Vol. 26 (1) 19–24 31 March 2014

Biokemistri
An International Journal of the Nigerian Society for Experimental Biology

Original Article

Purification and characterization of thermostable glucoamylase from *Rhizopus oligosporus* SK5 mutant obtained through UV radiation and chemical mutagenesis

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Received: 20 January 2014: Revised 13 March 2014; Accepted: 14 March 2014

ABSTRACT: Thermostable glucoamylase from *Rhizopus oligosporus* SK5 mutant was purified in a 3-step purification using *Imarsil*, activated charcoal and Sephadex-G-100 to achieve a 40-fold purification. The enzyme was optimally active at pH 5.0 and temperature of 80 °C. It exhibited a half-life of 60 minutes at 70 °C. Its stability was enhanced with addition of Soyabean flour or starch (3% w/w) leading to a retention of over 90% residual activity at 4 °C and 28 °C after 12 weeks of storage. SDS-PAGE analysis of purified enzyme showed two major bands with corresponding molecular weights of 36 kDa and 50 kDa. The study presents thermostable glucoamylase from *Rhizopus oligosporus* SK5 as a potential in the bioconversion of starch to glucose.

KEYWORDS: Enzyme purification, thermostability, *Rhizopus oligosporus*, glucoamylase,

INTRODUCTION

Glucoamylase (EC 3.2.1.3, α-1,4-glucanohydrolase) is an exo-acting enzyme which liberates glucose as the primary product of a step-wise hydrolysis of both α-1,6-glucosidie linkages and α-1,4 linkages of starch and its derivatives (Ali and Hossain, 1991). Glucoamylases have been characterized from different sources such as bacteria, fungi and yeast. A large number of filamentous fungi of mesophilic origin especially *Rhizopus spp* are usually employed in the production of glucoamylases due to their ubiquitous distribution and non-fastidious nutritional requirement (Akpan *et al*., 1996).

The greater industrial use of this enzyme is impaired by limited glucose yields owing to the slow hydrolysis of α-1,6-glucosidic bonds in starch, the formation of condensation products (mainly α-1,6-linked isomaltose-oligosaccharides), the poor stability of enzymes under standard conditions of pH and temperature (Ali and Hossain, 1991). Conventionally, bioconversion of starch to glucose and other oligosaccharides requires a two-step process: liquefaction and saccharification. Liquefaction process involves α-amylase at high temperature (80–95 °C) and pH 6.0 followed by saccharification process using glucoamylase at pH 4.5–5.0 and at temperature values ranging between 40 °C and 60 °C (Stamford *et al*., 2002). The need to lower process temperature and to bridge the gap of pH differential between the two enzymes always makes enzymatic hydrolysis of starch energy intensive, time consuming, and cost prohibitive.

The quest for starch-saccharifying enzymes with increased thermal and pH stability has inspired the search for bacterial glucoamylases from anaerobic thermophilic bacterium; *Clostridium thermosaccharolyticum* and actinomycetes such

as *Streptosporangium sp.* (Stamford *et al.*, 2002; Specka *et al.*, 1991). However, there are scanty information on the production of thermostable glucoamylase from *Rhizopus spp.* The improved characteristics of microbial glucoamylase in terms of high yield, thermostability and wide pH range could still be attainable through mutation and therefore facilitate starch bioprocesses (Enyioha *et al.* 2004). In this report, we describe the purification and characterization of a thermostable glucoamylase isolated from a mutant strain of *Rhizopus oligosporus* generated via UV radiation and chemical treatment.

MATERIALS AND METHODS

Preparation of Spore suspension

Amylolytic strain of *Rhizopus oligosporus* was obtained from the Culture Collection Unit of the Department of Microbiology, Federal University of Agriculture, Abeokuta, Nigeria. Spore suspensions of *R. oligosporus* were prepared by briefly washing 4-days old culture slants with sterilized saline solution (0.9% NaCl) with vigorous shaking. Spores were counted by a haemocytometer to adjust the count to approximately 10^7 spores/ml.

Mutagenesis

A spore suspension was prepared from a 24h-old slant culture of *Rhizopus oligosporus* (wild type) by suspending in sterile saline water. A 30 W Perkin Elmer UV-lamp was used as the source of radiation. Ultraviolet mutagenesis was applied as described by Dubey *et al.* (2000) with exposure times of 15, 30 and 45 min. UV mutation was followed by chemical mutagenesis using ethyl methane sulfonate (EMS) dissolved in phosphate buffer (pH 7) with a concentration of 50 μg/ml. Mutants were selected based on variations in colony morphology or sporulation on SDA and further selected for auxotrophic mutants on minimal medium as described by Valadares-Inglis and Azevedo (Valadares–Inglis and Azevedo, 1997). Purified mutated colonies were screened for glucoamylase production on Remarsol brilliant blue-starch agar plates at 30 °C for 24h. A leucine-dependent *Rhizopus oligosporus* SK5 mutant showing large clearance zones on RBB-starch plate was used for the study (Valadares–Inglis and Azevedo, 1997; Kareem, 2009). Cultures were maintained on Potato dextrose agar slants at 4 °C and sub-cultured bi-monthly

Glucoamylase Production

Glucoamylase was produced on a solid state medium containing rice bran, soyabean and cassava starch (10:3:1 w/w) as described by Akpan and Adelaja (2004). The pH of the medium was adjusted to 4.5 with 0.1M HCl and sterilized at 121 °C for 15 min. The sterilized medium in a Petri-dish was inoculated with a loopful of spores of a 24h–old culture of *R. oligosporus* SK5 and incubated at 30 °C for 72h. The crude glucoamylase was recovered by mixing fermented bran with acetate buffer (0.2 M, pH 4.5) in the ratio 1:4 (w/v) in a conical flask. The mixture was shaken on an orbital shaker at 150 rpm at 28 °C for 1h. The extract was then filtered using muslin cloth. The filtrate was used as the crude amylase and stored at 4 °C

Purification of glucoamylase

Imarsil (1.0 g) was mixed with 100 ml crude amylase (pH 4.5) and kept at 4 ºC for 4h according to the method of Kareem and Akpan (2003). The supernatant was gently siphoned and further purified by adding activated charcoal (3% w/v) at 50 °C for 20 minutes as described by Kareem *et al.* (2011), and then centrifuged at 2,500 rpm for 15 minutes. The supernatant, which contains the active enzyme, was applied to a Sephadex G-100 column (1.5 cm x 25 cm) which had been equilibrated with 0.2 M acetate buffer (pH 4.5). The column was eluted with the same buffer at a flow rate of 1 ml/min. Fractions that show amylase activity were qualitatively determined using the starch-layer plate method described by Akpan and Kareem (2004). Standard assay methods were thereafter used to quantify amylase activity and protein concentration.

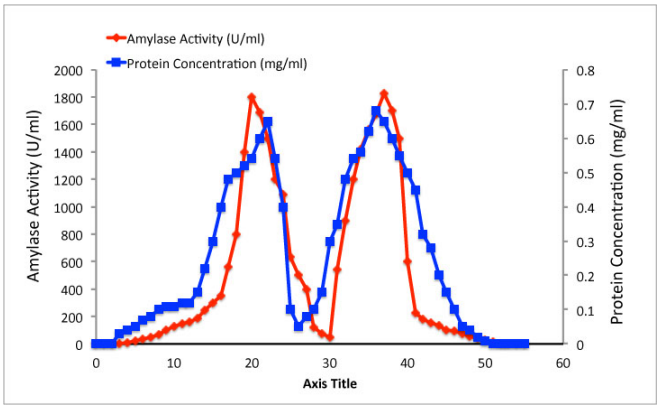

Figure 1: Elution pattern of *Rhizopus oligosporus* SK5 glucoamylase on Sephadex G-100

Determination of Glucoamylase Activity

The glucoamylase activity was determined according to the method of Akpan *et al.* (1996). Crude amylase was mixed with 4% w/v gelatinized cassava starch in 0.1 M acetate buffer (pH 4.5) in ratio (1:4 v/v) and incubated at 60 °C for 1h. The reaction was stopped by heating the tube at 100 °C for 2 minutes to inactivate the enzyme. Glucoamylase activity was determined using dinitrosalicylic acid method, and glucose was used as the standard (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme which liberate 1 micromole of glucose from starch in a 1-ml reaction mixture at 60 ºC for 1h. Specific activity was expressed as units of enzyme activity per mg of protein.

Protein Determination

The protein concentrations of the enzyme samples were determined using the method of Lowry *et al.* (1951) and bovine serum albumin was used as a standard. All measurements were means of triplicate determinations.

Table 1: Summary of purification of glucoamylase

Steps	Volume (ml)	Amylase activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude amylase	100	1702	50	33	—	100
Imarsil	90	1694	7.5	225.9	7.4	90
Activated charcoal	70	1625	1.8	930.5	28.2	76
Sephadex G-100	56	1512	0.9	1329	40.3	57

Molecular weight of purified enzyme

The result of electrophoretic analysis of purified glucoamylase showed that the purified glucoamylase has two major protein bands with corresponding molecular weight of 36 kDa and 50 kDa (Figure 2). Glucoamylases from *Rhizopus species* have been reported to exhibit two to three molecular mass units (Pandey, 1995).

In addition, previous studies had shown that molecular weights of fungal glucoamylases are reported to be in the range of 25–112 kDa (Takahashi *et al*., 1978; Koc and Metin, 2010). Although glucoamylase produced by *R. oligosporus* SK5 exhibited two distinct subunit molecular masses, active fractions yielded glucose as the sole end product on thin layer chromatography which indicated that the enzyme is glucoamylase (data not shown).

Effect of assay pH and Temperature on Enzyme Activity

The effect of temperature on glucoamylase activity was determined between 40 ºC and 90 ºC using Cassava starch (4% w/v) in 0.1 M acetate buffer (pH 4.5) as the substrate. The effect of pH on enzyme activity was evaluated by carrying out the reactions in 0.1 M acetate buffer between pH 3.0 and 5.5 at 60 ºC for 1h.

Enzyme Stability profile

The thermostability of the purified glucoamylase was determined by pre-incubating a solution of enzyme (5 ml) for 3h at various temperatures (50–70 °C), with or without either starch or soyabean flour (5% w/v). The pH stability of the purified glucoamylase was determined by mixing the enzyme solution with citrate phosphate buffer (0.1 M) with a pH range (3–7) and kept at 30 °C for 6h. The residual activity was determined at regular intervals.

Storage stability of purified glucoamylase was carried out using starch and soyabean flour as additives. Additives (5 g) were added to purified glucoamylase (100 ml) and stored at 4 °C and 28 °C. Enzyme activity of stabilized samples was determined at weekly intervals for a period of 12 weeks.

Effect of activators and inhibitors

The effects of various metals ions ($CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $FeCl_2$, $HgCl_2$, $PbCl_2$) and EDTA at a final concentration of 5 mM on the purified glucoamylase were evaluated. A mixture containing 0.5 ml of the metal ion solutions and 0.5 ml of enzyme solution was incubated at pH 4.5 for 30 min at room temperature. The relative enzyme assay was measured under standard assay.

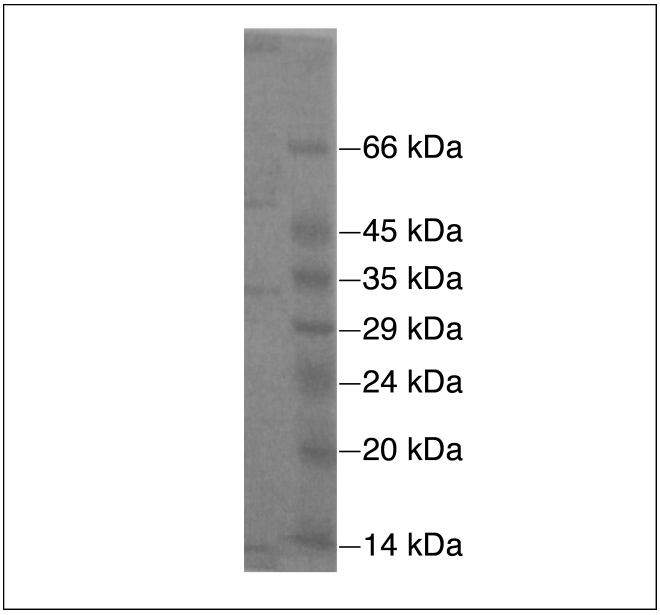

Figure 2: Sodium dodecyl sulphate polyacrylamide gel electrophoresis of purified thermostable glucoamylase isolated from *Rhizopus oligosporus* SK5

RESULTS AND DISCUSSION

Studies on the purification of crude fungal amylase indicated that a 40-fold purification was obtained with a final yield of 50% of total amylase in a 3-step purification procedure (Table1). The elution pattern of *R. oligosporus* SK5 amylase had two peaks at fractions (19-22) and (34-38) on Sephadex G-100 column (Figure 1). This purification fold value is considered higher than values reported in the literature (Kareem and Akpan, 2003; Selvakumar *et al*., 1996; Pandey, 1995). Kareem *et al.* (2011) reported that partial purification of enzyme with activated charcoal prior to gel filtration will ensure a high purification fold by circumventing some associated problems such as difficulty of scaling up and plugging when treating crude extracts which often contain viscous and particulate materials.

Effect of pH and Temperature on glucoamylase activity

The result presented in Figure 3 indicated that the enzyme was active over a broad pH value (4.0–6.0) with optimum at pH 5.0. This report agrees with the recommended pH values for hydrolysis of starch using commercially available fungal glucoamylases (Pandey, 1995). The temperature-dependence profile of the enzyme (Figure 4) showed that its optimum activity is at 80 °C which is higher than those of some known fungal glucoamylases (Pandey, 1995; Takahashi *et al*., 1978). A considerable success has been achieved in microbial selection and enhancement of enzyme production through mutation by UV irradiation or with chemical mutagens (Koc and Metin, 2010). Such mutant strains can show superior characteristics such as genetic stability and temperature preference (Koc and Metin, 2010). Commercial glucoamylase produced by *Rhizopus* species are totally inactive at temperature above 60 °C. Since gelatinization at 100 °C is key to industrial saccharification of starch, glucoamylase activity over a broad pH value (4.0–6.0) and at high temperature are of considerable biotechnological interest since these can be used in enhancing synergistic hydrolysis of starch with commercially available α-amylases (Suntornsuk and Hang, 1997).

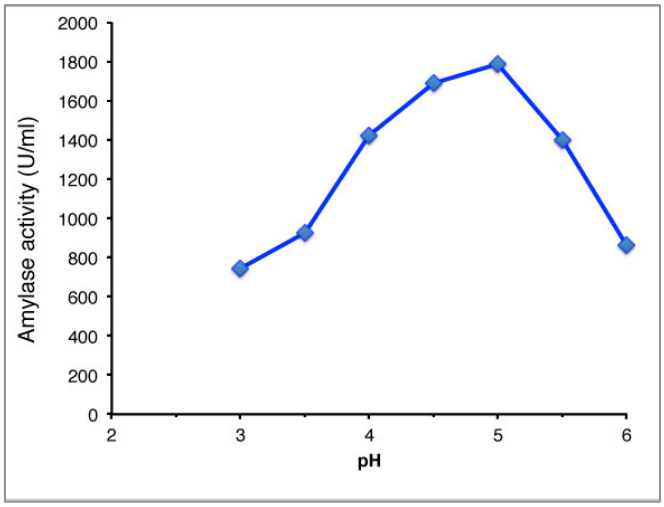

Figure 3: Effect of assay pH on amylase activity isolated from *R.oligosporus*.

Stability profile of glucoamylase

The effect of pH on the stability of glucoamylase showed that the enzyme was more stable at pH values 3.5–6.0 while retaining substantial enzyme activities of 88%, 97% and 85% at pH values 4.0, 5.0 and 6.0 respectively after 6 hours of incubation (Figure 5). This property might offer it a better placement in the industrial application by minimizing the use of acids and bases for pH adjustment between sequential liquefaction and saccharification processes (Suntornsuk and Hang, 1997).

The result of thermal stability of the purified enzyme showed that the enzyme was stable at 50 °C and 60 °C by retaining 92% and 82% residual activity respectively after 2 hours incubation (Figure 6). It also exhibited a half-life of 60 minutes at 70 °C. Addition of soybean flour and starch enhanced the stability of the enzyme yielding 78% and 95% residual activity after incubation at 70 °C for 2 hours. The result is in agreement with the findings of Akpan and Adelaja (Valadares–Inglis and Azevedo, 1997) that fortification of amylase with either soyabean flour or starch enhanced its stability against thermal deactivationwhich might be attributed to the carbohydrate or glycoprotein components of the stabilizers.

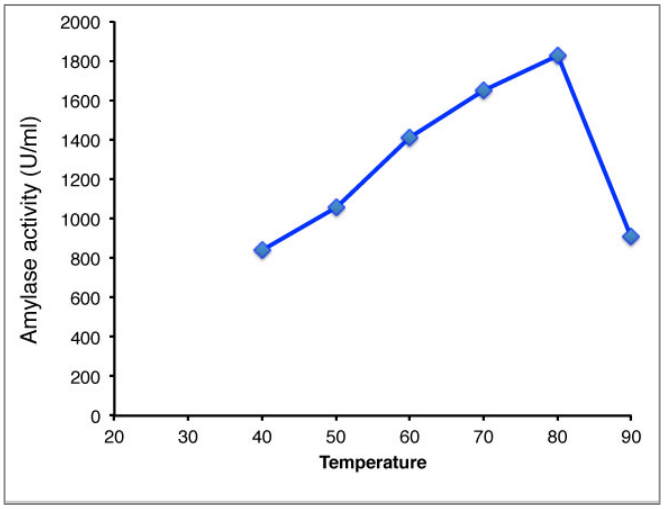

Figure 4: Effect of assay temperature on amylase activity isolated from *R. oligosporus*.

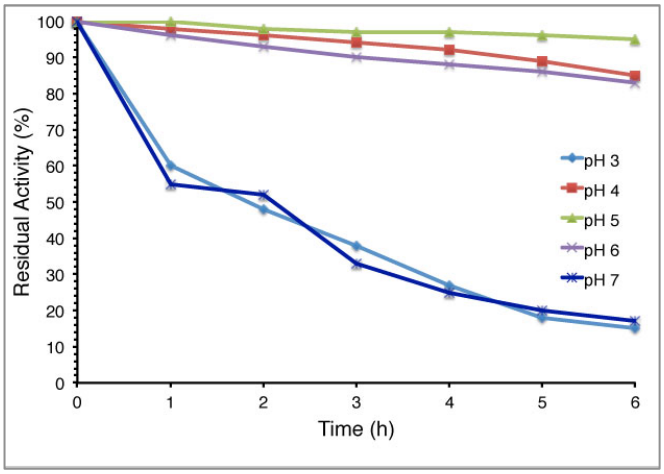

Figure 5: pH stability profile of glucoamylase from *R. oligosporus* SK5.

The optimum temperature and thermostability of the glucoamylase reported here are higher than those of some known fungal glucoamylases (Selvakumar *et al*., 1996; Campos and Felix, 1995). It has been reported that thermostability of glucoamylase enhances bioconversion of starch by ensuring maximal efficiency and reduction of microbial contamination (Stamford *et al*., 2002).

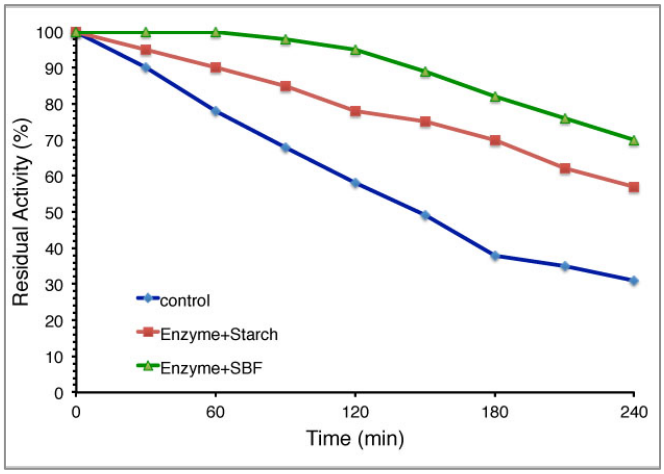

Figure 6: Thermostability profile of *R. oligosporus* SK5 glucoamylase after incubation at 70 °C for the time intervals indicated.

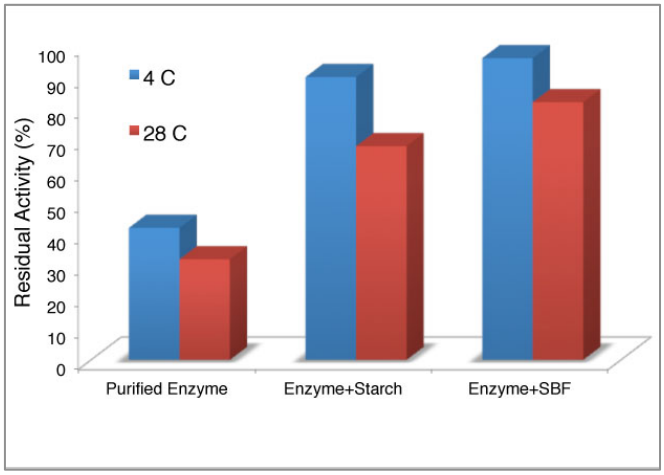

Figure 7: Effect of stabilizers on storage stability of *R. oligosporus* SK5 glucoamylase at 4 °C and 28 °C after 16 weeks.

The result presented in Figure 7 showed that addition of starch and soybean flour enhanced the stability of the enzyme by retaining more than 90% residual activity at 4 ºC while 55–60% was obtained at 28 ºC against the control with 30–40% residual activity after 12 weeks of storage. There was no significant difference in stability imparted by these two stabilizers at 4 ºC and 28 ºC after 12 weeks of storage (P>0.05). The results confirmed similar observations by Geisow (1991) that fortification of soluble enzymes with their substrates leads to improved storage stability by forming stable complexes. It has also been reported that such fortification will reduce activities of acid proteinases usually present in extracts of fungal glucoamylase thereby reducing the rate of proteolysis of enzyme preparation during storage and consequently increase the application efficiency of glucoamylases (Pandey *et al*., 2000; Rakhimova *et al*., 2006).

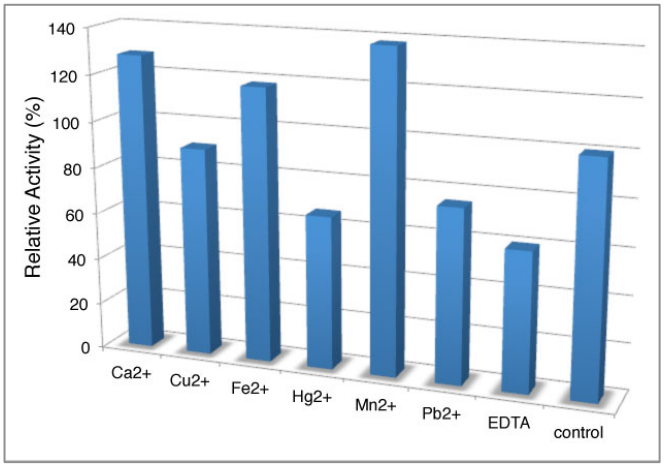

Figure 8: Effect of activators and inhibitors on purified glucoamylase.

Effect of activators and inhibitors on the amylase activity of fungal amylases

The effects of different metal ions and reagents on enzyme activity as presented in Figure 8 showed that Mn^{2+}, Ca^{2+}, and Fe^{2+} increased glucoamylase activity by 20–45%. Metal ions and chemical agents at concentrations between 1 and 5 mM are known to exert stimulatory effects on glucoamylase (Koc and Metin, 2010). Similar results were reported for glucoamylase from *R. oryzae* mutant (Suntornsuk and Hang, 1997). It has been suggested that metal ions probably force amylase to adopt a compact structure, by setting out the hydrophobic residues of the enzyme, thereby inducing resistance to extreme pH and temperatures (Soni *et al*., 2005; Regulapati *et al*., 2007). Since glucoamylases are used together with α-amylase, which requires Ca^{2+} ions in the liquefaction process, stimulation of glucoamylase activity by Ca^{2+} ions makes it more suitable for use in starch bioconversion process. The enzyme activity was strongly inhibited by Cu^{2+}, Hg^{2+}, Pb^{2+} and EDTA. This inhibitory effect has been reported for glucoamylases from *Aspergillus flavus* (Koc and Metin, 2010).

Conclusion

Our findings from this study showed that purified thermostable glucoamylase from *R. oligosporous* SK-5 was optimally active at 80 °C and pH 5, and that its stability at high temperature was further enhanced with addition of soybean and starch. It is hoped that this thermostability could be put to use in shortening the process time required for starch hydrolysis.

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