Production of Fuel Alcohol from Hull-less Barley by Very High Gravity Technology

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Cereal Chem. 72(4):360-364

ABSTRACT

Very high gravity mashes (>30 g dissolved solids per 100 ml) were prepared from an experimental hull-less barley (SB90354) and fermented with active dry yeast. A maximum ethanol concentration of 17.1% (v/v) was realized in fermented mash, and a total ethanol yield of 443 L per tonne of barley (dwb) was obtained. To prevent excess viscosity during mashing, it was necessary to hydrolyze β-glucan in ground barley using crude preparations of β-glucanase or Biocellulase. While both these preparations possessed an endoglucanase activity, no measurable exoglucanase activity was detected. A typical mash prepared at a water-to-grain ratio of 3:1 and without hydrolysis of β-glucan had a viscosity of 2,480 BU, while the viscosities of the mashes prepared after hydrolysis of β-glucan with β-glucanase or Biocellulase were 560 and 240 BU, respectively. Hydrolysis of β-glucan not only reduced the viscosity of the barley mash but also released water bound and trapped by the β-glucan gel. The free amino nitrogen (FAN) content of the barley mashes was high when compared to wheat mashes, and about 80% of this FAN was taken up by yeast. In spite of the high FAN content of the mash, an exogenously added nitrogen supplement stimulated yeast growth and fermentation.

A previous report from this laboratory showed that hull-less barley, although often high in β-glucan, can be mashed and fermented to yield 10.6% ethanol (v/v) using traditional fuel alcohol technology (Ingledew et al 1995). The mashing and fermentation procedures used were essentially the same as those reported for the production of fuel alcohol from wheat (Thomas and Ingledew 1990), and it was shown that normal gravity barley mashes containing ~20 g of dissolved solids per 100 ml of the liquid portion of the mash fermented at rates comparable to wheat mashes of equivalent dissolved solids content.

Wheat and corn mashes used in industry for the production of fuel alcohol usually have a dissolved solids content in the range of 20–24 g/100 ml of mash. Normally a water-to-grain ratio of 3:1 is used to prepare these mashes. A mash prepared from barley with this same water-to-grain ratio is very viscous. The high viscosity of barley mashes has hindered the development of a technology for the industrial-scale production of fuel alcohol from this grain. Also, there have been problems in mash run-off in brewing and in those distilleries where barley is used as a significant part of the mash grain bill. Because of the viscosity development, preparation of barley mashes with dissolved solids content >20 g/100 ml is difficult even under laboratory conditions. However, barley mash viscosity can be reduced considerably by treatment with a crude preparation of β-glucanase such as one derived from an *Aspergillus* species (Ingledew et al 1995). This observation led to further investigation into the use of alternate or additional enzyme preparations to aid in the mashing of barley and other grains. In this article, we report further on the use of crude β-glucanase preparations to reduce viscosity during the mashing of barley. In fact, by using one of these enzymes, and by adjusting the water-to-grain ratio, the dissolved solids content of the mashes can be raised to ≥30 g/100 ml. Such mashes are known as very high gravity (VHG) mashes and have been prepared and completely fermented in this laboratory. Use of VHG fermentation technology allows considerable saving of water, reduces distillation costs, and allows more alcohol to be made with given plant capacity and labor costs. In addition, VHG fermentation reduces capital costs, lowers energy cost per liter of alcohol, and reduces the risk of bacterial contamination. Industry has not yet adopted the VHG fermentation technology for fuel alcohol production, mainly because VHG mashes from grains are very viscous and difficult to handle. The methods described here provide VHG mashes of low viscosity and can be easily adapted for the preparation of VHG mashes from barley for the industrial-scale production of fuel alcohol.

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MATERIALS AND METHODS

Barley

A hull-less barley (SB90354, a high viscosity, high β-glucan experimental hull-less barley cultivar with normal starch content) was used throughout the study. The barley had a moisture content of 10.3 ± 0.1%. The percentage composition of the barley on a dry weight basis was: starch, 70.0 ± 1.4; protein, 15.5 ± 0.2; lipids, 2.3 ± 0.0; β-glucan, 6.8 ± 0.1; acid dietary fiber, 2.0 ± 0.0; and ash, 1.8 ± 0.0. Analytical methods were described previously (Ingledew et al 1995).

Enzymes, Reagents, and Chemicals

A crude powdered preparation of β-glucanase derived from an *Aspergillus* species, was obtained from GNC Bioferm, Saskatoon SK. Biocellulase TRI was supplied by Quest International, Sarasota, FL. High-temperature α-amylase (High-T), glucoamylase (Allcoholase II), and active dry yeast were all obtained from the Alltech Biotechnology Center, Nicholasville, KY. β-Glucan was isolated from barley as described by Bhatty (1993). All other chemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, MO, or obtained locally. All chemicals were of reagent grade.

Grinding and Mashing of the Barley

Hull-less barley was ground with a plate grinder (Disc Mill S.500, Glen Mills, Inc., Clifton, NJ) at a setting of 5. A sieve analysis showed that 83% of the ground barley had a particle size between 20 and 60 mesh, while the remainder was finer than 60 mesh.

The method of mashing the barley was a modification of the procedure used for mashing of wheat (Thomas and Ingledew 1990). The required amount of water was warmed to 45°C in a water bath and 0.02% (w/w) β-glucanase (based on the weight of barley used) was added to the water. Immediately after the addition of the enzyme, ground barley was added with vigorous stirring. The enzyme was allowed to react for 30 min at 45°C. The temperature of the slurry was then raised to 60°C. Ten milliliters of 100 m*M* calcium chloride solution was added for each liter of water used for mashing followed by 5.0 ml of high-temperature α-amylase (High-T) per kilogram of barley used. The starch was gelatinized by raising the temperature of the slurry to 90°C and holding for 45 min. The volume lost through evaporation was made up by adding sterile distilled water. No make-up water was added or only limited quantities were added when

the dissolved solids content was to be raised beyond the level possible with the original water-to-grain ratio. Liquefaction was continued after the starch was cooled to 80° C by adding another 5.0 ml of high-temperature α-amylase and letting it react for 30 min. In the studies reported here, mashes were prepared in 3-kg quantities.

Fermentation

The liquefied mash was then saccharified and fermented with active dry yeast as described for wheat (Thomas and Ingledew 1990, Thomas and Ingledew 1992b). In most of the experiments, 500-g samples of barley mashes were fermented at 20° C in duplicate with or without 16 m*M* urea as the nutrient supplement. Mashes were transferred to sterile Celstir fermentors (Wheaton Scientific, Millville, NJ) that contained either 10 ml of distilled water or 10 ml of urea solution. To each fermentor, the required amount of glucoamylase proportional to the dissolved solids contents of the mash (1.2 ml/500 g mash of 20 g dissolved solids/100 ml) was added to convert dextrin to fermentable sugars. Saccharification was conducted at 30° C. Thirty minutes after the addition of glucoamylase, the temperature of the mash was lowered. The mash was inoculated with preconditioned active dry yeast at a level of 10^8 cells/g of dissolved solids in the mash. This is equivalent to the recommended inoculation level under brewing conditions of 10^6 yeast cells/ml/° Plato (Casey and Ingledew 1985). When dealing with grain mashes, the former calculation method for inoculation level is preferred because the exact volume of the liquid portion of the grain mash is not known. The amount of fermentable sugar (roughly equivalent to dissolved solids content) from a given weight of grain can be predicted. The amount of inoculum is then independent of the volume or weight of the mash but dependent on the amount of fermentable sugar in the mash. As an example, it can be calculated that 3.5 kg of yeast would be required to inoculate a mash prepared from 1 tonne of hull-less barley containing 70% starch (700,000 g of starch in 1 tonne × 1.11 [to convert starch to sugar] × 10^8 cells/g of sugar ÷ 2.2 × 10^{10} viable cells/g of active dry yeast = 3,500 g of yeast). Fermentations were repeatable with several mashes.

Viscosity Measurements

Viscosities of the mashes at 30° C were measured with a starch tester (model AV30, Haake, Inc., Saddle Brook, NJ) and the relative viscosity expressed in Brabender Units (BU). Hydrolysis of β-glucan by β-glucanase and Biocellulase was tested by measuring reduction in viscosity of a 0.5% β-glucan solution. The viscosity was measured at 40° C with a viscometer (Cannon-Fenske 100, Cannon Instrument Co., State College, PA). All measurements were made in triplicate.

Fermentation Progress

Progress of fermentation was monitored by measuring the dissolved solids of the mash at regular intervals. Samples collected at various times were centrifuged (10,300 × *g*) for 15 min, and the specific gravity of the clear supernatant liquids were measured at 20° C with a digital density meter (DMA-45, Anton Paar, Graz, Austria). With the aid of appropriate tables, specific gravity readings were converted to dissolved solids (expressed as grams of sucrose per 100 ml).

Analyses

All analyses were done in duplicate. Total FAN in the supernatant liquid was determined by the ninhydrin method of the European Brewery Convention (1987). Ethanol was measured enzymatically using alcohol dehydrogenase (Thomas and Ingledew 1990). Known concentrations of ethanol were used as standards. Glycerol, sugars, and dextrins were measured by high-performance liquid chromatography (HPLC) (Thomas et al 1993b). Clear supernatant liquids obtained by centrifugation of mashes at 10,300 × *g* for 15 min were diluted with distilled water and injected into a column (FAM-PAK, Waters Chromatographic Div., Milford, MA) maintained at 65° C. This column separates sugars, organic acids, and alcohols. These components were eluted from the column with HPLC-grade water containing orthophosphoric acid at a concentration of 1.5 m*M*. The separated components were detected and quantified with a differential refractometer (model 410, Waters). The elution rate was 1 ml/min, and methanol was used as internal standard.

FAN in the mashes were measured by the HPLC method described previously for the determination of free amino acids in physiological samples (Thomas et al 1993a). The samples were derivatized by the Pico.Tag procedure and analyzed by injection into a Pico.Tag free amino acid column (3.5 mm × 30 cm) maintained at 46° C. Derivatized amino acids were separated by eluting with a proprietary gradient solvent system delivered at a rate of 1 ml/min, and were detected and quantified by measuring absorbance at 254 nm with a UV detector (model 410, Waters).

RESULTS

β-Glucan Hydrolyzing Enzymes and the Viscosity of Barley Mashes

A number of crude enzyme preparations were tested for their effectiveness in reducing barley mash viscosity. The two selected enzyme preparations were provided under the names β-glucanase and Biocellulase TRI. Barley mashes were prepared with a water-to-grain ratio of 3:1. The viscosity of the mashes prepared at different times varied slightly (by 30–50 BU) because of differences in the rates of evaporation during cooking and starch hydrolysis. The mashes were then treated with β-glucanase or Biocellulase, and changes in viscosity were monitored with a viscoamylograph. The average results from a typical experiment showed that a barley mash prepared with a water-to-grain ratio of 3:1 and without the aid of β—glucanase or Biocellulase had a viscosity of 2,460 BU and a dissolved solids content of 32.6 g/100 ml in the liquid portion. On treating this mash with β-glucanase (0.02%, w/w) the viscosity was reduced at the rate of 8.2 BU/sec to a minimum value of 420 BU. Biocellulase applied to the mash (0.02%, v/w) reduced the viscosity to a minimum value of 540 BU at the rate of 7.9 BU/sec.

The viscosity reduction of the mash appears to have occurred through the hydrolysis of β-glucan. Both these enzyme preparations possessed β-glucanase activity as measured by their ability to decrease the viscosity of pure β-glucan solutions. For example, the viscosity of a 0.5% solution of a barley β-glucan solution was reduced from 1.034 centistokes (cs) to 0.339 cs by a 0.02% solution of β-glucanase or Biocellulase. The viscosity of distilled water under the measuring condition was 0.323 cs. It is therefore reasonable to assume that the viscosity reduction in the barley mash by these two enzyme preparations occurred through the hydrolysis of β-glucan.

Because mashes prepared from barley are very viscous and are difficult to handle, the use of barley as a feed stock in the alcohol industry has always been a serious challenge. In the experiments reported here, however, we modified the mashing procedure such that the mashes made by this method had very reduced viscosities. The modification involved treating the barley slurry (prepared by mixing ground barley with water) with β-glucanase or with Biocellulase before gelatinization of starch as described above.

Several mashes were prepared by the modified procedure and by using different water-to-grain ratios. With this modification, the viscosity development during heating was reduced considerably, and mashes with low final viscosity resulted. As an example, a mash prepared with a grain-to-water ratio of 3:1 and without the aid of any viscosity-reducing enzyme had a viscosity of 2,480 BU. The viscosity of the same prepared mash was only 560 BU when the ground barley-water slurry was treated with β-glucanase (0.2 g/kg of barley) before mashing. Biocellulase (0.2 ml/kg of barley) reduced the viscosity of similarly produced mashes even further to a value of 240 BU. It appears that Biocellulase possesses β-glucanase activity as well as other enzymes that hydrolyze other compounds (perhaps proteins and pentosans), resulting in a further reduction in the viscosity. Application of Biocellulase before gelatinization of starch reduced the viscosity

of the final mash to 240 BU; however, the viscosity was reduced to only 540 BU when the enzyme was applied after the preparation of mash.

Carbohydrates in the Prepared Mash

Most of the carbohydrates produced during the mashing and saccharification of barley mashes are fermentable sugars. Carbohydrate analysis by HPLC of a typical industry mash prepared with a water-to-grain ratio of 3:1 and with β-glucanase (0.2 g of enzyme/kg of barley) led to the results shown in Table I. Of the dissolved solids in the mash, ~92% were carbohydrates; glucose accounted for 78.7%. The rest (8%) of the dissolved solids in the mash may be soluble proteins, pentosans, and products of the partial hydrolysis of β-glucan.

VHG Mashes Fermented to Completion

It is possible to raise the dissolved solids content of the mash from a typical 22.6 g/100 ml to almost 32 g/100 ml by changing the water-to-grain ratio from 3:1 to 1.8:1 (Table II). In such cases, the amount of β-glucan-degrading enzyme added per unit weight of the grain was held constant.

The dissolved solids and FAN contents of the mashes increased with decreasing water-to-grain ratio, although FAN did not rise in proportion to increased extract concentration (Table II). The amount of FAN released under strictly defined mashing conditions is proportional to the dissolved solids concentration of the mash (Thomas and Ingledew 1990). However, when mashing conditions were altered by changing water-to-grain ratios or changes were made to the cooking regime, the amounts of FAN detected in the mash became variable. Ethanol yield was, as expected, proportional to the fermentable sugar content of the mash.

Rate of Fermentation

Normal and VHG mashes were prepared by adjusting the water-to-grain ratio. Mashes were fermented with and without 16 m*M* urea as a nitrogenous supplement. Even without urea, a 32 g/100 ml VHG fermentation completed fermentation, although, in this case, it required eight days (Fig. 1). Fermentation completed within four days when urea was added. In normal gravity mash, a similar decrease in fermentation time was observed on supplementation of the media with urea (five days vs. two and one-half days). Further decreases in fermentation time are expected as temperature is increased to an optimum value dictated by the initial gravity or sugar concentration (Jones and Ingledew 1994) and as yeast pitching (inoculation) levels are increased (Thomas and Ingledew 1992b).

Maximal uptake of FAN occurred within the first 24 hr in normal gravity mash, whereas 48 hr was required in VHG mash (data not shown). Supplementing the media with urea did not affect the rate of FAN uptake nor the percentage of FAN taken up from the mash. Irrespective of the initial FAN content, 79.5% of the FAN was utilized. Near the end of fermentation, there was a slight increase in the FAN content of the urea-supplemented samples, perhaps resulting from autolysis of yeast cells. This may indicate release of peptidase enzyme from stressed yeast or excretion of FAN from yeasts due to protein degradation inside the cell. Free amino acid analysis by HPLC of the supernatant of a VHG mash showed that seven amino acids (asp, glu, asn, gln, ala, arg, pro) constituted two thirds of the original amino acid content in the mash (Table III). The total α-amino nitrogen content of the mash was close to the value determined by the ninhydrin method of FAN estimation. Although arginine constituted only 9.3% of the total amino acids in the mash, it provided 25% of the nitrogen for yeast growth. Of all of the nitrogen sources tested, arginine was the best in stimulating yeast growth under conditions of ethanolic fermentation (Thomas and Ingledew 1992a).

Ethanol Yield

While the concentration of ethanol in the fermented mash is a good indicator of the progress and completion of fermentation, it is not predictive of the ethanol yield from a given weight of the grain. This is especially true when the mashing conditions are changed, and the exact volumes of the liquid portions in any mash are unknown. In this study, fermentability and ethanol yield of barley mash were determined by preparing barley mashes with a water-to-grain ratio of 2:1 and with the aid of β-glucanase. The mash was supplemented with 16 m*M* urea and fermented in duplicate at 20° C with active dry yeast. At the end of fermentation (96 hr), the total mashes were distilled and ethanol concentra-

TABLE I
Carbohydrate Profile of a Barley Mash[a] at the Beginning and at the End of Fermentation

Component	Carbohydrate Concentration (g/100 ml)	
	At Zero Time	At End of Fermentation
Dextrins	1.67 (8.4)[b]	1.14
Maltotriose	0.97 (4.9)	0.09
Maltose	0.28 (1.4)	0.17
Glucose	17.1 (85.4)	0.84
Glycerol	0.05 (0.2)	0.55

[a]Contained 21.7 g of dissolved solids/100 ml.
[b]Component as a percentage of total carbohydrates and glycerol.

TABLE II
Dissolved Solids, Free Amino Nitrogen (FAN), and Ethanol Content of Mashes Prepared with Different Water-to-Grain Ratios

Ratio	Initial Dissolved Solids (g/100 ml)	FAN (mg N/L)	Ethanol (%, v/v)
3:1	22.6 ± 0.0	118.7 ± 0.0	12.85 ± 0.05
2:1	26.9 ± 0.2	161.6 ± 1.1	14.85 ± 0.25
1.8:1	31.4 ± 1.0	177.3 ± 4.2	17.10 ± 0.20

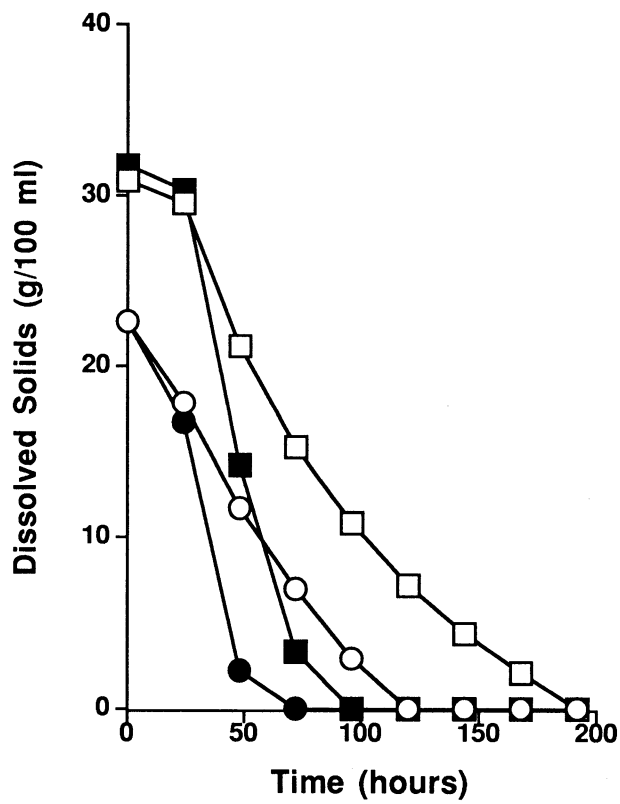

Fig. 1. Rate of fermentation of hull-less barley mashes prepared with different water-to-grain ratios: water-to-grain ratio of 1.8:1 (□, ■) or 3:1 (○, ●), and fermented with (■, ●) or without (□, ○) 16 m*M* urea as a nitrogen supplement.

tions of a measured volume of distillate were determined. From this, the total ethanol yield was calculated to be 443 L/tonne of barley (dwb). This observed value is close to the theoretical value of 468 L/tonne calculated on the basis of 70% starch and a fermentation efficiency of 93%.

DISCUSSION

Industrially, grain mashes used for fuel alcohol production normally provide about 20 g of fermentable sugars per 100 ml of mash. On fermentation, such a mash could yield ~12% ethanol by volume. A barley mash with such an amount of fermentable sugar is very viscous because of its high β-glucan content. Other components of the grain that contribute to the viscosity of mash are pentosans (Boros et al 1993), proteins, and gelatinized (but not hydrolyzed) starch. The mashing procedure employed in this study completely hydrolyzed starch to dextrins, oligosaccharides, and glucose. These water-soluble products of starch hydrolysis contributed very little to the viscosity of the prepared mash. Proteins could increase the viscosity of the mash, but to a great degree this depends on the nature of the protein and its solubility. For example, the primary cause of viscosity of wheat mash appears to be gluten. The results presented in this article show that much of the viscosity of the barley mash resulted from the gelation properties of β-glucan. Hydrolysis of β-glucan by enzymes added either before starch gelatinization or after preparation of the mash led to substantial reductions in viscosity. It was, of course, hoped that hydrolysis of β-glucan would go to completion and yield monomers (glucose) and that this would lead to increases in the ethanol yield. This, however, was not realized. The β-glucanase preparation used in this study was an endoglucanase, and, as expected, it did not liberate glucose, although the supplier of this enzyme had reported liberation of small amounts of glucose from dilute solutions of pure substrate. The barley mash contained substantial amounts of glucose resulting from hydrolysis of starch. This may have resulted in feedback inhibition of β-glucanase by glucose. Even increasing the β-glucanase level to 1 g/kg of barley did not increase the ethanol yield (data not shown). We do not know the degree of hydrolysis of β-glucan by this enzyme preparation, but it is clear that hydrolysis did not proceed past cellobiose. Biocellulase, which was equally effective in reducing the viscosity of the mash, also did not increase the glucose content in the mash. The development of glucose-liberating β-glucanases would clearly benefit the fuel alcohol industry.

The supernatant portion of the mash prepared with a water-to-grain ratio of 3:1 and without the application of any viscosity-reducing enzyme had a dissolved solids content of about 32 g/100 ml, and the mash had an initial viscosity of 2,460 BU. Mash prepared with the same grain-to-water ratio, but with the aid of viscosity-reducing enzymes, had 22.6 g of dissolved solids per 100 ml, but the viscosities of these mashes were below 500 BU. The total ethanol obtained from a given amount of grain was not affected by the viscosity reduction of the mash and the volumes of mash were equal. This suggested that the amounts of fermentable sugar present in both cases were the same, indicating that the quantity of free water available for dissolving the sugar was considerably less when β-glucan was not hydrolyzed. The high dissolved-solids concentration of the mash prepared without the aid of β-glucanase may be attributed to less water being available (being bound by β-glucan) for dissolution of soluble components. Obviously, hydrolysis of β-glucan released considerable amounts of bound water, which further diluted dissolved solids.

TABLE III
Free Amino Acid Composition of a Very High Gravity Mash Prepared from Hull-less Barley[a]

Amino Acid	μmoles/ml	%
Asp	0.85	9.3
Glu	0.71	7.8
Ser	0.38	4.2
Asn + Gln	1.30	14.3
Gly	0.39	4.3
His	0.14	1.5
Thr	0.12	1.3
Ala	1.36	14.9
Arg	0.85	9.3
Pro	0.97	10.6
Tyr	0.13	1.4
Val	0.44	4.8
Met	0.08	0.9
Ile	0.22	2.4
Leu	0.53	5.8
Phe	0.26	2.8
Trp	0.11	1.2
Lys	0.27	3.0
Total	9.11	100

[a]Total α-amino N = 127.4 μg/ml. Total N = 189.1 μg/ml.

The fermentability of barley mashes was at least as good as that of wheat mash, if not better. Although barley mashes contain almost double the amount of FAN as a wheat mash of equivalent gravity, fermentation rate is still stimulated by supplementation with assimilable nitrogen. Compared to wheat mashes, barley mashes contain higher percentages of lysine, an amino acid known to inhibit yeast growth and to retard the rate of fermentation under nitrogen-limiting growth conditions (Thomas and Ingledew 1992a). On raising the assimilable nitrogen concentration to nonlimiting levels, the inhibitory effect of lysine can be overcome. The stimulatory effect of added urea may, therefore, be the result of the elimination of the inhibitory effect of lysine on yeast growth by excess nitrogen. In addition, barley mashes contain relatively large amounts of glycine and proline (15% of the total free amino acids). Both these amino acids have been shown to offer osmoprotection to *Saccharomyces cerevisiae* (Thomas et al 1994).

We have shown previously that ethanol output at a given fermentor capacity can be elevated by increasing the fermentable sugar content of the mashes to VHG levels (>30 g of dissolved solids/100 ml of the liquid portion) (Thomas et al 1993b). Because of the development of very high viscosity during cooking, VHG mashes from barley could not be prepared without the application of viscosity-reducing enzymes (data not shown). However, by changing the water-to-grain ratio and by hydrolyzing β-glucans enzymatically before gelatinization of starch, VHG mashes from barley were easily prepared. Compared to VHG mash preparation from wheat, this is a considerable improvement in methodology. In the former case, the procedure requires a double mashing technique or the addition of freeze-dried wheat hydrolyzate (Thomas and Ingledew 1990, Jones and Ingledew 1994) or other adjuncts (Jones et al, *in press*). The insoluble nature and concentration of wheat proteins makes wheat mashes considerably thicker than a barley mash of equivalent gravity. VHG barley mashes are directly prepared, and the economics of their preparation may therefore be more attractive than VHG wheat mash. Although we have been able to prepare mashes with a water-to-grain ratio as low as 1.5:1, it is not yet clear whether at such low water availability starch gelatinization and hydrolysis would be complete (Muller 1989, Muller and Canterranne 1994). This aspect is now under investigation.

ACKNOWLEDGMENTS

We gratefully acknowledge technical support from R. S. Bhatty's laboratory, and research support provided by Western Grains Research Foundation and by the Natural Sciences and Engineering Research Council of Canada.

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[Received October 24, 1994. Accepted April 11, 1995]