J Ind Microbiol Biotechnol (2012) 39:605–612
DOI 10.1007/s10295-011-1053-1

Production of multiple extracellular enzyme activities by novel submerged culture of *Aspergillus kawachii* for ethanol production from raw cassava flour

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Received: 31 July 2011 / Accepted: 21 October 2011 / Published online: 10 November 2011

Abstract Cassava is a starch-containing root crop that is widely used as a raw material in a variety of industrial applications, most recently in the production of fuel ethanol. In the present study, ethanol production from raw (uncooked) cassava flour by simultaneous saccharification and fermentation (SSF) using a preparation consisting of multiple enzyme activities from *Aspergillus kawachii* FS005 was investigated. The multi-activity preparation was obtained from a novel submerged fermentation broth of *A. kawachii* FS005 grown on unmilled crude barley as a carbon source. The preparation was found to consist of glucoamylase, acid-stable α-amylase, acid carboxypeptidase, acid protease, cellulase and xylanase activities, and exhibited glucose and free amino nitrogen (FAN) production rates of 37.7 and 118.7 mg/l/h, respectively, during *A. kawachii* FS005-mediated saccharification of uncooked raw cassava flour. Ethanol production from 18.2% (w/v) dry uncooked solids of raw cassava flour by SSF with the multi-activity enzyme preparation yielded 9.0% (v/v) of ethanol and 92.3% fermentation efficiency. A feasibility study for ethanol production by SSF with a two-step mash using raw cassava flour and the multi-activity enzyme preparation manufactured on-site was verified on a pilot plant scale. The enzyme preparation obtained from the *A. kawachii* FS005 culture broth exhibited glucose and FAN production rates of 41.1 and 135.5 mg/l/h, respectively. SSF performed in a mash volume of about 1,612 l containing 20.6% (w/v) dry raw cassava solids and 106 l of on-site manufactured *A. kawachii* FS005 culture broth yielded 10.3% (v/v) ethanol and a fermentation efficiency of 92.7%.

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Keywords Cassava · Enzyme production · *Aspergillus kawachii* · Simultaneous saccharification and fermentation · Ethanol production

Introduction

Industrial ethanol production has been described based on use of a variety of starch-containing substrates, including inexpensive cassava. Fresh cassava has a very high starch content, up to 30%. Dried cassava contains 80% fermentable substrate [10]. While the technology of ethanol production using starch-based substrates is well known, on-going studies continue to focus on process improvements that increase efficiency and reduce costs. One of the most important innovations with respect to fermentation technology is the development of a simultaneous saccharification and fermentation (SSF) process based on raw (uncooked) substrate, which has reduced capital costs, simplified operations, reduced fermentation time, and allowed high loading of solids. Additional recent advances include a new generation of enzymes [e.g., α-amylase from *Aspergillus kawachii* and glucoamylase from *Aspergillus niger* (STARGEN™ 001, Genencor International Inc., USA)] for SSF processing of raw substrates. STARGEN™ 001 has the advantages of glucoamylase-mediated exo-activity, which creates sharp and deep pinholes, as well as endo-activity of α-amylase that widens the pinholes. This combination enhances continuous release of fermentable glucose from granular starches in a single step at moderate temperature well below the gelatinization temperature [23]. However, because the starch granules in cassava are large and complex structures, a high reactor loading of enzyme is required. To overcome this problem, on-going efforts to optimize the STARGEN-based SSF processing of raw

substrate are the focus of current experimental work [17, 22]. On the other hand, on-site production of multi-activity enzyme preparations from *Penicillium* sp. [11] and *A. niger* [20] for use in the hydrolysis of raw cassava starch has been reported as a potential improvement to the SSF process. However, because on-site manufacturing of multi-activity enzyme preparations is a relatively new concept, they have only been evaluated on a laboratory scale (e.g., 250-ml Erlenmeyer flasks). Clearly, expansion to a pilot plant scale is required to determine potential industrial feasibility.

Glucoamylase and acid-stable α-amylase from *A. kawachii* have granular starch-hydrolyzing activity [4, 7], and are important industrial enzymes used to produce products such as bio-ethanol from starch substrates derived from grains and cereals. However, production of acid-stable α-amylase by submerged culture is problematic because acid-stable α-amylase is recognized as a solid-state culture-specific product [16], and to our knowledge, no reports have been published on the simultaneous production of glucoamylase and acid-stable α-amylase by standard submerged culture of *A. kawachii*. Recently, we described a novel submerged culture technique to produce high levels of glucoamylase and acid-stable α-amylase in *A. kawachii* [14, 24]. Briefly, an unmilled cereal whose surface is completely covered with kernels is used in submerged culture as the carbon source. In general, the productivities of various enzymes, including amylolytic, proteolytic, and cellulolytic enzymes in *Aspergillus* sp. are strongly repressed in the presence of glucose via CreA [1–3, 6, 8, 12, 21]. To achieve efficient industrial production of these enzymes, the process must be operated at low glucose concentration or CreA-mediated repression must be reduced or abolished. When crude unmilled barley is used in the submerged culture of *A. kawachii*, the kernels with a hard outer layer enclose the soft endosperm and prevent leakage of large amounts of starch into the culture broth. Therefore, the glucose concentration in the culture broth is maintained at a low level, overcoming CreA-mediated repression and allowing simultaneous production of glucoamylase and acid-stable α-amylase. On the other hand, when milled crude barley was used, the glucose concentration in the culture broth rose, resulting in a decrease in the production of glucoamylase and acid-stable α-amylase [24].

In the present study, we first investigated the production of various enzymes by *A. kawachii* FS005 grown in the novel submerged culture, and evaluated the ability of the multi-activity preparation obtained to saccharify raw cassava flour on a laboratory scale. We then evaluated ethanol production from raw cassava flour in an SSF process using the *A. kawachii* FS005 enzyme preparation. Finally, a feasibility study of ethanol production was undertaken on a pilot plant scale using an SSF process, raw cassava flour as substrate, and the on-site manufactured enzyme preparation from *A. kawachii* FS005.

Materials and methods

Materials

Raw Indonesian cassava flour (starch content, 75.2% of dry weight) was obtained from Shinozaki Perfumery Co., Ltd., Japan. The fresh cassava was sun-dried to decrease water content to less than 10% (w/w), and was ground and sieved through a mesh screen (50–100, 300–150 μm).

Microorganisms

A. kawachii FS005 from our collection was maintained on potato dextrose agar plates (Becton–Dickinson, USA) and used to produce the multi-activity enzyme preparation. An active dry commercial *Saccharomyces cerevisiae* strain, Ethanol Red, was obtained from Lesaffre Company (France) and used for ethanol fermentations.

Submerged culture of *A. kawachii* FS005 on a laboratory scale

To produce a seed culture, pearled barley (crude barley was polished to a residual weight of 65% w/w, Australia-grown Starling spices) was used. The liquid culture medium contained 80 g pearled barley per liter and was sterilized at 121°C for 15 min. Conidia of *A. kawachii* were inoculated at 10^5 spores/ml into 40 ml of a seed culture medium in 200-ml baffled flasks and cultivated at 100 rpm and 37°C for 24 h. The culture for the production of enzyme was grown in 500-ml baffled flasks with a working volume of 100 ml. A modified Czapek-Dox medium containing (per liter): 25 g crude barley or milled crude barley, 3 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 g ferrous sulfate, 5 g wheat bran (Showa Sangyo Co., Ltd., Japan) was sterilized at 121°C for 15 min. Milled crude barley was produced by milling 100 g of crude barley for 2 min using a Magnum blender (Osaka Chemical, Japan), and sieved through a mesh screen (18–100, 1 mm–150 μm). Seed culture (2 ml) was inoculated into 100 ml of a liquid medium and cultivated at 100 rpm and 37°C for 72 h.

Enzymatic saccharification of raw (uncooked) cassava flour on a laboratory scale

Experiments for the production of raw cassava flour hydrolysates were carried out by mixing raw cassava flour with *A. kawachii* FS005 culture broth. A separate set of experiments was performed by mixing raw cassava flour with a commercial enzyme, STARGEN™ 001 in order to compare the hydrolysis of *A. kawachii* FS005 culture broth with an industrially relevant preparation. To prepare raw cassava flour slurry, 30 g of raw cassava flour was suspended in

140 ml of 10 mM sodium acetate buffer (pH 4.0) in 500-ml flasks. A 10-ml aliquot of *A. kawachii* FS005 culture broth or 10 ml of a diluted commercial enzyme (including 0.1 ml of STARGEN™ 001) were added into the slurry. The samples were incubated in an incubator shaker (MIR-220R, Sanyo, Japan) at 50°C and 150 rpm. After 1.0 h, the supernatant from 1.5 ml of hydrolysate was separated by centrifugation at 3,000 × *g* for 10 min and was assayed for glucose and free amino nitrogen (FAN).

Ethanol production from raw cassava flour by a laboratory-scale SSF process

Experiments to produce ethanol from raw cassava flour by SSF were carried out as follows. To prepare raw cassava flour mash, 30 g of raw cassava flour was mixed with 140 ml of water in 500-ml flasks. A 10-ml aliquot of *A. kawachii* FS005 culture broth using crude barley or a 10-ml aliquot of a diluted commercial enzyme (0.1 ml of STARGEN™ 001) was added to the mash. To prevent bacterial contamination, 0.15 ml of 90% (w/v) lactic acid (Musashino Chemical Laboratory, Ltd., Japan) was added to the mash whose pH was adjusted to 4.0, followed by the addition of 0.2 g of dry yeast suspended in 2 ml of water. Urea (0.15 g) was added as a yeast nitrogen source. The mashes were then incubated statically in an incubator (MIR-220R, Sanyo, Japan) at 37°C. After 18, 42, 66, and 85 h, 3 ml of the fermented mash was harvested and the supernatant was separated by centrifugation at 3,000 × *g* for 10 min and assayed for glucose, xylose, and ethanol.

Ethanol production from raw cassava flour by a pilot plant-scale SSF process using a multi-activity enzyme preparation from *A. kawachii* FS005 manufactured on-site

To manufacture a multi-activity enzyme preparation, a submerged culture of *A. kawachii* FS005 was grown on crude barley on a pilot plant scale. To produce a seed culture, a liquid medium was prepared containing 80 g of pearled barley per liter and was sterilized at 121°C for 15 min. *A. kawachii* conidia were inoculated at 10^5 spores/ml into 100 ml of seed culture medium in 500-ml baffled flasks and cultivated at 100 rpm and 37°C for 24 h. A pre-culture was performed in a 5,000-ml jar fermenter (Bioneer-N500, Marubishi Bioengineering Co., Ltd., Japan) with a working volume of 3,000 ml. A modified Czapek-Dox medium containing (per liter): 25 g crude barley, 3 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 g ferrous sulfate, 5 g wheat bran was sterilized at 121°C for 15 min. The seed culture (60 ml) was inoculated into 3,000 ml of pre-culture medium and grown at 250 rpm with an aeration rate of 3,000 ml/min at 37°C for 24 h. The main culture was performed in 90-l jar fermenters (Type KMJ-90MST, MBS Co., Ltd., Japan) with a working volume of 60 l. A liquid medium of the same composition as the pre-culture medium was sterilized at 121°C for 20 min. The pre-culture (1.2 l) was inoculated into 60 l of main-culture medium and cultivated at 150 rpm with an aeration rate of 60 l/min at 37°C for 66 h. Two lots of media were inoculated and cultured yielding 120 l of *A. kawachii* FS005 broth.

Ethanol production by the SSF process was carried out on a pilot plant scale by a two-step mash as follows. To obtain a primary mash, raw cassava flour (124 kg) was mixed with 731 l of water and 106 l of the *A. kawachii* FS005 culture broth in the mixing tank. The pH of the mash was adjusted to 4.0 using 0.98 l of 90% (w/v) lactic acid. Then, 20 g of dried yeast suspended in 0.8 l of water was added into the mash, and transferred to the fermentation tank. The primary mash was fermented at 37°C for 36 h under static conditions. In the secondary mash, raw cassava flour (208 kg) was mixed with 560 l of water, and the pH was adjusted to 4.0 using 0.62 l of 90% (w/v) lactic acid prior to immediate transfer to the fermentation tank. This secondary fermentation was carried out at 37°C for 114 h under static conditions.

Assays

Glucoamylase, acid-stable α-amylase, acid carboxypeptidase, acid protease, cellulase, and xylanase were measured in the supernatant of the submerged culture broth. Glucoamylase, acid carboxypeptidase, and acid protease assays were performed according to the official analytical methods of the National Tax Administration Agency of Japan [18]. Acid-stable α-amylase was assayed as described [24]. Cellulase and xylanase activities were analyzed as described [20] with the following minor modifications. The 300-µl reactions contained the appropriate dilution of the supernatant in 50 mM sodium acetate buffer, pH5.0 and the appropriate substrates: 1% (w/v) birchwood xylan for xylanase activity and 0.5% (w/v) carboxymethyl cellulose for cellulase activity. The reactions were incubated at 40°C for 10 min and the amount of reducing sugar was determined by the 3,5-dinitrosalisylic acid (DNS) method [15]. FAN concentration was determined by the ninhydrin colorimetric method [9]. Glucose, xylose and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) using a refractive index detector (RID-10A, Shimadzu, Japan) and a Aminex HPX-87H (300 × 7.8 mm I.D., Bio-Rad, Japan) column. The supernatants of fermented mash were diluted tenfold with distilled water and filtered (DISMIC-13cp filter with pore size of 0.45 µm, Advantec, Japan) prior to HPLC analysis. The mobile phase was 5 mM sulfuric acid, the flow rate was 0.6 ml/min, the oven

temperature was 50°C, and the injection volume was 10 µl. The yeast population in the fermented mash was determined using a Thoma haemacytometer. Yeast viability was determined by staining with 0.01% (w/v) methylene blue. Bacterial cells were counted on Difco lactobacilli MRS broth (Becton–Dickinson, USA) agar plates following platings of 100-fold dilutions of the fermented mash and incubation at 37°C for 2 days.

Results and discussion

Laboratory-scale production of multi-activity enzyme preparation by novel submerged culture of *A. kawachii* FS005

In a preliminary experiment, *A. kawachii* FS005 was found to be a better amylolytic enzyme-producing strain than the type strain *A. kawachii* NBRC4308, and was found to produce more activity in the modified Czapek-Dox medium than previously reported [24] as assessed by mycelial growth (data not shown). Therefore, *A. kawachii* FS005 and the modified Czapek-Dox medium were used throughout this study. Figure 1 shows amylolytic, proteolytic, and cellulolytic enzyme activities in the submerged culture broths of *A. kawachii* FS005 using crude barley or milled crude barley as a carbon source. As noted in our previous work with *A. kawachii* NBRC4308, higher simultaneous glucoamylase and acid-stable α-amylase production were observed in the culture broth of *A. kawachii* FS005 grown on crude barley. Further, significant acid carboxypeptidase, acid protease, cellulase, and xylanase activities were also detected in the crude barley culture broth. In contrast, milling of barley had a negative effect on enzyme productivity. Glucoamylase, acid carboxypeptidase, cellulase, and xylanase activities in the broth obtained from the milled crude

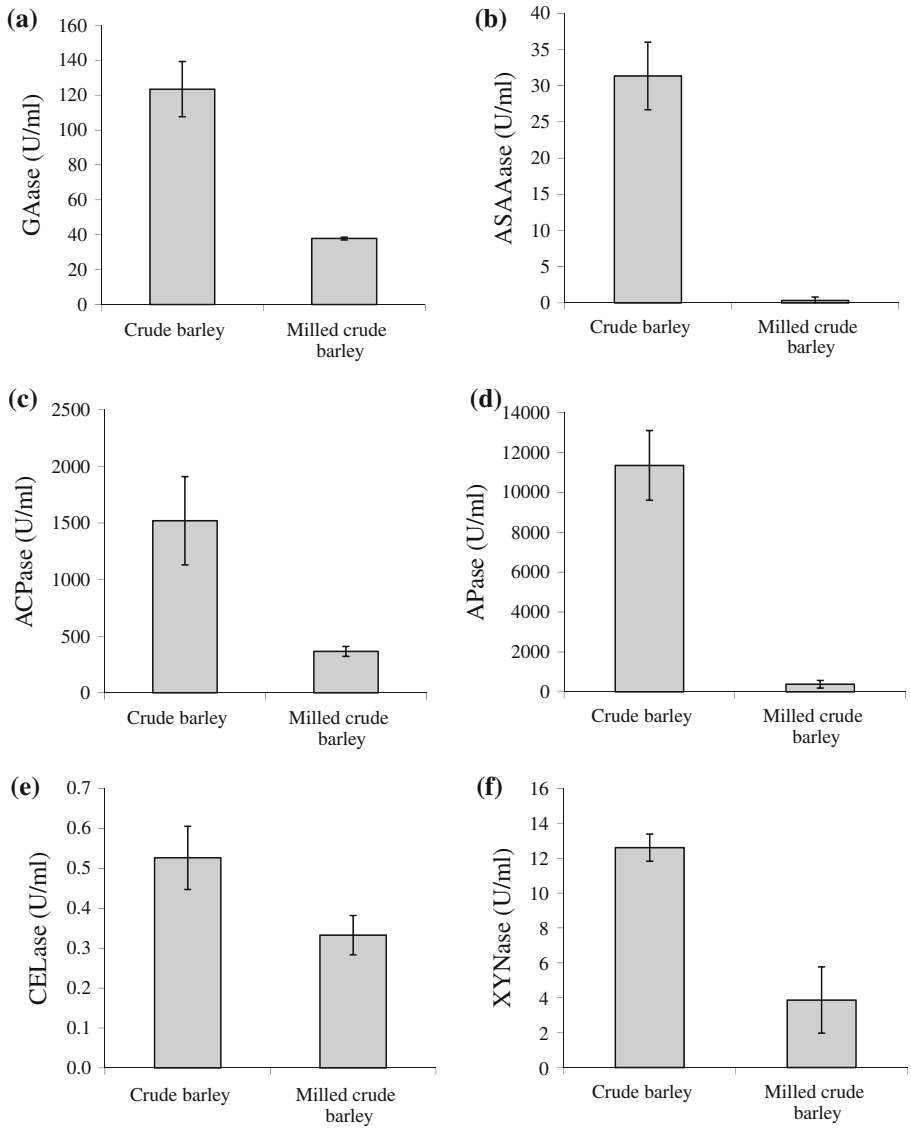

Fig. 1 Enzyme activities in the culture broth of *A. kawachii* FS005 grown on crude barley or milled crude barley. **a** glucoamylase; **b** acid-stable α-amylase; **c** acid carboxypeptidase; **d** acid protease; **e** cellulase; **f** xylanase. Values are means ± SD from three replications

barley culture were one-third of that obtained from the unmilled crude barley culture. In addition, low acid-stable α-amylase and acid protease activities were observed in the milled crude barley culture broth. Although the cultivation conditions are slightly different, the results of this experiment were similar to our previous results [13], and the following hypothesis that might explain the high enzyme production in the submerged culture with crude barley is considered. In the first half of the period of culture using milled crude barley, rapid glucose release from the substrate owing to milling activates glycolysis. In the case of culture using crude barley, carbohydrate metabolism is gradual owing to slow sugar release from crude barley. The carbon catabolite repression mediated by CreA might be reduced, and the enzyme production is greatly increased. In the second half of the period of culture using milled crude barley, the disappearance of carbohydrate occurs early. Therefore, a marked change in the metabolism of nitrogen compounds such as amino acids occurs, and this leads to the saturation of enzyme production. In the second half of the period of culture using crude barley, slow carbohydrate release makes it possible to supply sugar continuously for a long time. Consequently, nitrogen metabolism is maintained, and this allows the biosynthesis of amino acids, contributing to high enzyme production. For the above reasons, the submerged culture using crude unmilled barley as substrate was found superior for the simultaneous production of amylolytic, proteolytic and cellulolytic enzymes for use in raw cassava starch saccharification.

Laboratory-scale saccharification of raw cassava flour by use of the multi-activity enzyme preparation from submerged culture of *A. kawachii* FS005

Saccharification of raw cassava flour by the aforementioned culture broth of *A. kawachii* FS005 was evaluated. For comparison, the enzymatic saccharification of raw cassava flour by a novel commercial enzyme, STARGEN™ 001, was also performed. Table 1 shows glucose and FAN production rates during the saccharification of raw cassava flour. The glucose production rates of the crude barley-grown *A. kawachii* FS005 culture broth and STARGEN™ 001 were 37.7 and 42.2 mg/l/h, respectively. The glucose production rate obtained from the milled crude barley-grown culture broth was 20% of that of the crude barley-grown culture broth. As shown in Fig. 1, glucoamylase activity of the milled crude barley-grown culture broth was one-third of that of crude barley-grown culture broth. No acid-stable α-amylase activity was detected in the broth of the milled crude barley-grown culture. We speculate that the glucoamylase and acid-stable α-amylase in the *A. kawachii* FS005 culture broth functioned synergistically in saccharifying the raw cassava starch. FAN production rates for the crude barley-grown *A. kawachii* FS005 culture broth, the milled crude barley-grown *A. kawachii* FS005 culture broth and STARGEN™ 001 were 118.7, 48.6, and 58.1 mg/l/h, respectively. Recently, the addition of acid protease from *A. niger* was found to have a synergistic effect on the hydrolysis of various starch substrates including corn and barley under STARGEN-based enzymatic saccharification conditions [5, 25]. It is likely that the lower acid protease and acid carboxypeptidase activities in the milled crude barley-grown *A. kawachii* FS005 culture broth contributed to the inefficient hydrolysis of the raw cassava flour. Acid protease and acid carboxypeptidase activities in the crude barley-grown *A. kawachii* FS005 culture broth were found to have a profound effect on FAN production from raw cassava flour.

Table 1 Glucose and free amino nitrogen (FAN) production during enzymatic hydrolysis of raw cassava flour using *A. kawachii* FS005 culture broth and a commercial enzyme, STARGEN™ 001

	Production rate (mg/l/h)	
	Glucose	FAN
—	2.8 ± 0.3	28.7 ± 7.8
STARGEN™ 001	42.2 ± 1.7	58.1 ± 17.7
Crude barley-grown *A. kawachii* culture broth	37.7 ± 3.2	118.7 ± 19.6
Milled crude barley-grown *A. kawachii* culture broth	7.6 ± 1.3	48.6 ± 6.7

Saccharification in the absence of enzyme addition is indicated as (—). Values are means ± SD from three replications

Laboratory-scale ethanol production by SSF using raw cassava flour and the multi-activity enzyme preparation from *A. kawachii* FS005

As a next step, SSF in a mash volume of 165 ml containing 18.2% (w/v) dry cassava solids was investigated. Enzyme loading of the multi-activity preparation from *A. kawachii* FS005 and STARGEN™ 001 was 33% (v/w) and 0.33% (v/w) relative to the raw cassava flour, respectively. As shown in Table 1, these enzyme loadings resulted in about the same rate of glucose production during saccharification of raw cassava flour. However, as shown in Fig. 2a, the ethanol production using STARGEN™ 001 was lower than that obtained when the multi-activity enzyme preparation from *A. kawachii* FS005 was used. It seems reasonable to suppose that the difference of ethanol concentrations in the fermented mash is approximately equivalent to the difference of residual glucose in the mash (Fig. 2b). The fact that residual glucose in the STARGEN™ 001-derived fermenting mash was higher than that in the mash derived from the multi-activity enzyme preparation from *A. kawachii* FS005

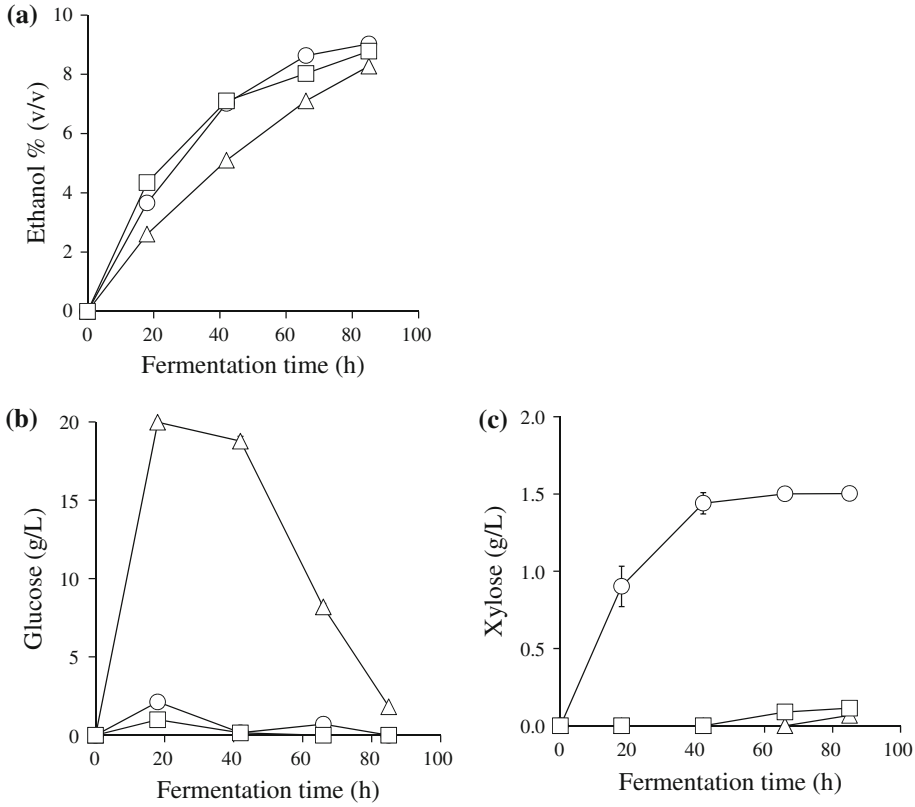

Fig. 2 Fermentation profiles for SSF of raw cassava flour. **a** ethanol; **b** glucose; **c** xylose; (*open circle*), multi-activity enzyme preparation from *A. kawachii* FS005; (*open square*), STARGEN™ 001; (*open square*), STARGEN™ 001 supplemented with 0.1% (w/v) urea. Values are means ± S.D from three replications

throughout the fermentation suggests that yeast growth and activation was delayed in the former mash. Consequently, the cassava starch-to-ethanol fermentation efficiencies for the fermented mash after 85 h using STARGEN™ 001 and that using the multi-activity enzyme preparation from *A. kawachii* FS005 were 84.6 and 92.3% of theoretical values, respectively (Table 2). In contrast, the ethanol production using STARGEN™ 001 with 0.1% w/v urea was significantly better (Fig. 2a), with the cassava starch-to-ethanol fermentation efficiency in the mash reaching 89.7%. Because cassava is low in nitrogen, nitrogen addition to the mash is generally required in order to maintain optimal yeast growth during fermentation [26]. We speculate that the nitrogen (urea) addition was essential for the growth and activation of yeast cells in the STARGEN™ 001-derived mash. On the other hand, it is likely that the proteolytic ability in the multi-activity enzyme preparation from *A. kawachii* FS005 generated sufficient FAN to allow the yeast to ferment efficiently without nitrogen addition. Rattanachomsri et al. [20] reported on the laboratory-scale saccharification of cassava pulp using a multi-activity enzyme preparation from *A. niger* BCC17849. The preparation consisted of non-starch polysaccharide-hydrolyzing activities including cellulase and xylanase, which act cooperatively to release starch granules trapped in the fibrous cell wall structure for subsequent saccharification by raw cassava starch-degrading activity. As shown in Fig. 2c, more xylose

Table 2 Ethanol yields during SSF processing of raw cassava flour using *A. kawachii* FS005 culture broth as a multi-activity enzyme preparation

	Ethanol % (v/v)	Fermentation efficiency (% theor.)
STARGEN™ 001	8.28 ± 0.04	84.6 ± 0.10
STARGEN™ 001 with urea	8.78 ± 0.10	89.7 ± 0.26
Multi-activity enzyme preparation from *A. kawachii*	9.03 ± 0.21	92.3 ± 0.55

was detected in the fermented mash using the multi-activity enzyme preparation from *A. kawachii* FS005 than STARGEN™ 001. The culture broth from crude barley-grown *A. kawachii* FS005 was found to have both cellulase and xylanase activity (Fig. 1e, 1f), which might contribute to unraveling the complicated fibrous cell wall structures. A significant amount of xylose in the fermented mash using the multi-activity enzyme preparation from *A. kawachii* FS005 suggests this speculation. It is possible that the higher fermentation efficiency observed in the mash using the multi-activity enzyme preparation from *A. kawachii* FS005 rather than STARGEN™ 001 supplemented with urea was due to these cell wall-degrading activities. Further experimental study about the accurate yield of glucose and xylose is needed in order to elucidate the cooperative

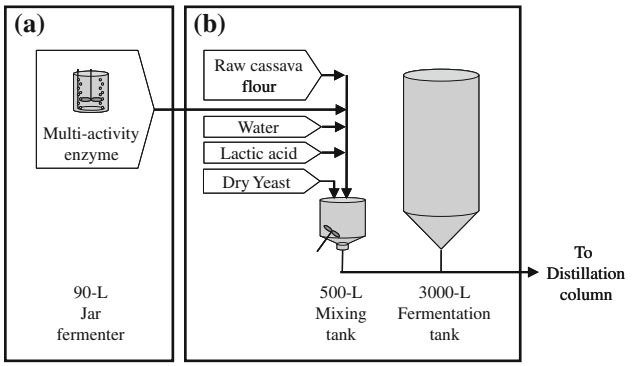

Fig. 3 Schematic of SSF processing of raw cassava flour to ethanol using a multi-activity enzyme preparation from *A. kawachii* on a pilot plant scale. **a** Production process for the multi-activity enzyme preparation. **b** SSF process for raw cassava flour

actions of the composite enzyme activities in the multi-enzyme in detail. In recent years, much research has focused on very high gravity (VHG) ethanolic fermentations [19]. With some starch substrates, a problem of high mash viscosity has been encountered during preparation of VHG mashes. Cell-wall-degrading enzymes such as cellulase, xylanase, arabinase, and pectinase have been employed to reduce mash viscosity. We anticipate that the multi-activity enzyme preparation from *A. kawachii* FS005 may also have application in reducing the viscosity of VHG cassava mashes.

A feasibility study of pilot plant-scale ethanol production by SSF of raw cassava flour using the multi-activity enzyme preparation from *A. kawachii* FS005 manufactured on site

To our knowledge, no studies have reported on pilot plant-scale ethanol production from SSF of raw cassava starch using multi-activity enzyme preparations manufactured on site. Figure 3 shows a schematic of such a process. Initially, *A. kawachii* FS005 was grown in a 90-l stirred jar fermenter (Fig. 3a). The following activities (U/ml) were measured in the culture broth after 66 h: glucoamylase, 144; acid-stable α-amylase, 36; acid carboxypeptidase, 3,974; acid protease, 13,277; cellulase, 0.42; and xylanase, 10.5. Further, this *A. kawachii* FS005 culture broth yielded 41.1 and 135.5 mg/l/h glucose and FAN, respectively, during saccharification of the raw cassava flour. Based on these results, the submerged culture was scaled up. Immediately after growth of *A. kawachii* FS005 ceased, the culture broth was harvested and used as a multi-activity enzyme preparation in an SSF process (Fig. 3b). One of the most significant technical problems with the use of a raw (uncooked) substrate in a large-scale study is inhibition of ethanol fermentation caused by bacterial contamination. In a preliminary experiment, significant inhibition by contaminating *Lactobacilli* was observed (data not shown). To avoid this problem, a two-step mash and fermentation were evaluated. Yeast growth was promoted in the primary fermentation as yeast was found to be the dominant microbial species. In spite of an initial low population of viable yeast cells in the primary mash, 5×10^5 cells/ml, the population reached 9×10^7 cells/ml after the primary fermentation. On the other hand, the initial population of viable bacteria in the primary mash was 5×10^4 cells/ml which increased modestly to 1×10^5 cells/ml after the primary fermentation. Figure 4 shows the fermentation profile after the secondary mash step of the SSF process. The low pH likely limited the type and number of bacterial contaminants as the maximum number of bacteria was about 10^3 smaller than the yeast population. Subsequent conversion of the cassava starch to ethanol was found to be accelerated during the secondary fermentation. The SSF process based on a mash volume of about 1,612 l per mash unit containing 20.6% (w/v) dry raw

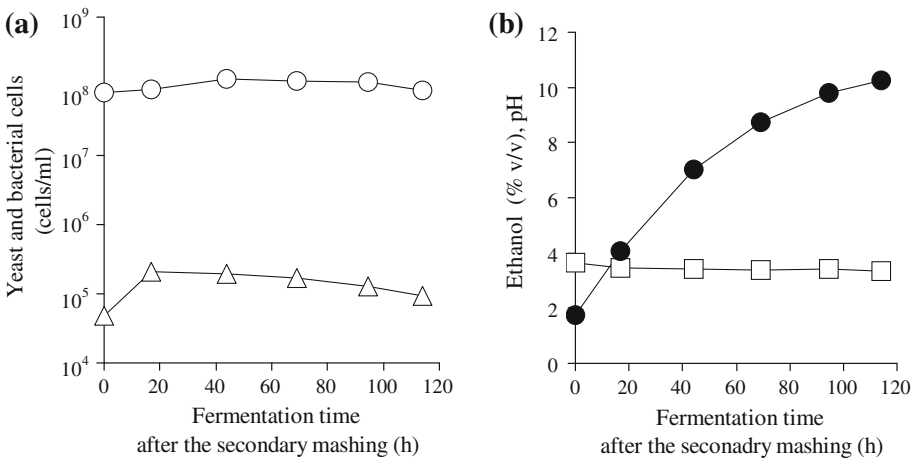

Fig. 4 Fermentation profiles of raw cassava flour subjected to SSF processing to ethanol using a multi-activity enzyme preparation from *A. kawachii* FS005 on a pilot plant scale. **a** Viable yeast and bacteria; **b** ethanol concentration and pH of the secondary mash; (*open circle*), viable yeast; (*open square*), viable bacteria; (*open square*), pH of fermenting mash; (*filled circle*), ethanol in fermenting mash

cassava solids resulted in a maximum ethanol yield of 10.3% (v/v) without significant spoilage. The cassava starch-to-ethanol fermentation efficiency was determined to be 92.7% of theoretical value.

In conclusion, ethanol production from raw cassava flour in an SSF process with a multi-activity enzyme preparation manufactured on site was demonstrated on a pilot plant scale. The multiple enzyme activities detected in the culture broth of *A. kawachii* FS005 appear to be particularly promising for SSF processing of a raw substrate. Clearly, further process development is necessary, focusing on cultivation conditions for *A. kawachii*, including medium composition and strain improvement, as well as all aspects of the SSF process. An evaluation of the economics of the entire process, including on-site production of the saccharifying enzymes is in progress.

Acknowledgments The authors are thankful to Genencor Kyowa Co., Ltd., Japan, for the gift of enzymes, and to Shinozaki Perfumery Co., Ltd., Japan, for the provision of raw cassava flour.

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