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Alleviation of the effects of nitrogen limitation in high gravity worts through increased inoculation rates

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SUMMARY

There are few inexpensive, practical methods to increase the usable nitrogen level in a substrate to be fermented to a potable alcohol product, but the provision of adequate assimilable nitrogen to a fermentation medium is critical for rapid and full "wort" attenuation. One practical solution to circumvent the problem may be to increase the inoculation rate to much higher than recommended levels. In this work, an increase in the pitching rate from 1.6×10^7 cfu/ml to 8×10^7 cfu/ml was shown to alleviate fermentation problems caused by nitrogen limitation. Attenuation and ethanol production rates became independent of the initial wort-free amino nitrogen (FAN) concentration, as did yeast viability and maximal yeast cell number. However, the final total cell mass was lower if the wort was nitrogen-deficient, regardless of the pitching rate. These cells were smaller and/or lighter and contained less protein at the end of fermentation. Such yeast could cause problems in subsequent fermentations if reuse of yeast (common in brewing) was considered.

INTRODUCTION

An inadequate supply of assimilable nitrogen in fermentation media can lead to poor yeast growth, protracted or "stuck" wine [13,19] or beer [12,14] fermentations, poor attenuation rates and consequent decreased productivity. In addition, the organoleptic qualities of the product may deteriorate due to the catabolism of amino acids and peptides causing H_2S formation [19,23], abnormal ester formation [3], and altered patterns of diacetyl formation [7].

There are a few practical ways by which the potable alcohol industry can alleviate the effects of nitrogen limitation. The brewer can alter mashing conditions, add enzymes to increase proteolysis, increase the malt to adjunct ratio in the wort, and/or provide high amounts of yeast foods containing assimilable nitrogen. The winemaker is limited to the last alternative. Where allowable by law, specific yeast foods containing ammonium ion and/or free amino nitrogen can be used as nutrient supplements. Unfortunately, allowable addition levels are often too low to achieve full stimulation of the wine fermentation [12], and some foods have been shown to be undesirable. An example of this is the recent ban on the use of urea as a yeast food in potable alcohol production due to its contribution to the formation of ethyl carbamate (a proven carcinogen) [11,20]. The major portion of nitrogen derived from free amino acids and short peptides is utilized to synthesize new cellular and enzymic proteins [21]. Most of the FAN is taken up during the period of yeast growth and division. Since growing cells attenuate sugar solutions at rates much greater than non-growing cells [15], any factor which limits yeast growth will consequently limit the rate of attenuation. In brewing worts, yeast growth is proportional to the FAN concentration up to a value of 100 mg/l [21]. Such observations have led to recommended minimal amounts of FAN of 140–150 mg/l for the adequate fermentation of normal gravity (10–12°Plato) worts [12,14]. Absolute concentrations of assimilable nitrogen required for high gravity brewing (14–18°P) have not yet been defined, although in the winery, the amount of nitrogen required for yeast growth has been correlated with the sugar content of the must [13,19,27].

The minimal requirements for nitrogen are therefore dictated by the amount of yeast growth desired in that medium. The linear growth rate, yeast mass increase and final cell number, and the rate of sugar catabolism in grape must are all directly influenced by the amino nitrogen content of a nitrogen-limited must [19,27]. Once minimal requirements for assimilable nitrogen are met, excess nitrogen stimulates only the fermentation rate [19,27].

A consistent and predictable inoculation (pitching)

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rate is important for uniform fermentations [16]. One practical way to achieve an adequate rate of attenuation is to increase that pitching rate. In brewing, the number of viable cells required is related to the initial gravity of the wort [16]. Brewers pitch 12 °P wort at 10 to 15 million viable cells per ml and increase this rate to 25 million cells per ml in higher gravity brewing [4,16]. A general rule is to add one to two million viable cells per ml per degree Plato [5,16]. Depending on the numbers of viable cells pitched, brewers yeasts will increase in number three- to five-fold during the fermentation.

Winemakers traditionally use lower pitching rates approximating 5 million cells/ml [13] even though carbohydrate in the juice can exceed 24 ° Brix. Final yeast levels in commercial winemaking are close to 10^8 cells/ml. This represents a 20-fold increase in cell number. The level of yeast growth which occurs in winemaking is therefore about four times that which is observed in the brewery. It is not surprising that reports of nitrogen-limited, sluggish, or protracted must fermentations outnumber those of wort fermentations, although this may change as more breweries adopt high gravity brewing — a process where worts are usually supplemented with a nitrogen-deficient adjunct such as corn syrup [5,6,10].

Ingledew and Kunkee [13] reported that increases in the inoculation rate in highly nitrogen-deficient grape juice led to faster fermentation. They suggested that in order to prevent sluggish fermentation in nitrogen-poor juices, one should inoculate with at least 10^7 yeasts/ml and employ nitrogen-containing yeast foods to the maximum allowable level. The suggested inoculation rate was at least double that normally employed by those wineries using active dry wine yeasts.

Higher pitching rates are advantageous in many respects. Attenuation begins more rapidly, and viability losses that occur immediately after pitching [4] are not apparent. Less dependence on nutrients for growth is likely since the overall growth level is reduced. It is well known that increasing the pitching rate results in decreased specific growth rates [25,26]. Griffin [9] and Slaughter and McKernan [24] confirmed that attenuation rate increased as the pitching rate was increased, and also reported that the profiles of volatile flavor and aroma producing compounds were relatively unaffected by increased inoculum size.

In this paper, the behavior of the lager yeast, *Saccharomyces uvarum*, was examined under conditions of severe nitrogen limitation in high gravity brewing fermentations. The fermentative performance was evaluated at recommended pitching rates and related to the initial FAN level of the wort. The pitching rate was then increased in an attempt to alleviate some of the nitrogen-limitation effects observed at the lower inoculation level.

MATERIALS AND METHODS

Yeast. A commercial *Saccharomyces uvarum* lager strain was used throughout (Labatt's Saskatchewan Brewery, Saskatoon, SK, Canada). Fresh slurry was obtained in a sterile 2-l flask 48 h prior to the start of each experiment. The yeast was then enumerated, stored at 4 °C, and then used directly without adapting the cells to high gravity or high ethanol environments.

Preparation of worts. Commercial high gravity 16 g dissolved solids/100 ml (approx. 16 °P) lager wort prepared from a malt/corn-grit adjunct mixture was provided by Molson's Saskatchewan Breweries Ltd., Regina, SK). The wort was autoclaved (121 °C for 30 min) and stored in 2-l quantities at 2 °C until required. The wort was coarsely filtered prior to use to remove precipitated protein. The original wort which contained 250 mg FAN/l was diluted with a maltose solution (16 g/100 ml but 0 mg/l FAN), so that the mixtures contained 50, 100, 150, 200, and 250 and 250 mg FAN/l. All of the prepared worts contained carbohydrate at 16 °P. These mixtures were fermented along with a control 16 °P maltose solution, and with a 16 °P wort which was supplemented with 1% (w/v) yeast extract (Difco Laboratories) adjusting its FAN content to 750 mg/l.

Fermentations. Sterile "worts" (400 ml) adjusted to various nitrogen levels were dispensed aseptically into 500 ml sterile Wheaton Celstir reactors (Wheaton Instruments, Millville, NJ, U.S.A.) The fermentor contents were agitated at 90 rpm throughout the fermentation by a Wheaton Model III Biostir 6. Temperature was maintained at 14 °C by a Haake Model D3 refrigerated circulator (Haake Mers-Technik Co., Berlin, F.R.G.).

Semi-anaerobic conditions were achieved as follows: first, the unpitched wort at 4 °C was saturated with oxygen from the air by agitating the contents overnight with foam plugs in the fermentor side-arm ports; the wort was pitched by introduction of the slurry through a side-arm opening. After fermentation had initiated, oxygen access to the wort was limited by the CO_2-blanket that formed during active fermentation.

Anaerobic conditions were achieved by flushing the wort with nitrogen gas to remove all dissolved oxygen, pitching the yeast through a port while flushing the headspace with nitrogen gas, and sealing the ports with rubber stoppers, venting needles, and Parafilm during the fermentation.

Viable counts and pitching procedures. The membrane filtration technique was used to enumerate yeasts in slurry [4]. Samples were filtered in triplicate and plated onto yeast extract-peptone-dextrose agar (YEPD). The slurry volume for the desired pitching level was then calculated. This volume was introduced aseptically through a fer-

mentor port. The two pitching rates compared were the recommended rate of 1.6×10^7 cfu/ml [5] and an increased rate of 8×10^7 cfu/ml (the expected maximal yeast number from a wort pitched at 1.6×10^7 cfu/ml). Membrane filtration was also used to enumerate yeasts in the fermentation samples.

Wort and beer analysis. Total dissolved solids after yeast removal (11670 × *g*, 15 min) were determined gravimetrically [6]. This method relies on the fact that non-volatile carbohydrate is converted to volatile alcohol and carbon dioxide—neither of which can be weighed after drying at 105 °C. Duplicate 4.0 ml aliquots of supernatant were transferred to preweighed aluminum pans, dried for 2 h at 105 °C, cooled and reweighed. The total dissolved solids in g per 100 ml were then calculated. Ethanol was determined in duplicate by use of the Sigma alcohol dehydrogenase assay (Sigma Technical Bulletin No. 331 U.V., Sigma Chemical Co., St. Louis, MO, U.S.A.). Free amino nitrogen in duplicate wort samples was determined colorimetrically by the European Brewing Convention's ninhydrin method [8] employing glycine (Sigma) as the standard.

Yeast analyses. Cell pellets obtained by centrifugation of 20 ml samples were used for yeast mass determinations. The pellets were washed twice with 20 ml of 0.1% peptone water and then resuspended to 10.0 ml of the same solution. Duplicate 3.0 ml samples of the concentrated suspensions and of the resuspending peptone water were transferred to preweighed aluminum pans. The pans were dried to constant weight at 105 °C and the cell dry weight per ml of fermenting liquid was calculated following correction for the weight of the peptone.

Yeast glycogen levels (in duplicate) were determined by the iodine/potassium iodide staining method of Quain and Tubb [22] using rabbit liver glycogen (Sigma) as standard. An adapted method of Lowry et al. [17] was used for yeast protein determinations (in duplicate). The sole adaptation consisted of first digesting the sample and solubilizing the protein by boiling the sample for 5 min with an equal volume of 0.5 N NaOH.

Statistical analysis and fermentation replication. All results are means of duplicate fermentations. Analysis of variance was used to determine if significant differences ($\alpha = 0.05$) existed between nitrogen treatments. To determine where these differences occurred, Duncan's multiple range test was employed [18]. Usually, the points at which maximal growth had occurred were compared. In the case of viable counts, points where maximal numbers were attained were chosen for comparison.

RESULTS AND DISCUSSION

Yeast viability

Figs. 1A and B show the effects of various levels of nitrogen on the ability of yeasts to divide and maintain viability when both pitching levels were employed. In the case of the recommended pitching rate (Fig. 1A), the wort nitrogen level affected the number of viable yeast present after the first 12 h. Higher nitrogen contents resulted in faster and significantly ($\alpha = 0.05$) more extensive cell division. The final level of viable cells was a function of the degree of nitrogen limitation as very low initial FAN levels lead to significantly ($\alpha = 0.05$) lower maximal viable counts. The sugar control was particularly inhibited with appreciable cell death occurring after 72 h. If FAN was present in excess, it promoted faster cell division although final cell numbers were similar (compare the 750 mg/l and 250 mg/l controls).

When a higher pitching rate of 80 million cfu/ml was employed, a different result was obtained (Fig. 1B). Viable counts were maintained throughout fermentation at or near the pitched level in all fermentors. Although some increase in mass occurred, and some new cells may have been made simultaneously with the death of others, the wort (even with added yeast extract) would not nutritionally support even one full cell division of this population. Thus, increasing the pitching rate from 1.6×10^7 to

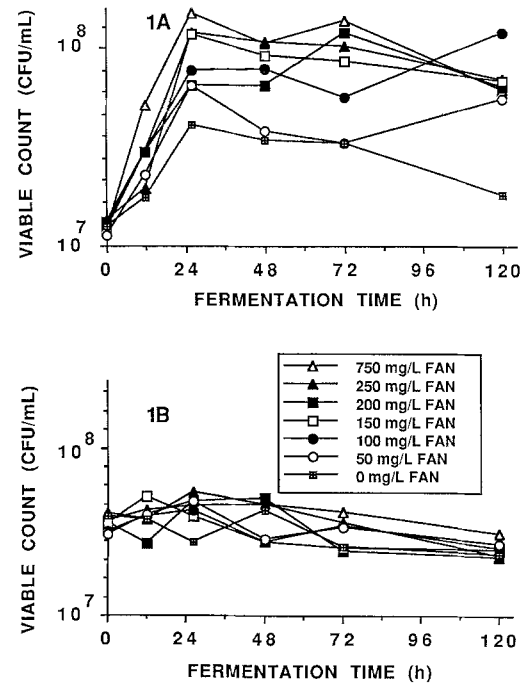

Fig. 1. The effect of FAN concentration on yeast viability over the course of the fermentation for (1A) the recommended pitching rate, and (1B) five-fold the recommended pitching rate.

8×10^7 yeasts/ml appeared to relieve the dependence of yeast cell division and viability on the initial FAN level of the wort.

Yeast mass accumulation

Fig. 2A shows the patterns of cell mass accumulation in the various worts when employing the lower pitching rate. As the initial FAN level was increased, the cell dry weight after the first 12 h accumulated significantly faster ($\alpha = 0.05$) and to a higher maximal level. This was in part due to increased cell division (as seen in Fig. 1A), but the same effect occurred under conditions of excess assimilable nitrogen where no such differences in cell number were observed.

Fig. 2B illustrates that the same trends were observed when the elevated pitching rate was used. At this inoculation rate, there were no significant increases in viable counts (Fig. 1B), but yeast dry weight did increase to a degree largely dictated by the initial FAN level in the wort. Since cell numbers were virtually unchanged, and cell death (by methylene blue) was not extensive enough to be noticeable, the variability in cell mass appeared to be due to the production of larger and/or heavier cells. Later decreases in dry weight were not associated with viability losses. Cells during these times became lighter but did not lyse.

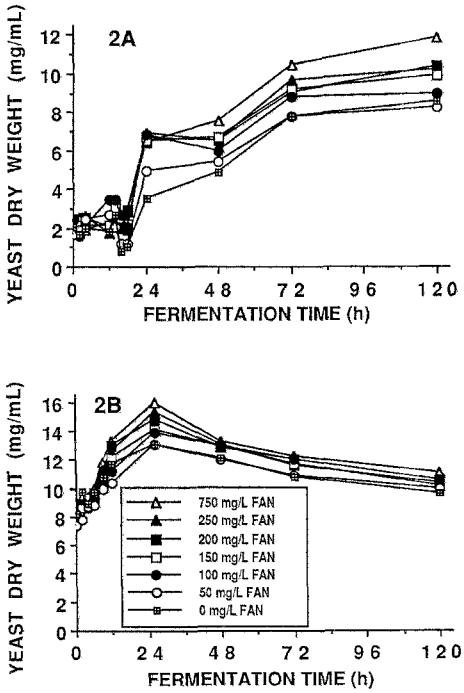

Fig. 2. The effect of FAN concentration on yeast dry weight over the course of the fermentation for (2A) the recommended pitching rate, and (2B) five-fold the recommended pitching rate.

Cell constituents were analyzed in order to determine whether there were qualitative differences in the composition of cells and whether this contributed to differences in cell size and/or mass. Final yeast glycogen concentrations at 120 h were not significantly ($\alpha = 0.05$) affected by the initial wort FAN level. However, yeast protein contents at end-fermentation were affected (Table 1); higher initial FAN concentrations resulted in significantly ($\alpha = 0.05$) more yeast protein at the end of attenuation. The protein content of the yeast was directly proportional to the initial concentration of FAN in the wort ($r = 0.99$).

Attenuation

Fig. 3A demonstrates the patterns of attenuation that resulted in the various worts when they were pitched at the recommended level. The wort plus YE control (750 mg/l FAN) started and finished at higher total solids levels due to the YE addition, but it is evident that this wort fermented the fastest overall. Yeast in the other fermentors consumed sugar at rates related to the initial wort nitrogen content. The sugar control fermented at the slowest rate. Results indicated that the attenuation rate (as measured gravimetrically) was controlled by the wort nitrogen content if it was present in limited amounts. Under conditions of nitrogen-limitation, slower attenuation rates were due in part to a reduction in the number of new actively-fermenting cells per unit volume.

When the pitching level was increased, attenuation rates in the fermentors were virtually the same (Fig. 3B).

TABLE 1

The effect of limitations in initial wort FAN on final yeast protein content[a]

Initial wort FAN level (mg/l)	Yeast protein content (g/100 g at 120 h)
750	36.9 ± 0.9^{b}
250	27.4 ± 1.6^{c}
200	24.6 ± 0.9^{c}
150	23.6 ± 0.7^{c2}
100	17.9 ± 1.6^{d}
50	15.8 ± 2.5^{d}
0	12.6 ± 0.8^{e}

[a] Mean of duplicate fermentations. All results are mean protein values ± S.D.

[b–e] Results bearing different subscripts are significantly different from each other ($\alpha = 0.05$) as evaluated by analysis of variance and Duncan's multiple range test.

[c2] Result is significantly different from all results other than the 200 mg/l FAN level.

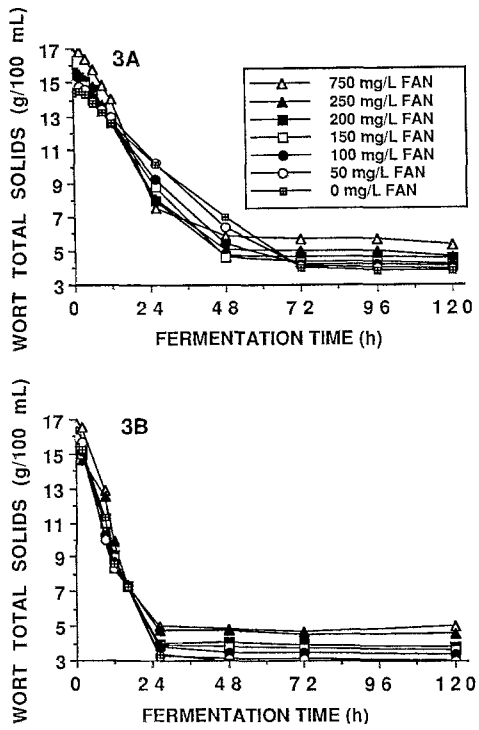

Fig. 3. The effect of FAN concentration on attenuation over the course of the fermentation for (3A) the recommended pitching rate, and (3B) five-fold the recommended pitching rate.

They all fermented at the same approximate rate and to the same end level. Even the wort containing 1% YE (750 mg/l FAN) demonstrated no significant difference in attenuation. Protraction of the attenuation rate due to FAN-limitation therefore is alleviated by the use of high pitching rates. This is important to any industry fermenting nitrogen-deficient substrates where the use of nitrogen additives is not a plausible or total solution.

Ethanol production

At both pitching levels, the ethanol production patterns reflected the attenuation rates. At the recommended inoculation level, the rate of ethanol formation was largely determined by the initial FAN content of the wort. At the high pitching rate, since attenuation was so rapid in all the worts, the rate of ethanol formation did not depend on the initial FAN content in the wort. Clearly, a higher pitching rate helped to alleviate ethanol formation problems observed at the recommended inoculation level.

Maximal ethanol concentrations for the lower pitching rate experiment ranged from 4.9 to 5.3% w/v. Maximal ethanol levels for the higher pitching rate experiments were 5.4 to 6.1% w/v. Higher nitrogen contents led to higher maximal ethanol concentrations. Slower attenuation led to less final ethanol as was the case when the lower pitching level was employed.

FAN utilization patterns

Fig. 4A shows the pattern of FAN utilization in the fermentors when the recommended pitching level was used. Most cell division took place during the first 24 h (Fig. 1A) and since FAN use and growth are related, one would expect the majority of FAN utilization in this period. This was the case. It is evident from this figure that FAN was limited by 24 h in most of these worts even though the FAN was not completely used due to the fact than FAN from peptide material larger than a di- or tripeptide is not metabolizable by the yeast. The difference between the final FAN level in the sugar solution (FAN present in the slurry that was used) and the final FAN in the worts of lower FAN concentration was the detectable nitrogen supplied by the amount of original wort used which was not usable by the yeast (the free amino groups on larger peptides and proteinaceous wort constituents).

When a high pitching rate was employed, FAN use was more rapid due to the higher number of added cells (Fig. 4B). In the low FAN worts, most FAN disappeared in 12 h or less. At either pitching rate there was a direct and proportional relationship between the amount of FAN used in the fermentation and the initial FAN supply in the wort ($y = 7.47 + 0.75x$, $r = 0.99$). This is further proof that these worts were nitrogen limiting.

Effects of oxygen and nitrogen limitation

The above described experiments were not performed under conditions of simultaneous nitrogen and oxygen limitations. Yeasts require dissolved oxygen in order to synthesize unsaturated fatty acids and ergosterol [1,2] both of which are required for continued yeast growth and viability [1,2,6]. When the added pressure of oxygen limitation was included and the recommended pitching rate used, the inhibitory effects of nitrogen limitation were further compounded (data not shown). Attenuation slowed and the sugar control would not end ferment (Fig. 5). Viability losses were significant in the lowest FAN content worts when oxygen was excluded. These viability losses corresponded to the time at which usable FAN from the medium had disappeared. Overall maximal cell numbers were significantly reduced ($\alpha = 0.05$) from the levels reached when oxygen was available to the yeast under the same conditions of nitrogen limitation. Increasing the wort gravity from 16 to 28 g dissolved solids/100 ml (very high gravity) while still limiting oxygen and nitrogen supplies did not further compound the noted effects (data not shown). These results obtained with wort closely parallel those for a must system reported earlier [13].

When pitching levels were raised to a point where no cell number increase occurred, attenuation and ethanol

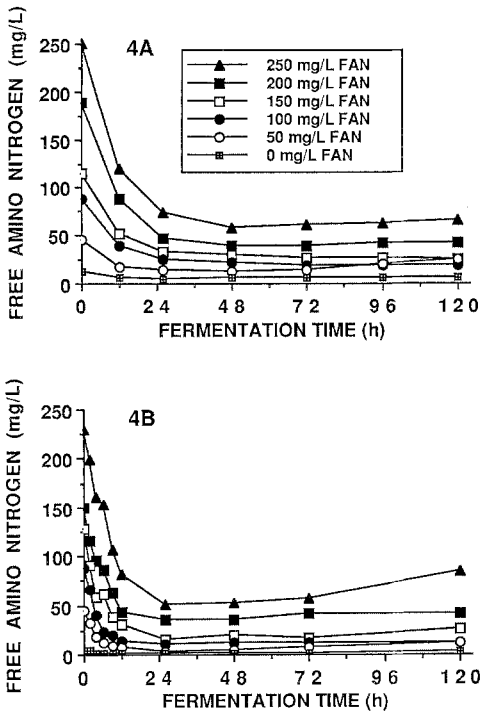

Fig. 4. The utilization of FAN over the course of the fermentation for (4A) the recommended pitching rate, and (4B) five-fold the recommended pitching rate. The 750 mg/l fermentations were not included due to scale considerations.

production rates were independent of dissolved oxygen supplies in high gravity wort (data not shown). Therefore, when one reduces the overall level of cell division, nutrients required for yeast growth such as oxygen and assimilable nitrogen, were found not to control attenuation or ethanol formation rates.

CONCLUSIONS

It is obvious from the results presented here that high pitching rates increase the rate of fermentation, not only when the amount of utilizable nitrogen is adequate, but even more importantly when the supply of assimilable nitrogen is low or growth limiting. Although the mechanism of stimulation of fermentation at high pitching rates is not clearly understood, two possibilities may be suggested. First, although no increase in viable cell count was observed, there was an increase in the biomass. This suggested that there was cell growth in terms of cell enlargement even though no new viable cells were seen. Alternatively, some cells equal to the number of newly formed cells might have lost viability, with the result that there was no measureable change in the viable counts. Moreover, although few were detected by methylene blue, dead cells might have lysed or excreted nutrients, such as amino acids, vitamins or minerals, stimulatory to the fermentation.

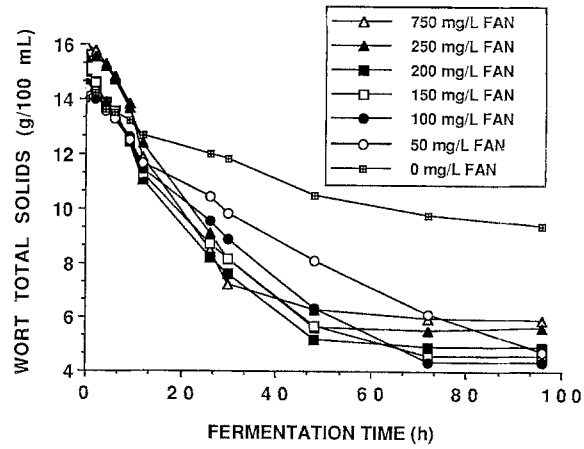

Fig. 5. Wort attenuation versus time using the recommended pitching rate when both oxygen and nitrogen were limiting yeast growth.

Whatever the mechanism, stimulation of fermentation at high pitching rates has important practical applications. By increasing pitching rate, one can stimulate the rate of fermentation and avoid "stuck" or "sluggish" fermentations. This is especially beneficial when it is not allowed by law to supplement the "wort" or "must" with growth-stimulatory nutrients in the form of yeast foods.

Such high levels of inoculation are probably not practically attainable using pitching yeast slurry (where approximately 160 ml of slurry of 1 liter for "wort" would be necessary based on a slurry cell count of 5×10^8 per ml) unless the slurry could be concentrated or unless the pitching level was somewhat decreased from the higher rate used in this work. However, any increase over the recommended pitching rate would help to alleviate the problems of nitrogen limitation.

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