

# Viscosity Concerns with Rye Mashers Used for Ethanol Production

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## ABSTRACT

Cereal Chem. 76(3):459–464

Rye cultivars leading to high-viscosity extracts were easily mashed—even under very high gravity (VHG) conditions—when enzymes were added to reduce viscosity caused by pentosans. The enzymes were so effective that for the fuel alcohol industry, the need for special genetic selections of rye with reduced extract viscosity was not indicated. High-starch cultivars of

rye, however, were still most beneficial when alcohol was the end product of value. Both normal and VHG mashers fermented out within 48 hr under the conditions described. Yields (L/t of starch) were, on average, 5.3% higher under VHG conditions than under normal gravity.

Rye is used in Canada for the production of potable alcohol, and occasionally as an adjunct in fuel alcohol manufacture. It is comparable to wheat in starch content and is relatively inexpensive. However, it is not often employed as a fermentation substrate because of its high levels of soluble, high molecular weight pentosans which lead to development of viscosity during mashing. High viscosity reduces throughput and leads to increased production costs. In recent years, for economic reasons, fuel alcohol manufacturers have considered alternate substrates such as rye, and raised carbohydrate concentrations in mashers. Resultant viscosity problems must therefore be addressed.

There are two general types of fermentation technology available for industrial-scale production of fuel alcohol from grain (Thomas et al 1996). In normal gravity fermentations, a grain-to-water ratio of  $\approx 1:3$  is used and mashers with 20–25 g of dissolved solids per 100 mL of the liquid portion of the mash result. Approximately 10% alcohol (v/v) is made, but it may be necessary to enzymatically hydrolyze viscosity-causing polymers (such as  $\beta$ -glucans, pentosans, celluloses, and proteins) before the mashing of ground grain-water slurries. In very high gravity (VHG) fermentation technology, mashers as high as 38 g of dissolved solids per 100 mL can be fermented to yield 23% (v/v) ethanol (at least in the laboratory). The maximum concentration of ethanol produced in commercial plants is  $\approx 14\%$  (v/v), indicating the use of a solids content (fermentable carbohydrate) midway between normal and VHG conditions. The strategies used for preparation of VHG mashers include an increased grain-to-water ratio, the use of enzymes to lower mash viscosity, and partial or complete removal of insoluble solids that do not benefit the fermentation but occupy significant volume.

The amount of ethanol attainable from a given weight of grain remains virtually the same regardless of which technology is used because ethanol yield is a function of the amount of starch and fermentable sugar in the original feedstock. Application of VHG technology, however, reduces the cost of production by increasing plant throughput; decreasing water usage, cooling, heating, and labor costs per liter of alcohol; and reducing energy input at the still—all due to an increased concentration of ethanol in the fermented mash and, where possible, removal of solids before fermentation (Ingledew 1993, 1995).

Plant breeding studies have differentiated cultivars and selections of rye on the basis of extract viscosity, which is an indirect measure of soluble pentosan concentration and molecular weight. However, in the fuel alcohol industry, it is not always possible to

select and use high-starch grain cultivars, let alone specially selected grains like rye with minimal levels of (in this case) pentosans. We wished to determine whether it was advantageous to select low pentosan rye cultivars for fuel alcohol production. The detailed objectives of this study were to determine the amount of starch in each rye sample selected, to assess ethanol production in relation to the theoretical yield; to develop methods to prepare normal gravity and VHG rye mashers with reduced viscosities; and to optimize fermentation conditions.

## MATERIALS AND METHODS

### Rye Selections

Samples of  $\approx 20$  kg were collected and cleaned with an air cleaner to remove any straw or chaf, and hand-picked to remove other contamination. Whole grain samples were equilibrated to  $\approx 100$  g of moisture / kg of grain by placing them in a controlled-environment room (22°C and 40% rh) for one week. Grain was ground using a cyclone mill (Udy Corp., Fort Collins, CO) fitted with a 0.5-mm screen. Samples were reequilibrated in the controlled-environment room for another week after grinding.

Ground samples (200 mg) were mixed with 1.0 mL of 1.0M Na-acetate buffer (pH 5.0) and were incubated for 30 min at 40°C on an Eppendorf thermomixer (model 5436, Hamburg, Germany). The slurries were centrifuged (10 min at 12,000  $\times$  g), the supernatant was decanted, and extract viscosity (in centipoise units) was determined at 30°C using a Brookfield cone-plate viscometer (model LVDV-II+, Brookfield Engineering Laboratories, Stoughton, MA).

### Starch

Starch contents were assessed directly on a dry basis by the method of Chiang and Johnson (1977) with slight modification. Starch analysis relies on the instant gelatinization of ground grain starch by NaOH followed by pH adjustment and complete hydrolysis of gelatinized starch with excess glucoamylase enzyme ( $\alpha$ -1,4 and  $\alpha$ -1,6 activity) to glucose. The starch-derived glucose is then measured against a glucose standard curve using hexokinase and glucose-6-P dehydrogenase enzymes in a spectrophotometric assay using the Sigma Diagnostics (St. Louis MO) glucose (HK) 10 kit.

### Mashing

The mashing process was conducted as described previously for wheat, hullless barley, oats, hullless oats, barley, rye, and triticale (Thomas and Ingledew 1990, 1992, 1995; Thomas et al 1993, 1995; Jones and Ingledew 1994a–c; Jones et al 1995; Ingledew et al 1995; Wang et al 1998). Fermentations were monitored for sugar consumption, total yeast cell number and yeast viability, bacterial contamination, free amino nitrogen (FAN), ethanol, and viscosity. All fermentations were performed with added yeast food (urea). A total of 14 fermentations (seven rye cultivars in duplicate) were performed at normal gravities. An additional 14 fermentations were performed to examine the feasibility of VHG rye fermentations and determine whether or not viscosity reduction through

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application of enzymes would permit fermentation of mashes with larger amounts of sugars. A coincident requirement was the determination of ethanol yield from specified amounts of the rye samples. This necessitated additional fermentations to be performed on normal and VHG mashes prepared from all seven cultivars of rye samples. These latter fermentations were conducted in duplicate with nutrient supplementation until all sugars were consumed. No samples were withdrawn during fermentation. The amount of ethanol obtained by distillation was related to the amount of grain used (its starch content measured by the direct method). Yield and efficiency were then determined.

Initially, the seven rye samples were mashed at a 1:3 grain-to-water ratio (a typical ratio in industry), in the absence of viscosity-reducing enzymes, and their viscosities were measured using an amylograph and reported in Brabender units (BU). The effect of viscosity-reducing enzymes was assessed after mashing.

To make normal gravity mashes, 700 g of ground grain (setting #5 in an S500 disk mill, Glen Mills, Inc., Clifton, NJ) was added to 2,100 mL of distilled water at 45°C. When desired, Roxazyme G or Novo 348 enzyme was added and the grain-water slurry was held at 45°C for 30 min. Roxazyme G (Hoffman-La Roche, Mississauga ON), a fungal enzyme from *Trichoderma viride* that contains cellulase activity (8,000 IU/g) and xylanase activity (43,350 IU/g), was added at 560 mg/2,800 g of mash. Novo 348 enzyme (Novo Nordisk, Franklinton, NC) is produced by *Humicola insolens* and is a complex carbohydrase mixture with  $\beta$ -glucanase (100 fungal  $\beta$ -glucanase activity units/g) and pentosanase activities. The enzyme was added at 700  $\mu$ L/2,800 g of mash. After enzyme pretreatment, mashing was conducted by the normal procedure—the temperature was increased to 60°C, CaCl<sub>2</sub>·2H<sub>2</sub>O (1 mM final concentration) and 3.5 mL of HT  $\alpha$ -amylase (Alltech Biotechnology Center, Nicholasville KY) were added, and the grain-water slurry was held at 60°C for 5 min. The temperature was then raised to 95°C and held at this temperature for 45 min, reduced to 80°C, and an additional 3.5 mL of HT  $\alpha$ -amylase was added. The mash was held at 80°C for 30 min. The mash was then weighed, and sterile distilled water was added to replace water lost through evaporation.

For VHG mash preparation, the grain-to-water ratio for mashing was changed from 1:3 to 1:1.8 to give a dissolved solids content in excess of 31 g/100 mL. A sample of 1,000 g of each ground grain was dispersed in 1,800 mL of distilled water at 45°C. Roxazyme G (0.02% by wt. of grain) was added and the grain-water slurry was held at 45°C for 30 min. After enzyme pretreatment, mashing was continued by the normal procedure. Temperature was increased to 60°C, CaCl<sub>2</sub>·2H<sub>2</sub>O (1 mM final concentration) and 5 mL of HT  $\alpha$ -amylase (Alltech) were added, and the grain-water slurry was held at 60°C for 5 min. The temperature was then raised to 95°C and held for 45 min for gelatinization of the starch. After reducing the temperature to 80°C, an additional 5 mL of HT  $\alpha$ -amylase was added and liquefaction was allowed to proceed for 30 min. The

mash was then cooled to 40°C, and an additional 0.02% (by grain weight) of Roxazyme G was added and allowed to react at 40°C for 30 min. The mash was then weighed and distilled water was added to the mash to replace water lost through evaporation.

### Fermentation

The prepared mashes, which contained insoluble grain components, were aseptically dispensed in 500-g aliquots into sterile fermentors to which filter-sterilized urea had been added to give a final concentration of 8 mM (or 16 mM in VHG conditions). The mash temperature was adjusted to 30°C, and saccharified by adding glucoamylase (Alltech Alcoholase II at 0.8 mL/500 g of mash, or 1.2 mL for VHG) 30 min before yeast inoculation. Preconditioned active dry yeast (11 g) (Alltech Superstart batch 103333) was rehydrated in 99 mL of 0.1% peptone at 38°C for 20 min. Rehydrated ADY (3 mL, 4.5 for VHG) was added per 500 g of mash to give  $\approx 20 \times 10^6$  yeast ( $30 \times 10^6$  for VHG) per gram of mash. The temperature was maintained at 27°C during fermentation. Up to 0.3 mL (0.5 mL for VHG) of sterile antifoam (10% dilution of Corning Emulsion C) was added to reduce foaming and rising of the rye mashes.

### Viscosity Reduction

A 300-g portion of ground rye was added to 600 mL of distilled water at 45°C. Roxazyme G (0.02%, or 0.04% by wt. of grain) was added, and the grain-water slurry was incubated at 45°C for 30 min. After enzyme pretreatment, mashing was performed by the normal procedure with additions scaled down in volume. The mash viscosity value was then determined, and additional Roxazyme G or Novo 348 was added after mashing to further reduce the viscosity.

## RESULTS AND DISCUSSION

The seven samples of rye, chosen for their range in extract viscosity (Table I), were analyzed for moisture, viscosity, crude protein, and starch content. Noteworthy are the fivefold range of extract viscosities and the smaller percentages of protein in the Kodiak and, especially, the Musketeer samples. All cultivars contained 60–62% starch except Kodiak and Musketeer at 63.28 and 67.27% (w/w, dry basis), respectively. Use of Musketeer rye should lead to significant increases in ethanol over other cultivars tested if this is a genetic trait and not an environmental influence due to climatic or soil conditions in the particular region where the crop was grown in 1994.

### Rye Mashed at Normal Gravity

*Viscosity development during normal gravity mashing.* Results show (Table II) that nonenzyme-treated rye mashes prepared from Kodiak AWP 1996 were the least viscous of the seven chosen rye samples. This rye can be mashed, without the aid of enzymes to

TABLE I  
Analysis of Rye Samples Used for Fermentation Studies<sup>a</sup>

Code	Rye Sample		Moisture (% w/w)		Extract Viscosity (cp)	Protein (%N $\times$ 5.7)	Starch (% w/w, db)
	Cultivar	Source <sup>b</sup>	As Received <sup>c</sup>	After Storage <sup>d</sup>			
Rye 1	Saratov-5	AWP 1996	8.28 $\pm$ 0.007	7.56 $\pm$ 0.03	8.0 $\pm$ 0.30	11.09 $\pm$ 0.086	62.15 $\pm$ 0.64
Rye 2	Kodiak	AWP 1996	8.22 $\pm$ 0.030	8.14 $\pm$ 0.17	12.8 $\pm$ 0.65	9.49 $\pm$ 0.086	63.28 $\pm$ 0.85
Rye 3	8591-SD	SPARC 1995	8.01 $\pm$ 0.010	7.93 $\pm$ 0.32	19.3 $\pm$ 2.00	11.20 $\pm$ 0.085	61.61 $\pm$ 2.59
Rye 4	9191-GT 100	SPARC 1996	7.73 $\pm$ 0.010	7.01 $\pm$ 0.12	24.1 $\pm$ 0.70	12.97 $\pm$ 0.028	59.88 $\pm$ 0.64
Rye 5	8591-SD Low	SPARC 1996	8.22 $\pm$ 0.020	7.27 $\pm$ 0.19	29.3 $\pm$ 0.80	11.54 $\pm$ 0.086	60.40 $\pm$ 1.10
Rye 6	Musketeer	L. Kruse 1994	8.31 $\pm$ 0.060	6.93 $\pm$ 0.07	35.0 $\pm$ 4.80	7.15 $\pm$ 0.143	67.27 $\pm$ 1.22
Rye 7	8591-SD High	SPARC 1996	8.17 $\pm$ 0.010	6.96 $\pm$ 0.10	41.4 $\pm$ 1.80	11.71 $\pm$ 0.143	61.89 $\pm$ 1.20

<sup>a</sup> All values are means of duplicate samples.

<sup>b</sup> AWP, Alberta Wheat Pool; SPARC, Semiarid Prairie Agricultural Research Center; L. Kruse, Swift Current, SK.

<sup>c</sup> Values of moisture at time of measurement of initial extract viscosity and protein.

<sup>d</sup> Values used for calculation of ethanol yield per tonne of grain obtained after 12 weeks of storage.

reduce viscosity, and fermented. We consider a viscosity in excess of 500 BU too high for normal handling and fermentation. The viscosities of all prepared rye mashes, except Kodiak, exceeded 500 BU (530–910 BU).

From a practical point of view, it is important to minimize viscosity development during mashing. Although the viscosity measurements were made by adding either Roxazyme G or Novo 348 after mashing (Table II), viscosity can also be reduced by adding enzyme during incubation before mashing. Both enzymes were effective in reducing viscosity, but Roxazyme G was chosen for all subsequent studies.

*Normal gravity mashes for fermentation.* Viscosities of mashes decreased during the course of fermentation, and final viscosities were remeasured after 72 hr of fermentation. Normal gravity mashes prepared after incubating grain-water slurries with Roxazyme G had considerably lower viscosities (Table III) than mashes prepared without enzyme treatment (Table II). These mashes could be fermented directly. Viscosities of treated mashes prepared from different samples differed significantly (Table III), although none of the viscosities were high enough to warrant further addition of enzyme. If desired, viscosities of the prepared mashes could be further reduced before fermentation (to 90–150 BU) by a second addition of Roxazyme G (0.02% by wt. of grain) after mashing (results not shown).

Two series of fermentations were conducted in duplicate for each rye sample. One set of duplicates was sampled at 0, 24, 48 and 72 hr for yeast cell counts, yeast viability, dissolved solids, ethanol, and FAN determinations. Fermentation of a second set of duplicates allowed to proceed to completion without sampling were distilled at end fermentation, and the ethanol was determined by HPLC analysis of the distillates. The final ethanol yield was determined (Wang et al 1998), and the yield obtained was compared to the theoretical maximum yield attainable based on the starch contents of each rye cultivar (Table IV).

*Rate of fermentation of normal gravity mashes.* Dissolved solids contents of the normal gravity mashes were  $\approx 20$ – $22$  g/100 mL of the liquid portion of the mash. The rates of fermentation of the normal gravity mashes were very rapid, and most of the fermentable sugars were consumed during the first 24 hr. All fermentations, except for the Musketeer sample which was higher in starch, were completed in 30 hr. Fermentation of the Musketeer rye took

**TABLE II**  
Viscosity<sup>a</sup> (BU) of Normal Gravity Rye Mashes Before and After Adding Viscosity-Reducing Enzymes<sup>b</sup>

Rye Cultivar	Initial Mash Viscosity	Roxazyme G	Novo 348
Saratov-5	530 ± 42.4	110	170
Kodiak	395 ± 14.1	105	140
8591-SD	660 ± 21.2	125	170
9191-GT100	905 ± 7.1	100	165
8591-SD Low	880 ± 0	120	180
Musketeer	750 ± 38.9	100	130
8591-SD High	910 ± 0	120	195

<sup>a</sup> Viscosity measured at 40°C.

<sup>b</sup> Enzymes added after mashing; 80 mg/400 g of mash.

**TABLE III**  
Viscosity of Seven Normal Gravity Mashes After Premashing Treatments with Viscosity-Reducing Roxazyme G Enzyme

Rye Cultivar	Mash Viscosity (BU)	
	Before Fermentation	After Fermentation
Saratov-5	180	75
Kodiak	120	90
8591-SD	240	75
9191-GT 100	365	80
8591-SD Low	345	80
Musketeer	360	75
8591-SD High	460	85

48 hr. The fast rates of fermentation may be attributed to the fermentation temperature used (27°C) and to a greater availability of nutrients for yeast growth in rye mashes than in mashes made from other grains. For example, FAN in normal gravity rye mashes varied between 112 and 143 mg/L, whereas a wheat mash of similar dissolved solids content would have an average FAN of only 60 mg/L. Some dissolved solids in these mashes were not fermentable sugars and remained unfermented at the end of fermentation. HPLC analysis showed that unused sugars were at low concentrations (0.2–0.4 g/100 mL of mash).

It should also be noted that all fermentations were conducted with nutrient supplementation. This was done because previous studies have shown that all grain mashes, with the exception of oats (Thomas and Ingledew 1995), are deficient in yeast-usable nitrogen, and that rates of fermentation are improved drastically by addition of urea or other suitable sources of nitrogen (Jones and Ingledew 1994a, Wang et al 1998).

*Yeast growth and viability in normal gravity mashes.* Normal gravity mashes were inoculated with 20 million viable yeast cells per milliliter of mash. Rapid multiplication of yeast corresponded to the rapid utilization of dissolved solids. The majority of growth occurred in the first 24 hr, by which time the cell number had reached  $\approx 250$  million yeast/mL. This means that slightly less than four generations of yeast division were supported by this medium, and that there were few, if any, perceptible differences in nutritive status of the seven extracts. Yeast remained viable for at least 72 hr after the start of fermentation, as estimated by the methylene blue technique and by counting the cells by microscope using an improved Neubauer hemacytometer counting chamber.

In spite of the availability of added urea in the mashes, maximum uptake of FAN from mash occurred within 24 hr. Residual FAN in the mashes consisted of amino nitrogen of peptides and proteins that yeast does not use as a nitrogen source. Small amounts of FAN were liberated toward the end of the fermentation—presumably due to lysis or cell leakage in a small proportion of the cells.

The rate and extent of alcohol production were measured in each rye fermentation. In many of the cultivars, few differences were seen, with the exception of the Musketeer which produced  $\approx 10\%$

**TABLE IV**  
Ethanol Yields from Normal Gravity Mash Fermentations

Rye Cultivar	Observed Yield <sup>a</sup>		
	L/t of Rye (wet wt.)	L/t of Rye (dry wt.)	% of Theoretical
Saratov-5	367.53 ± 2.58	397.56 ± 2.79	89.11
Kodiak	357.40 ± 5.44	389.07 ± 5.92	85.65
8591-SD	348.49 ± 5.44	378.51 ± 5.90	85.58
9191-GT 100	364.89 ± 2.83	392.40 ± 3.08	91.28
8591-SD Low	362.87 ± 0.86	391.32 ± 0.93	90.26
Musketeer	386.36 ± 0	415.13 ± 0	85.96
8591-SD High	356.80 ± 0	383.49 ± 0	86.31

<sup>a</sup> All values are means of duplicate analyses.

**TABLE V**  
Viscosity of Very High Gravity (VHG) Mashes After Treatments with Viscosity-Reducing Roxazyme Enzyme

Rye Cultivar	Mash Viscosity (BU)	
	Before Fermentation <sup>a</sup>	After Fermentation <sup>b</sup>
Saratov-5	205	70
Kodiak	205	60
8591-SD	250	85
9191-GT 100	270	120
8591-SD Low	270	100
Musketeer	250	60
8591-SD High	315	80

<sup>a</sup> Prepared mash was distributed in duplicate to two fermentors.

<sup>b</sup> Mashes fermented in duplicate but combined for final viscosity assay.

more ethanol than the other six samples. This is a reflection of the higher starch content of this cultivar. All other fermentors contained  $\approx 9.5\%$  ethanol (v/v), whereas the Musketeer rye fermentor produced  $\approx 10.5\%$ . Table IV indicates yield per tonne of grain (wet or dry). Observed yields had a range of 80–91% of the theoretical values.

### Rye Mashed at VHG

*Viscosity development during VHG mashing.* Preliminary studies were conducted to determine optimum conditions of grain-to-water ratio, preincubation, and application of viscosity-reducing enzymes for the preparation of VHG rye mash. The aim was to obtain a mash with a dissolved solids concentration of at least 30 g/100 mL and a viscosity of <500 BU. The rye sample 8591-SD High was chosen for this study because it had the maximum extract viscosity of the seven normal gravity mashes. The mash was prepared with a grain-to-water ratio of 1:2 and pretreated with 0.02% Roxazyme at 45°C for 30 min. The effect of viscosity-reducing enzymes was assessed.

The mash prepared after pretreating the grain-water slurry with 0.02% Roxazyme G had a viscosity of 1,090 BU. The viscosity of this mash was further reduced to 330 BU by adding 0.02% Roxazyme G again after mashing. Similar effects were observed when Novo 348 (100  $\mu\text{L}/100\text{ g}$  of grain) was used. Further additions of Roxazyme G or Novo 348 to the prepared mash after mashing did not reduce the viscosity any further. The mash pretreated with 0.04% Roxazyme G had a viscosity of 850 BU, and this was reduced to 305 BU with additional Roxazyme G added after mashing. Further addition of enzyme after mashing did not lead to a further reduction in viscosity. Although the mash pretreated with 0.02% Roxazyme G had a higher initial viscosity than that pretreated with 0.04%, subsequent treatment with 0.02% Roxazyme G after mashing reduced the viscosity of both of these mashes to approximately the same level.

There were noticeable problems with the VHG mash rising excessively during gelatinization, especially in the VHG mash pretreated with the highest level (0.04%) of Roxazyme G used. We do not know the cause for this phenomenon.

*Preparation of VHG rye mash for fermentation.* The volume of the liquid portion that can be separated by centrifugation is a crude measure of extractability and is an indication of the effectiveness of the viscosity-reducing enzymes. A known amount of mash was centrifuged at  $6,555 \times g$  for 15 min and the liquid portion was collected. Without enzyme treatment, very little of the liquid portion could be removed, even after centrifuging. Viscosities of the VHG mashes and volumes of supernatants that could be collected were determined.

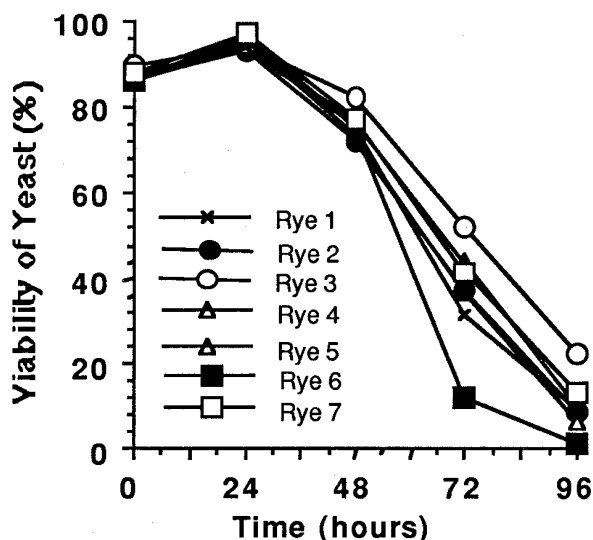


Fig. 1. Viability of yeast during fermentation of very high gravity (VHG) mashes made from seven rye selections. (See Table I for rye cultivar codes.)

Viscosities of VHG mashes prepared with double doses of Roxazyme G (0.02% during preincubation and 0.02% after mashing) were low (205–315 BU) for the seven rye samples processed (Table V). Viscosities decreased during fermentation and they were therefore remeasured after 96 hr of fermentation (Table V). The dissolved solids contents of these mashes before fermentation had a range of 32.66–34.64 g/100 mL. As expected, the FAN values of these mashes increased with increasing amounts of grain used in the mashings and ranged between 171 mg/L of mash (9191-GT 100) and 239 mg/L of mash (Musketeer). The extractability (quantity of liquid portion that can be poured out) was slightly lower in VHG mashes than in normal gravity mashes.

*Rate of fermentation in VHG mashes.* Yields of alcohol obtained in VHG level fermentations were compared to the theoretical maximum yields attainable based on the rye starch contents. HPLC analyses of clear supernatants were made after fermentation to determine the sugar profile and residual unfermented sugars. Dissolved solids (2–3 g/100 mL) remained at the end of fermentation. HPLC analysis showed that the 0.9–1.2 g/100 mL of “fermentable” sugars that remained were isomaltose and trehalose, both unfermentable. These sugars coeluted with maltose. VHG fermentations began at 32–34 g/100 mL and fermented within 48 hr to completion in all cases.

*Yeast growth and viability in VHG mashes.* As with the normal gravity fermentations, all yeast growth took place in the first 24 hr, but in VHG mashes, the maximum cell numbers rose to  $\approx 300$  million cells/mL. Four generations of cell growth were supported by the rye media. The time required to complete fermentations of VHG and normal gravity mashes were similar (48 hr), although the former contained an average 62% more dissolved solids. The increased yeast growth (300 million cells/mL vs. 250 million cells/mL) and the increased catalytic power afforded by 50 million extra growing cells/mL accounted for the more rapid fermentation of VHG mashes.

When the results of viability assessment were compared, it was very obvious that although the yeast cells were able to complete the ethanol fermentation, the viability reduced greatly during the last 50–60 hr of the experiments. Although cell viability as monitored by microscope and methylene blue vital stain (the accepted method in industry) is not a good method for viabilities of <80%, the viabilities dropped to almost zero by 96 hr (Fig. 1). Viabilities in normal gravity experiments were >90% throughout the fermentation (not shown).

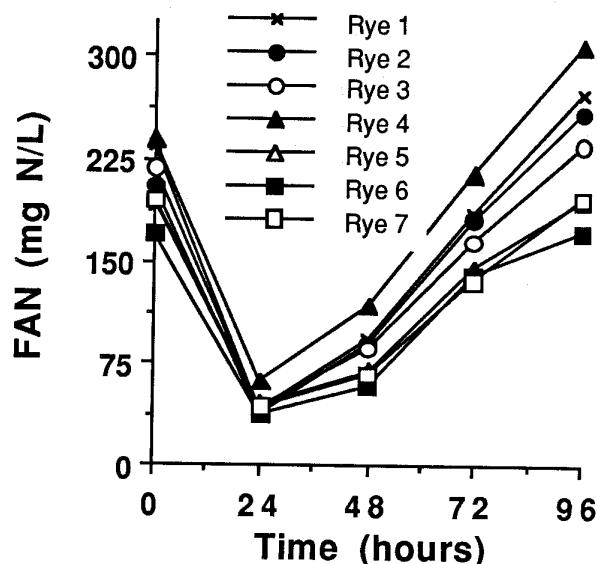


Fig. 2. Free amino nitrogen (FAN) utilization during fermentation of very high gravity (VHG) mashes made from seven rye selections. (See Table I for rye cultivar codes.)

As seen in normal gravity fermentations, the usable FAN in VHG fermentations was taken up in the first 24 hr (Fig. 2), but large amounts of FAN were liberated subsequent to growth. It is suspected that extensive lysis or cell leakage took place as the viability of the cells decreased. In normal gravity fermentations, the FAN was used up very quickly, but little liberation of FAN took place in the late stages of the experiments (not shown).

All rye samples fermented under VHG conditions yielded 14.29–15.59% (v/v) ethanol. Musketeer rye again led to higher levels of alcohol—in this case ≈16% (v/v). It should be noted that the levels of ethanol made in VHG conditions was ≈1.6-fold higher than levels under normal gravity. This demonstrates the industrially important observation that alcohol plants using VHG technology are able to increase productivity and decrease the volume of process water utilized.

The yields of alcohol per tonne of grain (wet and dry) were increased significantly by VHG production. The efficiencies of ethanol yield were also increased at 89.5–94.5% (Table VI). The yields of alcohol per tonne of starch and per tonne of rye grain were calculated to facilitate comparison of normal and VHG conditions (Table VII).

### CONCLUSIONS

Rye mashes prepared with a grain-to-water ratio of 1:3 (normal gravity) and without viscosity-reducing enzymes were very viscous, ranging from 395 BU for Kodiak to 910 BU for 8591-SD High. The method used for rye cultivar selection, however, had predicted a viscosity range of fivefold among the cultivars. The two methods of viscosity measurements used are obviously not strictly comparable. Viscosity development during mashing can be minimized by preincubating ground grain-water slurry at 45°C for 30 min with 0.02% (by wt. of grain used) Roxazyme G or Novo 348, and viscosity of the mash can be further reduced with additional enzyme after mashing (0.02% by wt. of grain). Apparently, the mashing procedure made the viscosity-causing polymers more available to the enzyme. The second enzyme treatment was deemed unnecessary for normal gravity fermentations of rye because mash viscosities were low.

All rye mashes contained significant amounts of FAN. Amounts were almost double that reported earlier for wheat mashes (Thomas and Ingledew 1990). In spite of relatively high FAN contents, fermentations of these mashes were stimulated further by urea supplementation. In all cases, fermentations were completed before 48 hr at 27°C. Most of the rapid fermentation occurred during the first 24 hr, coincident with nearly all of the FAN uptake and nearly all of the cell multiplication.

The rate of ethanol production corresponded with the rate of sugar uptake. The ethanol yield corresponded to 86–91% of the theoretical yield (based on starch content). Under ideal conditions, a yield of 93–94 % of theoretical is considered the maximum attainable.

VHG mashes of rye containing 33–35 g of dissolved solids/100 mL can be prepared by adjusting the grain-to water ratio to 1:1.8 and by preincubating grain-water slurry with 0.02% Roxazyme G at 45°C for 30 min. A second addition of Roxazyme G (0.02%) to the prepared mash was necessary to bring the viscosity of these mashes to a value low enough (<500 BU) for convenient handling. Viscosity was not a problem in these enzyme-treated fermentations in spite of the fivefold extract viscosity differences in the initial selected cultivars. The viscosities of the mashes did not exactly correspond to the magnitude of those measured during selection of the samples on dilute cultivar extracts free of insoluble solids. This surely indicates that alterations in the techniques and handling methods must affect the gel strength of each extract, and that interactions of grain components during mashing contribute to the viscosity of the final mash. These data support the use of viscosity-reducing enzymes whenever rye is used in fermentation. Although this work shows that 100% rye mashes can be used for fermentation—both in normal and VHG conditions, caution should be taken due to mash lifting. Care should be exercised in industrial exploitation of these results in case of the severity of this phenomenon under industrial conditions.

As with normal gravity mashes, fermentations of VHG mashes were completed within 48 hr at 27°C, although VHG mashes contained 62% more dissolved solids than normal gravity mashes. Cell multiplication occurred to a greater extent in VHG mashes, and the increased catalytic power provided by the increased cell mass accounts for the rapid rate of sugar utilization and ethanol production. Normal gravity fermentations may have terminated before the 48-hr sampling time. Cell viability declined rapidly after reaching its maximum. The FAN content of the supernatant increased at the same time, which may indicate cell lysis followed by proteolysis.

Ethanol yields for VHG alcohol production were 89.5–94.5% of theoretical, which was higher than that seen for normal gravity fermentation. This finding is also reflected in ethanol yield per tonne of starch (Table VII).

It was also noted that Musketeer rye fermented to higher alcohol concentrations than the other cultivars tested because of its higher starch content. Rye (or any other grain) with higher starch provides higher alcohol levels at end fermentation. For this industry, it would appear unnecessary to develop low viscosity cultivars, even though in livestock feeding, substantial benefit may be provided by the decreased pentosan content of these selected ryes. Attention should be given to developing, growing, and marketing specific grain cultivars higher in starch content, and work should be done to fully determine all environmental and cropping practices that optimize starch levels. Grain for this industry could be grown under contract if it could be shown that increased starch would increase profitability of fuel alcohol plants. This industry is now unable to control the type of grain, the price of grain, or the chemical quality of the grain it purchases.

**TABLE VI**  
Ethanol Yields from Very High Gravity (VHG) Mash Fermentations

Rye Cultivar	Observed Yield <sup>a</sup>		
	L / t of Grain (wet wt.)	L / t of Grain (dry wt.)	% of Theoretical
Saratov-5	389.56 ± 1.81	421.42 ± 1.95	94.46
Kodiak	390.70 ± 3.41	425.32 ± 3.71	93.63
8591-SD	382.18 ± 0.20	415.10 ± 0.21	93.85
9191-GT 100	370.12 ± 1.61	398.02 ± 1.70	92.59
8591-SD Low	371.68 ± 1.41	400.82 ± 1.51	92.45
Musketeer	404.46 ± 4.82	434.58 ± 5.18	90.00
8591-SD High	369.83 ± 3.21	397.50 ± 3.45	89.47

<sup>a</sup> All values are means of duplicate analyses.

**TABLE VII**  
Actual and Theoretical Yields of Ethanol per tonne of Starch or Rye Grain in Normal and Very High Gravity (VHG) Conditions

Rye Samples	Observed Yield <sup>a</sup>		
	Normal Gravity	VHG Gravity	Theoretical Yield <sup>b</sup>
Saratov-5	639.7 ± 4.48	678.1 ± 3.14	446.13
Kodiak	614.8 ± 9.19	672.1 ± 5.85	454.24
8591-SD	614.4 ± 9.61	673.8 ± 0.34	442.29
9191-GT 100	655.3 ± 5.14	664.7 ± 2.83	429.87
8591-SD Low	647.9 ± 1.58	663.6 ± 2.51	433.57
Musketeer	617.1 ± 0	646.0 ± 7.69	482.92
8591-SD High	619.6 ± 0	642.3 ± 5.57	444.30

<sup>a</sup> L/t of of starch. Theoretical yield of ethanol per tonne of starch is 717.88 L.

<sup>b</sup> L/t of of rye (dry wt).

## ACKNOWLEDGMENTS

This work was supported by the Green Plan Ethanol Program of Agriculture and Agri-Food Canada and, in part, by the Western Grains Research Foundation and the Natural Sciences and Engineering Research Council of Canada. We wish to thank the Alberta Wheat Pool and Lionel Kruse for providing samples of rye.

## LITERATURE CITED

- Chiang, B.-Y., and Johnson, J. A. 1977. Measurement of total and gelatinized starch by glucoamylase and *o*-toluidine reagent. *Cereal Chem.* 54:429-435.
- Ingledeu, W. M. 1993. Yeasts for production of fuel alcohol. Pages 245-291 in: *The Yeasts*, Vol 5, 2nd ed. A. H. Rose and J. S. Harrison, eds. Academic Press: New York.
- Ingledeu, W. M. 1995. The Biochemistry of Ethanol Production. Pages 55-80 in: *The Alcohol Textbook*. T. P. Lyons, D. R. Kelsall, and J. E. Murtagh, eds. Nottingham University Press: Nottingham, UK.
- Ingledeu, W. M., Jones, A. M., Bhatta, R. S., and Rossnagel, B. G. 1995. Fuel alcohol production from hull-less barley. *Cereal Chem.* 72:147-150.
- Jones, A. M., and Ingledeu, W. M. 1994a. Fuel alcohol production: Appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. *Process Biochem.* 29:483-488.
- Jones, A. M., and Ingledeu, W. M. 1994b. Fuel alcohol production: Optimization of temperature for efficient very-high-gravity fermentation. *Appl. Environ. Microbiol.* 60:1048-1051.
- Jones, A. M., and Ingledeu, W. M. 1994c. Fuel alcohol production: Assessment of selected commercial proteases for very high gravity wheat mash fermentation. *Enzyme Microbial Technol.* 16:683-687.
- Jones, A. M., Thomas, K. C., and Ingledeu, W. M. 1995. VHG fermentation: Fuel alcohol production from wheat mashes fortified with sugar adjuncts. *Int. Sugar J.* 97:606-610.
- Thomas, K. C., and Ingledeu, W. M. 1990. Fuel alcohol production: Effects of free amino nitrogen on fermentation of very high gravity wheat mashes. *Appl. Environ. Microbiol.* 56:2046-2050.
- Thomas, K. C., and Ingledeu, W. M. 1992. Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. *J. Ind. Microbiol.* 10:61-68.
- Thomas, K. C., and Ingledeu, W. M. 1995. Production of fuel alcohol from oats by fermentation. *J. Ind. Microbiol.* 15:125-130.
- Thomas, K. C., Hynes, S. H., Jones, A. M., and Ingledeu, W. M. 1993. Production of fuel alcohol from wheat by VHG technology: Effect of sugar concentration and fermentation temperature. *Appl. Biochem. Biotechnol.* 43:211-226.
- Thomas, K. C., Dhas, A., Rossnagel, B. G., and Ingledeu, W. M. 1995. Production of fuel alcohol from hullless barley by VHG technology. *Cereal Chem.* 72:360-364.
- Thomas, K. C., Hynes, S. J., and Ingledeu, W. M. 1996. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Proc. Biochem.* 31:321-331.
- Wang, S., Thomas, K. C., Ingledeu, W. M., Sosulski, K., and Sosulski, F. W. 1998. Production of fuel alcohol from rye and triticale by very-high-gravity (VHG) fermentation. *Appl. Biochem. Biotechnol.* 69:23-41.

[Received July 9, 1998. Accepted January 12, 1999.]