane 9, hydrolysis products by 25 mg partially purified TK-PUL.

TK-PUL. The values of DE increased with the increase in the concentration of TK-PUL and Termamyl (Table 5).

3.11 Saccharide profiling

The compositions of saccharides in the glucose syrups, prepared from simultaneous liquefaction and saccharification 30% (w/v) corn starch using TK-PUL, were determined by TLC. Major reaction-end products were maltose, maltotriose, and maltodextrins along with traces of glucose (Fig. 7). The saccharides concentrations increased with the increase in the amount of enzyme (TK-PUL or Termamyl). These results indicated that TK-PUL is quite efficient enzyme for starch hydrolysis.

3.12 Conclusion

TK-PUL, a metal ion independent type III pullulan hydrolase, displays the potential to be considered as a candidate for starch-processing industries. TK-PUL may be employed for simultaneous liquefaction and saccharification of corn starch to a level comparable to Termamyl. Further studies on optimization and scale up of simultaneous liquefaction and saccharification are needed to economize the process.

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Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The conducted research is not related to either human or animals use.

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