High-Gravity Brewing: Influence of High-Ethanol Beer on the Viability of Contaminating Brewing Bacteria

C. A. Magnus, W. M. Ingledew, and G. P. Casey, *Food Biotechnology Laboratory, Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Canada S7N 0W0*

ABSTRACT

It has been reported that when high-gravity brewers' worts were supplemented with a source of nitrogen and unsaturated lipids and sterol, ethanol concentrations up to 16.4% v/v could be achieved within normal fermentation times. As the resultant harvested yeast can be repitched over a number of generations, there appears to be no reason in industry to limit the gravities of commercial worts to 16° Plato, especially when the ester and fusel oil patterns of resultant beers may not be as elevated as previously thought. In this report the influence of high-alcohol beer made from 28° P wort on the viability of traditional bacterial brewing contaminants was examined. *Lactobacillus* and *Pediococcus*, *Acetomonas*, *Acetobacter*, and *Zymomonas* contaminants were able to survive levels of ethanol of 12–13% v/v. *Hafnia* (*Flavobacterium* or *Obesumbacterium*), *Enterobacter* (including *E. agglomerans*), *Citrobacter*, and *Klebsiella* species, known to exist through most stages of the fermentation of traditional gravity wort, were completely eliminated by the elevated ethanol levels. Very high gravity fermentations therefore narrow the range of bacteria capable of spoiling the beer, thereby reducing the risk of bacterial spoilage problems. This work illustrates an additional production advantage in favor of increasing original gravities of worts.

Key words: Bacterial contaminants, Cell death, Ethanol, High-gravity brewing

High-gravity brewing, the fermentation of worts higher in original gravity than 11–12° P, offers numerous product quality and economic advantages (9,16,35). Until recently, high-gravity brewing was stated to be limited to the production of beers with 7–8% v/v ethanol (16,24,28,36,37). This limit has been ascribed to problems of protracted and incomplete fermentations resulting from the low tolerance of brewers' yeasts to ethanol (12,34) and to high osmotic pressure (22).

Research in this laboratory, however, demonstrated that a combination of increased pitching rates (7) and nutritional supplementation (8–10) will permit the production of lager beer containing up to 16.4% v/v ethanol at 14° C within the time of a normal brewery fermentation. This occurs without excessive production of acetate esters (11), and yeast viability remains high (8–10).

In traditional brewing, with the production of ethanol concentrations of 4–5% v/v, the most troublesome bacterial contaminants are members of the lactic acid bacteria, i.e., *Lactobacillus* spp. and *Pediococcus* spp. (2,17,25,27). However, *Flavobacterium proteus* (29) and *Enterobacter agglomerans* (32) can also tolerate ethanol concentrations greater than 5% v/v. They can therefore contaminate harvested pitching yeast, and spread throughout the production plant. Spoilage may also arise from the presence of acetic acid bacteria (*Acetomonas* and *Acetobacter*), *Zymomonas* spp., and strict anaerobes (*Megasphera* sp. [33], *Pectinatus cerevisiophilus* [3], and *Bacteroides serpens* [21]).

In the present study, the influence of strong beer (containing ethanol up to 12–13% v/v) on the viability of traditional bacterial brewing contaminants was studied.

EXPERIMENTAL

Commercial Yeast Strain

A production strain of *Saccharomyces uvarum* (*carlsbergensis*) was used. Fresh slurries of this commercial lager yeast were collected just before use. There was no requirement to adapt this yeast to an increased ethanol tolerance (10). Yeast was monitored by plating the slurry on actidione-containing media to ensure that only low levels of bacterial contaminants were present.

Bacterial Strains

Representatives of the four main groups of bacterial beer contaminants—coliforms, acetic acid bacteria, anaerobes, and lactic acid bacteria—were chosen as test organisms to determine their sensitivity to increased levels of end products in high-gravity brews. *Citrobacter freundii* ATCC 8090, *Enterobacter agglomerans* #127, *Flavobacterium proteus* ATCC 12841, *Hafnia* sp. BSO 105, and *Klebsiella oxytoca* #52 were chosen to represent the coliform group; *Acetobacter* sp. BSO 5 and *Gluconobacter* (*Acetomonas*) *oxydans* subsp. *oxydans* NCIB 9013 to represent the acetic acid group; *Zymomonas anaerobia* BSO 57 to represent the anaerobic group of beer spoilers; and *Lactobacillus brevis* BSO 31, *L. frigidus* NCIB 8518, *Pediococcus acidilactici* NCIB 6990, and *Pediococcus* sp. BSO 77 were selected as representatives of the lactic acid group. These organisms were originally obtained from the National Collection of Industrial Bacteria in Aberdeen (NCIB strains), the Agricultural Research Council Food Research Institute in Norwich (BSO strains), the American Type Culture Collection in Rockville, MA (ATCC strains), or from J. De Ley (Belgium).

Preparation and Enumeration of Inocula

Cultures of the coliform and lactic acid groups were initially inoculated into 50.0 ml of wort broth (no longer commercially available but consisting of, per L: 12.75 g of technical maltose, 15.0 g of malt extract, 2.75 g of dextrin, 1.0 g of dipotassium phosphate, and 0.78 g of peptone), and *Zymomonas* was inoculated into tomato juice broth (Difco). All were incubated at 27° C in an anaerobic incubator (that had been twice evacuated and filled with beverage-grade CO_2). After 24 hr, the cultures were subcultured into wort broth containing 10% v/v beer (to partly acclimatize these bacteria to the more unfavorable environment) and incubated under CO_2 as above. *Acetobacter* and *Acetomonas* bacteria were cultivated aerobically in tomato juice broth. Growth of all cultures was followed by using a model 800-3 Klett-Summerson colorimeter containing a no. 66 red filter (Klett Manufacturing Co., Ltd., New York, NY) until stationary phase was reached. At this time enough culture was added to 12° P worts and to very high gravity worts of approximately 28° P (28% w/v dissolved solids) to result in bacterial loads of approximately 10^7 colony-forming units (CFU)/ml. Bacterial viability was followed by daily membrane filtration (in triplicate) with incubation of membranes at 27° C in a carbon dioxide environment (18). *Acetobacter* and *Acetomonas* samples were aerobically incubated at 27° C.

The coliforms *Enterobacter agglomerans* #127, *Klebsiella oxytoca* #52, *Hafnia* sp. BSO 105, *Citrobacter freundii* ATCC 8090, and *Flavobacterium proteus* ATCC 12841 were grown by placing the membranes onto MacConkey's agar containing 10 mg/L of actidione. Acetic acid bacteria were enumerated on Carr's medium with actidione, *Zymomonas* on Dodds and Martin's MYGP (malt extract, yeast extract, glucose, and peptone) medium with actidione, and lactic acid bacteria on MRS (de Man, Rogosa, Sharpe) medium, with actidione. Media formulations have been described previously (4–6).

Worts were pitched with 1×10^7/ml (12° P) or 3×10^7/ml (28° P) yeast (7). Yeast viable counts were also determined by membrane filtration (18), except that oxytetracycline-gentamycin agar was used to enumerate yeast viability by inhibiting the growth of the bacterial contaminants (19,20).

High-gravity worts, nutritional supplements, and fermentation conditions have been described in previous publications (8,9). The 28° P wort was supplemented with ergosterol, oleic acid, and assimilable nitrogen, as described earlier.

Ethanol Assays

Final ethanol values for each experiment were measured enzymatically with alcohol dehydrogenase as described in Sigma Technical Bulletin 331 UV (Sigma Chemical Co., St. Louis, MO). Daily ethanol values were estimated from the daily change in apparent extract in each flask, assuming 51 g of ethanol produced from 100 g of sugar (as glucose) utilized and assuming a conversion of 95% of theoretical.

Dissolved Solids

Wort and beer dissolved solids (grams of solids per 100 ml) were determined as described previously (7).

RESULTS AND DISCUSSION

Both 12 and 28° P worts were shown to support growth of all bacteria used. In the absence of yeast, inocula of approximately 10^3 cells/ml were able to multiply to at least 10^6/ml and as high as 10^9/ml before further growth was restricted by end products of metabolism.

As expected, in traditional 12° P worts, *C. freundii* (Fig. 1) was unable to tolerate beer with normal ethanol levels of approximately 5% v/v, and died prior to full yeast attenuation of the wort. *K. oxytoca* death kinetics (not shown) were similar to those for *C. freundii*. *Hafnia* (Fig. 1) experienced a notable decrease in cell viability within two days of fermentation, at which point cell numbers became constant at a final cell concentration of 10^3 CFU/ml. *F. proteus* and *E. agglomerans* (Fig. 1) were not affected by 5% v/v ethanol beer, and at the end of the fermentation, 1.1×10^6 and 3.7×10^6 CFU/ml of *F. proteus* and *E. agglomerans*, respectively, remained in the fermentations. This bacterial population would remain in the yeast, and these viable cells would be able to contaminate subsequently pitched fermentations.

In the high-gravity brews, however, all species of the coliform group tested were completely killed by the time ethanol concentrations had increased to 11–12% v/v (Fig. 2). This would eliminate any possibility of further product contamination by coliforms in high-gravity brews. Contamination problems with *Hafnia* and *E. agglomerans* have often been described but would be completely eliminated by fermentation of such very high-gravity worts.

Lactic acid bacteria were much more resistant to the inhibiting effect of the fermentation products. *Pediococcus* sp. BSO 77 (not shown) and *P. acidilactici* NCIB 6990 (Figs. 3 and 4) were able to tolerate and survive the ethanol levels produced in 12 and 28° P worts. *L. brevis* (not shown) and *L. frigidus* (Figs. 3 and 4) also survived the high-gravity fermentation but were unable to live in the presence of low (5% v/v) levels of ethanol. This surprising but reproducible finding may have been caused by a protective effect provided by the ergosterol-fatty acid and nitrogen growth factors provided for the yeast in the 28° P wort. Experiments have shown that these compounds protect lactobacilli from death when added to either 12 or 28° P worts. If they are not added (as in the 12° P worts described here), the lactobacilli die.

Survival of lactic acid bacteria in beers made from very high gravity worts was not unexpected. In wine fermentations, where ethanol concentrations of 11–12% v/v are common, lactic acid bacteria are the only bacteria known to grow in wines that are stored anaerobically and that contain proper concentrations of free sulfur dioxide (11,13,18). Likewise in distilling fermentations,

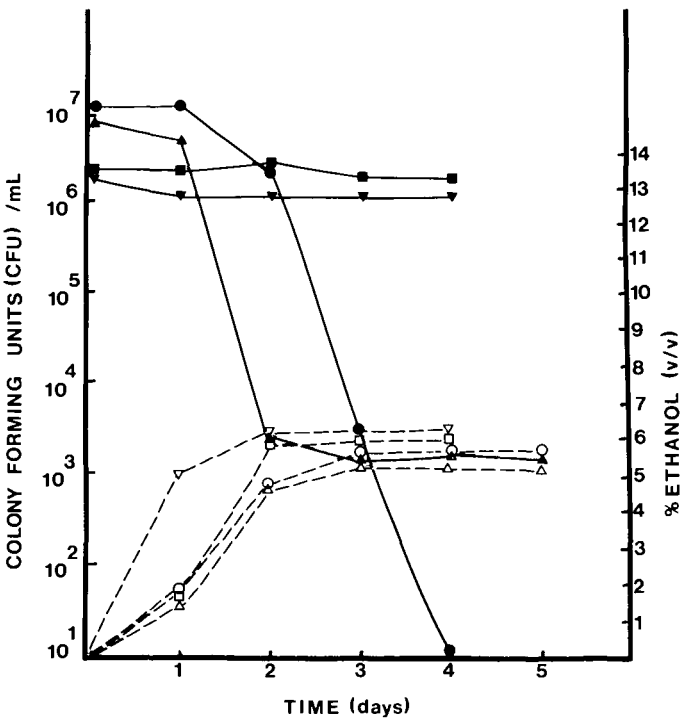

Fig. 1. Survivor curves of bacterial brewing contaminants (closed symbols) in 12° P wort vs. increasing concentrations of ethanol in the beer fermentation (open symbols). *Citrobacter freundii* ATCC 8090, ● ○ ; *Enterobacter agglomerans* #127, ■ □; *Flavobacterium proteus* ATCC 12841, ▼ ▽; and *Hafnia* sp. BSO 105 ▲ △ .

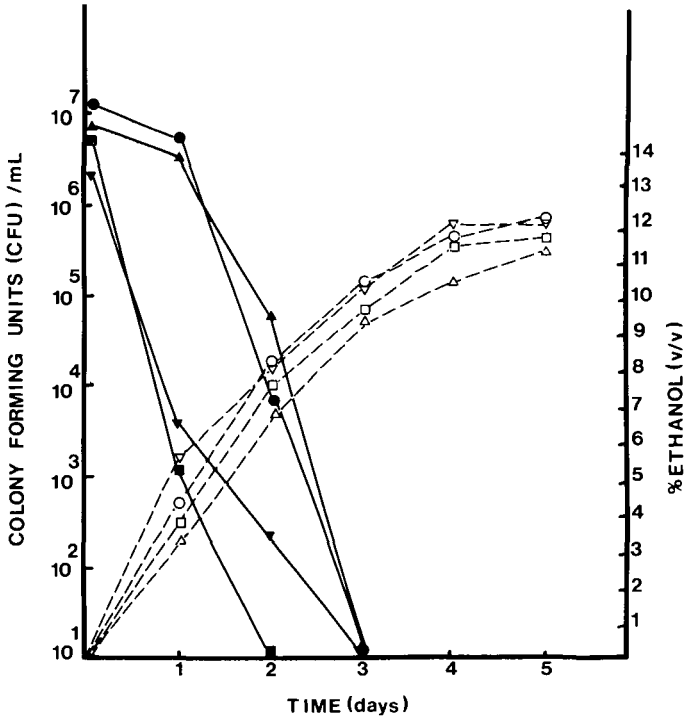

Fig. 2. Survivor curves of bacterial brewing contaminants (closed symbols) in 28° P wort vs. increasing concentrations of ethanol in the beer fermentation (open symbols). *Citrobacter freundii* ATCC 8090, ● ○; *Enterobacter agglomerans* #127, ■ □; *Flavobacterium proteus* ATCC 12841, ▼ ▽; and *Hafnia* sp. BSO 105, ▲ △ .

where peak ethanol concentrations of 12–13% v/v are sometimes achieved, lactic acid bacteria are the most commonly found contaminants (24). It seems unlikely that ethanol concentrations even higher than those reported here would eliminate contamination problems by lactic acid bacteria. In fact, *L. trichodes* (15) has been reported to spoil 17% v/v ethanol-fortified wines, and *L. heterohiochii* and *L. fermentum* were even found in 20% v/v ethanol sake (30,31).

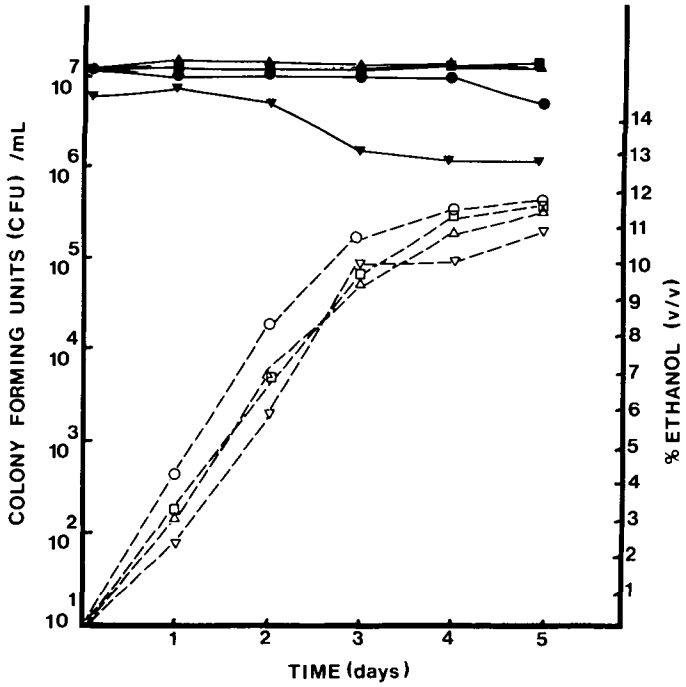

Fig. 3. Survivor curves of bacterial brewing contaminants (closed symbols) in 12° P wort vs. increasing concentrations of ethanol in the beer fermentation (open symbols). *Lactobacillus frigidus* NCIB 8518, ● O; *Pediococcus acidilactici* NCIB 6990, ■ □; *Acetobacter* sp. BSO 8, ▲ △; and *Zymomonas anaerobia* BSO 57, ▼ ▽ .

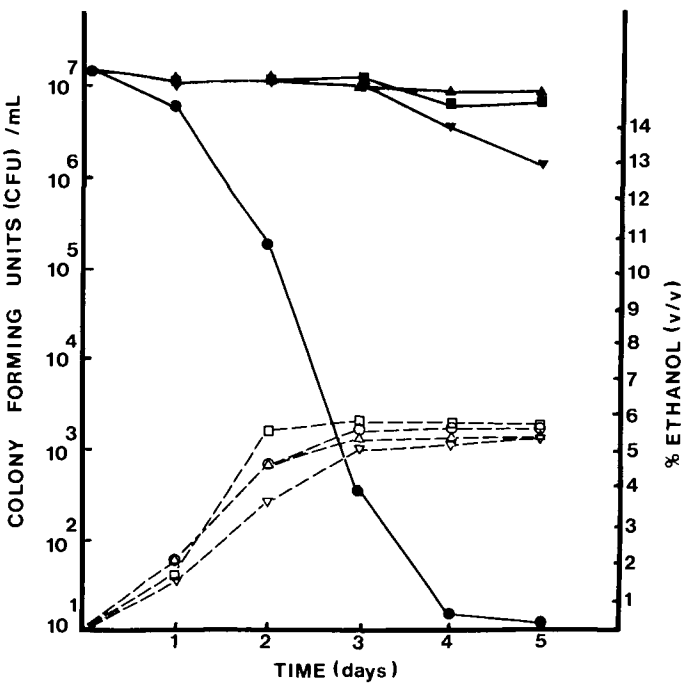

Fig. 4. Survivor curves of bacterial brewing contaminants (closed symbols) in 28° P wort vs. increasing concentrations of ethanol in the beer fermentation (open symbols). *Lactobacillus frigidus* NCIB 8518, ● O; *Pediococcus acidilactici* NCIB 6990, ■ □; *Acetobacter* sp. BSO 8, ▲ △; and *Zymomonas anaerobia* BSO 57, ▼ ▽ .

Within the acetic acid group, both *Acetomonas oxydans* subsp. *oxydans* (data not shown) and *Acetobacter* sp. BSO 8 (Figs. 3 and 4) were able to tolerate ethanol levels of 5 and 10% v/v. No death of these bacteria was evident over the course of the fermentations. Although most species of acetic acid bacteria are strict aerobes, some strains are micro-aerophilic and can survive in fermentations. In fact, wine research has shown that these organisms require only small amounts of oxygen for growth and are always present in must and wine (23,24). However, as these organisms are seldom a problem inside breweries, survival here is of little consequence.

Z. anaerobia (Figs. 3 and 4) was also able to survive the fermentations of 12 and 28° P worts, although one log unit of cell death was noted under both conditions. Current literature indicates that contaminating strains of *Zymomonas* can only tolerate up to 6% ethanol (14). However, as shown with selected strains under optimum laboratory conditions (26), *Zymomonas* is able to produce and therefore tolerate the higher ethanol levels found in these high-gravity fermentations.

In conclusion, the survival of these bacterial brewing contaminants followed the basic pattern anticipated. As all of the bacteria are capable of growing in 28 and 12° P worts, they all appear to be osmotolerant. The alcohol produced is therefore the factor most likely to kill those cells. The coliforms were not able to survive the final ethanol concentrations obtained in these very high gravity brews; this should be a significant advantage for the brewing industry, especially in countries like the United Kingdom, where coliforms have at times been troublesome (2). The other groups of bacterial contaminants—acetic acid bacteria, lactic acid bacteria, and *Zymomonas*—were capable of surviving the high gravity fermentations and could still cause spoilage problems during beer production. The resultant somewhat smaller spectrum of microorganisms resembles that described for wines of 10–14% alcohol (1,13).

ACKNOWLEDGMENTS

The authors thank Molson Breweries of Canada, Ltd., and the National Science and Engineering Research Council for research grants and a scholarship (G.P.C.), which partially supported this work.

LITERATURE CITED

1. Amerine, M. A., and Kunkee, R. E. *Ann. Rev. Microbiol.* 22:323, 1968.
2. Ault, R. G. *J. Inst. Brew.* 71:376, 1965.
3. Back, W., Weis, N., and Seidel, H. *Brauwissenschaft* 32:233, 1979.
4. Casey, G. P., and Ingledew, W. M. *Brew. Dig.* 56(2):26, 1981.
5. Casey, G. P., and Ingledew, W. M. *Brew. Dig.* 56(3):38, 1981.
6. Casey, G. P., and Ingledew, W. M. *Brew. Dig.* 56(4):24, 1981.
7. Casey, G. P., and Ingledew, W. M. *J. Am. Soc. Brew. Chem.* 41:148, 1983.
8. Casey, G. P., Magnus, C. A., and Ingledew, W. M. *Biotech. Lett.* 5:429, 1983.
9. Casey, G. P., Magnus, C. A., and Ingledew, W. M. *Appl. Environ. Microbiol.* 48:639, 1984.
10. Casey, G. P. Ph.D. Thesis. University of Saskatchewan, 1984.
11. Casey, G. P., Chen, E. C., and Ingledew, W. M. *J. Am. Soc. Brew. Chem.* 43:179, 1985.
12. Day, A., Anderson, E., and Martin, P. A. *Eur. Brew. Conv., Proc. Cong. 15th, Nice, 1975,* p. 377.
13. Fleet, G. H., Lafon-Lafourcade, S., and Ribereau-Gayon, P. *Appl. Environ. Microbiol.* 48:1034, 1984.
14. Gilliland, R. B. *Inst. Brew. Aust. NZ Sect., Proc. Conv.* 16:135, 1980.
15. Goswell, R. W., and Kunkee, R. E. Page 478 in: Economic Microbiology. Vol. 1. A. H. Rose, ed. Academic Press: New York, NY, 1977.
16. Hackstaff, B. W. *Tech. Q. Master Brew. Assoc. Am.* 15:1, 1978.
17. Ingledew, W. M. *J. Am. Soc. Brew. Chem.* 37:145, 1979.
18. Ingledew, W. M., Burton, J. D., Hysert, D. W., and Van Gheluwe, G. *J. Am. Soc. Brew. Chem.* 38:125, 1980.
19. Ingledew, W. M., and Casey, G. A. *Brew. Dig.* 57(3):18, 1982.

20. Ingledew, W. M., and Casey, G. A. *Brew. Dig.* 57(4):22, 1982.
21. Kirchner, G., Lurz, R., and Matsuzawa, K. *Monatsschr. Brau.* 33:461. 1980.
22. Kunkee, R. E., and Goswell, R. W. Page 315 in: Economic Microbiology. Vol. 1. S. H. Rose, ed. Academic Press: New York, NY, 1977.
23. Lafon-Lafourcade, S., and Ribereau-Gayon, P. Page 1 in: Progress in Industrial Microbiology. Vol. 19. M. E. Bushell, ed. Elsevier: Amsterdam, 1984.
24. Makanjuola, D. B., and Springham, D. G. *J. Inst. Brew.* 90:13, 1984.
25. Rainbow, C. *Proc. Biochem.* 6:15, 1971.
26. Rogers, P. L., Lee, K. J., and Tribe, D. E. *Proc. Biochem.* 15:7, 1980.
27. Shimwell, J. L. *Wallerstein Lab. Commun.* 12:267, 1949.
28. Skinner, K. *The Brewer* 63:3, 1977.
29. Strandskov, F. B., Baker, H. W., and Bockelman, J. B. *Wallerstein Lab. Commun.* 16:261, 1953.
30. Tanigawa, T., and Umezu, M. *J. Gen. Appl. Microbiol.* 25:31, 1979.
31. Uchida, K. *Biochim. Biophys. Acta* 348:86, 1974.
32. van Vuueren, H. J. J., Kersters, K., De Ley, J., Toerien, D. F., and Meisel, R. *J. Inst. Brew.* 84:315, 1978.
33. Weis, N., Seidel, H., and Back, W. *Brauwissenschaft* 37:189, 1979.
34. White, F. H. *Inst. Brew. Aust. NZ Sect., Proc. Conv.* 15:133, 1978.
35. Whitear, A. L., and Crabb, D. *The Brewer* 63:60, 1977.
36. Whitworth, C. Page 155 in: Eur. Brew. Conv. Fermentation Storage Symp. 1978.
37. Witt, P. R., and Blythe, P. *J. Am. Soc. Brew. Chem.* 34:76, 1976.

[Received October 7, 1985. Accepted April 25, 1986.]