Research Article

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Valorization of waste foods using pullulan hydrolase from *Thermococcus kodakarensis*

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Abstract: The starchy foods including staled or leftover bread, insect infested or damaged cereal grains and bakery wastes are usually discarded, which is a threat to public health due to extensive fungal growth. In the present study we have utilized them as raw materials for synthesis of maltose and glucose syrups using pullulan hydrolase from *Thermococcus kodakarensis* (TK-PUL). The novelty of the process was that whole process was completed: (i) in the absence of any liquefying amylase; (ii) without pre-gelatinization of starch; and (iii) using undisrupted *E. coli* cells, expressing TK-PUL gene, as a source of extremely thermostable TK-PUL protein. Since glucose and maltose can serve as precursors for a variety of biotechnological products, it is therefore anticipated that hydrolysis of starchy food wastes by TK-PUL would be highly beneficial. It will serve as cost effective measure not only to extract valuables from rubbish but also lower the level of environmental pollutants.

Keywords: valorization; waste foods; pullulan hydrolase; glucose syrup; maltose syrup; *Thermococcus kodakarensis*.

Abbreviations: FAO, Food and Agriculture Organization; TK-PUL, pullulan hydrolase from *Thermococcus kodakarensis*.

1 Introduction

Food waste can be defined as the end products of various food processing industries that have not been recycled or used for other purposes. Their economic value is less than the cost of collection and recovery for reuse. Therefore, they are discarded as waste [1]. Food and Agriculture Organization (FAO) of the United Nations reported that food is wasted at all levels during processing. FAO estimated around 1.3 billion tons of the food produced for human consumption is wasted yearly, which represents one third of the total production around the globe. It includes cereals, roots and tubers, oilseed and pulses, fruits and vegetables, meat, seafood, milk and eggs (<http://www.fao.org/save-food/resources/keyfindings/en/>). The waste food is either dumped in the landfill sites or treated to avoid environmental pollution [2].

The scientists have generated energy through anaerobic digestion or incineration of waste food materials [3-5]. In developing countries waste food is used as cattle feed. A more productive approach of the waste food management is to use it as feed stock for the growth of microorganisms. The microbes consume carbon, nitrogen and phosphorous from food wastes, assimilate them into new biomass and yield valuable metabolites [5].

Enzymatic hydrolysis of waste food is a relatively recent approach. Bakery leftovers including bread, pastry and other starchy materials like waste cereals (damaged and insect infested wheat, corn, rice, etc.) and tubers (potato, sweet potato, yam and cassava) can serve as raw materials. Starch content of these leftovers is converted into reducing sugars, which in turn are fermented into valuable biotechnological products [6,7]. Various hydrolytic enzymes (cellulases, amylases, glucoamylases and proteases) have been employed for this purpose [8-10].

Previously, we have cloned and characterized a pullulan hydrolase type III from hyperthermophilic archaeon *Thermococcus kodakarensis* (TK-PUL). This novel enzyme has the capacity to break down both the α -1,4- and α -1,6-glucosidic linkages in starch under industrial conditions [11,12]. The present study was

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designed to hydrolyze starchy food wastes including damaged corn grains, damaged wheat grains and waste chapattis (unleavened flatbread) by TK-PUL to produce maltose and glucose syrups.

2 Materials and methods

2.1 Chemicals, reagents and enzymes

Waste foods, including damaged wheat grains, damaged corn grains and waste chapattis, were procured from local market of Lahore, Pakistan. The gene encoding TK-PUL was previously cloned in expression vector pET-21a (+) [11]. *Escherichia coli* BL-21 Codon Plus (DE3)-RIL (Novagen, Madison, WI) was used as a host for gene expression and production of recombinant TK-PUL. All chemicals and reagents used were of high purity. They were purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), or Fisher Scientific (Leicestershire, UK). Amyloglucosidase from *Aspergillus niger* and maltose were from Sigma-Aldrich (Taufkirchen, Germany) whereas glucose oxidase/oxidase kit (GLUCOSE liquicolor®) was purchased from HUMAN Diagnostics (HUMAN GmbH, Wiesbaden, Germany).

2.2 Production of TK-PUL

Recombinant TK-PUL was produced in *E. coli* BL-21 Codon Plus (DE3)-RIL cells that were transformed with pET-PUL, a recombinant plasmid containing TK-PUL encoding gene ligated in pET-21a (+) [11]. *E. coli* cells harboring recombinant plasmid pET-PUL were grown in Luria-Bertani (LB) medium containing ampicillin (100 µg/mL) at 37 °C with shaking at 100 rpm. Overnight grown culture was then inoculated (2% v/v) into fresh LB medium (3.5 L) containing ampicillin (100 µg/mL) plus 10 mM lactose and further grown for 16 h under the same conditions. The cells were harvested by centrifugation at 6,500 rpm for 10 min at 4 °C. Harvested cell pellet was washed with 50 mM Tris-HCl buffer (pH 8.8) and re-suspended in 10 mL of the same buffer. Finally, the optical density of cell suspension at 660 nm was 0.980. These re-suspended cells were subsequently used as source of TK-PUL.

In order to confirm the production and release of TK-PUL from *E. coli*, the prepared cells suspension was aliquoted and incubated at 90±2 °C for various periods of time (10, 20, 30, 60, 90 min). Heated samples were centrifuged at 12,000 rpm for 10 min, supernatants were separated and pellets were re-suspended in equal volumes of 50 mM Tris-HCl buffer. All of the samples were analyzed

by activity assay as well as 12% SDS-PAGE as described earlier [11].

2.3 Enzymatic hydrolysis of waste food materials

Waste food materials including wheat grains, corn grains and dried chapattis were ground to fine powder, using laboratory grinder, and sieved to get homogenous mixture. Finally, ground powder of these food wastes (6 g each) were suspended separately in 50 mM sodium citrate buffer, pH 4.2 (in a total volume of 20 mL), to get slurries (30% w/v solid content). Suspension (900 µL each) of *E. coli* BL-21 Codon Plus (DE3)-RIL cells, containing recombinant TK-PUL, was then added separately into each flask containing slurry. Control treatments (30% w/v slurries without enzyme) were also prepared and processed similarly. All flasks were then incubated at 90±2 °C in water bath for 60 h. Samples (100 µL) were taken at regular intervals, diluted ten times (up to 1 mL) with distilled water. Centrifuged at 10,000 rpm for 3 min and supernatants were analyzed for increase in reducing ends by dinitrosalicylic acid method [13]. Maltose was used as standard during determination of reducing sugars.

After 60 h of incubation at 90±2 °C all of the hydrolyzed samples were centrifuged at 6,500 rpm for 5 min. Supernatants were transferred (separately) to new flasks. Amyloglucosidase (34 µL ~ 9 U) from *A. niger* (A7095 SIGMA) was added into each flask and incubated for further 24 h at 60±2 °C in water bath. After 24 h, hydrolysates were centrifuged at 6,500 rpm for 5 min and supernatants were stored at -20 °C till further use.

2.4 Determination of saccharide profile

Composition of saccharides obtained in the hydrolysis products were analyzed by high-performance liquid chromatography on an Aminex HPX-42A column (300 × 78 mm; Bio-Rad Laboratories, Inc., Hercules, CA) as described earlier [11].

2.5 Determination of glucose content

Glucose content in the samples collected at various intervals was determined by glucose oxidase/oxidase kit (GLUCOSE liquicolor®; HUMAN GmbH, Germany). Samples collected were centrifuged and clear supernatants (50 µL) were mixed separately with Glucose Liquicolor reagent (1 mL) and incubated at 37 °C for 5 min. Optical density at 500 nm was measured for each sample. Controls and blanks were set up as per supplier's

guidelines. Glucose content was calculated from standard curve. All the assays were performed in triplicate.

3 Results and discussion

3.1 Synthesis of maltose and glucose syrups from waste foods

The term glucose syrup refers to a concentrated solution of starch hydrolysates, in which hydrolysis of starch has not been completed. As a result of partial breakdown, these starch hydrolysates (glucose syrups) contain a mixture of glucose, maltose, maltotriose and higher oligosaccharides. Degree of starch hydrolysis in glucose syrups is monitored in terms of dextrose equivalent (DE) that should be at least 20 [14,15]. Maltose syrup describes a concentrated and purified solution containing major proportion of maltose with respect to other saccharides and maltose content not less than 30% of the solution on dry basis (<https://www.cargill.com/food-beverage/lat/high-maltose-syrups-latam>).

Three different approaches have previously been reported to extract glucose from starchy food wastes, which include: (i) use of crude amylolytic enzymes from fungal sources [8,16,17]; (ii) direct use of fungal biomass rich in carbohydrases (produced via solid state fermentation on food wastes) [6,18,19]; and (iii) use of commercial enzyme preparations [10,20,21].

All of these approaches had their own limitations.

Crude enzymes cannot be stored for long time, therefore, fresh preparations are always needed. Use of fungal biomass cannot provide consistent results because fungal growth depends upon the nature of substrate (type of food waste used). Moreover, possible risks of contaminations always remain there during fungal growth. Use of commercial enzymes is expensive. Additionally, the available fungal enzymes are not thermostable. Use of thermostable enzymes can help not only in completing the starch hydrolysis at elevated temperature, but also in avoiding the risk of contamination. Furthermore, at elevated temperature starch remains completely solubilized and efficient hydrolysis is possible.

TK-PUL can completely hydrolyze starch into maltose at 90 ± 2 °C [11]. Using TK-PUL hydrolysis of starchy food wastes was performed at 90 ± 2 °C. Since TK-PUL has previously been proven an extremely thermostable pullulanase, undisrupted whole *E. coli* BL-21 Codon Plus (DE3)-RIL cells, containing recombinant TK-PUL, were therefore employed as a source of enzyme in order to save labor, energy and time consumed in lysis of host cells by sonication or any other means. Host cells released the heat-stable TK-PUL into the medium at 90 ± 2 °C within 10 min (Fig. 1). As anticipated, TK-PUL efficiently converted starch content of food wastes into smaller saccharides, as observed from gradual increase in reducing sugar content. The reducing sugars increased at almost steady rate and reached at peak level after 56 h (Fig. 2). A decline in reducing sugars was observed after 56 h probably due to the interaction of these reducing sugars with the

Figure 1. Coomassie Brilliant Blue stained SDS-PAGE showing effect of incubation at 90 ± 2 °C on lysis of *E. coli* cells. Lane 1, whole cells before heating; lane 2, pellet after 10 min incubation; lane 3, supernatant after 10 min incubation; lane 4, pellet after 20 min incubation; lane 5, supernatant after 20 min incubation; lane 6, pellet after 30 min incubation; lane 7, supernatant after 30 min incubation, lane 8, pellet after 60 min incubation; lane 9, supernatant after 60 min incubation; lane 10, pellet after 90 min incubation; lane 11, supernatant after 90 min incubation.

amino groups of proteins contained in waste foods. Such a reaction is very well known as carbonyl-amine or Maillard's reaction [22]. Keeping in view the onset of Maillard's reaction, hydrolysis was terminated after 60 h and suspended solids were removed by centrifugation. Saccharide profiling of starch hydrolysates obtained in the supernatants revealed that the starch content of food wastes was converted into saccharides of varying lengths. Maltose and maltotriose were in the largest proportion among the saccharides smaller than maltopentaose (Table 1, Fig. 3). Starch hydrolysates obtained in the supernatants were further treated with amyloglucosidase to convert all saccharides into glucose, as evidenced from high-performance liquid chromatography analysis (data not shown). Final concentrations of glucose in hydrolysates (glucose syrups) of wheat, corn and chapatties were 201.32, 227.12 and 218.32 g/L, respectively. As per our knowledge, these were the highest ever reported yields of glucose from hydrolysis of food wastes. Pleissner and co-workers used fungal mash (*Aspergillus awamori* and *Aspergillus oryzae*) for hydrolysis of food and bakery wastes and obtained 52.89 g glucose/100 g food wastes [18]. Zhang and co-workers [6] valorized cake and pastry wastes into succinic acid. During pretreatment, they hydrolyzed these wastes with fungal mash containing *A. awamori* and *A. oryzae*, and reported a glucose yield of 35.6 g/L and 54.2 g/L from cake and pastry wastes, respectively [6]. Yan and co-workers used fungal α -amylase for hydrolysis of dining room wastes and obtained 194.43 g reducing sugars/L [10]. Cekmecelioglu and Uncu obtained about 65 g reducing sugars per 100 g kitchen wastes with the combined action of commercially obtained fungal enzymes (α -amylase, glucoamylase, cellulase and β -glucosidase) [20]. Hong and Yoon also employed a mixture of commercial enzymes (α -amylase, glucoamylase and protease) for hydrolysis of food residues from University Cafeteria and got 60 g reducing sugars per 100 g food waste [21].

Figure 2. Time course release of reducing sugars during hydrolysis of wheat (triangles), corn (circles) and chapatti (squares). Maltose was used as standard during dinitrosalicylic acid assay [13]. Data are the average values of three experiments.

Figure 3. Saccharide profile of starch hydrolysates from various food wastes after 60 h incubation with TK-PUL. A, Corn hydrolysates; B, chapatti hydrolysates; C, wheat hydrolysates.

Table 1. Saccharide composition of starch hydrolysates from various food wastes after 60 h incubation with TK-PUL.

Saccharides	Percent composition		
	Wheat	Chapatti	Corn
Maltose	21.7	17.2	20.6
Maltotriose	17.4	11.1	15.5
Maltotetraose	8.7	5.2	-
Maltopentaose	-	3.3	-
Higher saccharides	52.1	63.2	63.9

3.2 Conclusion

We conclude that TK-PUL can successfully be employed for valorization of food wastes. Being highly thermostable, it can be used immediately after its production in *E. coli* without any need of cell lysis or purification. It is anticipated that the process developed in this study will help to devise effective strategy of bio-refining. However, further optimizations are required that are under way in our laboratory. The hydrolysates obtained from the waste food materials were rich in fermentable sugars and may potentially be converted into other valuable products.

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Conflict of interest: The authors declare no conflict of interests.

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