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Fuel Alcohol Production: Optimization of Temperature for Efficient Very-High-Gravity Fermentation

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The time required to end ferment wheat mash decreased as the temperature was increased from 17 to 33°C, but it increased as the concentration of dissolved solids was raised from 14.0 to 36.5 g/100 ml. Ethanol yield was not appreciably affected. Over the range of fermentation temperatures tested, the addition of urea accelerated the rate of fermentation, decreased the time required to complete fermentation at all dissolved-solid concentrations, and stimulated the production of slightly more ethanol than was produced by the corresponding unsupplemented control mashes. The optimum temperature for maximum ethanol production in urea-supplemented very-high-gravity wheat mash was 27°C. These data are important for the industrial assessment of very-high-gravity fermentation technology.

Fermentation ethanol is being widely investigated as a renewable fuel source because in many respects it is superior to gasoline as a liquid fuel (10, 11). There continue to be times, however, when ethanol cannot compete economically with gasoline or petroleum derivatives of fossil fuels. Opportunity therefore exists for process improvements in the conversion of biomass to fuel alcohol which will result in more favorable production economics. Very-high-gravity (VHG) fermentation is one such process improvement aimed at increasing both the rate of fermentation and the final ethanol concentration and thereby reducing processing costs (5). This novel technology relies on the preparation and fermentation of mashes containing concentrations of carbohydrate in excess of 30% (wt/vol).

In industry, fermentation of high-sugar mashes has been considered impractical because of problems with yeast viability and incomplete or stuck fermentations (12). Laboratory-scale studies have indicated, however, that complete fermentation of VHG wheat mash, containing as much as 38% (wt/vol) dissolved solids, is possible with the concomitant production of up to 23% (vol/vol) ethanol (6, 15–17). In contrast to industrial fuel ethanol fermentations, which are typically conducted at 30 to 35°C (5), fermentations of VHG grain mashes in those studies were conducted at 20°C. This lower temperature was chosen because, as discussed in reviews of the topic (1, 2, 7, 18, 19), high fermentation temperatures can result in stuck fermentation, and ethanol tolerance is known to decrease with increased temperatures. Such problems are more pronounced as the carbohydrate concentration in the fermentation mash is increased (4, 9, 14, 15).

The ability to conduct VHG fermentation on an industrial scale at temperatures greater than 20°C would make this novel technology even more economically viable since fermentation times would be reduced, less cooling would be required to maintain the temperature of the fermenting mash, and the technology would be appropriate in tropical climates where cooling is less practical. Thomas et al. (15) have recently reported the effect of temperature on the fermentation of VHG wheat mashes containing 37 to 38% (wt/vol) sugar with and without yeast extract supplementation. However, no comprehensive report delineating the optimum fermentation temperature and substrate concentration for the production of high levels of bioethanol is available. In addition, while the provision of adequate assimilable nitrogen in a fermentation is critical for rapid and complete sugar utilization (6, 13, 16, 17), yeast extract such as that used as a yeast-assimilable nitrogen source in the study of Thomas et al. (15) is uneconomical for routine use in the fuel alcohol industry. Urea, a cost-effective yeast food for nonpotable-alcohol production (6), was employed in the present study.

Wheat mash and fermentation. VHG wheat mash, containing 36.5 g of dissolved solids per 100 ml, was prepared by the double mashing procedure outlined previously (6) and was diluted with sterile distilled water to obtain all other dissolved-solid concentrations. It is important to note that after completion of the double mashing procedure, only the grain residues from the second mash remained. For this reason, such mash had double the dextrin level but normal levels of undissolved solids compared with a mash at normal specific gravity. The mashes were chemically sterilized with 0.02% (vol/wt) diethyl pyrocarbonate (6) and then stored frozen at −40°C until needed. Wheat mashes (500-g quantities) were added to sterile jacketed 500-ml Celstir bioreactors (Wheaton Scientific, Millville, N.J.) containing either 10 ml of sterile distilled water or 10-ml solutions of filter-sterilized urea to obtain a final urea concentration in the mash of 16 mM. The bioreactors were connected to a 30°C circulating water bath, and mash dextrins were saccharified to fermentable sugars by adding 0.39 to 1.2 ml of glucoamylase (Allcoholase II; Alltech Biotechnology Centre, Nicholasville, Ky.) to 500-g portions of mash ranging from 14 to 36.5 g of total solids per 100 ml. After 30 min the temperature was adjusted to that desired for fermentation. A commercial active dry *Saccharomyces cerevisiae* yeast (Alltech) was preconditioned in 0.1% peptone at 38°C for 20 min prior to inoculation at the recommended rate of 10^6 viable cells per ml per °P of mash (1, 8). Fermentations were monitored at predetermined intervals for total dissolved-solid and ethanol concentrations.

Analyses. Analyses of clarified supernatant samples (10,300 × *g*; 15 min) of the fermenting mash were conducted. Total

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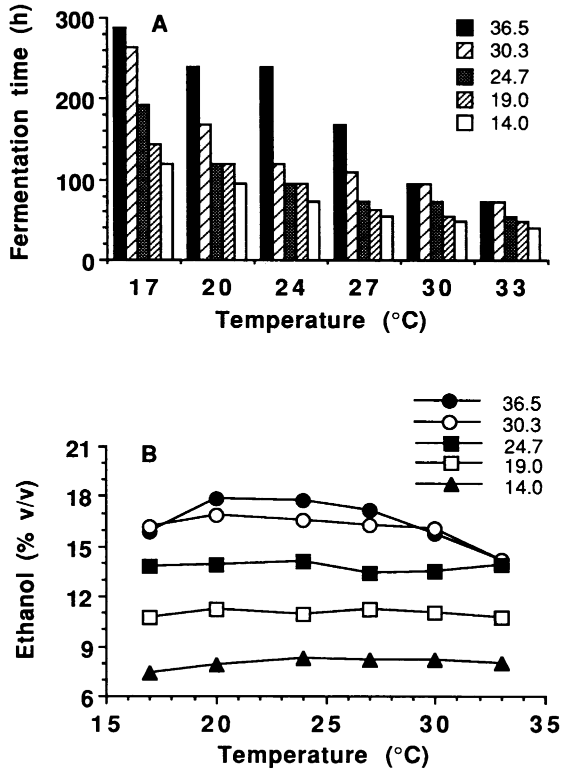

FIG. 1. Effects of temperature and initial substrate concentration on the time taken to end ferment unsupplemented wheat mash (A) and on ethanol yield (B). Wheat mashes initially contained 14.0 to 36.5 g of dissolved solids per 100 ml.

concentrations of dissolved solids were determined at 20°C with a digital density meter (DMA-45; Anton Paar KG, Graz, Austria). Specific-gravity or corresponding Plato readings (in grams per 100 g) were converted into grams of dissolved solids per 100 ml by comparison with the densities of sucrose solutions of the same gravity. At the high gravities used in this work, weight-by-volume readings are considerably different from weight-by-weight readings. Dry-weight determinations were made by drying 4-ml aliquots of clarified supernatants at 105°C for 3 h. Ethanol was measured by using alcohol dehydrogenase (Sigma technical bulletin no. 331-uv). Analyses were conducted in duplicate. Representative results are presented.

Influence of temperature, substrate concentration, and nutrient addition. To assess the combined effects of fermentation temperature and initial substrate concentration on ethanol production, wheat mashes ranging from normal gravity (14 to 20 g of dissolved solids per 100 ml) to VHG (>30 g of dissolved solids per 100 ml) were fermented with active dry yeast at temperatures between 17 and 33°C. Figure 1 summarizes the fermentation performance of wheat mashes not supplemented with urea at the various temperatures and sugar concentrations employed. As expected, as the initial wheat mash was increased from 14.0 to 36.5% (wt/vol), the time required to complete sugar utilization increased. Although this study did not monitor the viability of the yeast cell populations, this decrease in fermentation rate (and increase in the time necessary to complete the fermentation) is attributed to the inhibition of yeast growth and increased loss of yeast cell viability which occur under conditions of alcoholic and osmotic stress (4, 9, 14, 15, 18, 19). These effects become more pronounced with increasing fermentation temperature (3, 4, 7, 15). Furthermore, increased end ethanol levels reduce the maximum temperature of yeast growth and cause yeast death (18, 19). With the exception of the mash which initially contained 36.5 g of dissolved solids per 100 ml (data for residual sugar not shown), all wheat mashes were fermentable to completion despite the fermentation temperature employed. Regardless of the initial sugar concentration over the range of 14.0 to 36.5% (wt/vol), as fermentation temperature was increased, the time required to complete the fermentation decreased (Fig. 1A). For example, complete conversion of the fermentable sugar to ethanol in the unsupplemented 14.0-g/100-ml mash was observed within 120 h at 17°C, but only 40 h was required at 33°C (Fig. 1A). At 17°C, 7.4% (vol/vol) ethanol was achieved, while 8.0% (vol/vol) was realized at 33°C (Fig. 1B). The low fermentation temperature, 17°C, appeared to depress slightly the fermentation efficiency (data not shown) and ethanol yield of this normal-gravity wheat mash. VHG wheat mash containing 36.5 g of dissolved solids per 100 ml required 12 days at 17°C to end ferment (Fig. 1A) but required only 3 days when the fermentation temperature was increased to 33°C. Slightly more dissolved solids remained unfermented at 33°C (37%) than at 17°C (29%), but at both temperature extremes, the fermentation efficiency was 95% of the theoretical value, with ethanol yields of 14.2 and 15.9% (vol/vol) at 33 and 17°C, respectively (Fig. 1B).

Fermentation efficiency was increased to 100 to 104% and more ethanol was produced (17.8% [vol/vol]; Fig. 1B) when VHG mash was fermented at an intermediate temperature (either 20 or 24°C). However, significantly more time (10 days) was required for maximum ethanol production (Fig. 1A) than the 3 days required at 33°C. Thus, fermentation of 36.5% (wt/vol) sugar at 20 or 24°C yielded 3.6% (vol/vol) more ethanol than was produced at 33°C, but an additional 7 days of fermentation was required.

While the choice of fermentation temperature significantly affected the time required to complete the conversion of sugar to ethanol in unsupplemented wheat mash, there was little effect on ethanol yield, at least with substrate concentrations of 30% (wt/vol) dissolved solids and concentrations below 30% (Fig. 1B). Moreover, the fermentation efficiency was never less than 94% when calculated on the basis of dissolved solids utilized in the mashes. The optimum temperature for maximum ethanol production in unsupplemented VHG wheat mash containing 36.5 g of dissolved solids per 100 ml was 20°C (Fig. 1B).

Our results support previous observations that nutrient supplementation significantly improves fermentation of high-gravity media (3, 4, 6, 13, 15–17). As shown in Fig. 2, urea at 16 mM decreased the time required to complete fermentation of all wheat mashes at between 17 and 33°C and allowed more carbohydrate in VHG mashes to be converted to ethanol than was converted in the case of unsupplemented mashes (Fig. 1). This was probably due to the elimination of sluggish or stuck fermentations. The yeast was able to ferment all available sugar. The rate of fermentation of 14.0-g/100-ml mashes was accelerated to completion within 48 h at 17°C, producing 7.5% (vol/vol) ethanol, while only 17 h was required at 33°C to yield 7.7% (vol/vol) ethanol. Similarly, the addition of 16 mM urea to VHG wheat mash containing 36.5 g of dissolved solids per 100 ml reduced the fermentation time to only 48 h (Fig. 2A) and resulted in the fermentation of more sugar, producing an additional 1.5% (vol/vol) ethanol (Fig. 2B) compared with fermentation of unsupplemented 36.5% (wt/vol) mashes. When 36.5% (wt/vol) mashes were supplemented with urea and fermented at 20, 24, or 27°C, fermentation was accelerated

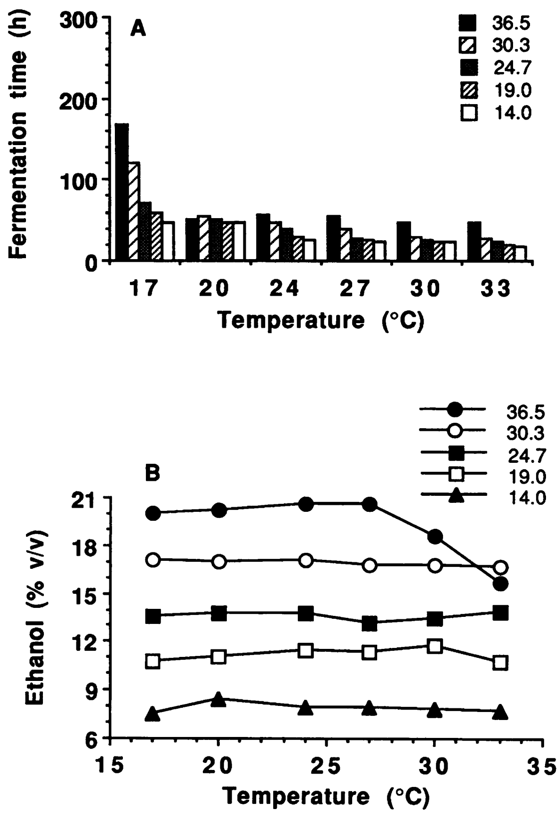

FIG. 2. Effects of temperature and initial substrate concentration on the time required to end ferment wheat mashes supplemented with 16 mM urea (A) and on ethanol yield (B). Wheat mashes initially contained 14.0 to 36.5 g of dissolved solids per 100 ml.

to completion within 48 to 52 h (Fig. 2A), yielding 20.6% (vol/vol) ethanol at each temperature (Fig. 2B). Supplementation with urea did not, however, increase the fermentation efficiency. The optimum temperature for maximum ethanol production in urea-supplemented VHG wheat mash was 27°C (Fig. 2B).

In contrast to the observation of Thomas et al. (15) that the addition of yeast extract to VHG wheat mash at temperatures above 20°C did not increase the total amount of sugar consumed by the yeast, more sugar was fermented in the 36.5% (wt/vol) mashes with urea than in those without the supplement at all temperatures tested in the present study. For example, in the control fermentation at 17°C, 10.6 g of dissolved solids (as determined by dry weight) per 100 ml remained unutilized at the end of fermentation (288 h), while in the presence of 16 mM urea only 6.4 g/100 ml remained unfermented (168 h). At 33°C, 13.8 g of dissolved solids per 100 ml remained unused at 72 h in the absence of urea, compared with 10.1 g/100 ml in the presence of urea after 48 h. This apparent contradiction in results may be due to the differences between urea and yeast extract as assimilable nitrogen sources in VHG fermentation, and thus it may be another reason for choosing urea for routine use in the industrial manufacture of fuel ethanol.

The combined effects of nutrient supplementation with urea and an increased fermentation temperature greatly decreased the time required to end ferment wheat mashes over the range of 14.0 to 36.5 g of dissolved solids per 100 ml. Independent of fermentation temperature, ethanol yields from VHG wheat mashes (30.3 and 36.5 g/100 ml) were also somewhat enhanced compared with those from the unsupplemented controls. This study indicates that in the industrial production of fuel alcohol, fermentation performance and ethanol production will be greatly affected by process variables, in particular, fermentation temperature, nutrient supplementation, and (because of osmotic phenomena) the choice of mash dissolved-solid concentration. In view of our results, for optimum and efficient industrial production of fuel alcohol from wheat mashes over the range of normal gravity to VHG, nutrient supplementation with urea and fermentation temperatures up to 30°C should be considered along with the particular yeast strain used in each industrial plant. Our laboratory-scale studies further suggest that VHG wheat mashes containing 36.5 g of dissolved solids per 100 ml can be readily fermented at 27°C within 55 h in the presence of 16 mM urea, with the concomitant production of 20.6% (vol/vol) ethanol. Although under these fermentation conditions 15% of the total dissolved solids remain unfermented, the fermentation is still 101% efficient when efficiency is calculated on the basis of the sugar consumed. In addition, both the time taken to end ferment and the cost of cooling the fermenting mash are significantly reduced; these are both of potential importance to industry. Although fermentation of mashes with the highest concentrations of dissolved solids used here would not likely be considered for use in industry, alcohol concentrations of 18% (vol/vol) should be easily attainable from wheat mashes by using VHG fermentation technology.

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REFERENCES

1. **Casey, G. P., and W. M. Ingledew.** 1985. Reevaluation of alcohol synthesis and tolerance in brewer's yeast. J. Am. Soc. Brew. Chem. **43:**75–83.
2. **Casey, G. P., and W. M. Ingledew.** 1986. Ethanol tolerance in yeasts. Crit. Rev. Microbiol. **13:**219–280.
3. **Casey, G. P., C. A. Magnus, and W. M. Ingledew.** 1984. High-gravity brewing: effects of nutrition on yeast composition, fermentative ability, and alcohol production. Appl. Environ. Microbiol. **48:**639–646.
4. **D'Amore, T., G. Celotto, and G. G. Stewart.** 1991. Advances in the fermentation of high gravity wort, p. 337–344. *In* European Brewery Convention Proceedings, 23rd Congress—Lisbon. IRL Press, Oxford.
5. **Ingledew, W. M.** 1993. Yeasts for production of fuel ethanol, p. 245–291. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, 2nd ed., vol. 5. Yeast technology. Academic Press, New York.
6. **Jones, A. M., and W. M. Ingledew.** Fuel alcohol production: appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. Process Biochem., in press.
7. **Jones, R. P., N. Pamment, and P. F. Greenfield.** 1981. Alcohol fermentation by yeasts—the effect of environmental and other variables. Process Biochem. **16:**42–49.
8. **Knudsen, F. B.** 1985. Fermentation variables and their control. J. Am. Soc. Brew. Chem. **43:**91–95.
9. **Laluce, C., M. C. Palmieri, and R. C. L. Cruz.** 1991. Growth and fermentation characteristics of new selected strains of *Saccharomyces* at high temperatures and high cell densities. Biotechnol. Bioeng. **37:**528–536.
10. **Lynd, L. R., J. H. Cushman, R. J. Nichols, and C. E. Wyman.** 1991. Fuel ethanol from cellulosic biomass. Science **251:**1318–1323.
11. **Maiorella, B. L.** 1985. Ethanol, p. 861–914. *In* M. Moo-Young (ed.), Comprehensive biotechnology. The principles, applications and regulations of biotechnology in industry, agriculture and medicine, vol. 3. Current commodity products. Pergamon, Oxford.

12. **Nagodawithana, T. W.** 1986. Yeasts: their role in modified cereal fermentations. Adv. Cereal Sci. Technol. **VIII:**15–104.
13. **O'Connor-Cox, E. S. C., J. Paik, and W. M. Ingledew.** 1991. Improved ethanol yields through supplementation with excess assimilable nitrogen. J. Ind. Microbiol. **8:**45–52.
14. **Panchal, C. J., and G. G. Stewart.** 1980. The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain. J. Inst. Brew. **86:**207–210.
15. **Thomas, K. C., S. H. Hynes, A. M. Jones, and W. M. Ingledew.** 1993. Production of fuel alcohol from wheat by VHG technology: effect of sugar concentration and fermentation temperature. Appl. Biochem. Biotechnol. **43:**211–226.
16. **Thomas, K. C., and W. M. Ingledew.** 1990. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. Appl. Environ. Microbiol. **56:**2046–2050.
17. **Thomas, K. C., and W. M. Ingledew.** 1992. Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. J. Ind. Microbiol. **10:**61–68.
18. **van Uden, N.** 1984. Effects of ethanol on the temperature relations of viability and growth in yeast. Crit. Rev. Biotechnol. **1:**263–272.
19. **van Uden, N.** 1989. Effect of alcohols on the temperature relations of growth and death in yeasts, p. 77–88. *In* N. van Uden (ed.), Alcohol toxicity in yeasts and bacteria. CRC Press, Inc., Boca Raton, Fla.