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Production of Fuel Ethanol from Rye and Triticale by Very-High-Gravity (VHG) Fermentation

S. WANG,[1] K. C. THOMAS,[1] W. M. INGLEDEW,*[1] K. SOSULSKI,[3] AND F. W. SOSULSKI[2]

[1]*Department of Applied Microbiology and Food Science, and* [2]*Department of Crop Science and Plant Ecology, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK Canada S7N 5A8; and* [3]*Saskatchewan Research Council, 15 Innovation Boulevard, Saskatoon, SK Canada S7N 2X8*

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ABSTRACT

Very-high-gravity (VHG) rye and triticale mashes, containing about 28.5 g dissolved solids/100 mL of mash supernatant, were prepared by adjusting water:grain ratios to 2:1. Because of high viscosity, which develops during mashing, it was necessary to pretreat ground rye-water slurries with viscosity-reducing enzymes. There were no viscosity problems during the preparation of triticale mashes. Fermentations were conducted at 20°C, with and without 16 m*M* urea as a nitrogenous supplement. All fermentations were completed within 120–144 h. Supplementation with urea shortened the times required for completion of fermentation by 33% for triticale and by 40% for rye. The fermentation efficiencies for both grains ranged between 90 and 93%. These values are comparable to those reported for wheat, implying competitiveness of rye and triticale as fermentation feedstocks to replace wheat. The final ethanol yields were 409 L for rye and 417–435 L for triticale/t (dry basis). For a given size of fermentation vessel, 33% more grain was used in the VHG fermentation process than in normal gravity fermentation. This resulted in a 35–56% increase in ethanol concentration in the beer, when fermentors were filled to a constant volume. The corresponding reduction in water use by about one-third would result in savings in energy

*Author to whom all correspondence and reprint requests should be addressed.

consumption in mash heating, mash cooling, and ethanol distillation. Fermentation efficiencies and final ethanol yields obtained per unit weight of grain fermented were not significantly different from the normal gravity fermentations.

Index Entries: Rye; Triticale; Fuel alcohol; Very-high-gravity fermentation technology; VHG; Urea; Stillage.

INTRODUCTION

Distillers and fuel alcohol manufacturers normally ferment grain mashes containing 20–24 g of dissolved solids/100 g *(1)*. Dissolved solids concentrations in this range have been considered normal gravity in these industries. Very-high-gravity (VHG) fermentation for fuel alcohol production has been defined as the use of mashes with 300 g or more dissolved solids/L *(2)*. High gravity and VHG fermentation technology is of commercial interest because of its potential to increase the final ethanol concentration, and to reduce processing costs *(3)*. Research shows that this technology can be applied to the production of fuel alcohol from wheat *(2,4–8)*, from barley *(9,10)*, and from oats *(11)*. The highest reported ethanol concentration in the fermented mash was 23.8% (v/v), when a wheat mash containing 38% (w/v) dissolved solids was fermented at 20°C without nutrient supplementation *(2)*. Ethanol concentrations range in industry between 7 and 14% v/v. VHG fermentation almost doubles the ethanol concentration obtained in a normal gravity fermentation, but preparation of feedstock containing such high levels of dissolved solids necessitates several technical modifications. Adjustment of the water:grain ratio from 3:1 to 2:1, or lower, during mashing is the most economic and simplest way to increase the ultimate sugar concentration in a grain mash *(1)*.

Previous study showed that less expensive crops, such as rye and triticale, could be fermented to completion at the normal gravity level of 20–21 g dissolved solids/100 mL of mash liquid *(12)*. These two crops could be used by the alcohol industry to replace wheat, whose price as the basic raw material for fuel ethanol production in western Canada has increased significantly during recent years. High or VHG rye and triticale fermentations would further increase ethanol concentrations in fermentation vessels and throughput rates in ethanol plants.

Although the gelatinization and hydrolysis of starch are not major concerns during the preparation of normal-gravity rye and triticale mashes with a water:grain ratio of 3:1, reduction of the water:grain ratio is said to cause nonavailability of sufficient amounts of water *(1)*, so that incomplete starch gelatinization and hydrolysis, or inhibition of complete hydrolysis of the gelatinized starch, might occur *(13)*. This could be a major concern for VHG rye mashes, because rye is higher in pentosan con-

tent than other cereals *(14)*, and water-soluble pentosans are chiefly responsible for forming viscous solutions in concentrated rye flour-water slurries *(14–17)*. If lower water:grain ratios were employed, treatments of concentrated mashes with a viscosity-reducing enzyme would be necessary to break down the carbohydrates responsible for absorbing large amounts of free water.

The objectives of this study were to determine the effects of the reduction of water:grain ratio from 3:1 to 2:1 on mash viscosities, fermentation rates, ethanol yields, and fermentation efficiencies. The study involved fermenting rye and triticale mashes containing ~28.5 g of dissolved solids/100 mL of mash liquid at 20°C. Results are compared with those obtained from normal gravity rye and triticale fermentations *(12)*, to further assess the economics of rye or triticale as basic raw materials for fuel alcohol production.

MATERIALS AND METHODS

Rye and Triticale Samples

Fall rye (PRIMA) and triticale (AC COPIA) were both developed by, and obtained from, Agriculture and Agri-Food Canada (Saskatoon, SK). The fall rye had a moisture content of 12.26±0.01%, and a starch content of 63.11±0.79% (dry basis). The triticale had a moisture content of 10.19±0.01%, and a starch content of 64.95±0.10% (dry basis).

Enzymes, Reagents, and Chemicals

A powdered fungal enzyme preparation from *Trichoderma viride*, Roxazyme G, was provided by Hoffman-La Roche (Mississauga, ON). The Roxazyme G contained cellulase activity of 8000 IU/g and xylanase activity of 43,350 IU/g. The high-temperature (HT) α-amylase, glucoamylase (Allcoholase II), and active dry yeast were all obtained from the Alltech Biotechnology Center (Nicholasville, KY). The HT α-amylase preparation contained 10 mg protein/mL, and had a specific activity of ~1.14 g starch (hydrolyzed) min/mg protein between 80 and 90°C. The glucoamylase preparation contained 98.5 mg protein/mL, and had a specific activity of ~1 mg of glucose (produced) min/mg protein at 30°C. All other chemicals and enzymes were purchased from Sigma (St. Louis, MO), or were obtained locally. All chemicals were of reagent grade.

Grinding and Mashing

Rye and triticale grains were ground with a plate grinder (GlenMills, Clifton, NJ) at setting number 5. For mashing, 1 part of ground whole

grain, as-is basis, was dispersed with constant agitation into 2 parts of dH_2O containing 1 m*M* calcium chloride, prewarmed at 40°C. For rye mashes, 0.0002 g of Roxazyme G was added per g of ground cereal used (as-is basis). The slurries were incubated with stirring at 40°C for 30 min for mash viscosity reduction. An enzyme pretreatment was not necessary for triticale mashes, because the viscosities of these mashes were as low as 570 Brabender Units (BU). To each mash, 0.005 mL of HT α-amylase preparation was added per g of cereal. After 5 min, the temperature was raised to about 96°C, and held at this temperature for 45 min, with continuous agitation at ~200 rpm. The temperature was then lowered to 80°C. The gelatinized starch was liquefied further by the addition of another 0.005 mL of HT α-amylase per g of cereal, and incubated at 80°C for 30 min. The weight lost because of evaporation was made up by the addition of sterile dH_2O. The rye mashes were again treated at 40°C for 30 min with the viscosity-reducing enzyme, Roxazyme G, at a level of 0.0002 g enzyme/g of ground cereal mashed (as-is basis). Four hundred g of mash were then transferred to each jacketed Celstir fermenter (Wheaton Instruments, Millville, NJ). The mashes were cooled to 4°C by connecting fermenters to a D3-G water bath circulator (Haake, Saddle Brook, NJ). Then, 40 μL of the chemical sterilant, diethyl pyrocarbonate (DEPC); (Sigma), was added to each fermenter, and the jars were put in a refrigerator at 4°C for 68 h before fermentation. Under such conditions, DEPC completely decomposes, and fermenters are then ready for saccharification and fermentation.

Viscosity Measurements

In separate experiments, the viscosities of these mashes were measured with a starch tester (Model AV 30, Haake) at 40°C, and the relative viscosity expressed in Brabender Units (BUs).

Fermentation

The fermenters were connected to a D3-G water bath circulator (Haake), and equilibrated to 30°C for 15 min. Either 5 mL of sterile dH_2O, or 5 mL of filter-sterilized urea solution, a common and effective yeast food, were added to each fermenter to provide a final concentration of 0 or 16 m*M*, respectively. Then, 0.008 mL of Allcoholase II preparation was added per g of cereal mashed to saccharify the dextrins to fermentable sugars. After 30 min, the temperature of the fermenter was lowered and maintained at 20°C throughout the fermentation, and the fermenter was inoculated with 6 mL of active dry yeast inocula (inoculated at approx 5×10^7 viable cells/g of dissolved solids). The fermentations were monitored until all fermentable sugars were consumed.

Preparation of Inocula

Eleven grams of active dry yeast were dispersed in 99 mL of 0.1% sterile peptone water, prewarmed at 38°C. The yeast solution prepared contained approx 2×10^9 viable cells/mL. After 20 min at 38°C, the required amount of inoculum was added to each fermenter.

Fermentation Progress and Analyses

Progress of fermentation was monitored by measuring a number of fermentation parameters at 0, 12, and 24h, and every 24 h thereafter, until all dissolved solids were consumed. Total yeast cell counts and viable counts were determined by the direct microscopic method at a magnification of ×400, with an improved Neubauer counting chamber. One part of mash was diluted with 9 parts of methylene blue stain. Living cells (clear) were distinguished from dead cells, which stained blue *(4)*. Other samples collected at various times were centrifuged (10,300*g*) at 4°C for 15 min, and the clear supernatant liquids were measured for pH. The content of dissolved solids, expressed as g sugar/100 mL fermenting liquid, was measured at 20°C with a digital density meter (DMA-45, Anton Paar, Graz, Austria). Total free amino nitrogen (FAN) in the supernatant liquid was determined by the ninhydrin method of the European Brewery Convention *(18)*. Ethanol was measured by the alcohol dehydrogenase assay (Sigma Bulletin no. 331 U.V., Sigma). Glycerol and sugars (maltotriose, maltose, and glucose) were measured, and dextrins estimated, by high-performance liquid chromatography (HPLC). Clear supernatant liquids obtained by centrifugation were diluted with dH_2O and injected into a column (FAM-PAK, Waters Chromatographic Division, 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 with a differential refractometer (model 410, Waters), and quantified with a computer program (Maxima, Waters). The elution rate was 1 mL/min, and methanol was used as internal standard. Clear supernatant liquids obtained by centrifugation of mashes (10,300*g* for 15 min) prior to glucoamylase saccharification and yeast inoculation were used for the determination of free amino acids. 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 441, Waters).

For the determination of ethanol yield and fermentation efficiencies, parallel duplicate fermentations without sampling were set up with a known quantity of grain. The ethanol in these mashes was distilled at the end of fermentation. Fermentation efficiencies were calculated as ratios between actual alcohol yield (weight basis) and theoretical yield of ethanol from a given weight of starch. Starch in the grains used was determined directly by the method of Budke *(19)* and used for the above calculations of theoretical yield, assuming conversion of starch to glucose (factor of 1.11), and conversion of glucose to ethanol (factor of 0.51).

Fermentation stillages were analyzed by the standard AACC *(20)* procedures for moisture (Method 44-15A), crude protein (Method 46-13), crude fat (Method 30-25), and ash (Method 08-03), and by the AOAC *(21)* method for total dietary fiber (TDF) (Method 991-43). Protein contents of the stillages were calculated using the nitrogen:protein ratio of 5.7, based on the recommendation of Sosulski and Imafidon *(22)*.

All analyses, except for mash viscosity, were conducted in duplicate, and results are presented as the mean of duplicate analyses.

RESULTS AND DISCUSSION

Viscosities of Mashes

At the end of mashing, the viscosity of triticale mash, prepared with a water:grain ratio of 2:1 by weight, was 570 BU, when measured at 40°C. Enzyme pretreatment was considered unnecessary for viscosity reduction of triticale mashes. A mash viscosity in the 500 BU range was considered low enough to avoid problems in stirring during mashing, and mash lifting during fermentation. However, the postmashing viscosity of rye mash was 1980 BU, which implied that treatments of mash with viscosity-reducing enzyme were necessary in order to reduce stirring and foaming problems. The enzyme treatment of rye mash was carried out in two stages. The first stage was intended to reduce viscosity of rye-water slurry prior to mashing by pretreating with a viscosity-reducing enzyme, Roxazyme G, at 40°C for 30 min, at a level of 0.0002 g of the enzyme/g of rye (as-is basis). Following mashing, the mash viscosity of 785 BU was high enough to warrant a second enzyme treatment. The second stage involved a treatment with Roxazyme G at 40°C for 30 min, at a level of 0.0002 g of the enzyme/g of rye. Mash viscosity was then reduced from 785 to 300 BU in 4 min, and from 785 to 200 BU in 24 min. The initial viscosity reduction rate was 285 BU/min. It was found that Roxazyme G promoted a greater mash viscosity reduction when it was added after mashing was completed. Nonstarch carbohydrates appear to be more susceptible to Roxazyme G attack after they are subjected to heat treatment during mashing.

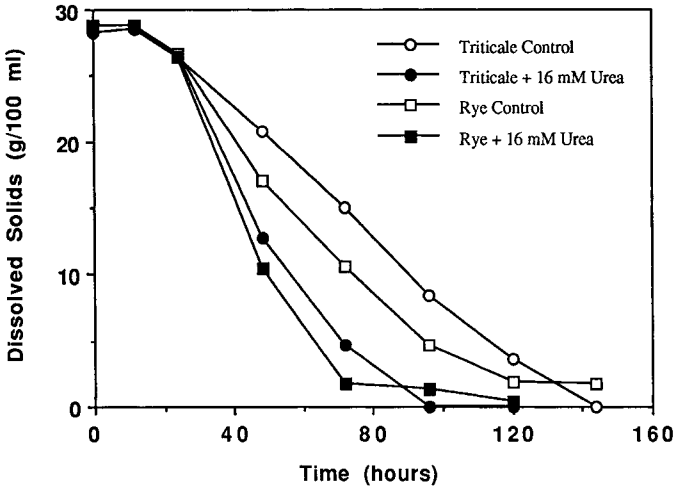

Fig. 1. Rates of utilization of dissolved solids in VHG rye and triticale mashes.

Rates of Fermentation

Mashes were fermented with and without the 16 m*M* urea nitrogenous supplement. It was found that fermentations containing as much as 28.5 g dissolved solids/100 mL of mash supernatant could be completed in 120 h (rye) to 144 h (triticale) without urea supplementation (Fig. 1). Rye fermented faster than triticale because of a higher level of FAN in rye. The supplementation of mash with urea at 16 m*M* accelerated sugar-consumption rates (the rate of fermentation), and reduced fermentation time by about 48 h for both triticale and rye. The fermentation rates were similar in rye and triticale mashes when both were supplemented with urea. This work reinforces the findings from this laboratory, that virtually all grain extracts are deficient in yeast-usable nitrogen. About 0.4–1.7 g/100 mL of dissolved solids remained unused during rye fermentations. The rye mash contained a higher amount of nonsugar components that were not fermentable. Longer times were required for the completion of the high-gravity fermentations. This was mostly because of higher sugars content (28.5 g/100 mL), and the lower fermentation temperature (20°C) used. Fermentations at this gravity are possible at 27°C *(6)*, but work was done at 20°C, to avoid any possibility of ethanol toxicity at elevated temperatures.

Carbohydrate Composition, and Glycerol and Ethanol Concentrations of Mashes

HPLC analyses illustrated that, at the beginning of fermentation, the fermenting rye and triticale mashes were composed of dextrins (estimated

at 6.7–8.0 g/100 mL), maltose (5.3–8.1 g/100 mL), and glucose (12.5–16.2 g/100 mL) (Tables 1–4). The glucose content at inoculation accounted for about 50% of total saccharified carbohydrates. Such high concentrations of glucose were caused by high initial dissolved solids content and the 50 min of glucoamylase saccharification prior to yeast inoculation, of which 30 min were used for saccharification at 30°C, and 20 min were used for cooling of the fermenter from 30°C to the fermentation temperature of 20°C before inoculation. As the fermentations continued, dextrins were gradually hydrolyzed to low-mol-wt sugars, which were then consumed by the yeast to produce ethanol, yeast cells, and smaller amounts of related fermentation byproducts, such as glycerol and organic acids. Consequently, reductions in dextrin and maltose concentrations were observed. Maltotriose, one of the hydrolysis products of dextrin, emerged in midfermentation, as did a peak concentration of glucose at 24 h because of hydrolysis of dextrins to low-mol-wt sugars, which were then consumed. The rates of glucose consumption were faster after 24 h in urea-supplemented mashes. However, the rates of dextrins and maltose consumption were similar regardless of urea supplementation. This may be explained by the already high glucose content in the media to be used by the yeast. The high glucose content may limit the rate of hydrolysis of dextrins and maltose. Relatively higher amounts of dextrins and maltose were observed at the end of rye fermentations. However, mash-dissolved solids include, in addition to dextrins or maltose, substances that are not fermentable. Also, reduced glucoamylase activity toward the end of fermentation may leave a certain amount of unhydrolyzed dextrins. Glycerol concentration increased to 0.7–1.0 g/100 mL level toward the end of fermentation, which represented 3.1–3.4% (w/w) of total carbohydrate used. Ethanol concentrations in fermenting liquids ranged from 13–15 mL/100 mL of mash liquid at the end of fermentations.

Yeast Cell Numbers and Viabilities

Fermentations were inoculated at about 50 million cells/g of mash. The total cell number increased 4–8-fold within the first 48 h (Fig. 2). With urea supplementation, the rates of cell multiplication were faster, and the final cell numbers were much higher. This led to increased catalytic activity, and therefore faster fermentation rates. After 48 h, cell numbers started to decline slightly. In an earlier report *(12)*, it was found that the increase in cell biomass was much higher (10–15-fold) in normal-gravity mashes than in the VHG fermentations reported here. Limited amounts of nutrients in these mashes will allow only the growth of a specific number of cells, regardless of the number of cells added at inoculation. It was also found that cells after inoculation were ~75% viable, as assessed with

Table 1
Carbohydrate Composition, and Glycerol and Ethanol Concentrations in Triticale Fermentations Without Urea Supplementation

Hours	Dextrins[a] (g/100 ml)	Maltotriose (g/100 ml)	Maltose (g/100 ml)	Glucose (g/100 ml)	Glycerol (g/100 ml)	Ethanol[b] (ml/100 ml)
0	6.67±0.04	0	7.62±0.08	16.25±0.14	0	0
12	5.13±0.11	0.09±0.01	1.63±0.01	22.16±0.25	0	0.14±0.03
24	2.38±0.05	1.11±0.04	0.31±0.01	22.52±0.52	0.10±0.00	1.07±0.00
48	1.67±0.04	1.13±0.04	0.59±0.07	17.72±0.33	0.42±0.01	4.14±0.04
72	1.57±0.04	0.75±0.03	0.74±0.08	13.14±0.44	0.68±0.01	7.22±0.06
96	1.44±0.07	0.34±0.02	0.70±0.10	7.78±0.27	0.86±0.04	10.28±0.43
120	1.52±0.06	0	0.92±0.02	4.33±0.80	1.01±0.13	12.87±0.18
144	1.42±0.01	0	0.73±0.00	0.02±0.00	0.97±0.02	14.75±0.16

[a]Dextrins include maltotetraose, maltopentaose, and other soluble saccharides of higher mol wt.

[b]Ethanol concentration was expressed as mL ethanol/100 mL fermenting supernatant. Concentration in w/v can be calculated by dividing by 0.789.

Table 2
Carbohydrate Composition, and Glycerol and Ethanol Concentrations in Triticale Fermentations Supplemented with 16 m*M* Urea

Hours	Dextrins[a] (g/100 ml)	Maltotriose (g/100 ml)	Maltose (g/100 ml)	Glucose (g/100 ml)	Glycerol (g/100 ml)	Ethanol[b] (ml/100 ml)
0	8.02±0.38	0	8.09±0.28	15.39±0.58	0	0
12	5.54±0.12	0	2.20±0.10	20.75±0.56	0.02±0.00	0.14±0.01
24	2.61±0.09	1.36±0.04	0.56±0.09	21.00±0.42	0.10±0.00	1.03±0.01
48	1.57±0.04	0.92±0.01	0.57±0.02	11.01±0.12	0.63±0.02	7.81±0.25
72	1.40±0.01	0.32±0.02	0.58±0.08	4.78±0.16	0.93±0.01	12.27±0.23
96	1.37±0.12	0	0.56±0.04	0.03±0.01	0.88±0.05	14.75±0.00
120	1.46±0.05	0	0.56±0.02	0.08±0.01	0.94±0.02	15.08±0.28

[a]Dextrins include maltotetraose, maltopentaose, and other soluble saccharides of higher mol wt.

[b]Ethanol concentration was expressed as mL ethanol/100 mL fermenting supernatant. Concentration in w/v can be calculated by dividing by 0.789.

Table 3
Carbohydrate Composition, and Glycerol and Ethanol Concentrations in Rye Fermentations Without Urea Supplementation

Hours	Dextrins[a] (g/100 ml)	Maltotriose (g/100 ml)	Maltose (g/100 ml)	Glucose (g/100 ml)	Glycerol (g/100 ml)	Ethanol[b] (ml/100 ml)
0	7.95±0.34	0	5.77±0.19	12.55±0.68	0	0
12	6.45±0.35	0	1.61±0.13	17.10±0.45	0.03±0.00	0.11±0.00
24	3.56±0.04	1.42±0.02	0.49±0.01	17.37±0.02	0.08±0.00	1.11±0.00
48	3.09±0.10	1.72±0.03	0.30±0.00	12.62±0.38	0.48±0.03	6.10±0.20
72	2.98±0.07	0.16±0.01	0.65±0.04	6.84±0.28	0.57±0.01	8.40±0.09
96	2.85±0.02	0.03±0.00	0.78±0.01	1.81±0.01	0.70±0.01	11.84±0.08
120	2.54±0.06	0	0.77±0.02	0.14±0.02	0.70±0.01	12.85±0.21
144	2.39±0.02	0	0.85±0.02	0.00±0.00	0.74±0.01	12.91±0.08

[a]Dextrins include maltotetraose, maltopentaose, and other soluble saccharides of higher mol wt.

[b]Ethanol concentration was expressed as mL ethanol/100 mL of fermenting supernatant. Concentration in w/v can be calculated by dividing by 0.789.

Table 4
Carbohydrate Composition, and Glycerol and Ethanol Concentrations in Rye Fermentation Supplemented with 16 m*M* Urea

Hours	Dextrins[a] (g/100 ml)	Maltotriose (g/100 ml)	Maltose (g/100 ml)	Glucose (g/100 ml)	Glycerol (g/100 ml)	Ethanol[b] (ml/100 ml)
0	7.30±0.02	0	5.33±0.09	13.86±0.13	0	0
12	6.48±0.05	0.15±0.00	1.02±0.02	19.97±0.06	0.03±0.00	0.17±0.05
24	3.90±0.10	1.86±0.06	0.28±0.03	19.73±0.29	0.10±0.00	1.31±0.03
48	2.96±0.05	1.41±0.04	0.27±0.00	7.44±0.08	0.62±0.00	9.21±0.06
72	3.05±0.04	0	0.66±0.00	0.24±0.04	0.80±0.02	13.58±0.25
96	2.93±0.30	0	0.66±0.02	0.12±0.06	0.78±0.03	13.80±0.18
120	2.54±0.04	0	0.65±0.00	0.04±0.00	0.72±0.00	14.33±0.41

[a]Dextrins include maltotetraose, maltopentaose, and other soluble saccharides of higher mol wt.

[b]Ethanol concentration was expressed as mL ethanol/100 mL fermenting supernatant. Concentration in w/v can be calculated by dividing by 0.789.

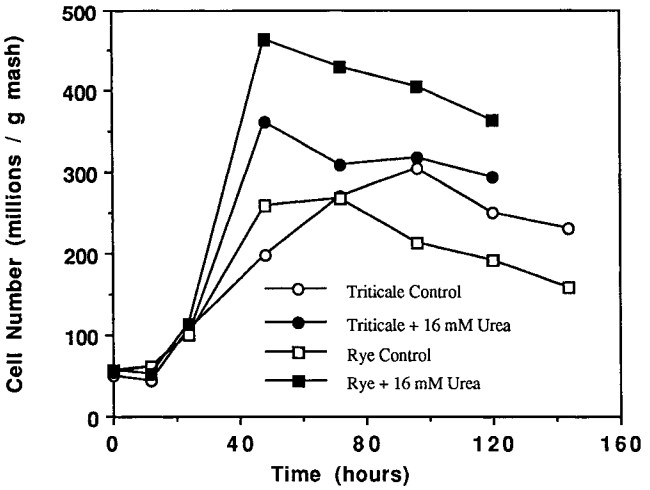

Fig. 2. Yeast cell numbers during VHG rye and triticale fermentations.

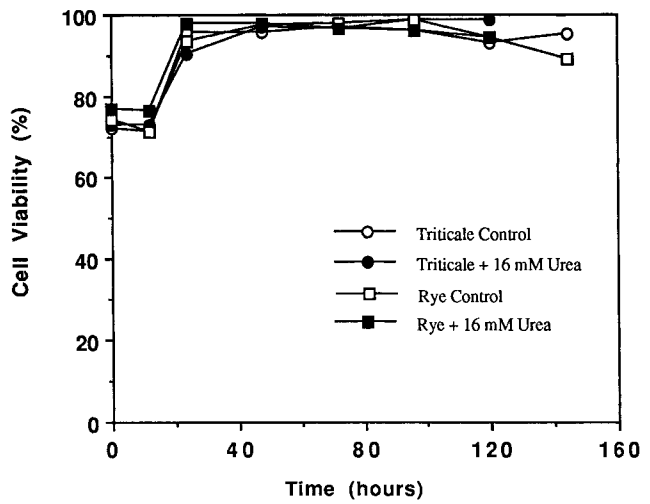

Fig. 3. Yeast cell viability during VHG rye and triticale fermentations.

methylene blue dye (Fig. 3). Such initial low viabilities could be explained by high osmotic pressures in the fermentation media, because of high glucose concentrations that inhibited or adversely affected cell growth *(2)*. On a weight basis, the osmotic effect exerted by glucose is greater than that offered by disaccharides and higher-mol-wt sugars, such as dextrins *(23)*. This may lead to some damage to cell membranes, but nutrient limitation could also have contributed to lower cell viabilities. In the current study, a 50-min glucoamylase saccharification prior to yeast inoculation (which yielded high amounts of glucose) may not be beneficial to maintenance of high initial rates of cell viability. Instead, simultaneous saccharification and yeast inoculation might benefit the yeast in these VHG

fermentations. Nevertheless, cell viabilities increased to 90–97% within the first 24 h (Fig. 3), and viabilities remained relatively constant, or only decreased slightly, during the rest of the fermentation. Supplementation with urea did not significantly effect cell viability.

pH Values of Fermenting Mashes

The pH values of fermenting mashes decreased from an initial 5.5–5.6 to 4.7–4.9 toward the end of fermentation, as acidic products were produced during fermentations. The rates of pH decrease were similar regardless of the grain used, or urea supplementation. The final pH values around 4.7–4.9, however, indicated that no major contamination occurred during these laboratory fermentations; pH values below 4.0 normally indicate severe contamination with lactic acid bacteria. In single fermentations without yeast recycle, contamination is never seen, despite the use of active dry yeast, which will contain a small but significant bacterial content. The final pH values of VHG fermentations were about 0.5 units higher than the final pH values seen in normal-gravity fermentations *(12)*. Theoretically, the concentrations of acidic byproducts produced would be relatively higher in the VHG fermentations, because less water was present in the media. However, higher concentrations of mash proteins are also present, which have strong buffering capacity and tend to resist the change in medium pH values.

Free Amino Nitrogen and Free Amino Acid Concentrations

Rye mashes had FAN contents of 160 mg/L, which was significantly higher than that of triticale at 100 mg/L. The higher FAN level in rye explains the faster fermentation rates because of the effect of the extra nitrogen on yeast growth. It was also found that most FAN uptake occurred within the first 24 h for both rye and triticale (Fig. 4). During the first 12 h of fermentation, the levels of FAN were virtually unchanged, because virtually no cell growth occurred within this period. Almost all growth and all FAN uptake took place between 12 and 36 h. After 24 h, the FAN contents did not change for triticale, and decreased only slightly for rye, indicating that usable FAN was exhausted. This is the case in all grain mashes tested in this laboratory. Near the end of fermentation, there was slight increase in the FAN content, especially when urea was supplemented. This properly resulted from partial autolysis of yeast cells, the release of peptidase enzyme from stressed yeast, or the excretion of FAN from yeasts because of protein degradation inside the cell.

The FAN contents reflected the general levels of usable nitrogen, but it is also important to examine levels of individual free amino acids, the main sources of nitrogen in unsupplemented mashes. Experimental results

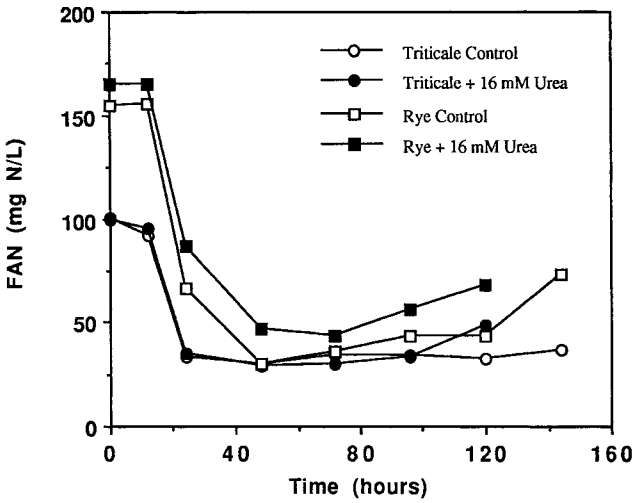

Fig. 4. Changes in FAN during VHG fermentation of triticale and rye mashes.

obtained from this laboratory, and surveys made by other researchers, have shown that not all amino acids are used effectively by *Saccharomyces* yeasts *(4,24,25)*. The Schultz and Pomper survey *(26)* showed that amino acids, such as alanine, arginine, asparagine, aspartic acid, glutamic acid, leucine, and valine, promote good growth of yeast when used as sole nitrogen sources. Although isoleucine, methionine, phenylalanine, serine, and tryptophan allowed moderate-to-good growth, cystine, glycine, histidine, lysine, proline, and threonine were poor sources of nitrogen. Thomas and Ingledew *(25)* added amino acids individually to wheat mashes, and measured their effects on fermentation rates. In descending order with respect to shortening fermentation time from 57 to 13%, the amino acid arginine was the most effective in promoting fermentation, followed by leucine, valine, phenylalanine, aspartic acid, threonine, glutamic acid, serine, cysteine, methionine, tyrosine, alanine, isoleucine, and histidine. Glycine and lysine were found to delay fermentation time by 20 and 52%, respectively. The results obtained from Thomas and Ingledew *(25)* were basically in accordance with the early survey made by Schultz and Pomper *(26)* especially in identifying the right kind of amino acids that promote yeast growth and fermentation rate. It is rather difficult to describe the ability of each individual free amino acid to promote growth and fermentation because of variations in media used, the profile of amino acids present, and their amounts relative to each other. These experimental results, however, provided valuable information, which was especially helpful in determining and comparing the nutritional profile of fermentation media.

Analysis on free-amino-acid contents, expressed in μmol/mL in rye and triticale mashes, both after mashing and prior to glucoamylase sac-

Table 5
Free Amino Acid Content (μmol/mL of Mash Liquid) in Rye and Triticale Mashes[a] After Mashing, but Prior to Glucoamylase Saccharification and Yeast Inoculation

Abbreviation	Component	Rye Mash	Triticale Mash	Rye/Triticale
ASP	Aspartic Acid	1.511±0.215	0.536±0.138	2.82
GLU	Glutamic Acid	0.658±0.172	0.696±0.099	0.94
SER	Serine	0.339±0.106	0.155±0.044	2.18
ASN	Asparagine	1.916±0.264	0.803±0.088	2.37
GLN	Glutamine	0.230±0.032	0.114±0.004	2.02
GLY	Glycine	0.092±0.033	0.101±0.011	0.91
HIS	Histidine	0.439±0.134	0.261±0.013	1.68
THR	Threonine	0.159±0.075	0.138±0.131	1.15
ALA	Alanine	0.900±0.133	0.410±0.015	2.20
ARG	Arginine	0.522±0.115	0.274±0.061	1.90
PRO	Proline	0.371±0.042	0.374±0.079	0.99
TYR	Tyrosine	0.458±0.092	0.468±0.067	0.98
VAL	Valine	0.239±0.007	0.150±0.003	1.59
MET	Methionine	0.041±0.002	0.018±0.001	2.28
CYS_2	Cystine	0.032±0.027	0.049±0.017	0.65
ILE	Isoleucine	0.104±0.000	0.066±0.001	1.58
LEU	Leucine	0.188±0.003	0.106±0.002	1.77
PHE	Phenylanlanine	0.122±0.004	0.083±0.004	1.47
ORN	Orninthine	0.168±0.006	0.215±0.001	0.78
LYS	Lysine	0.120±0.042	0.061±0.003	1.97
TOTAL		8.609	5.078	1.70

[a]Mashes were prepared with water:grain ratio 2:1, by weight.

charification and yeast inoculation, showed that rye mashes had a higher overall free-amino-acid content (8.61 μmol/mL of mash liquid) than those of triticale mashes (5.08 μmol/mL of mash liquid) (Table 5). These results were proportional to the levels of FAN presented previously, although expression in moles for each amino acid does not quantify the total moles of nitrogen obtained by FAN analysis. The concentrations of the right free amino acids, which promote cell growth and stimulate fermentation rate, were significantly higher in rye mashes, compared to those in triticale mashes. The fermentation-promoting free amino acids in rye mashes, such as arginine, alanine, asparagine, aspartic acid, leucine, and valine, were present at 1.6–2.8× those found in triticale mashes; the levels of glutamic

Table 6
Ethanol Yields and Fermentation Efficiencies

Cereal Grains	Ethanol Yields (L/Tonne)		Fermentation Efficiencies[a]
	Dry Basis	14% Moisture	(% theoretical)
Triticale Control	435±1	374±1	93.0±0.1
Triticale+16 mM Urea	417±14	359±12	89.4±3.1
Rye Control	409±9	352±8	90.1±2.1
Rye+16 mM Urea	409±3	352±3	90.1±0.9

[a]Fermentation efficiencies were calculated as ratios between actual ethanol yield and theoretical ethanol yield, assuming all starch was converted to glucose, and then to ethanol.

acid were about the same in both mashes. Poor or inhibitory amino acids, such as cystine and glycine, were in relatively low concentrations in both mashes. Although levels of inhibitory lysine and histidine in rye mash were 1.7–2.0× those in triticale mash, the amounts of other promoting amino acids were high enough to minimize any inhibitory effect *(25)*. Other poor amino acids, such as proline and threonine, were found in about the same levels in both rye and triticale mashes. Based on these results, it was concluded that rye had a better profile of free amino acids than triticale, and this finding helped to explain its faster sugar consumption and fermentation rate.

Ethanol Yields, Fermentation Efficiencies, and Ethanol Concentrations

In fermentations conducted for yield and efficiency calculations (no sampling was carried out to maintain accuracy), the alcoholic mashes were distilled at the end of fermentation. The weights of alcohol obtained from a known weight of grain starch were determined, and compared to calculated theoretical values. The ethanol yields were 409 L/tonne of rye grain (dry basis), and 417–435 L/tonne of triticale grain (dry basis) (Table 6). Supplementation with urea did not affect fermentation efficiencies of rye, which were about 90.1%; however, urea addition appeared to cause a reduction in triticale fermentation efficiency from 93.0 to 89.4%. This could be caused by an increased growth of yeast, resulting in additional consumption of sugars for biomass production. Both the ethanol yields and fermentation efficiencies obtained by the high-gravity fermentations were close to those obtained by normal-gravity fermentations *(12)*.

Table 7
Chemical Composition of Fermentation Stillages (w/w, Dry Basis)

Fermentation Stillages	Starch (%)	Ash (%)	Fat (%)	Crude Protein[a] (%)	TDF (%)
Rye, No Urea	1.52±0.08	5.66±0.01	3.32±0.01	27.72±0.20	28.24±0.72
Rye + 16mM Urea	1.47±0.09	5.42±0.01	3.24±0.13	29.27±0.09	27.89±0.18
Triticale, No Urea	1.67±0.51	5.04±0.03	3.93±0.06	34.54±0.71	32.61±1.03
Triticale + 16mM Urea	0.86±0.24	4.88±0.07	4.22±0.13	35.90±0.16	33.66±0.26

[a]N (Kjedahl) × 5.7 (22)

For a 28.5 g dissolved solids/100 mL mash, VHG fermentation, the ethanol concentrations in the beer were found to be 12.9–14.3% (v/v) for rye, and 14.7–15.1% (v/v) for triticale, respectively. A 35–56% increase in ethanol concentration was seen, when compared to similar volumes of normal-gravity fermentations *(12)*. The corresponding reduction in water use (by one-third) in the case of high-gravity fermentation would lead to savings in energy consumption.

Chemical Compositions of Stillages

Stillage samples (spent grain) from VHG rye and triticale fermentation contained mostly protein and total dietary fiber (TDF) (Table 7). Protein and TDF each accounted for about 28%, on a dry basis, of rye stillages; triticale stillages were composed of about 33% protein and 33% TDF, on a dry basis. The remaining constituents of each stillage were ash, fat, residual recalcitrant carbohydrates, yeast cells, and nonvolatile fermentation byproducts. The fact that stillages from rye were lower in protein content was a result of the initial lower protein content in rye grain. Lower-than-expected TDF content in rye stillages was the result of viscosity-reducing enzyme treatments on rye mashes which partially hydrolyzed pentosans prior to fermentation. Supplementation with urea during fermentation did not alter the chemical composition of the stillages, except for the increased protein from yeast. Stillages obtained from a single grain in high-gravity fermentation were similar to those obtained from the same grain at normal gravity, and would be of similar benefit nutritionally as animal feeds.

CONCLUSIONS

Studies on VHG triticale fermentations carried out by reducing water: grain ratios from 3:1 to 2:1 showed that a 28.5 g dissolved solids/100 mL

fermentation could be completed within 144 h. Fermentation time was reduced from 144 to 96 h, when the triticale mashes were supplemented with urea to a final level of 16 m*M*. VHG rye fermentations were completed within 120 h with urea present. However, about 0.4–1.7 g/100 mL of residual dissolved solids remained, a portion of which could be soluble pentosans, or nonfermentable sugars and dextrins, which were unused during fermentation. The fact that longer times were required for the completion of VHG fermentations, compared to normal-gravity mashes, was, in fact, related to their higher sugar contents (28.5 g/100 mL) and the lower fermentation temperature (20°C) used. Although it may not be necessary to ferment at 20°C *(6)*, temperatures above 27°C will progressively lead to problems, because ethanol levels, in combination with high temperatures, lead to injury of the yeast *(27,28)*. It is suggested that the time required for completion could be reduced (for wheat data, *see* ref. *6*). A further reduction in time is also anticipated as yeast addition levels are increased. Reduction in water:grain ratios from 3:1 to 2:1 led to an increase in sugar content by one-third but did not affect fermentation efficiencies, or final ethanol yields per unit weight of grain fermented. The fact that fermentation efficiencies achieved were in the range of 90–93% for both rye and triticale, regardless of normal- or high-gravity fermentation conditions, showed the potential for rye and triticale to replace wheat as basic raw materials for fuel alcohol production. The increase in sugar content in the fermentation feedstock by one-third was realized simply by reducing water use by one-third. This resulted not only in an increase in ethanol concentration in the fermentation vessel (operating at constant volume) by 35–56%, but would lead to substantial savings in water usage, and in energy input for mash heating, mash cooling, and ethanol distillation.

It was also noted that a water:grain ratio of 2:1 appeared to be close to the limit that could be applied in this industry. Further reduction in water:grain ratio could result in incomplete starch liquifaction and problems caused by high mash viscosity. To further raise the sugar content, it would be necessary to use postmashing separation, a double mashing method, an addition of a high sugar adjunct *(1)*, or to reduce the amount of mash insolubles by grain preprocessing *(29)*.

Because of their less expensive costs per kg starch, rye and triticale would appear to be economic as replacements for wheat as raw materials for fuel alcohol production. Rye provides a higher nutritional level for yeast and a better free-amino-acid profile, which explains its faster fermentation rate. Although high mash viscosity could be a problem, these mashes can be treated effectively with viscosity-reducing enzyme(s). Meanwhile, triticale is attractive especially for its low mash viscosity. After supplementation with external nitrogenous sources, such as urea, triticale fermented as fast as rye.

This work also indicates that stillage obtained from different grains should not be expected to be equal as animal feeds. In addition, mash pretreatments of grains during mashing and fermentations may affect greatly the final nutritional value of such stillages.

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