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Enzymatic production of glucose from different qualities of grain sorghum and application of ultrasound to enhance the yield

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ABSTRACT

The objective of the present work was to add value to three different qualities of grain sorghum namely normal healthy, germinated, and blackened through production of glucose, and to intensify glucose production (yield) by means of ultrasound treatment. Liquefaction (using *Bacillus licheniformis* α-amylase) and saccharification (using amyloglucosidase) processes were optimized with use of normal sorghum flour as a starting material for the production of glucose. The effect of ultrasound treatment on the sorghum slurry prior to liquefaction was studied on the processes of liquefaction and saccharification under optimized conditions. Due to ultrasound treatment, liquefact DE increased by 10–25% depending upon sonication time and the intensity. Ultrasound treatment of 1 min at 100% amplitude was found to decrease the average particle size of the slurry from 302 μm to 115 μm, which resulted in an increased percentage of saccharification by about 8%. The reason for the increase in the percentage of saccharification was attributed to the availability of additional starch for hydrolysis due to ultrasound-assisted disruption of the protein matrix (surrounding starch granules) and the amylose–lipid complex. Integration of ultrasound treatment in the state of art of the production of glucose from dry-milled sorghum and its possible subsequent use in the bioethanol production may improve the overall economics of the process.

1. Introduction

Sorghum (*Sorghum bicolor* L. Moench) is an important drought-resistant cereal crop and is ranked as the fifth largest produced cereal in the world after wheat, rice, barley, and maize. Production of sorghum in 2007–2008 in the world was 64 million metric tons (www.fas.usda.gov). Leading sorghum-producing countries include the United States (19.9%), Nigeria (15.5%), India (11.3%), Mexico (9.8%), Sudan (7%), and Argentina (5.4%) (www.fas.usda.gov). Sorghum ranks third in the major food grain crops in India. Sorghum is valued because of its ability to grow in areas with marginal rainfall and high temperatures (that is, in semiarid tropics and subtropical regions of the world), where it is difficult to grow any other cereal. Also, because of its relatively short growing season requirement, it is suitable for double cropping and crop rotation systems.[1] The average percentages of starch, proteins, moisture, fibers, lipids, and ash in sorghum are 70.1, 11.2, 11.6, 1.82, 3.54, and 1.8, respectively.[2]

Although production of grain sorghum is high in India, its demand is decreasing with change in the way of living due to increased urbanization, increased per capita income of the population, and easy availability of other preferred cereals in sufficient quantities at affordable prices. Hence, in addition to being a major source of staple food for humans, it also serves as a source of feed for cattle and other livestock during scarcity of maize, but at lower prices. Also, about 10–20% of the production gets wasted due to damage and inadequate transport and storage facilities. Industrial-grade damaged sorghum grains (containing 30–55% sound grains) are available in large quantity at the Food Corporation of India (FCI) at 10 times lower rate than the fresh grains.[3] Damage includes grain with a chalky appearance, as well as grain that is cracked, broken, and infected with mold. These damaged grains are not suitable for human consumption. Several mold-causing fungi are producers of potent mycotoxins that are harmful to the health and productivity of both humans and animals.[4] Hence, damage caused by insect infection and by attack of fungus (blackened sorghum or grain mold) because of wet and humid weather makes sorghum grains unfit even for animal consumption.

Hence, an industrial application is needed for normal and blackened sorghums in order to make sorghum cultivation economically viable for farmers through value-added products. There is a very small amount of research being done on value addition to sorghum through production of glucose[5,6], production of ethanol,[2,3,7–9] and isolation of starch.[10–14] The reason for the lower level of industrial exploitation could be attributed to reduced sorghum starch digestibility[15–20] and reduced protein digestibility[21] after cooking, that is, heat–moisture treatment of sorghum flour. There is no literature available on value-added products from blackened sorghum.

Application of ultrasound is reported in the wet-milling process for isolation of starch from corn,[22] sorghum,[14] and rice.[23] However, there is very little work available on the integration of ultrasound

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treatment in the production of glucose or ethanol (dry corn milling ethanol production[24,25]).

This paper reports the use of sorghum flour directly for liquefaction and saccharification instead of isolated sorghum starch. Research on the direct hydrolysis of sorghum flour was undertaken because of the low yields in the isolation of starch from sorghum (50–60%), that is, the rest of the unisolated starch gets wasted or does not fetch a respectable price. Direct hydrolysis was first used by Kroyer[26] in 1966 for the production of glucose from corn grits. Direct hydrolysis of flour from maize,[27,28] broken rice,[29] and sorghum[5,6,29] was reported for the production of glucose.

Objectives of the present work were to optimize enzymatic liquefaction and saccharification processes to produce glucose from three qualities of grain sorghum, that is, from healthy, blackened sorghum and germinated seeds, and to study the effect of ultrasound treatment prior to liquefaction on the performance of the liquefaction and saccharification processes.

2. Experimental

2.1. Materials

3,5-Dinitrosalicylic acid (DNSA), soluble starch, maltose, dextrose, MeCN for chromatography LiChrosolv, and other chemicals were purchased from E. Merck Ltd (India). Commercial preparations in liquid formulation of *Bacillus licheniformis* α-amylase (BLA) (EC number 3.2.1.1), amyloglucosidase (AG) (EC number 3.2.1.3), and pullulanase (PL) (EC number 3.2.1.41) were gifted by Advance Enzyme Technologies Pvt. Ltd (India). Healthy sorghum and blackened sorghum were purchased from the local market.

2.2. Methods

2.2.1. Measurement of protein concentration, concentration of reducing sugars and concentrations of malto-oligosaccharides

The protein concentration of the free enzyme was determined using the Folin–Lowry method[30] using BSA (0–0.6 mg/mL) as a standard. Concentration of reducing sugars was measured using the DNSA method[31] with dextrose (0–1 mg/mL) as a standard. Concentrations of glucose and malto-oligosaccharides up to maltoheptaose were measured using the HPTLC method, which is described elsewhere.[32]

2.2.2. Measurement of moisture content and particle size distribution of sorghum flour

Sorghum flour was kept at 80 °C until constant weight was obtained, and the moisture content was measured using mass balance. Particle size distribution of the ground sorghum flour was determined by using the Coulter Counter Particle Size Analyzer (LS 230) based on laser light diffraction.

2.2.3. Measurement of the starch content of sorghum

Sorghum grains were finely ground to the flour. Sorghum slurry (1% w/v, pH 4.5, 50 mM acetate buffer) was gelatinized for 10 min in boiling water. Then 200 units of BLA and 180 units of AG were added to the gelatinized solution, and the reaction mixture was kept under shaking conditions (180 rpm) at 55 °C for 24 h. Starch content in the sorghum flour was calculated by multiplying the total reducing sugars (glucose equiv) produced upon complete hydrolysis (that is, at the end of 24 h) by a factor of 0.9.

2.2.4. Amylolytic activity measurement, thermostability of amyloglucosidase and optimization of the amyloglucosidase:pullulanase (AG:PL) ratio for saccharification

The procedure for measurement of activity of free *B. licheniformis* α-amylase (BLA) is described elsewhere.[32]

2.2.4.1. Activity of free amyloglucosidase (AG). Gelatinized soluble starch solution (0.9 mL, 1% w/v, pH 4.5, 50 mM citrate buffer) was incubated with 0.1 mL of 10,000-fold diluted commercial amyloglucosidase (AG) solution at 65 °C for 10 min. Then 1 mL of DNSA reagent was added to reaction mixture to stop the reaction. The resulting solution was heated in a boiling water bath for 10 min. The variation in the concentration of reducing sugars was measured by the DNSA method using glucose as a standard. One unit of amyloglucosidase (AGU) was defined as that required to liberate one micromole of reducing sugars (glucose equiv) per min under the assay conditions.

2.2.4.2. Activity of free pullulanase (PL). Gelatinized soluble starch solution (0.9 mL, 1% w/v, pH 5.5, 50 mM citrate buffer) was incubated with 0.1 mL of 500-fold diluted commercial pullulanase (PL) solution at 60 °C for 10 min. Then 1 mL of DNSA reagent was added to the reaction mixture to stop the reaction. The resulting solution was heated in a boiling water bath for 10 min. The variation in the concentration of reducing sugars was measured by the DNSA method using glucose as a standard. One unit of pullulanase (PLU) was defined as that required to liberate one micromole of reducing sugars (glucose equiv) per min under the assay conditions.

2.2.4.3. Thermostability of free AG. Amyloglucosidase solution (3.63 AGU/mL, 0.05 M acetate buffer, pH 4.5) was incubated at desired temperature under shaking conditions (180 rpm) in the absence of substrate for 24 h. Samples of AG solution were taken at various time intervals for the measurement of its activity.

2.2.4.4. Optimization of AG: PL ratio for saccharification. Gelatinized soluble starch solution (5 mL, 2% (w/v), pH 4.5, 0.05 M acetate buffer) was incubated with 18 U of amyloglucosidase with varying units of pullulanase (0–4.5) separately for 1 h at 55 °C. Then samples were withdrawn and diluted ten times using 0.1 N HCl. These samples were analyzed for concentration of reducing sugars (glucose equiv), and optimum AG: PL ratio was obtained.

2.2.5. Studies on the liquefaction of sorghum

Sorghum grains were milled using a horizontally mounted stone flour mill (two stones of 38 cm diameter × 5.1 cm height dimensions, weighing about 10 kg each), still commonly used in rural India, and this flour was used for liquefaction, followed by its saccharification.

2.2.5.1. Optimization of liquefaction of sorghum. Liquefaction of sorghum flour was performed in a 250-mL stoppered conical flask containing 100 mL of magnetically stirred sorghum slurry. Sorghum slurry of 30% w/v concentration (100 mL) was obtained by mixing 30 g of sorghum flour and 80 mL of acetate buffer. Liquefaction of sorghum slurry (30% w/v in 0.05 M acetate buffer, BLA concentration of 0.08% v/w of flour) was performed using BLA at 85 °C (maintained by immersing the conical flask containing slurry in hot oil bath). Samples were withdrawn at time intervals of 15 min and diluted to approximately 1% (w/v) using 0.1 N HCl. The progress of liquefaction was monitored using the starch–iodine colorimetric reaction. Few drops of KI–I_2 reagent (0.05% w/v I_2 and 0.5% w/v KI solution) were added to few drops of a diluted sample, and the color of the mixture was observed. As the liquefac-

tion progresses, the color of the mixture changes according to the following sequence: deep blue, bluish violet, violet with tinge of dark red, and dark red with a tinge of violet.[33] When this last color was observed, liquefaction was considered to be complete (DE of the liquefact was approximately 15 at this stage). Samples (~1%w/v) were then centrifuged at 270 g for 10 min and supernatant was analyzed for the concentration of reducing sugars. The liquefaction process was optimized for a liquefaction time of 1.5 h by varying pH of buffered slurry (pH 5.2–6.7), concentration of BLA (0.04–0.16% v/w of sorghum flour), concentration of $CaCl_2$ (0–500 ppm), sorghum slurry concentration (10–35% w/v), and temperature (75–95 °C).

2.2.5.2. Liquefaction of sorghum of different qualities. Healthy sorghum grains were steeped in the ordinary tap water for 12 h and then separately germinated for 12 h, 24 h, 36 h, and 48 h. Germinated sorghum grains were first dried at 50 °C. Germinated sorghum and blackened sorghum were milled separately using a horizontally mounted stone flour mill. Liquefaction of geminated sorghum and blackened sorghum was performed at optimized liquefaction conditions and compared with liquefaction of normal healthy sorghum.

2.2.5.3. Effect of prior ultrasound treatment on the liquefaction of sorghum. An ultrasound horn was dipped into sorghum slurry (30% w/v in 0.05 M acetate buffer of pH 6, and $CaCl_2$ concentration of 200 ppm) to a depth of 1 cm, and the slurry was sonicated for different spans of time and at different ultrasound intensities. An ultrasound horn (Vibra-cell, Sonics and Materials Inc., USA) having a maximum power output of 750 W and operating at a frequency of 20 kHz was used in the study. The diameter of the probe was 1.3 cm. A short ultrasound treatment of sorghum slurry was followed by the addition of an optimized amount of BLA, and then subsequent liquefaction for 1.5 h was performed under optimized conditions. After liquefaction, the reaction mixture (30% w/v) was centrifuged at 5000 *g* for 20 min, and then the supernatant was collected. This supernatant was then analyzed for dry solid wt concentration and concentration of reducing sugars (glucose equiv). Dextrose equivalent (DE) is defined as follows:

$$\text{Dextrose equivalent(DE)} = \frac{C_{RS}\text{ in the starch hydrolysate, mg/mL}}{\text{dry solid wt concn of starch hydrolysate, mg/mL}} \times 100 \quad (1)$$

where C_{RS} is concentration of reducing sugars (glucose equiv).

2.2.6. Optimization of saccharification

The saccharification process was optimized for the processing temperature by performing a thermostability study of AG and also by performing saccharification of maltodextrins at different temperatures.

At the end of the liquefaction (that is, 1.5 h), the pH of the reaction mixture was reduced to pH 4.5 using acetic acid. Then sorghum liquefact was filtered hot using a muslin cloth, and the filtrate (F1) was collected. The cake obtained after the hot filtration was mixed well with 10 mL of distilled water and filtered. This second filtrate (F2) was mixed with first filtrate (F1). The optimized quantity of amyloglucosidase was added to the filtrate (F1) or to the mixture of F1 and F2. Then this reaction mixture was kept under shaking conditions (150 rpm) for 24 h under optimized conditions of temperature and enzyme concentration. At regular time intervals, samples were withdrawn, and 0.1 N HCl solution was added to the sample for dilution to approximately 1% w/v and for deactivation of AG in the sample. Samples (1% w/v) were then centrifuged at 270 g for 10 min, and the supernatant was analyzed for the concentration of reducing sugars (glucose equiv). The percentage of saccharification on the basis of original starch content in the sorghum flour is defined as follows;

$$\%\text{ saccharification} = \frac{C_f\text{ in mg/mL} \times \text{volume of reaction mixture in mL}}{\text{amount of sorghum flour in mg} \times Q} \times 100 \quad (2)$$

where C_f is concentration of reducing sugars (glucose equiv) at steady state (that is, 24 h); Q is mg of reducing sugars (glucose equiv) produced per mg of sorghum flour using method described in 2.2.3., that is (% starch content /100) × 1.11.

Since calculation of % saccharification is based on the original starch content in the sorghum flour, the term % saccharification can be also regarded as % yield of glucose.

The production of glucose from sorghum flour consists of the following five steps in sequence: 1. Ultrasound treatment of sorghum slurry, 2. Liquefaction of the sorghum slurry using *B. licheniformis* α-amylase, 3. Hot filtration of the liquefied sorghum slurry, 4. Washing of the cake (obtained after the hot filtration), followed by a second filtration and mixing of both filtrates, and 5. Saccharification of filtrate F1 or of the mixture of F1 and F2 using amyloglucosidase. The experiments were performed for glucose production using healthy, blackened, and germinated sorghum grains without or with step 1 or 4.

3. Results and discussion

Production of glucose from sorghum flour consists of two steps namely viz. 1. Liquefaction using *B. licheniformis* α-amylase (BLA) and 2. Saccharification using amyloglucosidase (AG).

3.1. Studies on the liquefaction process

Flour of healthy sorghum grains was used for optimization of liquefaction process. The starch content and moisture content of the healthy sorghum flour were estimated to be 69–70% and 10–11%, respectively. Average particle size of the sorghum flour used was 302 μm.

3.1.1. Optimization of the liquefaction process

3.1.1.1. Effect of pH. Optimum pH was observed to be 6 for liquefaction of sorghum flour (85 °C, 0.05 M acetate buffer, 10% w/v sorghum slurry, BLA concentration of 0.08% v/w of sorghum flour). At the same pH (6.0), further studies on the liquefaction of sorghum flour were carried out.

3.1.1.2. Effect of BLA concentration and $CaCl_2$ concentration. It can be seen from Figure 1a that liquefaction of sorghum slurry (25% w/v) was completed in 60 min with a BLA concentration of 0.08% v/w of flour in the absence of $CaCl_2$ supplementation. It can be also seen from Figure 1a that when concentration of reducing sugars was around 35 mg/mL (that is, DE in the range of 15–17), liquefaction of the slurry was completed according to the starch–iodine reaction. The region above dotted line in Figure 1a is liquefied region, that is, at all points in this region, liquefaction was observed to be complete. As the BLA concentration increased, the time at which the liquefaction was completed decreased.

It is well known that, since BLA is an organometallic enzyme, supplementation of Ca^{2+} ions improves the performance of BLA. Hence, liquefaction was performed at a BLA concentration of 0.06% v/w of flour at different concentrations of $CaCl_2$ to find the optimum $CaCl_2$ concentration. It can be seen from Figure 1b that liquefaction of sorghum slurry (25% w/v) was completed in 60 min, and all the hydrolysis curves corresponding to $CaCl_2$ con-

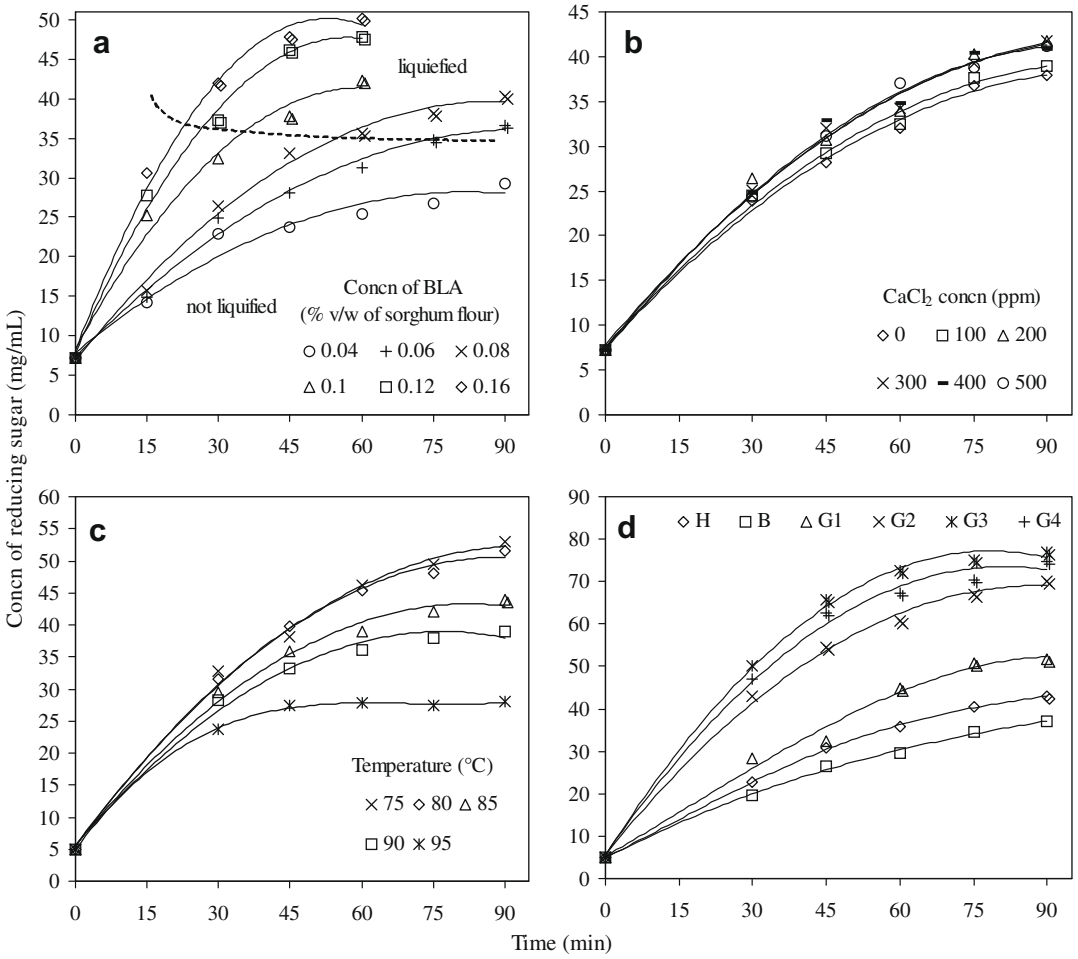

Figure 1. Effect of (a) BLA concentration (25% w/v sorghum slurry, 85 °C, pH 6 and $CaCl_2$ concn = 0 ppm), (b) $CaCl_2$ concn (25% w/v sorghum slurry, 85 °C, pH 6 and BLA concn = 0.06% v/w of sorghum flour), (c) temperature (30% w/v sorghum slurry, pH 6, BLA concn = 0.06% v/w of sorghum flour, and $CaCl_2$ concn = 200 ppm) and (d) quality of sorghum (30% w/v sorghum slurry, 85 °C, pH 6, BLA concn = 0.06% v/w of sorghum flour, and $CaCl_2$ concn = 200 ppm) on concentration of reducing sugars (glucose equiv) versus time curve. H—healthy sorghum; B—blackened sorghum; G1, G2, G3, G4—healthy sorghum germinated for 12 h, 24 h, 36 h, and 48 h, respectively, after steeping for 12 h in plain water. Data points without shadow—blue color on starch–iodine reaction. Data points with shadow—disappearance of blue color (that is, dark red with tinge of violet) on starch–iodine reaction.

centration greater than 200 ppm overlapped one another (Fig. 1b). This indicates that $CaCl_2$ concentration of 200 ppm was optimum for liquefaction. It is worth noting that liquefaction performance at a BLA concentration of 0.08% v/w without a supplement of $CaCl_2$ is similar to that obtained at a BLA concentration of 0.06% v/w with a $CaCl_2$ concentration of 200 ppm (Fig. 1a and b).

Though *B. licheniformis* α-amylase was used in the present work with supplementation of $CaCl_2$, high-temperature stable *B. licheniformis* α-amylases (genetically modified) are also available that require very little or no calcium addition. α-Amylases from *Bacillus steareothermophilus* (genetically modified) are very stable even at high temperature and require no calcium addition, but they are not yet commercially available.

3.1.1.3. Effect of sorghum slurry concentration. Liquefaction was performed at various concentrations (10–35% w/v) of sorghum slurry. The maximum concentration of sorghum slurry that can be used for liquefaction was observed to be 30% w/v, at which mixing of the reaction mixture was experimentally possible and liquefaction was also successfully completed within 1.5 h. Mixing and homogenization of the reaction mixture were visually much less efficient at 35% w/v due to its very high viscosity, and it took around 2.5–3 h to complete liquefaction. Hence, it was decided to use 30% w/v as the optimum concentration of sorghum flour in the slurry for all further experiments. Though 30% w/v slurry concentration was found optimum, the situation may be little different at the pilot demonstration and commercial scales.

3.1.1.4. Effect of liquefaction temperature. The gelatinization temperature of sorghum starch is reported to be in the range of 75–80 °C[34] and 60–80 °C.[2] Hence, the temperature was varied in the range of 75–95 °C at 30% w/v sorghum slurry concentration for liquefaction. Liquefaction is a combination of two processes: one is gelatinization of free starch granules, and the other is dextrinization of gelatinized starch molecules.[35] As the temperature of liquefaction was increased from 75 to 95 °C, two effects occur simultaneously: one is when the rate of gelatinization of starch increases, and the other is when the rate of dextrinization of the starch molecules decreases due to enzyme deactivation at elevated temperatures. Hence, there exists an optimum temperature for the liquefaction process. A temperature of 85 °C was observed to be optimum (Fig. 1c) for the liquefaction of sorghum flour, at which liquefaction was completed within 1.5 h (according to the starch–iodine reaction). Although the concentration of reducing sugars produced at temperatures of 75 and 80 °C was higher than that produced at a temperature of 85 °C, liquefaction was not com-

Table 1
Properties of amyloglucosidase and pullulanase used in the work

Parameter	Amyloglucosidase	Pullulanase
Optimum pH	4.5	3.8–4.3
Optimum temperature (°C)	65	60
Activity	36,300 AGU/mL	2950 PLU/mL
Protein content (mg/mL)	370	50
Specific activity	98 (AGU/mg of protein)	59 (PLU/mg of protein)

Table 2
Optimized parameters for liquefaction and saccharification

Parameter	Liquefaction	Saccharification
Temperature (°C)	85	55
pH	6	4.5
Slurry concentration	30% w of sorghum flour/v of slurry	n.a.
BLA concentration	0.06% v/w of sorghum flour, that is, 0.086% v/w of sorghum starch	n.a.
AG concentration	n.a.	6 AGU/mL of liquefact, that is, 0.058% v/w of starch
$CaCl_2$ concentration	200 ppm	n.a.

pleted in 1.5 h (according to the starch–iodine reaction). This could be because at 75 °C and 80 °C starch granules are not completely gelatinized (hence giving a blue color with the iodine reagent). However, higher concentration of reducing sugars could be attributed to continued depolymerization of gelatinized starch molecules. At temperatures of 90 °C and 95 °C, liquefaction performance was significantly poorer than its counterparts at 85 °C (Fig. 1c). Optimum conditions for liquefaction of sorghum flour are summarized in Table 2.

3.1.2. Liquefaction of sorghum of different qualities

Healthy, germinated, and blackened sorghum grains were found to contain 69–70%, 69–70%, and 70–71% starch, respectively. The high starch content in the blackened sorghum indicates that fungus has infected only the pericarp of the sorghum grain and not the endosperm.

It can be seen from Figure 1d that liquefaction of healthy sorghum was completed in 1.5 h under optimized conditions. However, liquefaction progress of blackened sorghum is slightly (7%) slower as compared to that of healthy sorghum. This could be attributed to enzyme inhibition due to mycotoxins present in the blackened pericarp.

It can be seen from Figure 1d that as the germination time increases, liquefaction performance also improves. However, beyond a germination time of 24 h, there is no significant improvement in the liquefaction performance. However, liquefaction of germinated sorghum (germination time of 24 h) was observed to be complete in 1 h only. This could be attributed to the loosening of the protein cage surrounding starch granules due to proteases produced (that is, protein matrix-degrading enzymes) during the process of germination.

3.1.3. Effect of ultrasound pretreatment on the extent of liquefaction

It can be seen from Figure 2 that as the sonication time increases at constant ultrasound intensity, DE of the liquefact also increases. Figure 3 shows that an increase in the DE is approximately the same at constant power consumption, irrespective of the ultrasound intensity used. Since ultrasound treatment consumes a large amount of energy, it was decided to keep sonication time low, that is, 1 min. If 1 min is considered as the optimum time for ultrasound treatment, 100% amplitude gives a maximum increase in the DE of the liquefact. The reason for the increase in the DE of the liquefact due to prior ultrasound treatment will be discussed in detail later in the text.

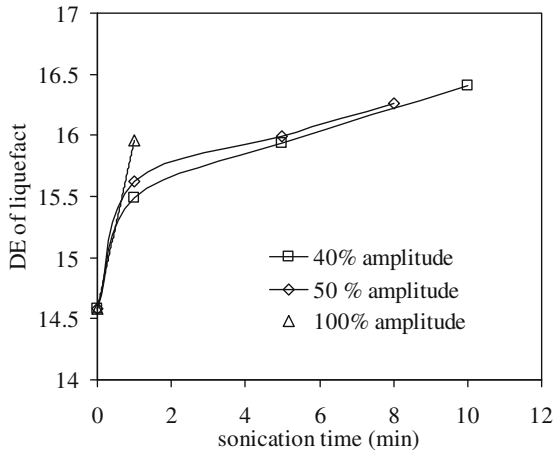

Figure 2. Effect of sonication time and ultrasound intensity on DE of liquefact.

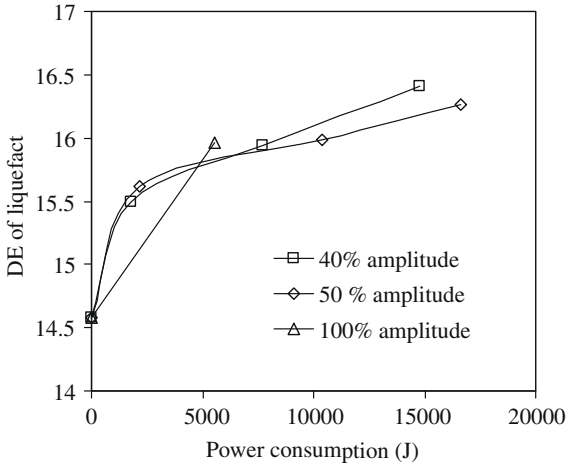

Figure 3. Variation in DE of liquefact with power consumption in sonication.

3.2. Saccharification of sorghum liquefact

3.2.1. Optimization of saccharification

Properties of amyloglucosidase (AG) and pullulanase (PL) are given in Table 1. pH versus % relative activity profile and temperature versus % relative activity profile of amyloglucosidase and pullulanase are given in Supplementary data.

The optimum temperature of AG using the specified assay procedure was found to be 65 °C (Table 1). However, the optimum operating temperature for saccharification using AG may not be the same as that obtained using the assay procedure. Hence, a study of the thermostability of AG was essential at an optimum pH of 4.5. The thermostability of AG was first checked at 65 °C. It was observed that the per cent relative activity of AG decreased to 5% (Fig. 4) at 65 °C within the first 3 h, only. Hence, it was decided to check the thermostability of AG at lower temperatures also, to find the optimum operating temperature for the saccharifi-

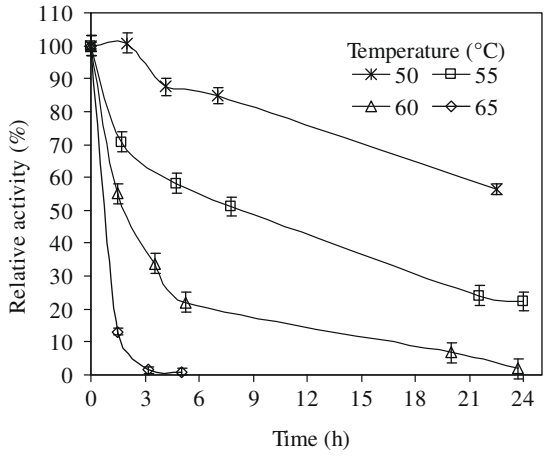

Figure 4. Thermostability of AG at pH 4.5 at different temperatures.

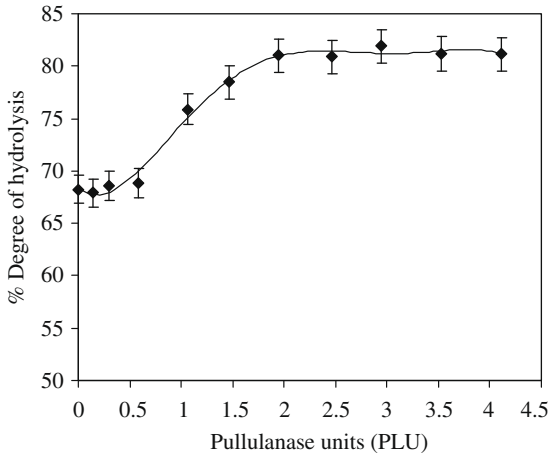

Figure 6. Optimization of ratio of AG units to PL units for hydrolysis of soluble starch.

fication of maltodextrins using AG. In addition to thermostability check at different temperatures, experimental runs utilizing AG and maltodextrins (DE 15, Sigma Aldrich) at different temperatures were also performed. It can be seen from Figures 4 and 5 that the optimum operating temperature for the saccharification of maltodextrins to glucose is around 55–60 °C.

The optimum ratio of AGU:PLU was found to be 18:2, and about 25% increase in the concentration of reducing sugars was observed due to the addition of optimum quantity of pullulanase (Fig. 6) using starch as substrate. During the saccharification of sorghum liquefact using amyloglucosidase with and without the optimum quantity of pullulanase, an increase in the concentration of reducing sugars was observed in the initial stages of reaction. However, the concentrations of reducing sugars at the end of 24 h were the same (Fig. 7). Hence, it was decided to use only amyloglucosidase for saccharification of sorghum liquefact, eliminating the use of pullulanase for a saccharification time of 24 h. If saccharification time needs to be reduced to 8 h, pullulanase can be used along with amyloglucosidase, but obviously at the added cost of the new enzyme.

The saccharification of sorghum liquefact (pH 4.5) was performed at different concentrations of amyloglucosidase. It can be seen from Figure 8 that saccharification was completed in 24 h with an AG concentration of 0.052% v/w of starch, and the concentration of reducing sugars obtained was 235 mg/mL, which was

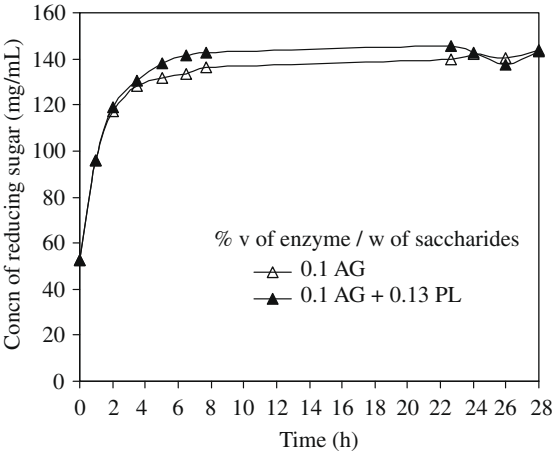

Figure 7. Saccharification of sorghum liquefact using amyloglucosidase with and without pullulanase (14% w/v, pH 4.5, 55 °C).

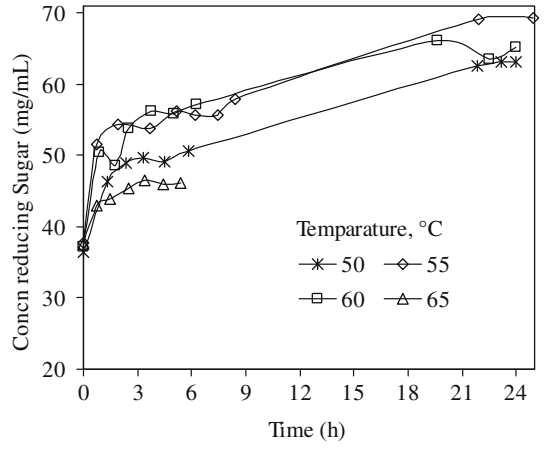

Figure 5. Change in concn of reducing sugars with time. 25 mL of 10% maltooligosaccharides (DE 15) solution in 50 mM acetate buffer, pH 4.5, 1.1 AGU/mL.

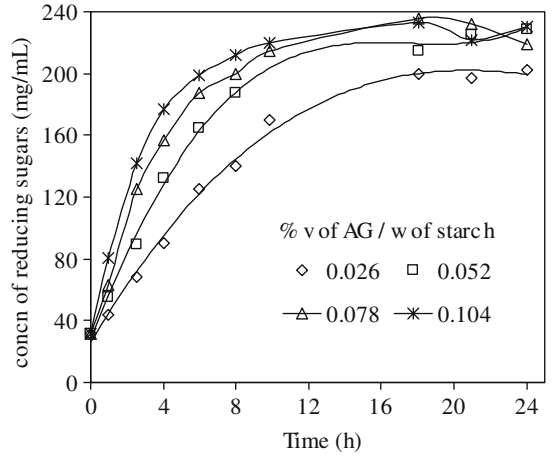

Figure 8. Variation in the concentration of reducing sugars versus time with amyloglucosidase concentration as a parameter.

same as that attained at higher concentrations of AG. HPTLC of the starch hydrolysate at 24 h showed the presence of glucose as a principal sugar with more than 95% selectivity.

Optimized conditions for saccharification of sorghum liquefact are summarized in Table 2.

3.2.2. Saccharification of sorghum liquefact

The percentage saccharification after treatment with AG for 24 h, for the three qualities of sorghum, increased with the use of the ultrasound treatment on sorghum slurry before liquefaction and by thoroughly washing the cake obtained after hot filtration. These data are summarized in Table 3. The recovery of more oligosaccharides after washing the cake, which otherwise were trapped inside the slurry filter cake and were unavailable for further saccharification, increased the overall percentage saccharification in the case of healthy sorghum and germinated sorghum (compare the values of H1 and H2, H3 and H4, G1 and G2, and G3 and G4 in Table 3). In the case of blackened sorghum, this effect was observed for both low and high BLA concentrations (compare values of B1 and B2, B3 and B4, B5 and B6, and B7 and B8 in Table 3), but the effect was more significant at high BLA concentration.

3.2.2.1. Effect of ultrasound treatment on particle size distribution. Particle size distribution of sorghum slurry was compared for three conditions: 1. Without ultrasound treatment; 2. slurry sonicated for 1 min at 40% amplitude, and 3. slurry sonicated for 1 min at 100% amplitude, and is shown in Figure 9. Average particle size was found to decrease from 302 μm to 163 μm (slurry sonicated for 1 min at 40% amplitude) and 115 μm (slurry sonicated for 1 min at 100% amplitude) due to cavitationally induced particle fragmentation. It can be also seen from Figure 9 that there are three major inflection points in the particle size distribution, which correspond to ~800 μm, 10–20 μm and ~1 μm. Due to the ultrasound treatment of sorghum slurry prior to liquefaction, the peak area corresponding to ~800 μm decreases; whereas, peak areas corresponding to 10–20 μm and ~1 μm increase. The inflection point of ~800 μm must be a function of the milling process, and mainly corresponds to large pericarp particles. However, the inflection point of ~1 μm may correspond to cell debris produced due to disintegration of the cells of the endosperm of sorghum. Such a decrease in the particle size distribution due to ultrasound is in agreement with the results reported for corn slurry[24] and for uranium ore slurry.[36] Particle fragmentation increasing the solid–liquid interfacial area and enhancement in the convective diffusivity of the leach solvent through micropores of the ore structure were attributed to a convective motion created by an acoustic cavitation (that is, shock-wave propagation and microjet formation) at the solid–liquid interface, which results in the enhancement of the rate of the leaching of uranium.[36]

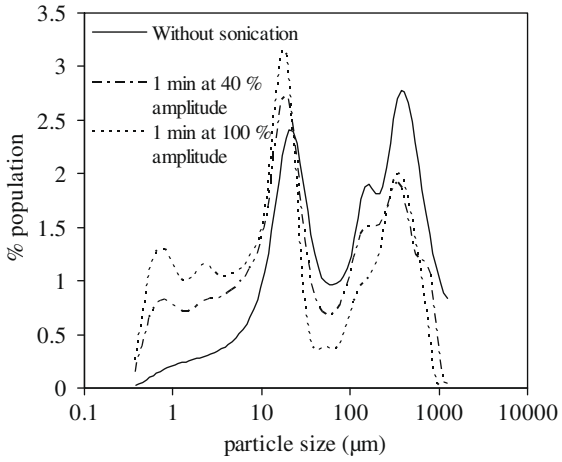

Figure 9. Effect of prior ultrasound treatment on the particle size distribution in 30% w/v sorghum flour slurry.

3.2.2.2. Effect of ultrasound treatment on per cent saccharification. In healthy and germinated sorghum grains, ultrasound treatment prior to liquefaction improved filterability of the liquefied slurry because of either an increase in the DE of liquefact (Table 3) or particle disintegration due to acoustic cavitation (Fig. 9). These results lead to an increase in the volume of F1 and in the subsequent per cent saccharification (compare the values of H1 and H3, H2 and H4, G1 and G3, and G2 and G4 in Table 3). The increase in the percentage of saccharification was highest upon inclusion of both ultrasound pretreatment (through release of additional starch granules, discussed in detail later in this section) and the step of washing during filtration (compare values of H1 and H4, G1 and G4 in Table 3). During germination and seedling growth of sorghum, different enzymes, including proteases, endoprotease, limit dextrinase, α-amylase, and endo-β-gluconase, are produced.[37] In case of germinated sorghum (germination time 24 h), there is significant increase (~30%) in liquefact DE due to prior ultrasound treatment. This could be attributed to weakening of the cell wall and protein matrix around the starch granules due

Table 3
Summary of effect of ultrasound treatment and washing on liquefaction and saccharification performance

Run No	BLA concn, % v/w	C_{RS}, (DE)	mg/mL	F1[a] (mL)	F2[a] (mL)	F1 + F2 (mL)	C_f (mg/mL)	% Saccharification[b]
H1	0.086	28.9	(13–14)	72	0	72	240	74 (S N; W N)
H2	0.086	27.7	(13–14)	71	15	86	229	84 (S N; W Y)
H3	0.086	31.2	(14–15)	75	0	75	251	81 (S Y; W N)
H4	0.086	28.5	(14–15)	74	15	89	235	90 (S Y; W Y)
G1[c]	0.086	29.6	(13–14)	73	0	73	240	75 (S N; W N)
G2[c]	0.086	26.7	(13–14)	73	15	88	225	85 (S N; W Y)
G3[c]	0.086	39.4	(16–17)	79	0	79	242	82 (S Y; W N)
G4[c]	0.086	36.7	(16–17)	79	11	90	232	90 (S Y; W Y)
B1	0.093	23.4	(9–10)	67	0	67	245	69 (S N; W N)
B2	0.093	24.6	(9–10)	65	17	82	231	79 (S N; W Y)
B3	0.093	22.7	(9–10)	70	0	70	240	70 (S Y; W N)
B4	0.093	24.4	(9–10)	69	16	85	226	80 (S Y; W Y)
B5	0.13	33.4	(16–17)	73	0	73	245	74 (S N; W N)
B6	0.13	32.1	(16–17)	73	15	88	233	86 (S N; W Y)
B7	0.13	32.1	(16–17)	75	0	75	239	75 (S Y; W N)
B8	0.13	30.4	(16–17)	75	14	89	234	87 (S Y; W Y)

[a] F1: Volume of filtrate after first filtration. F2: Volume of filtrate after second filtration.
[b] S N: No ultrasound treatment of sorghum slurry before liquefaction. S Y: Ultrasound treatment of sorghum slurry before liquefaction for 1 min at 100% amplitude. W N: No washing of cake after first filtration. W Y: Washing of cake after first filtration with 10 mL distilled water, followed by second filtration.
[c] Liquefaction time 1 h, Sorghum germinated for 24 h.

to an attack of the endoprotease[37] during germination, and hence effective release of starch granules from protein matrix by ultrasound treatment. The percentage of saccharification, however, did not go above 90%, even after germination.

In the case of blackened sorghum, a marginal increase in the per cent saccharification was observed due to ultrasound pretreatment (compare values of B1 and B3, and B2 and B4 in Table 3). It can be seen from Table 3 that at the optimized concentration of BLA, DE of the liquefact obtained with blackened sorghum was 9–10, which was much less than expected (DE, that is, 15). Hence, the BLA concentration was increased by about 40% (from 0.093% to 0.13% v/w of starch) to get a liquefact value of DE 16–17 (Table 3). The reason for this could be attributed to the release of mycotoxins and phenolic compounds in the blackened pericarp upon ultrasound pretreatment of sorghum slurry that can act as inhibitors of the starch-degrading enzymes, requiring higher doses of BLA for liquefaction and of AG for saccharification. Thus, the low DE values obtained for blackened sorghum are partially offset by higher BLA dosage.

It is first necessary to understand the sorghum grain structure in order to understand the reason behind the observed increase in the per cent saccharification due to ultrasound treatment. Cells of inner floury endosperm are round with round starch granules, whereas, the cells of the outer corneous endosperm are elongated with polygonal starch granules and are filled with protein bodies.[38] Also, the multicellular pericarp of sorghum grain, unlike other cereals, consists of small starch granules.[34] Starch granules of the floury endosperm of sorghum are loosely associated with paperlike sheets of protein material, while in the corneous endosperm they are tightly packed within rigid protein matrix.[38]

Treatment of sorghum flour (before cooking) with proteolytic enzymes like pronase[18] and pepsin[16] has shown an increase in the starch digestibility by pancreatic α-amylase[18] and also an increase in the rate of starch hydrolysis by amyloglucosidase[16] due to hydrolysis of the protein matrix surrounding the starch granules. Cooking of sorghum flour with reducing agents like sodium metabisulfite[18] or 2-mercaptoethanol[17] also increased starch digestibility with pancreatic α-amylase[18] or the degree of starch gelatinization[17] due to cleavage of disulfide bonds linking the protein surrounding the starch granules. Sorghum proteins are also reported to produce high-molecular-weight polymers by polymerization through disulfide bonding of prolamins[20] and large extended web-like microstructure[39,2] during the cooking of sorghum flour, into which small starch granules (~5 μm) remain tightly trapped.[2] These changes related to protein structure during cooking of sorghum flour with amylase contribute to subsequent incomplete gelatinization, hydrolysis of starch, and negative impact of protein content on the conversion efficiency of sorghum to ethanol.[2]

Since the lipid fraction within starch granules is insufficient to saturate the entire quantity of amylose, amylose exists in two forms: free amylose and an amylose–lipid complex.[40] In the case of normal sorghum, this amylose–lipid complex is reported to have endotherm or a temperature range of gelatinization (onset, peak, and ending of gelatinization) between 90 and 105 °C, whereas the remaining starch, that is, free amylose and amylopectin, has a major endotherm between 60 and 80 °C.[2] The use of lysophospholipase along with amyloglucosidase in saccharification resulted in a higher degree of degradation of the amylose–lipid complex (due to hydrolysis of lysophospholipids), and in an improvement in the rate and yield of filtration of hydrolysate produced after completion of saccharification (due to partial hydrolysis of micelles, which can clog pores of filter media).[41] These findings and the presence of amylose–lipid complex in the hydrolysate (after saccharification for 72 h using amyloglucosidase), proved by a differential scanning calorimetry (DSC) study,[41] indicate that the amylose–lipid complex affects the final degree of saccharification that can be practically achieved.

Hence, it seems that the amylose–lipid complex and the starch granules encased in the protein matrix do not get fully gelatinized, and the ungelatinized fraction of starch remains inaccessible for action of BLA. However, the cavitation phenomena caused by the ultrasound treatment prior to liquefaction may be releasing starch granules by disrupting both the protein matrix encasing the starch granules and the amylose–lipid complex. This can be also depicted by an increase in the peak area corresponding to the particle diameter of 10–20 μm (Fig. 9), which is the diameter of the sorghum starch granule.[40] These additional free starch granules get gelatinized and are available for liquefaction and further saccharification. This must be the reason for an increase in the liquefaction performance and an increase in the per cent saccharification due to ultrasound treatment prior to liquefaction. Similar enhancement in the glucose release using 14% w/v corn slurry (raw and cooked) due to prior sonication for 20 or 40 s at amplitudes ranging from 180 to 299 μm has been reported.[24] The increase in the glucose released from the sonicated samples was attributed to particle size reduction, better mixing due to micro streaming effects, and the release of additional lipid-bound starch.[24] The higher conversion efficiency of waxy sorghum to ethanol than that of normal sorghum[2] also supports the hypothesis that a larger quantity of amylose, and hence amylose–lipid complex, affects liquefaction and saccharification, and hence conversion efficiency of sorghum to glucose and hence to ethanol. A 1–10% increase in the DE of liquefact and ethanol yield has been reported[25] due to cavitation resulting from sonication for 1–7.5 min before cooking. It was claimed, but not experimentally proved,[25] that cavitational forces produced by sonication breaks complex proteins (that is, proteins not susceptible to hydrolysis to amino acids by proteolytic enzymes) to less complex proteins, which are more bioavailable to the digestive systems of animals.

For normal healthy sorghum 90% saccharification was reported.[6] However, it should be clarified that experiments were performed for the production of glucose[6] from sorghum (25% w/v slurry) without any filtration step after liquefaction, as the process was optimized for bioethanol production. Whereas, in the present work, filtration was done after liquefaction, which resulted in the trapping of oligosaccharides in the wet cake even after the washing procedure, hence giving lower per cent saccharification. When experiments were performed in the present work for the production of glucose from healthy sorghum without the filtration step, the values of per cent saccharification were observed to be 87–89 and 93–95 without and with ultrasound treatment, respectively.

4. Conclusions

Production of glucose from sorghum flour involves two steps namely 1. Liquefaction of flour using *B. licheniformis* α-amylase and 2. Saccharification using amyloglucosidase. In this work, the use of ultrasound in the production of glucose from sorghum flour has been explored. The value of per cent saccharification to glucose using normal sorghum flour (at optimized reaction conditions for liquefaction and saccharification) was increased from 74% to 90% due to the inclusion of the following two steps: 1. Ultrasound treatment of the sorghum slurry before liquefaction, and 2. Washing of the cake (obtained after the hot filtration) followed by a second filtration and mixing of both filtrates. Ultrasound treatment of the sorghum slurry prior to liquefaction appears to disrupt the hydrophobic protein matrix surrounding the starch granules and the amylose–lipid complex due to physical effects of acoustic cavitation, like shock-wave propagation and microjet formation in the

vicinity of a liquid–solid interface. This frees more starch granules and makes available more of these starch granules for further action of α-amylase and amyloglucosidase, which is responsible for an increase in the overall % saccharification. The effect of washing was through the recovery of oligosaccharides, which otherwise were unavailable for further saccharification. In this work, both blackened and germinated sorghum were also used for the production of glucose, and the values of per cent saccharification were 85% and 90%, respectively, with ultrasound pretreatment and a washing step integrated in the overall process. This means that integration of short ultrasound treatment (about 1 min) in the production of glucose from dry-milled sorghum and its possible subsequent use in bioethanol production will result in an increase in the production of glucose and subsequently ethanol, and hence may improve the economic feasibility of the process.

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Supplementary data

The enzyme activity-pH profile and the enzyme activity–temperature profile for AG are shown in Figures S1 and S2, respectively. The enzyme activity–pH profile and the enzyme activity–temperature profile for PL are shown in Figures S3 and S4, respectively. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.10.006.

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