

Ethanol Production from Pearl Millet Using *Saccharomyces cerevisiae*¹

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ABSTRACT

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Four pearl millet genotypes were tested for their potential as raw material for fuel ethanol production in this study. Ethanol fermentation was performed both in flasks on a rotary shaker and in a 5-L bioreactor using *Saccharomyces cerevisiae* (ATCC 24860). For rotary-shaker fermentation, the final ethanol yields were 8.7–16.8% (v/v) at dry mass concentrations of 20–35%, and the ethanol fermentation efficiencies were 90.0–95.6%. Ethanol fermentation efficiency at 30% dry mass on a 5-L bioreactor reached 94.2%, which was greater than that from fermentation

in the rotary shaker (92.9%). Results showed that the fermentation efficiencies of pearl millets, on a starch basis, were comparable to those of corn and grain sorghum. Because pearl millets have greater protein and lipid contents, distillers dried grains with solubles (DDGS) from pearl millets also had greater protein content and energy levels than did DDGS from corn and grain sorghum. Therefore, pearl millets could be a potential feedstock for fuel ethanol production in areas too dry to grow corn and grain sorghum.

Since the late 1970s, fuel ethanol production from renewable resources has grown into a huge industry and provides several billion gallons of ethanol for formulated gasoline in Canada, Brazil, the United States, and some other countries (Wheals et al 1999). The annual production of ethanol in the United States was 3.4 billion gallons in 2004 and is expected to reach 5.5 billion gallons by year 2005. About 30% of the gasoline in the United States currently is blended with ethanol and the percentage is still growing (MacDonald et al 2003). This makes the fuel ethanol industry the fastest growing energy industry in the world.

A great amount of research has been conducted on corn to achieve higher ethanol yields or to increase values of the by-products. Seed companies have made a great effort to develop corn hybrids with higher starch contents or higher extractable starch contents to increase ethanol yields (Bothast and Schlicher 2005). Utilizing both starch and fiber in the grains and increasing starch loading are also the major focus to achieve high ethanol yields. Bruce et al (2004) reported that a modified corn dry-grind process using both starch and fiber can increase ethanol yield by 7%. Ponnampalam et al (2004) reported that the integration of germ and fiber removal in the dry-grind ethanol industry could raise fermentation capacity, add value to by-products, and increase ethanol yield by 11%. Corn is an excellent source of starch for a glucose platform. However, corn alone can not meet the demand for fuel ethanol. The United States consumes more than 150 billion gallons of fuel for automobiles per year (Hicks et al 2005). Even if 100% of the 2004 corn crop were used for ethanol production, the produced ethanol would have only met ≈23% of our demands. Obviously, other small grains are needed for ethanol production, especially in areas without corn.

Although the biological process for ethanol production is the same in all the distilleries (conversion of glucose to ethanol), the major raw materials used in ethanol plants at different locations may be quite different. The price and availability of the raw materials and the price of by-products are critical factors for ethanol plants to maintain profitability. Sugarcane juice and molasses are the dominant materials for ethanol production in Brazil. In the United States and Canada, fuel ethanol is produced primarily from

corn and sorghum. Wheat is used in Western Canada (Wang et al 1997; Wheals et al 1999). Because the fluctuation in the market price and availability of corn has a great impact on the operation and profit margin of fuel ethanol plants, many fuel ethanol plants would use alternative grains as feedstocks for ethanol production. Since the 1990s, a great amount of research has been conducted on fuel ethanol production from other major cereal grains such as wheat, barley, oats, rye, and triticale (Thomas et al 1995; Thomas and Ingledew 1995; Sosulski et al 1997; Wang et al 1997, 1998; Hicks et al 2005). Fermentation efficiencies of ≈90% have been reached using those cereal grains (Sosulski et al 1997; Wang et al 1997, 1998) but specific problems may exist for a raw material such as high-viscosity mashes with oats, barley, and rye (Thomas et al 1995, 1996; Thomas and Ingledew 1995; Wang et al 1997, 1998) and low protein content in distillers dried grains with solubles (DDGS) from barley (Thomas et al 1995). Some ethanol plants already run solely on locally available grains such as wheat, rye, or sorghum instead of corn (Wang et al 1997). Therefore, study of the fermentability of locally available feedstocks in normal ethanol fermentation could provide more choices when the availability of materials is limited. This could be especially helpful for small ethanol facilities in rural areas to choose the most economic raw materials.

Pearl millet (*Pennisetum glaucum* L., R. Br.) is a warm season annual grass with ≈1.5 million planted acres in the United States. Pearl millet can grow in semiarid conditions with very low (≤300 mm) or inconsistent rainfall and can survive in areas where sorghum and corn will suffer more severe yield reductions or total crop failure (Dendy 1995). The chemical composition of pearl millet is similar to that of the other major cereals (Shelton and Lee 2000). Most pearl millet produced currently is used for poultry and livestock feed and bird feed. Feeding tests in cattle, swine, layer hens, ducks, and catfish showed that pearl millet is either superior to, or as good as, feed corn (Andrews et al 1996). The potential for industrial applications of pearl millet has not been studied.

The current study was conducted to evaluate the performance of pearl millet for ethanol production using both rotary-shaker and bioreactor fermentation systems. This work will not only benefit the fuel ethanol industry in semiarid rural areas by finding alternative raw materials but will also provide valuable information for breeders to modify existing pearl millet genotypes and develop new pearl millet hybrids for industrial applications.

MATERIALS AND METHODS

Pearl Millet Samples

The pearl millet samples used in this study included one released cultivar (Tifgrain102) and three advanced experimental

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breeding lines (04F-303, 04F-106, 04F-2304) that were produced in the field at Tifton, GA, under standard management practices (Lee et al 2004). These samples were chosen based on diverse genetic backgrounds and potential for contributing to new varietal releases. The pearl millet samples were ground into fine meal on a cyclone sample mill with a 2-mm screen (model 3010-018, Udy, Fort Collins, CO). The total starch, crude protein, crude fat, crude fiber, ash, and moisture content of these samples are listed in Table I.

Microorganism

The *S. cerevisiae* strain ATCC 24860 was used for ethanol fermentation and was maintained on yeast extract/peptone/dextrose (YPD) agar slants sealed with sterile mineral oil at room temperature ($\approx 25^\circ\text{C}$). The strain was subcultured to YPD agar slants and incubated at 25°C for three days and then used to inoculate pre-culture broths.

Preparation of Mashers from Ground Pearl Millet

Liquefaction. An aliquot of 100 mL of fermentation solution (containing 3.0 g of peptone, 1.0 g of KH_2PO_4 , and 1.0 g of $(\text{NH}_4)_2\text{SO}_4$ per liter) was added to each 250-mL flask with a designated amount of ground pearl millet (20, 25, 30, or 35 g, db). High-temperature α -amylase (Liquozyme, 240 Kilo Novo Unit (KNU)/g, Novozymes, Franklinton, NC) was added at ≈ 3 KNU/g of starch. One KNU is defined as the amount of enzyme that dextrinizes 5.26 g of starch dry substance (Merck Amylum soluble) per hour under Novo Nordisk's standard conditions for α -amylase determination ($37 \pm 0.05^\circ\text{C}$, 0.3 mM Ca^{2+} , and pH 5.6). After the sample materials were evenly wetted and dispersed in the fermentation solution, flasks were maintained at 95°C for 45 min in a water bath shaker (Labline microprocessor, Melrose Park, IL) operating at 120 rpm. Temperature of the contents in the flasks was then reduced to 80°C . The gelatinized and partly liquefied grain was further liquefied by adding a second dose of Liquozyme (≈ 3 KNU/g of starch) to each flask and maintained at 80°C for 30 min.

Saccharification. After the temperature of the liquefied mash was reduced to 60°C , glucoamylase (Spirizyme, 750 Novo Glucoamylase Unit (AGU)/g, Novozymes, Franklinton, NC) was added into each flask at 150 AGU/g of starch. The AGU is defined as the amount of enzyme that hydrolyzes 1 μM maltose/min under the standard conditions (37°C , 0.1M, pH 4.3 acetate buffer, 23.2 mM maltose, reaction time of 5 min). The flasks were maintained at 60°C for 30 min with the shaker running at 120 rpm. Then the flasks with finished pearl millet mashers were removed from the water bath and cooled to $\approx 30^\circ\text{C}$. The mashers were adjusted to pH 4.2 to 4.3 with 2N HCl before inoculation.

Fermentation Processes

The prepared mashers with substrate concentrations of 20, 25, 30, or 35% were inoculated with 5 mL of yeast preculture. The yeast preculture was prepared as described by Suresh et al (1999) and Zhan et al (2003). The cell concentration of the yeast preculture was checked by both its A_{600} value on a spectrophotometer and a counting chamber (BioRite, Fisher Scientific, Fairlawn, NJ). The

A_{600} values of the precultures were 2.20–2.60 with cell concentrations of $2\text{--}2.8 \times 10^8$ cells/mL; this ensured that the inoculated pearl millet mashers had a cell concentration of $1\text{--}1.4 \times 10^7$ cells/mL.

The ethanol fermentation was performed in an incubator shaker (model I2400, New Brunswick Scientific, Edison, NJ) at 30°C for 72 hr with a shaking rate of 150 rpm. Because ethanol fermentation is an anaerobic process, the fermentation flasks were sealed with S-bubblers filled with ≈ 2 mL of mineral oil. To compare the fermentation efficiency of pearl millet with that of corn, ethanol fermentation on mash made from yellow dent corn used the same procedures as for pearl millet.

The ethanol concentrations in fermentation broths were measured at different time intervals during the fermentation and were also monitored by measuring the total weights of the fermentation flasks because the weight loss by CO_2 evolution is proportional to the amount of ethanol produced during ethanol fermentation (Vieira et al 1992; Joekes et al 1998).

A 5-L bioreactor (New Brunswick Scientific, Edison, NJ) was used to confirm the results from fermentation on rotary shakers and to study the kinetics of ethanol fermentation at a 30% dry mass level (a total volume of ≈ 4 L in the 5-L vessel). Samples were collected at different times and analyzed for ethanol yields. The final fermentation efficiency on a starch basis was determined based on two replicates.

Analytical Methods

The pearl millet samples were analyzed following the AOAC Official Methods (AOAC International 1999) for moisture content (925.10), crude fat (920.39), and ash (942.05). Phosphorous was determined by a spectrophotometric method. Calcium was determined according to AOAC method 968.08. Crude fiber was analyzed using a filter bag technique (available at http://www.ankom.com/09_procedures/procedures3.shtml; ANKOM Technology). Approved Method 76-13 (AACC International 2000) was used to determine total starch contents of all the samples and to determine glucose concentrations in the fermentation mashers using commercially available Megazyme kits (Bray, Ireland; AOAC method 996.11). Crude protein was determined by the combustion method (LECO FP-428; AOAC method 990.03). Protein contents were calculated as $\text{N} \times 6.25$.

The ethanol concentration was determined by the specific gravity AOAC method 942.06. Fermentation efficiencies were calculated as a ratio of the actual ethanol yield to the theoretical ethanol yield. The total starch contents in the samples were used to calculate the theoretical ethanol yields, assuming 1 g of starch converts to 1.11 g of glucose and that 1 g of glucose may generate 0.511 g of ethanol (Thomas et al 1996).

Statistics

All experiments were conducted in triplicate. Results were presented as averages of replicates. ANOVA was conducted to determine the significant differences at 5% significant level ($P < 0.05$) in fermentation efficiency among the pearl millet samples at different dry mass levels.

TABLE I
Chemical Composition of Pearl Millet and Corn Samples

Samples	Moisture (%)	Starch (% db)	Protein (% db)	Crude Fat (% db)	Crude Fiber (% db)	Ash (% db)
Millet						
04F-303	11.27 \pm 0.17 ^a	65.30 \pm 0.13	13.68 \pm 0.02	6.27 \pm 0.07	1.10 \pm 0.06	1.76 \pm 0.02
Tifgrain-102	11.77 \pm 0.07	68.07 \pm 0.69	10.08 \pm 0.03	6.48 \pm 0.06	1.87 \pm 0.07	1.96 \pm 0.03
04F-2304	11.23 \pm 0.09	69.92 \pm 0.78	9.72 \pm 0.01	6.80 \pm 0.07	1.73 \pm 0.03	1.73 \pm 0.03
04F-106	10.90 \pm 0.05	70.39 \pm 0.94	10.86 \pm 0.01	6.68 \pm 0.07	1.00 \pm 0.07	1.53 \pm 0.03
Corn	13.03 \pm 0.05	73.00 \pm 0.82	8.35 \pm 0.04	4.05 \pm 0.05	1.97 \pm 0.06	1.54 \pm 0.04

^a Mean \pm standard deviation.

RESULTS AND DISCUSSION

Ethanol Production from Pearl Millets

Ethanol yields on mashes made from 20, 25, 30, and 35 g of pearl millet powder were ≈ 9 , 11, 13–14, and 16–17% (v/v), respectively. At each dry mass percentage, the ethanol yields were proportional to the starch content of the pearl millet samples; that is, the ethanol yields increased in the order of 04F-303, Tifgrain-102, 04F-2304, and 04F-106 as shown in Table II. The efficiency of ethanol yields on a starch basis was 90.0–95.6%; efficiency had an upward trend as the solid content in the mashes increased from 20 to 35%. Overall, the ethanol fermentation efficiencies on a starch basis from pearl millet mashes were greater than the reported 82–87% fermentation efficiencies from oats (Thomas and Ingledew 1995) and were comparable to the values of 89–94.6% from other cereals such as barley (Thomas et al 1995), rye, and triticale (Sosulski et al 1997; Wang et al 1997, 1998). The optimal efficiency for batch ethanol fermentation by yeast is ≈ 93 –95% of the theoretical value (O'Connor-Cox et al 1991). Our results showed that the fermentation efficiency with pearl millet at dry mass percentages for normal fermentation (≈ 30 –35% DM) could easily reach the optimal fermentation efficiency. Therefore, pearl millet could be a good raw material for fuel ethanol production.

There was no significant difference in ethanol fermentation performance among the four tested pearl millet samples when judged by their fermentation efficiencies. This was probably because the tested genotypes have similar chemical composition, chemical structure, and physical properties, especially starch contents. More samples with diverse chemical compositions need to be tested to identify the effect of genotype on the ethanol fermentation properties of pearl millets.

Kinetics of Ethanol Fermentation from Pearl Millets

Kinetics of ethanol production was studied using fermentation on rotary shakers and in a 5-L bioreactor. Glucose content, ethanol concentration, weight loss, and pH value were measured during the fermentation process.

The ethanol fermentation was conducted in three phases, based on fermentation results from the 5-L bioreactor. During the first fermentation phase (0–8 hr), both glucose reduction and ethanol production were very slow (Fig. 1). The glucose and ethanol yield curves reveal that, during the first couple of hours, the inoculated yeast cells went through a process of adjusting themselves to the new environment of the fermentation mash and exponential reproduction; little glucose was consumed and ethanol was barely detectable. The pH value stayed constant for the first 6 hr of fermentation. In the second phase (8–32 hr), the ethanol yield increased linearly. The amount of glucose consumed and the ethanol concentration noticeably progressed after 8 hr of fermentation. Most of the glucose in the mash was consumed during the second phase, but the proportional increase in ethanol concentration did

not end until ≈ 32 hr. This indicated that other fermentable sugars such as maltose, maltotriose, and dextrans were hydrolyzed into glucose and sustained rapid ethanol generation after the original glucose was consumed. At the beginning of the third phase (32–72 hr), the easily fermentable sugars were exhausted but the ethanol content still increased slowly by fermentation of the slowly released glucose from residual dextrans. After 48 hr, hydrolysis of the residual dextrans by glucoamylase was so slow that the increase in ethanol content was negligible. Therefore, the ethanol fermentation process on 30% pearl millet mash by *S. cerevisiae* essentially ends 48 hr after inoculation. The fermentation process on mashes with less dry mass could end earlier, and those with greater dry mass may take longer (≈ 24 hr for 20% mash, 36 hr for 25% mash, and 60 hr for 35% mash). The pH curve has a pattern similar to the glucose curve; pH was stable at ≈ 4.2 during the first few hours and then decreased to and stayed at ≈ 3.9 after ≈ 20 hr of fermentation, which indicated that the ethanol fermentation process was normal. Lower pH values usually indicate contamination of lactic acid bacteria.

The ethanol fermentation process generates equal moles of CO₂ and ethanol; therefore, the weight loss from CO₂ evolution could be a useful indicator for ethanol yield, especially in laboratory-scale fermentation tests in Erlenmeyer flasks on rotary shakers. Several researchers reported the use of weight loss from escaped CO₂ to monitor the ethanol fermentation process (Vieira et al 1992; Chi and Liu 1994; Joekes et al 1998; Fujita et al 2001; Dien et al 2002). Joekes et al (1998) showed that weight of fermentation mashes did not decrease any further after 30 hr of fermentation, which is in agreement with our data on pearl millet mashes (Fig. 2). No significant weight loss was observed during the final 24 hr of

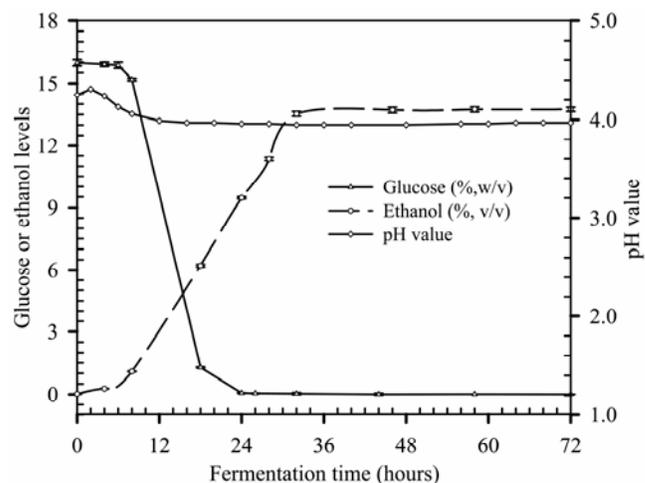


Fig. 1. Kinetics of ethanol fermentation from 30% dry mass of millet Tifgrain-102 on a BioFlo 3000 fermenter.

TABLE II
Ethanol Yields and Fermentation Efficiencies of Mashes Made from Pearl Millets with Different Dry Mass (% DM) Contents

Samples	Substrate Concentrations (% DM)			
	20	25	30	35
Ethanol yield (% v/v)				
04F-303	8.70 \pm 0.12 ^a	11.02 \pm 0.11	13.25 \pm 0.05	15.70 \pm 0.07
Tifgrain-102	8.81 \pm 0.10	11.16 \pm 0.13	13.64 \pm 0.06	16.30 \pm 0.08
04F-2304	9.23 \pm 0.11	11.67 \pm 0.12	14.05 \pm 0.05	16.59 \pm 0.10
04F-106	9.13 \pm 0.12	11.79 \pm 0.14	14.17 \pm 0.12	16.80 \pm 0.11
Fermentation efficiency (%) ^b				
04F-303	92.7 \pm 1.31	93.9 \pm 0.95	94.1 \pm 0.36	95.6 \pm 0.44
Tifgrain-102	90.0 \pm 1.06	91.2 \pm 1.03	92.9 \pm 0.44	95.2 \pm 0.46
04F-2304	91.8 \pm 1.08	92.9 \pm 0.93	93.2 \pm 0.36	94.3 \pm 0.56
04F-106	90.2 \pm 1.22	93.2 \pm 1.03	93.4 \pm 0.78	94.9 \pm 0.62

^a Mean \pm standard deviation.

^b Fermentation efficiency (%) = actual ethanol yield/theoretical ethanol yield \times 100.

TABLE III
Chemical Composition of Distillers Dried Grains with Solubles (DDGS) from Different Grains (% , db)

Cereals	Protein	Fat	Starch	Fiber	Ash	Phosphorous	Calcium
Wheat ^{a,b}	19.6–38.4	3.88–7.66	–	5.56–7.6	7.4–9.4	0.96	0.15
Corn ^{a,c}	23.0–31.3	9.0–11.9	5.10	6.3–10.2	4.6–12.1	0.84	
Sorghum ^{b,c}	30.3–45.3	12.3–12.5	5.70	10.7–11.6	2.1–5.3	0.84	0.10
Millet	30.74 ± 0.05	19.22 ± 0.08	3.45 ± 0.05	4.28 ± 0.11	5.22 ± 0.02	0.92 ± 0.01	0.07

^a Rasco et al (1987).

^b Nutrient composition of wheat DDGS. Available online at http://www.ddgs.umn.edu/wheat/wheat_ddgs.pdf. Mohawk Canada (2002).

^c Belyea et al (2004).

^d Wu and Sexson (1984).

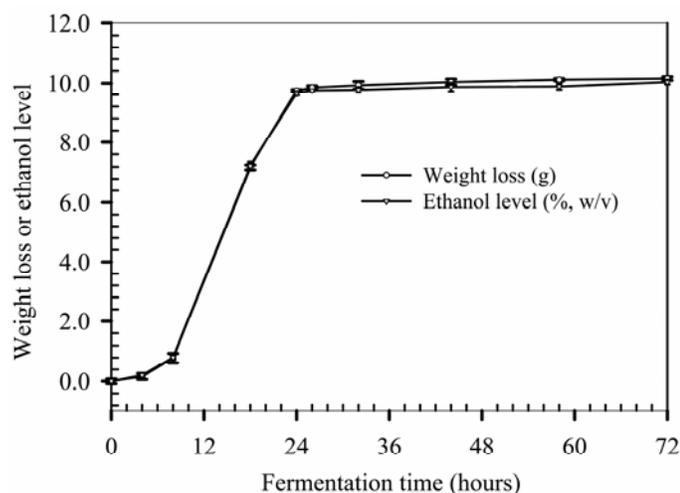


Fig. 2. Curves of weight loss (g) from CO₂ evolution and ethanol yields (% , w/v) during ethanol fermentation of pearl millet mashes.

fermentation (weight loss of ≈0.1 g). Figure 2 also shows that the curve of weight loss from CO₂ evolution is very similar to the ethanol yield curve. The ethanol yield curve and the curve of weight loss from CO₂ evolution almost overlapped with each other. Thus, the weight loss during ethanol fermentation also reveals the rate of the fermentation process. Monitoring the weight loss during a shaking-flask fermentation process can be a convenient way to predict the ethanol yield and determine the end point of the fermentation process. It is especially helpful when we evaluate new samples for ethanol yield using the shaking-flask test and do not have enough information about the chemical compositions or history of pretreatment.

The ethanol fermentation efficiency of pearl millets has been compared with that of corn (Fig. 3). Efficiency curves for mashes from pearl millet and corn show that pearl millet took less time to complete its fermentation than did corn. Pearl millet finished ethanol fermentation sometime between 24 and 36 hr after inoculation, whereas the efficiency curve of corn mash suggests that the ethanol fermentation process ended ≈36 to 48 hr after inoculation. The reason for the faster fermentation on mashes from pearl millet and slower fermentation on corn mash could be the difference in free α-amino nitrogen contents (FAN). As reported by O'Connor-Cox et al (1991), FAN content in mashes significantly affects the rate of ethanol fermentation. At the same glucose concentration, higher FAN concentrations not only accelerated the fermentation rate but also increased the ethanol yield. In our study, because pearl millet has a greater protein content than corn, FAN content in mash from pearl millet must also be greater than that in corn mash.

Chemical Composition of DDGS

Because fuel ethanol plants usually run on very limited profit margins, revenues from DDGS could be an important part of a plant's commercial viability. Nutrient composition determines the sale price of DDGS and therefore contributes significantly to

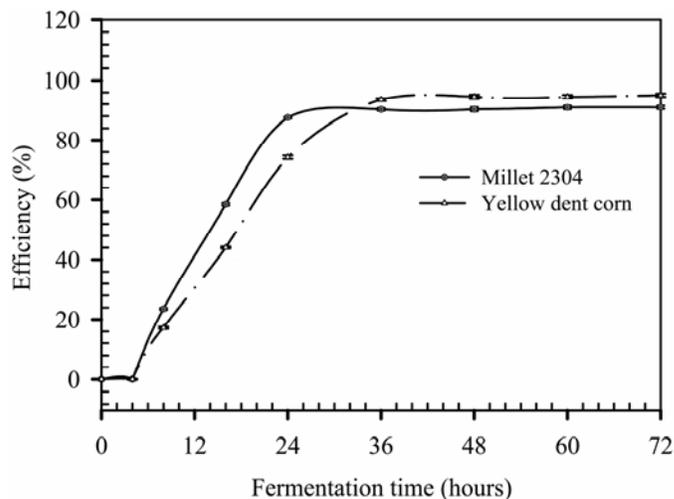


Fig. 3. Comparison of ethanol fermentation efficiency between mashes made from pearl millet and yellow dent corn with 20% dry mass.

maintaining the profitability of an ethanol plant. Table III shows the composition of DDGS from pearl millet and some other cereals. Greater protein and fat contents make the DDGS from pearl millet mash a better nutrient and energy source for animal feed than the DDGS from other grains. Pearl millet protein has higher essential amino acid contents than other feedstock cereals, and animal feeding tests have proved that the quality of proteins from pearl millets is superior to those of corn and sorghum (Andrews et al 1996), although the quality of DDGS from pearl millet, compared with other sources, needs to be confirmed by animal feeding tests. The greater energy content, greater protein content, and likely quality of pearl millet DDGS could be favorable elements encouraging fuel ethanol plants to choose pearl millet as an alternative feedstock.

CONCLUSIONS

Ethanol fermentation results from shaking-flask tests showed that ethanol yields from pearl millet mashes containing 20, 25, 30, and 35% dry mass were ≈9, 11, 13–14, and 16–17% (v/v), respectively; their corresponding fermentation efficiencies were between 90.0 and 95.6%. There is no significant difference between fermentation efficiencies of mashes made from different pearl millet samples at the same dry mass content at *P* < 0.05. Weight loss from CO₂ evolution during fermentation is a useful parameter in monitoring fermentation rate and predicting ethanol yield. Although ethanol fermentation by the shaking-flask method usually has lower fermentation efficiency than a fermentor, the shaking-flask test is a convenient way to evaluate ethanol fermentation properties of a material with efficiency comparable to that of a fermentor. Because its fermentation efficiency is comparable to that of corn and because it has good protein and fat content, and probable high-quality DDGS protein, pearl millet could be used as an alternative feedstock for fuel ethanol production.

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Erratum

Corrections were made to this article on April 10, 2006.
Table I on page 128 was corrected to show the fat content of corn as 4.05%.