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ORIGINAL PAPER

Production of raw cassava starch-degrading enzyme by *Penicillium* and its use in conversion of raw cassava flour to ethanol

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Abstract A newly isolated strain *Penicillium* sp. GXU20 produced a raw starch-degrading enzyme which showed optimum activity towards raw cassava starch at pH 4.5 and 50°C. Maximum raw cassava starch-degrading enzyme (RCSDE) activity of 20 U/ml was achieved when GXU20 was cultivated under optimized conditions using wheat bran (3.0% w/v) and soybean meal (2.5% w/v) as carbon and nitrogen sources at pH 5.0 and 28°C. This represented about a sixfold increment as compared with the activity obtained under basal conditions. Starch hydrolysis degree of 95% of raw cassava flour (150 g/l) was achieved after 72 h of digestion by crude RCSDE (30 U/g flour). Ethanol yield reached 53.3 g/l with fermentation efficiency of 92% after 48 h of simultaneous saccharification and fermentation of raw cassava flour at 150 g/l using the RCSDE (30 U/g flour), carried out at pH 4.0 and 40°C. This strain and its RCSDE have potential applications in processing of raw cassava starch to ethanol.

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Keywords Cassava · Raw cassava starch-degrading enzyme · *Penicillium* sp. · Raw starch hydrolysis · Simultaneous saccharification and fermentation

Introduction

Cassava (*Manihot esculenta* Cranz) is the world's fourth most important crop and is grown in many countries in Africa, Asia, and Latin America. Cassava is a starch-containing root crop and is one of the most important sources of calories in the tropics. Cassava is also widely employed as a raw material for many industrial applications in the animal feed industry and starch industry, and more recently for production of fuel ethanol. Cassava can be cultivated on arid and semiarid land where other crops, such as corn, do not thrive [5].

In Guangxi, China, approximately 200,000 hectares of cassava is under cultivation with production of about 6,000,000 tons of fresh cassava annually, accounting for around 60% of cassava production in China [3, 5]. The starch content in fresh cassava is higher than 30%, and it is estimated that 3.0 tons of dry cassava or 7.2 tons of fresh cassava can produce 1 ton of fuel ethanol [10]. Guangxi COFCO Bio-energy Co. Ltd. of Beihai, China, is now producing 200,000 tons of fuel ethanol from cassava starch annually [17]. Currently in Guangxi, China, 10% fuel ethanol is used in cars.

Both amylose and amylopectin in cassava starch can be hydrolyzed by amylolytic enzymes to release fermentable sugars which can be converted into ethanol by *Saccharomyces cerevisiae* [24]. In conventional cassava starch-to-ethanol processing, the starch is first cooked at 90°C or higher temperature with α-amylase (EC 3.2.1.1) [4]. The liquefied starch is then separately or simultaneously

saccharified by glucoamylase (EC 3.2.1.3) to glucose, which is then fermented by *S. cerevisiae*. This traditional process requires a large amount of energy for cooking the starch, thus resulting in high cost of ethanol production [25]. At the Guangxi COFCO Bio-energy Co. Ltd. at Beihai, pulp of manioc flour at concentration of 30–40% (w/v) is first liquefied at about 105°C by spraying enzyme. The liquors without saccharification are converted to ethanol by simultaneous action of glucoamylase and *S. cerevisiae* in fermentors. To conserve energy, identification of enzymes capable of hydrolyzing raw cassava starch granules into glucose in a single step without cooking for utilization in simultaneous saccharification and fermentation (SSF) would be an important advance towards increasing the efficiency and profitability of fuel ethanol production from cassava starch.

It is more difficult for amylolytic enzymes to digest raw starch granules than gelatinized starch granules because of particle size and the densely compacted polycrystalline structure present in raw starch granules [18, 20, 27]. Previous research has reported that many fungi such as *Aspergillus* sp. [22, 23] and *Rhizopus* sp. [15, 26] possess the ability to produce raw starch-degrading enzymes which can act on raw corn starch [11, 21–23] and raw potato starch [8] effectively. Production of a raw sago starch-degrading enzyme by the fungus *Acremonium* sp. has also been reported [13]. However, to our knowledge, no enzymes capable of digesting raw cassava starch have been reported.

In this study, a fungal strain capable of producing high yield of raw cassava starch-degrading enzyme (RCSDE) was isolated and identified as *Penicillium* sp. The culture conditions for producing RCSDE were optimized, and then the crude enzyme preparation was tested for hydrolysis of raw cassava flour at concentration of 150 g/l, which is the amount typically used in the starch industry. SSF of raw cassava flour (150 and 250 g/l) to ethanol at low enzyme load was also investigated.

Materials and methods

Materials

Soil samples for microbial isolation were collected from forest in Shiwandashan Mountain of Guangxi Zhuang Autonomous Region, China and fields close to a starch processing factory in Nanning, China. Raw starch materials (cassava, corn, potato, sweet potato, glutinous rice, rice, and buckwheat) and wheat bran were obtained from a local market in Nanning, China. The starch content of the raw cassava flour used was determined to be 76%. The yeast (*Saccharomyces cerevisiae*) used in SSF for ethanol production was a thermally resistant dried yeast from Angel Yeast Co., Ltd., China. All chemicals used were of analytical grade.

Isolation and identification of microorganism

Medium containing 10 g/l raw cassava flour, 3 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, and 15 g/l agar was used for isolating raw cassava starch-degrading microorganisms. Raw cassava flour was separately sterilized by Co^{60} radiation at dose of 20 kGy and was added to the isolation medium when it had cooled down to about 45°C. Three isolation agar plates without inoculation were found to be negative for microbial growth after incubation at 28°C for 5 days along with plates spread with 50 µl of different dilutions of soil suspensions, indicating complete sterilization of the raw cassava flour. Starch hydrolysis was assessed as clearing zones around the colonies in the isolation agar plates by staining with KI/I_2 solution. Colonies showing high starch hydrolysis activities on the plates were further evaluated by measuring their enzyme production levels in liquid fermentation in basal fermentation medium of 50 ml contained in 250-ml shake flasks and by checking the product after enzyme hydrolysis of raw cassava flour by high-performance liquid chromatography (HPLC). The basal fermentation medium contained 10 g/l raw cassava flour, 2 g/l tryptone, 2.5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.025 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l $FeSO_4 \cdot 7H_2O$, and 0.13 g/l $CaCl_2$ at pH 5.5. Flask fermentation was carried out at 28°C at 180 rpm for 5 days. One strain, named GXU20, which was isolated from forest soil of Shiwandashan Mountain, Guangxi, China, was found to produce the highest amount of RCSDE and was chosen for further study.

Strain GXU20 was further identified based on the method described by Dritsa et al. [6]. The internal transcribed spacer (ITS) region of strain GXU20 was amplified by polymerase chain reaction (PCR), and the PCR product was sequenced and analyzed by searching GenBank. Strain GXU20 was identified as *Penicillium* sp. The primers used in the PCR reaction were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′). The PCR reaction conditions were as follows: 95°C for 4.5 min, then 30 cycles at 95°C for 30 s, 40°C for 30 s, and 72°C for 1 min, with a final extension of 10 min at 72°C.

Enzyme assay

RCSDE activity was determined by measuring the reducing sugars released on hydrolysis of raw cassava starch. Raw cassava starch flour (2% w/v) in 450 µl, pH 4.0 citrate/phosphate buffer (a mixture of 100 mM citric acid and

200 mM Na_2HPO_4) was kept at 40°C for 5 min, and then an appropriate dilution of 50 µl of crude enzyme was added. After incubating for 30 min, the amount of reducing sugars was determined by the dinitrosalicylic acid (DNS) method as described by Miller [14]. One unit of RCSDE activity was defined as the amount of enzyme releasing 1 µmol glucose equivalent per min under the assay conditions.

The compounds produced by the enzyme from strain GXU20 upon hydrolysis of raw cassava starch were analyzed by HPLC. A reaction mixture containing 2 ml crude preparation of RCSDE enzyme solution and 6 ml 2% (w/v) raw cassava flour (suspended in pH 4.0 citrate/phosphate buffer) was kept at 40°C. A 500 µl aliquot was removed and heated in a boiling water bath for 5 min at different time intervals of 5 min, 10 min, 30 min, and 4 h. The supernatants were analyzed by HPLC using a Hypersil NH_2 column. The column was run at room temperature with 70% (v/v) acetonitrile in deionized water as the mobile phase at flow rate of 1.0 ml/min. The sugar standards used were glucose, maltose, and maltotriose.

Optimization of RCSDE production by *Penicillium* sp. GXU20 in liquid fermentation

RCSDE production was optimized by altering various environmental (pH and temperature) and culture (carbon and nitrogen sources) conditions in the basal fermentation medium. The effect of initial pH on RCSDE production was determined by growing *Penicillium* sp. GXU20 in basal fermentation medium at varying pH (4.0, 4.5, 5.0, 5.5, 6.0) at 28°C. To study the effect of temperature on production of the enzyme by strain GXU20, the culture was incubated in basal fermentation medium at 26°C, 28°C, 30°C, 32°C or 34°C at optimal pH.

The effect of carbon sources was studied by replacing raw cassava flour in the basal fermentation medium with other gelatinized and raw natural crude starch sources at final concentration of 1% (w/v). Similarly, the effect of nitrogen sources was studied by replacing tryptone and $(NH_4)_2SO_4$ with various nitrogen sources (1% w/v) in the basal fermentation medium. The optimal concentration of the best carbon source (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0% w/v) and nitrogen source (1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% w/v) for RCSDE production were also investigated. All optimization experiments were carried out on an orbital shaker at 200 rpm. The crude enzyme preparations were analyzed for RCSDE activity as described in the "Enzyme assay" section.

Effect of pH and temperature on crude RCSDE activity

The effect of pH on RCSDE activity was studied by assaying the crude enzyme using 2% (w/v) raw cassava flour solution in the pH range of 3.0–7.0 (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0) using citrate/phosphate buffer at 40°C. The optimum temperature for enzyme activity was analyzed from 20°C to 60°C (20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C) in citrate/phosphate buffer (pH 4.5).

Substrate specificity

The substrate specificity of RCSDE from *Penicillium* sp. GXU20 was determined by using different raw starches (soluble starch, cassava, glutinous rice, sweet potato, rice, corn, buckwheat, and potato) as substrate. Enzyme activity towards various substrates was determined using the enzyme assay. Results are shown as percentages, with the RCSDE activity set at 100% for comparison with other raw starch-degrading enzyme activities.

Determination of raw cassava starch adsorbability

Enzyme affinity towards raw cassava starch granules was studied by mixing 1 ml crude enzyme with raw cassava flour (2%, 4%, 8%, 16%, or 20% w/v) in 1 ml citrate/phosphate buffer (pH 4.0) and incubating at 40°C. A control, to exclude the possibility of enzyme precipitation or adsorption of the enzyme to the wall of the tube during the reaction, was included by incubating the enzyme without raw cassava flour in the reaction buffer. Samples were drawn out at different times of 15, 30, and 60 min. After centrifugation at 12,000 rpm for 5 min, the RCSDE activity of the supernatants was determined, and then the adsorption percentage was calculated as

$$\text{Adsorption}(\%) = (A - B) \times 100/A,$$

where A is the RCSDE activity of the control and B is the residual RCSDE activity in the supernatant after adsorption on raw cassava starch granules.

Scanning electron microscopy

Scanning electron micrographs were obtained for native cassava starch granules and raw cassava starch granules treated with RCSDE prepared from *Penicillium* sp. GXU20. Raw cassava starch flour (2% w/v) was suspended in 25 ml citrate/phosphate buffer (pH 4.0), and then 5 ml crude RCSDE (30 U) was added. The reaction was incubated for 6 h at 40°C, stopped by the addition of 10 ml absolute ethanol [9], and then the supernatant was removed by centrifugation for 5 min at 4,200 rpm. The sample was washed with absolute ethanol twice. The treated starch granules were dried at 35°C to constant weight. The micrographs were obtained by using a S-3400N scanning electron microscope (Hitachi, Ltd.).

Hydrolysis of raw cassava flour by crude enzyme preparation from *Penicillium* sp. GXU20

Raw cassava flour slurry (50, 100, 150, 200, or 250 g/l) was prepared in citrate/phosphate buffer (pH 4.0), and RCSDE was added at 30 U per g of cassava flour. The reaction mixture had a final volume of 20 ml. The extent of raw cassava starch hydrolysis was determined at 12 h intervals after the mixtures were incubated at 40°C with shaking at 150 rpm. The effect of enzyme dose on hydrolysis of raw cassava flour at concentration of 200 g/l was studied by varying its dose from 15 to 60 U/g raw cassava flour. To determine the degree of hydrolysis of starch, the starch residues were washed with sterile water twice and then dried at 40°C to constant weight [20].

The degree of hydrolysis of raw cassava starch was calculated by the following formula:

$$\text{Degree of hydrolysis}(\%) = (W_1 - W_2) \times 100/W_3,$$

where W_1 is the amount of dry cassava flour before the hydrolysis reaction, W_2 is the amount of flour after the hydrolysis reaction, and W_3 is the amount of starch in cassava flour before the hydrolysis reaction.

Simultaneous saccharification and fermentation of raw cassava flour to ethanol

SSF of raw cassava flour to ethanol was investigated. The reaction system consisted of crude RCSDE (30 U/g cassava flour) from *Penicillium* sp. GXU20, cassava flour 150 g/l (or 250 g/l), urea 3 g/l, and yeast 1 g/l. The pH of the mixture was adjusted to 4.0 using 2 M HCl, and studies were carried out in 250-ml conical flasks with 100 ml working volume at 40°C. Ethanol content, reducing sugars, and residual starch were assayed every 12 h.

Fermentation efficiency was calculated as the percentage of experimental to maximum theoretical yield.

Measurement of starch

The starch content of raw cassava flour was measured by treating raw cassava flour or samples in 2 M HCl in a boiling water bath for 15 min. The pH of the mixture was neutralized with 2 M NaOH after it cooled down to room temperature [7]. Reducing sugars were determined by the DNS method [14].

Protein determination

Protein concentration was determined by using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

Nucleotide sequence accession number

The ITS sequence of *Penicillium* sp. GXU20 was deposited in the GenBank database with accession number GU726770.

Statistical analysis

Each experiment was repeated three times. Average values with standard deviation from two or three replications in one experiment are presented.

Results

Isolation and identification of microorganism producing RCSDE

During the screening process, clearing zones were observed around the growing colonies of the isolated strains after the media were flooded with iodine solution. This suggests that the isolated strains secrete RCSDE into the isolation medium in the plates. Based on the results from the clearing zones and enzyme production in liquid fermentation, one isolated strain, designated GXU20, was found to produce the highest yield of RCSDE at 3 U/ml and was selected for further study.

The ITS sequence of strain GXU20 (accession number GU726770) shared 100% identity with the ITS sequences of *Penicillium* sp. (GenBank accession numbers EU301633, FJ977097, and DQ123663). The GXU20 colony on potato dextrose agar (PDA) was light green in color and around 5.0 cm in diameter on the 7th day of incubation at 28°C. Microscopic examination showed that the penicilli were biverticillate and the conidia were smooth with the shape of a sphere or ellipse, which are the characteristics of *Penicillium* sp. Based on the ITS sequence and microscopic features, the strain GXU20 was identified as a *Penicillium* sp.

HPLC analysis revealed that the product of hydrolysis of raw cassava flour by crude enzyme produced by *Penicillium* sp. GXU20 was solely glucose (Fig. 1), even early on in the first 5 min of the reaction. This result indicated that the extracellular RCSDE produced by strain GXU20 was mainly glucoamylase.

Optimization of RCSDE production by *Penicillium* sp. GXU20 during liquid fermentation

Under the operative conditions tested, the optimum pH and temperature for maximum production of RCSDE by *Penicillium* sp. GXU20 in cultivation were pH 5.0 (Fig. 2a) and 28°C (Fig. 2b). The RCSDE yield remained constant when GXU20 was cultivated in the pH range from 4.5 to 5.5.

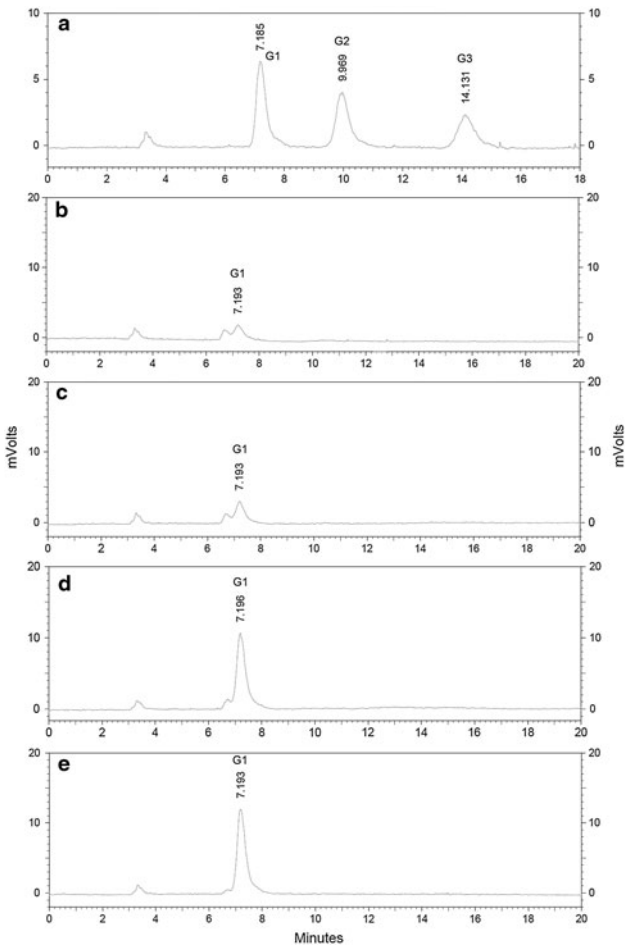

Fig. 1 HPLC chromatograms of hydrolysate of raw cassava starch hydrolyzed by crude RCSDE preparation from *Penicillium* sp. GXU20: (**a**) sugar standards glucose (G1), maltose (G2), and maltotriose (G3), and products of raw cassava starch hydrolyzation by crude RCSDE from strain GXU20 after: (**b**) 5 min of hydrolysis, (**c**) 10 min of hydrolysis, (**d**) 30 min of hydrolysis, and (**e**) 4 h of hydrolysis

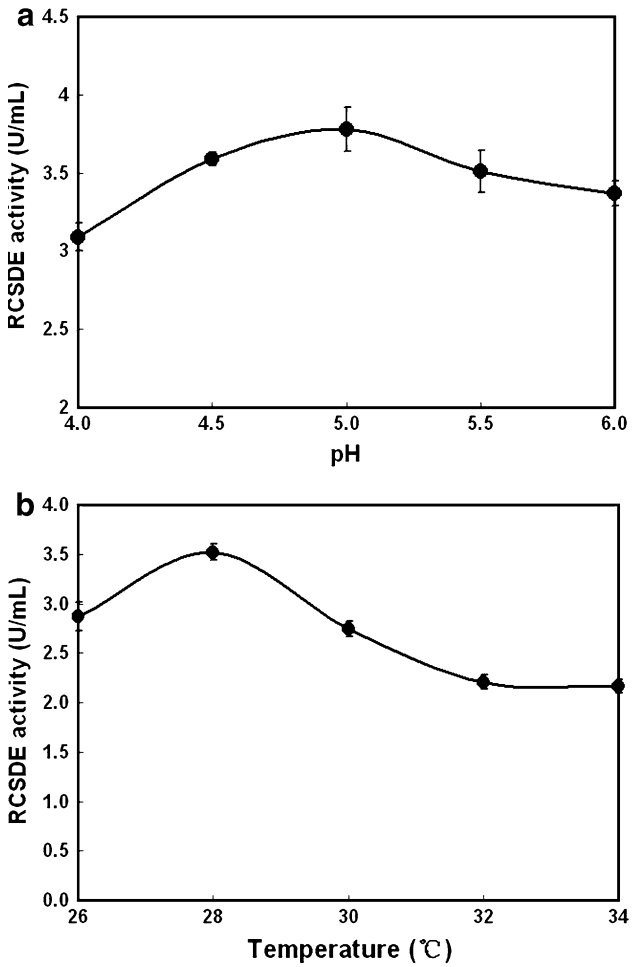

Fig. 2 Effects of different culture factors on RCSDE production by *Penicillium* sp. GXU20 in liquid fermentation. The basal medium at pH 5.5 and cultivation at 28°C was used as a control, producing 3 U/ml RCSDE. The role of pH and temperature was studied using the basal medium. Data are means ± standard deviation from three replications. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (**a**) Effect of initial pH on RCSDE production. (**b**) Effect of temperature on RCSDE production

Among the different starch materials tested, gelatinized starch sources rather than raw starch sources supported higher enzyme production by strain GXU20 (Fig. 3a). In particular 3.5% (w/v) wheat bran yielded a maximum RCSDE production of 17 U/ml (Fig. 3b), while soybean meal (Fig. 3c) at 2.5% (w/v) was found to be the best nitrogen source for RCSDE production, achieving productivity of 9.6 U/ml (Fig. 3d).

When wheat bran and soybean meal were synergistically employed together as the carbon and nitrogen sources at the optimum, pH 5.0 and 28°C, the RCSDE yield was 20 U/ml, representing approximately a sixfold increase as compared with the yield under the basal cultivation conditions.

Properties of the crude RCSDE

The optimum pH of the crude RCSDE produced by *Penicillium* GXU20 was pH 4.5 (Fig. 4a), which is close to the pH of 4.0 used for *S. cerevisiae* fermentation in the ethanol process. The enzyme was optimally active at 50°C (Fig. 4b). It showed about 87% relative activity at 40°C.

In determining the substrate specificity, it was found that crude enzyme preparation from GXU20 had the ability to hydrolyze all the raw starch flours to varying extents (Table 1). Results indicated that crude enzyme hydrolyzed raw starch materials in the following order: corn, rice, cassava, potato, sweet potato, buckwheat, glutinous rice, soluble starch. Raw corn and raw rice showed relatively higher enzyme susceptibility than other starches to the crude RCSDE.

Studies on the adsorbability of the crude RCSDE preparation from *Penicillium* sp. GXU20 towards raw cassava starch granules showed that the adsorption increased with

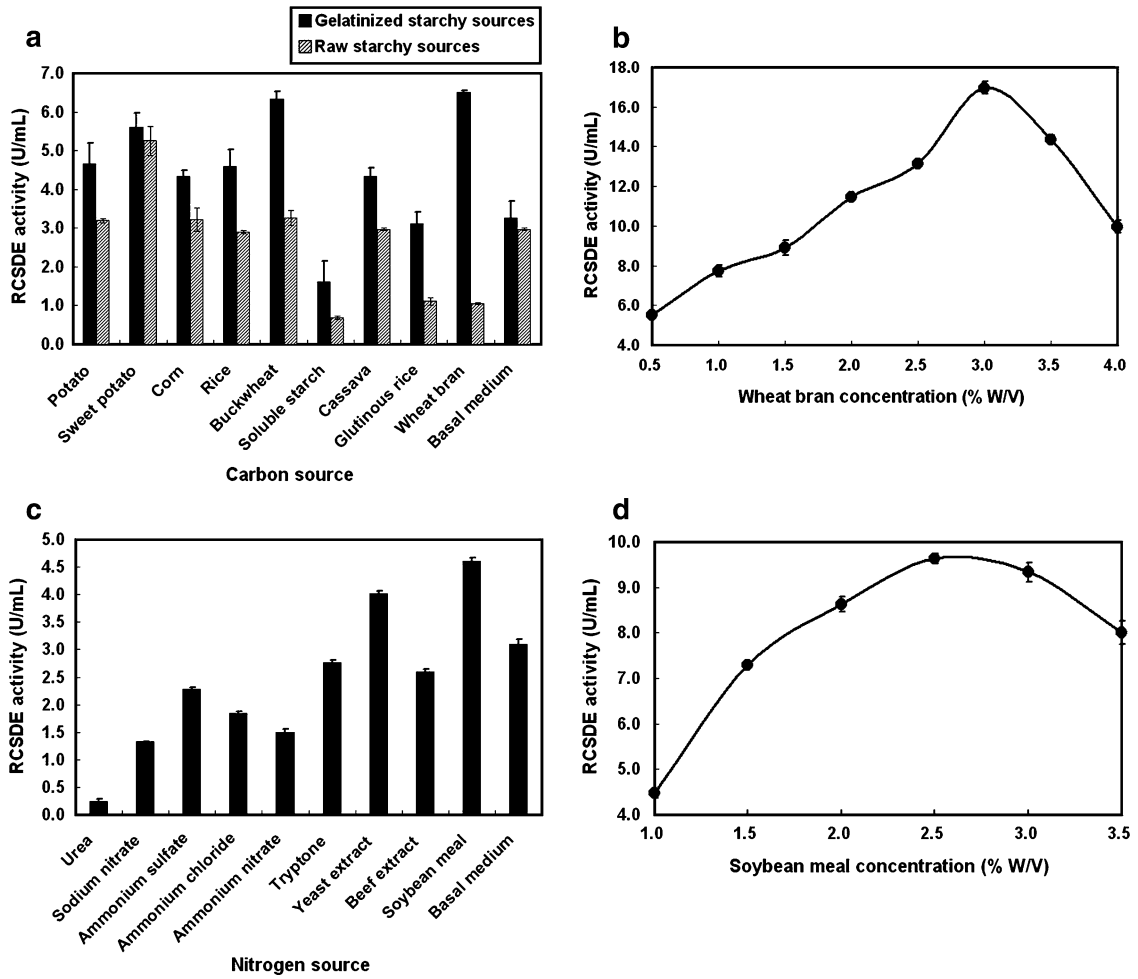

Fig. 3 Effect of carbon and nitrogen sources on RCSDE production by strain GXU20. The basal medium at pH 5.5 and cultivation at 28°C was used as a control, producing 3 U/ml RCSDE. The carbon and nitrogen components in the basal medium were replaced with different carbon and nitrogen sources while keeping the other ingredients the same. Data are means ± standard deviation from three replications. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (**a**) Effect of various carbon sources on RCSDE production. (**b**) Raw cassava flour in the basal medium was replaced with varying concentrations of wheat bran. (**c**) Effect of various nitrogen sources on RCSDE production. (**d**) Tryptone and $(NH_4)_2SO_4$ in the basal medium were replaced with varying concentrations of soybean meal

increasing raw cassava starch concentration from 2% to 20%, but not with increased incubation time at pH 4.0 and 40°C (Table 2).

Scanning electron microscopy (SEM)

The scanning electron micrographs revealed that treated cassava starch granules were severely damaged, suggesting that the granules were significantly hydrolyzed by RCSDE from *Penicillium* sp. GXU20. As shown in Fig. 5, the surface of untreated cassava granules was smooth (Fig. 5a). However, a rough surface and eroded granules were observed (Fig. 5b) in those hydrolyzed by RCSDE from *Penicillium* sp. GXU20. The surface of the treated cassava starch granules appeared to be composed of a number of small pores. The random pinholes on the surface of the granules may allow the RCSDE to penetrate into the inner granule more extensively.

Hydrolysis of raw cassava flour by crude RCSDE from *Penicillium* sp. GXU20

As shown in Fig. 6, the RCSDE preparation could hydrolyze raw cassava starch in a short incubation time of 36 h. Approximately 100% of the cassava starch (at 50 g/l) was hydrolyzed after 36 h of incubation. With a constant RCSDE dose of 30 U/g cassava flour, the degree of starch hydrolysis decreased as the cassava flour concentration increased. At cassava flour concentrations of 100, 150, 200, and 250 g/l, the degree of starch hydrolysis after 36 h of incubation was 90%, 80%, 74%, and 68%, respectively. When the incubation time was increased from 36 to 72 h, the degree of starch

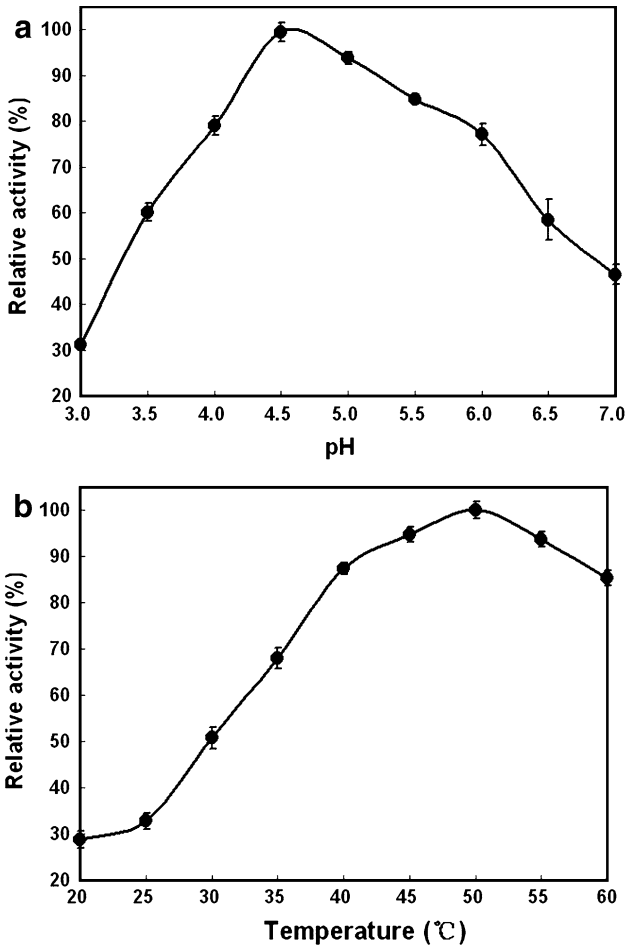

Fig. 4 Effect of pH and temperature on activity of crude RCSDE produced by *Penicillium* sp. GXU20. Data are means ± standard deviation from three replications. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (**a**) Influence of pH on crude RCSDE activity. (**b**) Influence of temperature on crude RCSDE activity

Table 1 Substrate specificity of crude RCSDE from strain GXU20 towards various raw starches at pH 4.0 and 40°C

Substrate	Specific activity (U/mg protein)	Relative activity (%)
Cassava	6.65 ± 0.095	100 ± 1.4
Corn	14.05 ± 0.64	211 ± 9.7
Rice	13.73 ± 0.34	206 ± 5.2
Potato	6.37 ± 0.026	96 ± 0.4
Sweet potato	4.22 ± 0.07	63 ± 1.0
Buckwheat	3.62 ± 0.069	54 ± 1.0
Glutinous rice	2.26 ± 0.018	34 ± 0.3
Soluble starch	0.26 ± 0.006	4 ± 0.09

Data are means ± standard deviation from three replications. The experiment was repeated three times, and similar results were obtained each time

hydrolysis increased to 100%, 95%, 87%, and 80%, respectively (Fig. 6a). These results indicate that the majority of the hydrolysis occurred during the first 36 h.

When 200 g/l raw cassava flour was incubated with an RCSDE dose from 15 to 60 U/g cassava flour, a similar degree of starch hydrolysis was observed, except for the enzyme dose of 15 U/g cassava flour (Fig. 6b).

Simultaneous saccharification and fermentation of raw cassava flour to ethanol

At flour concentration of 150 g/l, an amount typically used in the starch industry, ethanol yield of 53.3 g/l and fermentation efficiency of 92% were achieved after 48 h of SSF (Fig. 7a). No significant increase in ethanol yield was observed after 60 and 72 h of fermentation. At flour concentration of 250 g/l, ethanol yield of 75.6 g/l (Fig. 7b) and fermentation efficiency of 76% were obtained after 48 h of SSF. Prolonging the duration of SSF to 72 h resulted in little increase in ethanol content (80.5 g/l) with fermentation efficiency of 84%.

Discussion

RCSDE secreted by strain GXU20 showed high activity towards raw cassava starch. Production of this enzyme was significantly enhanced using wheat bran and soybean meal as the carbon and nitrogen sources for strain GXU20 cultivated at pH 5.0 and 28°C. An earlier study also indicated that wheat bran and soybean meal were effective for raw starch-degrading glucoamylase production by *Aspergillus niger* [19]. Wheat bran and soybean meal as cheap energy sources are generally preferred for industrial processes compared with more expensive, chemically refined ingredients such as tryptone and yeast extract.

Compared with traditional cassava starch-to-ethanol processing, enzyme that is capable of degrading raw cassava starch is economically attractive, since it can save energy costs. The crude RCSDE produced by strain GXU20 is effectively active towards raw cassava flour at pH 4.0 and 40°C, similar to the conditions used for fermentation of the thermally resistant dried yeast (*S. cerevisiae*) from Angel Yeast Co., Ltd., China. This is an important advantage for the potential application of RCSDE for use in digesting raw cassava flour in SSF. In addition, the SEM observation strongly supports the effective action of the crude RCSDE towards raw cassava starch granules. Aggarwal [2] reported a similar SEM observation pattern after hydrolysis of raw corn starch by glucoamylase from *Rhizopus*.

In the RCSDE adsorbability study, it was not observed that the amount of reducing sugars formed would be

Table 2 Adsorbability of RCSDE towards raw cassava starch granules

Data are means ± standard deviation from three replications. The experiment was repeated three times, and similar results were obtained each time

Concentration of raw cassava flour (% w/v)	Adsorption to raw cassava starch granules (%)		
	15 min	30 min	60 min
2	3.1 ± 1.9	14.4 ± 0.9	10.0 ± 1.1
4	9.2 ± 0.7	16.4 ± 1.7	13.1 ± 0.4
8	13.2 ± 0.4	18.0 ± 1.3	14.9 ± 0.7
16	18.8 ± 0.6	27.3 ± 1.6	23.7 ± 0.7
20	21.1 ± 1.2	29.8 ± 0.6	27.2 ± 1.6

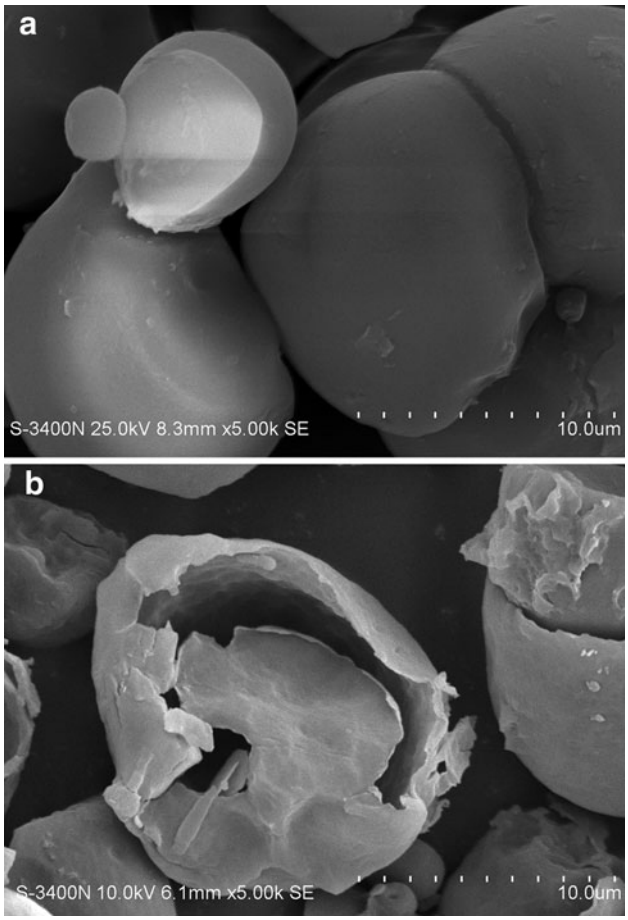

Fig. 5 Scanning electron micrographs of raw cassava starch granules: (**a**) untreated, and (**b**) treated with crude RCSDE preparation from *Penicillium* sp. GXU20

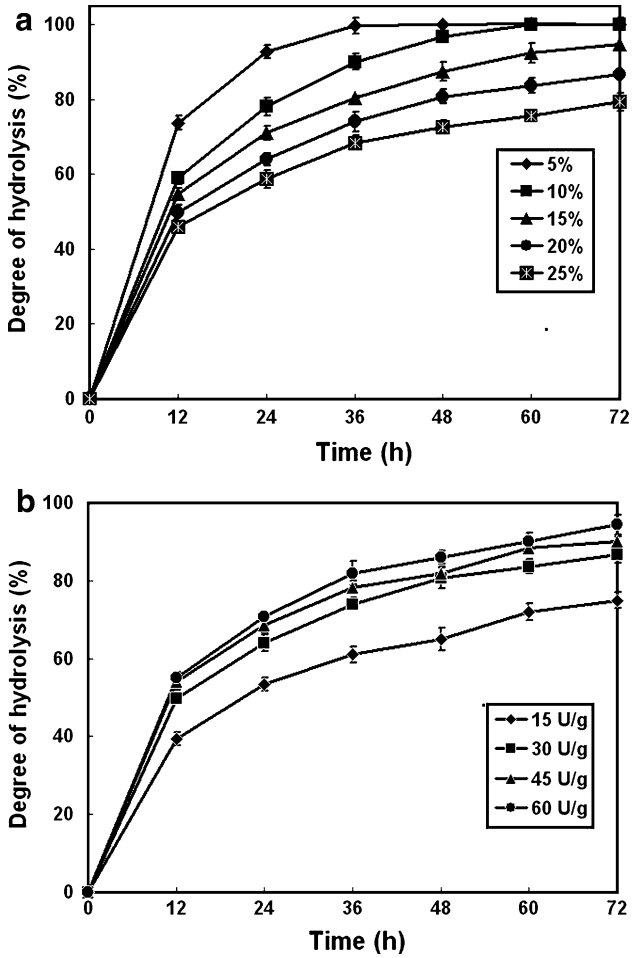

Fig. 6 Effect of substrate concentration and enzyme dose on hydrolysis of raw cassava flour by crude RCSDE produced by *Penicillium* sp. GXU20. All hydrolyses were carried out at pH 4.0 and 40°C. Data are means ± standard deviation from two replications. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (**a**) Hydrolysis of raw cassava flour at different concentrations with constant RCSDE dose (30 U/g cassava flour). (**b**) Hydrolysis of raw cassava flour (200 g/l) with varying doses of crude RCSDE preparation

proportional to the amount of RCSDE adsorption. A similar result was also observed by Kimura and Robyt [9], who noted that the enzyme could adsorb onto raw potato starch. Binding of raw starch-degrading enzyme to starch granules has been shown to be necessary for degrading starch granules by fungi glucoamylase [16]. Mamo and Gessesse [12] found that over 70% of the glucoamylase from *Aspergillus* sp. GP-21 could strongly absorb onto raw corn and raw potato starch after incubation for 15 min at room temperature. However, Sun et al. [21] found that the raw starch-degrading glucoamylase from *Penicillium* sp. X-1 showed no adsorbability to raw corn starch, despite varying the incubation conditions considerably.

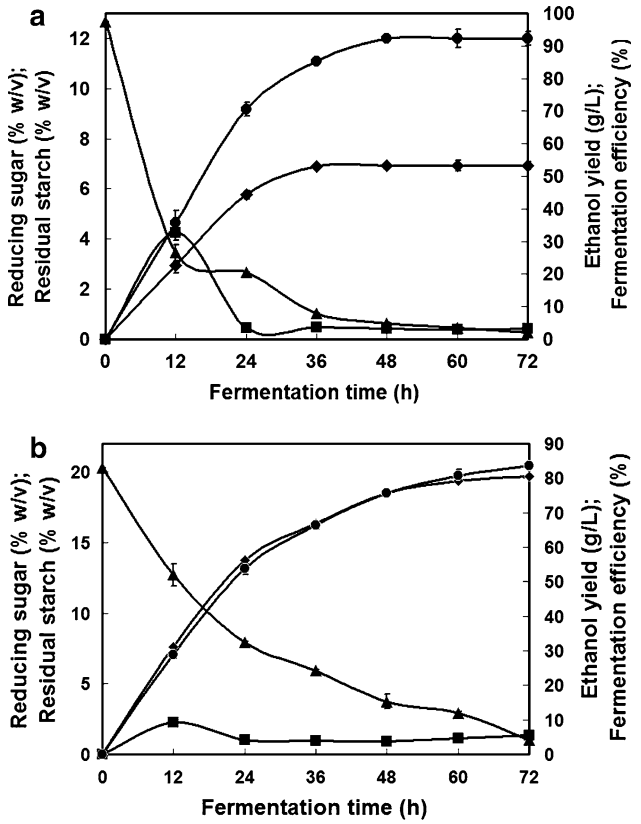

Fig. 7 Ethanol production from raw cassava flour by SSF using crude RCSDE from *Penicillium* sp. GXU20. Data are means ± standard deviation from two replications. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (**a**) SSF of raw cassava flour to ethanol at starch concentration of 150 g/l. (**b**) SSF of raw cassava flour to ethanol at higher starch concentration of 250 g/l. (*filled diamonds* ethanol yield, *filled squares* reducing sugar, *filled triangles* residual starch, *filled circles* fermentation efficiency)

The potential application of the crude RCSDE preparation from *Penicillium* sp. GXU20 was evaluated by studying the degree of hydrolysis of raw cassava flour at pH 4.0 and 40°C. The hydrolysis degree of 95% attained after 72 h of incubation strongly suggests that low temperature (40°C) and concentration of enzyme (30 U/g cassava flour) are sufficient for RCSDE to hydrolyze raw cassava flour at substrate concentration of 150 g/l. Using fungi crude enzyme, Abe et al. [1] reported that 86% of corn was hydrolyzed by raw starch-digesting amylase from *Aspergillus* sp. K27 at 30°C after 24 h with 250 U of enzyme (for 250 mg raw corn starch). Bacterial amylase was previously reported to show similar degradation ability towards raw corn and raw potato starch mashes at a higher enzyme dose [8, 11]. Goyal et al. [8] reported that a thermostable purified α-amylase showed digestion of raw potato starch at concentration of 12.5% (w/v), while Liu and Xu [11] reported a purified α-amylase from *Bacillus* sp. for which hydrolysis degree of 50% was obtained at raw corn starch concentration of 20% (w/v) with enzyme dose of 250 U per gram substrate after 12 h of incubation. For synergistic action of commercial α-amylase with purified glucoamylase, Sun [21] reported that hydrolysis content of 92.4% was obtained in hydrolysis of 15% (w/v) raw corn starch slurry after 2 h of incubation (500 U α-amylase plus 1,000 U raw starch-degrading glucoamylase for 15 g raw corn starch). The *Penicillium* sp. GXU20 RCSDE used in this study may have potential applications in direct degradation of raw cassava starch in the food and fermentation industries.

To further explore the potential use of the crude RCSDE preparation from strain GXU20, crude RCSDE was tested in SSF of raw cassava flour to ethanol. SSF of raw cassava flour (150 g/l) to ethanol was carried out effectively in a short time of 48 h using the crude RCSDE. The residual starch of 0.63% (w/v) and fermentation efficiency of 92% indicated that the SSF process was completed successfully. During the SSF process, raw cassava starch was easily digested by the RCSDE, since the level of reducing sugars was kept low (<0.5% w/v) after 24 h, when end-product inhibition would be likely. The cassava-to-ethanol process presented herein has a distinct advantage over the conventional process using cooked cassava starch, as the new process makes it unnecessary to consume energy for starch cooking.

Since this research was carried out in a 250-ml Erlenmeyer flask, it is necessary to scale up production and carry out application trials of the RCSDE before any conclusions can be drawn regarding industrial utilization of this crude enzyme. Further work on purification and application scale-up is in progress.

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