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Improved ethanol yields through supplementation with excess assimilable nitrogen

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SUMMARY

Very high gravity (VHG) worts of 24° Plato supplemented with various amounts of yeast extract (YE) were inoculated at two different levels with a commercial lager strain of *Saccharomyces cerevisiae*, and the resultant beers and yeast crops were analyzed. Excess assimilable nitrogen did not result in higher viable yeast counts but did lead to increased rates of growth and increased accumulation of cell mass (partly due to higher cellular protein content). Fermentation (ethanol production) rates were significantly enhanced by YE supplementation up to 1.25% w/v, and the maximum ethanol concentrations seen were significantly higher as YE levels were increased (maximum effect at 0.75%), although YE itself was largely unfermentable. Lower ethanol yields in unsupplemented control worts could not be explained by increased carbon diversion to acetaldehyde, glycerol, or glycogen.

INTRODUCTION

Provision of adequate assimilable nitrogen in a fermentation is critical for rapid and full sugar utilization. The major portion of free α-amino nitrogen (FAN) is utilized by yeasts to synthesize new cellular and enzymic proteins [20]. Minimal nitrogen requirements are usually dictated by the amount of yeast growth which must occur in the medium to achieve a satisfactory fermentation [17]. Nitrogen 'limitation', then, is a condition of inadequate usable nitrogen such that fermentation becomes protracted and/or incomplete (sluggish or stuck). Growing yeasts ferment sugar solutions at much higher rates than do resting cells [10,11] and any factor which prematurely limits yeast growth will cause an associated decline in the fermentation rate.

In potable alcohol production, adequate assimilable nitrogen is considered to be that amount that results in a normal fermentation rate and level. In normal gravity wort (10–12° Plato where 1° P = 1 g of sugar – as sucrose – per 100 g wort at 20 °C), adequate usable nitrogen must be in the 140–150 mg FAN/l range [6,9]. In higher gravity situations, the recommended concentration increases [2]. In enology, the recommended level is also related to the sugar concentration of the must [8,15,26].

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Nitrogen concentrations above the recommended level could be considered nitrogen 'excess', but they lead to more rapid fermentations [8,15,26] and increased yeast mass [1]. Only a few investigators have studied the effects of excess assimilable nitrogen [1,8,15,19,25,26]. The rapid fermentation rates experienced are of extreme importance to industrial alcohol producers. Fuel alcohol producers do not have to adhere to regulations regarding additive levels, and fermentation is done at elevated temperatures often employing high inoculation rates [14]. Most fuel alcohol producers know that adequate concentrations of assimilable nitrogen must be present for a normal fermentation but have ignored the possibility of increasing productivity through supply of excess assimilable nitrogen. Chen [4] was able to approach the ethanol productivity levels obtained in continuous fermentation by supplementing a batch fermentation with 2.8% YE and by using a higher pitching rate. In our laboratory, alcohol in batch cultures using brewing yeasts reach as high as 16% v/v [2,3], and during production of fuel alcohol from wheat mash, even greater concentrations of ethanol have been obtained [24].

Traditional batch culture systems are most commonly used in the alcohol industry [14]. It is important to examine further optimization of batch productivity by altering the nutritional status of the fermentable substrate. This paper examines the effects of excess assimilable nitrogen on the ethanol production rates and the final ethanol concentrations in traditional batch brewing fermentations.

MATERIALS AND METHODS

Yeast

A slurry of commercial *Saccharomyces cerevisiae* lager yeast was obtained 48 hours prior to the start of each experiment. The yeast was enumerated and used directly without adaptation to VHG or high ethanol environments.

Media preparation

Commercial high gravity (HG) 16° Plato lager wort prepared from a malt/corn-grit adjunct mixture was autoclaved (121 °C for 30 min) and stored in 2 l quantities at 2 °C until use. Prior to use the wort was coarsely filtered to remove precipitated protein. To the wort base, Casco 1639 high maltose syrup (Canada Starch Co., Inc., Cardinal, ON.) was added until the desired starting gravity was reached. The adjusted gravity was monitored using a hydrometer calibrated in °Plato. The prepared VHG wort was then supplemented with various amounts of YE (Difco Laboratories, Detroit, MI). The YE was dissolved by agitation, and the worts were reautoclaved (121 °C, 15 min). Unsupplemented VHG worts were fermented as controls. In the experiments employing a glucose medium, 5% w/v glucose (analytical grade, BDH Chemicals, Toronto, ON.) solutions were prepared in double-distilled, deionized water. These solutions were supplemented with YE and sterilized as above.

Fermentations

Supplemented and control media (400 ml) were dispensed aseptically into 500 ml sterile Wheaton Celstir reactors (Wheaton Instruments, Millville, NJ). 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 D3G refrigerated circulator (Haake Mers-Technik Co., Berlin, F.R.G.). Semi-anaerobic and anaerobic conditions were achieved as previously described [18].

Fermentation replication and statistical analysis

The YE-supplemented wort fermentations were conducted in triplicate; controls for these experiments and glucose fermentations were done in duplicate. 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 at the same significance level.

Viable counts and pitching procedures

Membrane filtration was used to enumerate viable organisms in yeast slurry and in fermentation samples [7]. The experiments inoculated at low cell densities were pitched through a sidearm at 2×10^6 colony forming units (CFU)/ml; other experiments were pitched at 20×10^6 CFU/ml, the recommended level for this VHG wort of 10^6 CFU/ml per °P [3,12]. In the glucose solution experiments, the pitching rate was 10^6 CFU/ml.

Medium and beer analyses

Total dissolved solids after yeast removal by centrifugation (11 670 × g, 15 min) were determined gravimetrically [2]. Ethanol was determined by alcohol dehydrogenase (Technical Bulletin No. 331 UV, Sigma Chemical Co., St Louis, MO). Free amino nitrogen (FAN) was determined colorimetrically by the ninhydrin method [5] employing glycine (Sigma) as the standard. Acetaldehyde concentrations were measured using aldehyde dehydrogenase (Boehringer Mannheim, Laval, PQ). Glycerol concentrations were determined using a glycerokinase-pyruvate kinase-lactate dehydrogenase method for the determination of glycerol in foodstuffs (Boehringer Mannheim). All analyses were done at least in duplicate.

Yeast analyses

Cell dry weights per ml of fermenting liquid were calculated as previously described [2,17]. An adapted method of Lowry et al. [13] 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. Yeast glycogen concentrations were determined by the method of Quain and Tubb [21,22] using a rabbit liver glycogen (Sigma) standard.

Carbohydrate analyses

Aliquots (2.5 ml) of wort were diluted to 250 ml. Diluted samples were passed through 3 cm^3 of 20–50 mesh AG 50W-X8 (H^+) cation exchange resin (Bio-Rad Laboratories, Mississauga, ON.), through 3 cm^3 of 100–200 mesh AG 1-X4 anion exchange resin (formate) and then through a C-18 Sep Pak cartridge (Waters Associates, Milford, MA) and a 0.45 μm membrane filter (Gelman Sciences, Laval PQ).

HPLC analyses

Wort and syrup samples were analysed on a Dionex (Sunnyvale, CA) Bio LC 4000 gradient HPLC with a 100 μl loop. Separation of the carbohydrates was carried out on a Dionex 10 μm Carbo Pac PA1 pellicular anion exchange column (4 × 250 mm). The flow rate was 1.0 ml/min and the carbohydrates were detected by a pulsed amperometric detector at a sensitivity of 10K. The electrode was maintained at the following potentials and durations: $E_1 = 0.05$ V, $t_1 = 120$ msec; $E_2 = 0.80$ V, $t_2 = 120$ msec; $E_3 = -0.60$ V; $t_3 = 420$ msec. A post-

column delivery system of 300 mM NaOH at a flow rate of 0.80 ml/min was used to prevent baseline drift. To achieve separation, an elution of 100 mM NaOH for 8 min was followed by a gradient established such that at 40 min, the mobile phase was 100 mM NaOH/250 mM sodium acetate. Over the next minute, the mobile phase was changed to 300 mM NaOH (to remove acetate ions from the columns). After 30 min of washing, the column was re-equilibrated with 100 mM NaOH in preparation for the next injection. The carbohydrates eluting from the columns were plotted by a Spectra Physics model 4290 integrator.

RESULTS

Starting assimilable nitrogen concentrations

Unsupplemented syrup-adjusted VHG wort contained 250 mg/l of FAN. Each 0.25% w/v increment of YE added to wort or glucose solution added approximately 125 mg FAN/l. Syrups contain no significant amounts of FAN.

Very high gravity wort inoculated at the lower inoculation rate

In these experiments, 24°P worts supplemented with 0.25 and 0.75% YE were pitched at 2×10^6 CFU/ml. The amounts of FAN taken up in the various worts were: 165 mg/l in control worts; 300 mg/l in 0.25% YE worts, and 400 mg/l in 0.75% YE worts. The relationship between the initial FAN supplied in the wort and the amount of FAN taken up by the yeast was linear ($r = 0.96$). The maximum viable cell count rose at the same rate in all of the worts to a maximum of

TABLE 1

Effect of YE supplementation on the yeast protein content at the end of fermentation

YE supplement (% w/v)	Protein g/100 g
0[a]	26.0 ± 2.0*
0.25[b]	35.7 ± 1.3**
0.75[b]	49.7 ± 2.1***

[a] Duplicate fermentations.
[b] Triplicate fermentations.
Each value represents the mean protein determination ± the standard deviation. Protein analyses were conducted in duplicate. Results bearing different superscripts (*, ** or ***) are significantly different from each other as evaluated by analysis of variance and Duncan's Multiple Range Test ($\alpha = 0.05$).

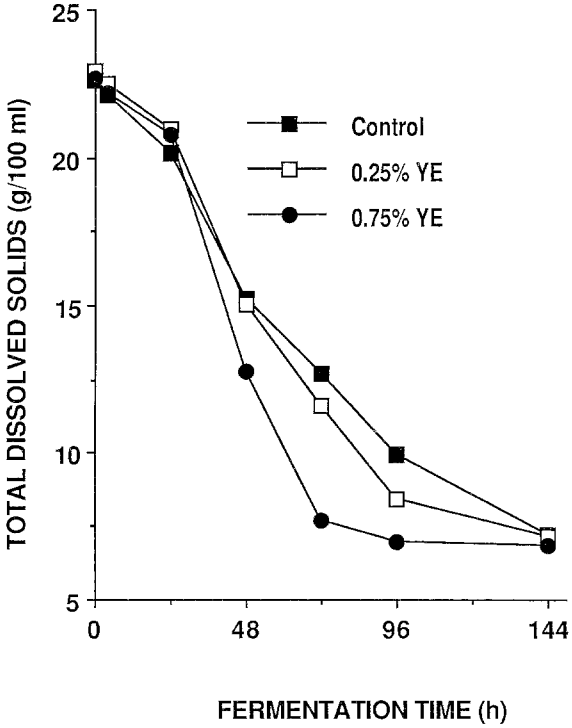

Fig. 1. The effect of supplemental assimilable nitrogen on attenuation of 24°P wort pitched at 2×10^6 CFU/ml. Points represent the means of triplicate fermentations except for the control points (duplicate).

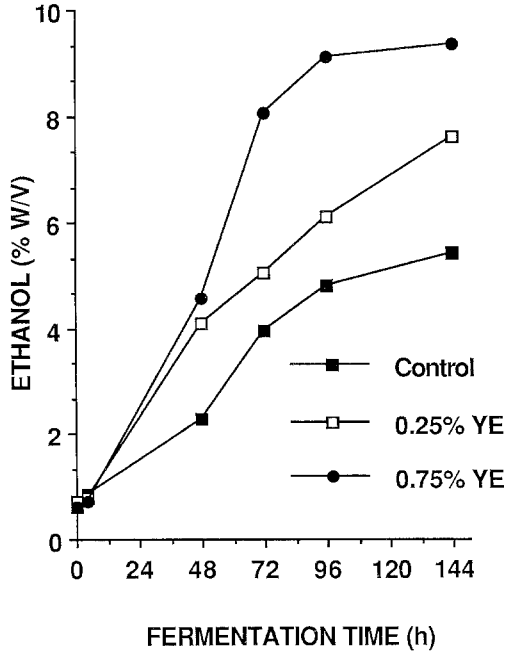

Fig. 2. The effect of supplemental assimilable nitrogen on ethanol formation and concentration (24°P wort, pitched at 2×10^6 CFU/ml). Points represent the means of triplicate fermentations except for the control points (duplicate). All values at 144 h were maximum ethanol values obtained, as fermentable carbohydrate was completely used at this time (Fig. 1).

80×10^6 CFU/ml. The cell mass increased from an initial level of 0.4 mg/ml to a maximum of 10–12 mg/ml. Both cell mass and cell number increased by a similar magnitude. The rate of mass increase was dependent on the initial FAN concentration of the wort. Yeast cells in the YE-supplemented fermentors (Table 1) contained significantly more protein at the end of fermentation. Fermentation rates also reflected the initial wort FAN content – with higher FAN concentrations resulting in significantly higher ($\alpha = 0.05$) rates (Fig. 1). Variations in dissolved solids in duplicate fermentations were always less than 0.8 g/100 ml, and usually less than 0.5 g/100 ml. Rapid ethanol production was seen (Fig. 2) with maximum ethanol concentrations dependent on the starting FAN content of the wort. Table 2 shows the theoretical maximum amounts of ethanol that could arise from syrup and wort of the same compositions as those employed in these experiments. The syrup-adjusted VHG wort results in a combination of sugars that could theoretically yield a maximum ethanol concentration of 53.2 to 53.6 g per 100 g of sugars.

At 24 h, YE-supplemented worts contained significantly higher amounts of acetaldehyde than did the control worts (16–17 mg/l vs. 11.5 mg/l). After this time, the acetaldehyde concentrations in the supplemented worts dropped to the same concentrations found in the controls – presumably due to the reduction of acetaldehyde to ethanol. Unsupplemented fermented wort contained higher amounts of glycerol than YE-supplemented wort (0.58 g/l vs. 0.42–0.48 g/l).

Very high gravity wort inoculated at the recommended inoculation rate

For this work, 24°P wort (250 mg FAN/l) was supplemented with 0.25, 0.5, and 0.75% w/v YE and pitched at 20×10^6 CFU/ml. Unsupplemented worts were used as controls. The viable cell count rose from 20×10^6 CFU/ml to a maximum of 80×10^6 CFU/ml in

TABLE 2

Ethanol yields from different fermentable sugars

Sugar	Sugar content[1] (% total sugar w/w[2])	Theoretical ethanol yield	
		g/100 g sugar	% w/w
A. High maltose brewing syrup			
Glucose	6.9	51	3.5
Fructose	n.d.	51	–
Isomaltose	0.2	53.7	0.1
Maltose	51.5	53.7	27.7
Maltotriose	12.2	54.6	6.7
Sucrose	n.d.	53.7	–
Totals	70.8		38.0
Yield calculation		38.0/70.8 × 100 = 53.6%	
B. Lager wort			
Glucose	10.4	51.0	5.3
Fructose	1.3	51.0	0.7
Isomaltose	0.5	53.7	0.3
Maltose	54.2	53.7	29.1
Maltotriose	12.8	54.6	7.0
Sucrose	1.7	53.7	0.9
Totals	81.3		43.2
Yield calculation		43.2/81.3 × 100 = 53.2%	

Sample calculation: maltose (342 MW) is hydrolyzed to 2 glucose ($2 \times 180 = 360$ MW). Then 100 g maltose is converted to 105.3 g of glucose and through glycolysis, $105.3 \times 0.51 = 53.7$ g of ethanol is produced (theoretical yield).

[1] Sugar concentrations determined by HPLC analysis. Values are the means of duplicate analyses.

[2] Dry basis

n.d. Not detected by HPLC analysis of the product.

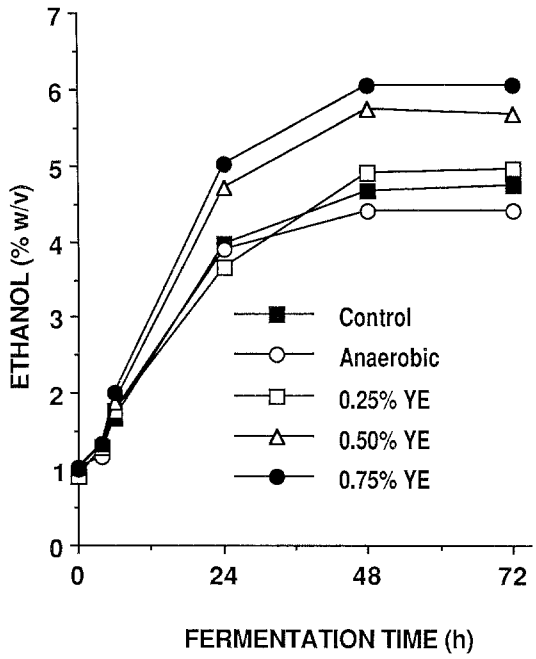

Fig. 3. The effect of supplemental assimilable nitrogen on ethanol formation and concentration (24°P wort, pitched at 20×10^6 CFU/ml). Points represent the means of triplicate fermentations except for the control points (duplicate).

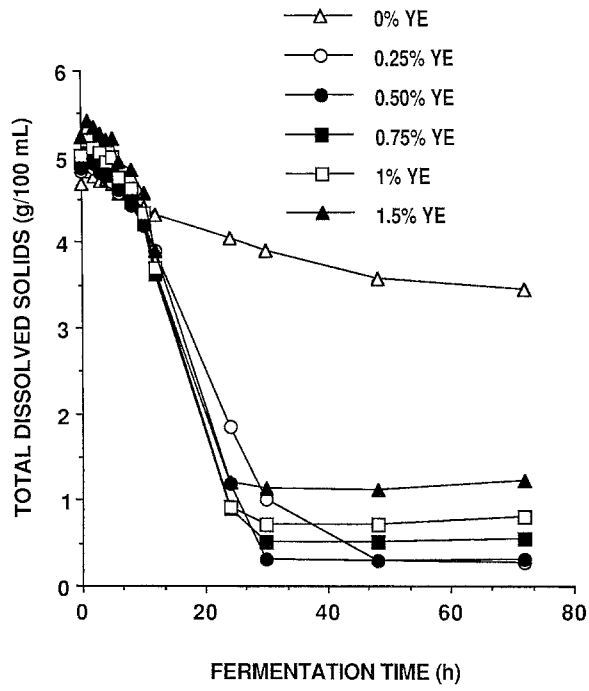

Fig. 4. The effect of supplemental assimilable nitrogen on the attenuation rate of a 5% glucose solution pitched at 10^6 CFU/ml. Points are the means of duplicate fermentations.

all worts. Maximum cell numbers were reached after 48 h, 24 h sooner than in the previous experiments inoculated at the lower level, and the rates of cell division were now almost independent of the initial FAN content. Cell mass rose from 4 mg/ml to a maximum of 12 mg/ml and the rates of mass accumulation were only slightly increased by added FAN. Again, cell mass increases were consistent with increases in cell number. Ethanol production rates and final concentrations were once again related to the initial wort FAN content (Fig. 3). Anaerobiosis led to slightly lower final ethanol concentrations. This was presumably due to less favorable growth conditions. Yeast protein contents, and glycerol and acetaldehyde levels in beer were unaffected by the initial FAN content of the wort when recommended pitching levels were used.

Glucose fermentations and the fermentability of yeast extract

A 5% w/v solution of glucose in water was supplemented with various concentrations of YE (0–1.5% w/v in 0.25% increments) and fermented by inoculating at a rate of 10^6 CFU/ml. As expected, the 5% glucose solutions with no added YE did not ferment satisfactorily (Fig. 4). The 0.25% YE fermentations (115 mg FAN/l) were protracted but end-fermented after 48 h. Glucose solutions with higher YE concentrations (0.5–1.5% w/v) end-fermented after 30 h. Variations in the end total solids

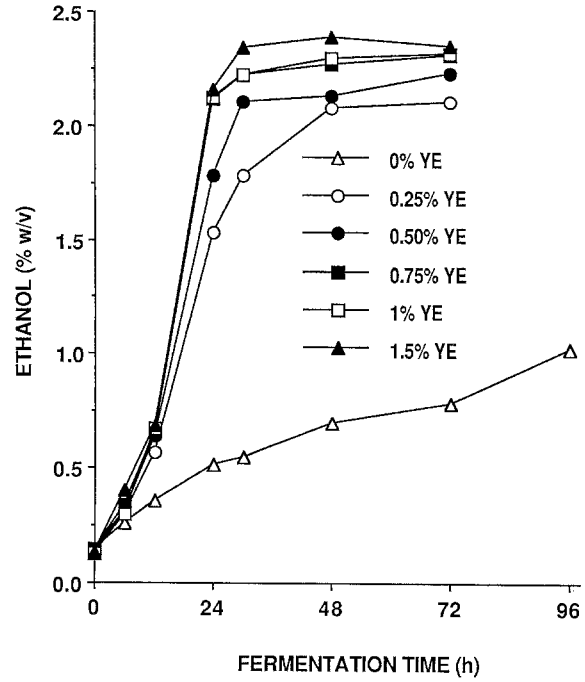

Fig. 5. The effect of supplemental assimilable nitrogen on ethanol formation and concentration in a 5% glucose solution pitched at 10^6 CFU/ml. Points are the means of duplicate fermentations.

(Fig. 4) were caused by residual YE remaining after completion of the fermentations. Fig. 5 demonstrates the stimulation provided by excess assimilable nitrogen on the rate of formation and the final concentration of ethanol. As more YE was added to the glucose solution, the ethanol production rate and end ethanol concentration were increased. The relation between the total dissolved solids used for ethanol production and the amount of YE added was linear ($r = 0.993$).

DISCUSSION

Yeast extract stimulated wort fermentation through its action as a source of FAN. Even though YE contains many substances known to promote yeast growth (vitamins, minerals, cofactors), previous work in this laboratory [2] has demonstrated that Casamino acids (Difco Laboratories, Detroit, MI), a purified casein hydrolysed for use as an amino acid supplement, resulted in the same level of stimulation of VHG fermentations. Thomas and Ingledew [24] have recently found stimulation of VHG fuel alcohol fermentations when hydrolyzed wheat proteins were used as a source of FAN. In the case of the glucose fermentations, other growth factors in YE obviously played a role in growth and multiplication.

At either pitching rate, the yeast cells used similar amounts of FAN. This occurred at each supplementation level. Each experiment gave rise to similar maximum cell numbers. Therefore, the amount of FAN used in a fermentation is not so much a function of the number of cell divisions which take place as it is related to the final cell mass supported by the wort. However, when a higher pitching rate was employed, FAN was taken up more quickly (24 h) than when fewer cells were pitched (48–72 h). All FAN uptake took place during the periods of active cell growth, but the period of cell growth was extended when the pitching rate was lower.

If available, the cells took up more FAN than was necessary for full fermentation of the sugar solutions. This phenomenon occurred in both wort and the glucose solution. In the basal wort, 250 mg FAN/l was originally available; a level considered to be more than adequate for the full fermentation of normal gravity wort [6,9]. VHG worts such as those used here have been reported to require higher concentrations of FAN for rapid attenuation [2]. In our experiments, using semianaerobic conditions, 250 mg FAN/l were sufficient to permit end-fermentation of VHG wort.

As reported by others [1,15,26], excess assimilable nitrogen did not promote increased numbers of cells or rates of cell division. Excess nitrogen did lead to increased rates and amounts of cell mass accumulation, particularly if a small inoculum was used. These increases were seen until FAN reached 870 mg/l, a level higher than the plateau of 500 mg FAN/l reported by Agenbach [1] but found optimal by Vos [25]. Since viable counts were not found to be different at the higher supplementation levels, the supplemented cells must have been larger and/or heavier than the unsupplemented yeast. An increased number of dead cells would also explain these results, although methylene blue staining failed to detect significant numbers. When the lower pitching rate was used, protein contents of the yeast increased in proportion to additions of YE. This helps to explain some of the mass differences as well as the stimulation of fermentation since some of this protein would be enzymic in nature. Saita and Slaughter [23] previously attributed stimulation by nitrogen to its function as a substrate for protein synthesis.

At either pitching rate, YE addition stimulated the wort fermentation rates. The stimulatory effect of YE was particularly apparent at the lower inoculation level. Increasing the pitching rate significantly increases the rate of fermentation. At a YE-supplementation level of 0.75%, the VHG fermentation pitched at the lower rate was completed in slightly more than 3 days, while the higher pitching rate resulted end-fermentation in 48 h. In fact, we have reported that higher pitching rates can alleviate some effects caused by nitrogen limitation [18].

Regardless of the inoculation rate, the addition of YE led to higher final ethanol concentrations in the fermented medium. Since cell numbers were unchanged by YE addition, and dead cell numbers did not rise, the actual ethanol production rate on a per cell basis was greatly increased. In the wort fermentations approximately 16 g dissolved solids/100 ml were utilized. Based on the theoretical maximum ethanol yields calculated for the syrup-adjusted wort (53.2–53.6%), 8.51 to 8.58% ethanol (w/v) could be produced. At the highest YE-supplementation level of 0.75% (no further increases in final ethanol concentrations after this point), 9.1% w/v ethanol was the maximum concentration attained. However, of this, 0.7% originated from the yeast slurry inoculum. Therefore, the net amount of ethanol formed was approximately 8.4% w/v. This was 98–99% of the theoretical maximum level. This is an extremely high yield; normal batch fermentations by yeasts optimally give rise to 93–95% of the theoretical value [14]. In contrast, the unsupplemented control worts gave rise to only 5.5% w/v ethanol, only 64–65% of the theoretical maximum. The low yields in unsupplemented conditions were likely due to the unfavorable VHG environment. This illustrates the importance of nutritionally optimizing these fermentations.

Attempts were made in control fermentations to determine the fate of the carbon not synthesized into ethanol.

Three possible products were evaluated; glycogen, acetaldehyde and glycerol. None of these compounds was found to be significantly elevated in fermentations demonstrating lowered ethanol yields. For this reason, it is believed that under unsupplemented conditions, carbon from glucose was diverted to a number of cell components via alternate pathways of metabolism.

The fermentation of glucose solutions was conducted in order to determine the ethanol yields resulting from a fully fermentable sugar and to see if some component of the YE was being fermented to extra ethanol. FAN concentrations of 250 mg/l (0.50% YE) were sufficient to promote full and rapid sugar fermentation. When fully fermented, 5% glucose could theoretically result in 2.6% ethanol by weight. YE-supplementation levels of 0.75–1.5% w/v gave rise to maximum ethanol concentrations of 2.3% (corrected for inoculum contribution). This represented 88% of the theoretical yield.

The extremely high ethanol yields in supplemented worts were disconcerting. Further investigation revealed that the gravimetric method used to measure total dissolved solids recovered only about 30% of the added YE. An investigation with a variety of sugars and worts (data not shown) showed that this was at least in part attributed to the combining of glucose and YE in the presence of heat (105 °C) forming volatile components lost at the elevated temperatures used for gravimetric analysis. Such compounds are commonly formed by Maillard type reactions [16]. Both the initial and final total solids levels were affected; however, the initial level of total solids was disproportionately lowered due to the presence of more amino and simple sugar groups early in the fermentation. Examples of this phenomenon may be noted in Figs. 1 and 4. When a given weight of YE was added, the initial gravity did not increase by the calculated amount. Gravimetric measurement by hydrometer does measure the dissolved YE since no heat treatment is involved in the analysis. Therefore, more total dissolved solids than what were gravimetrically determined existed in the prepared medium. This falsely elevated the calculated ethanol yields per unit weight of sugar but did not detract from the trends observed. Correction factors based on these gravimetric errors were applied to the calculated values dropping yields to 93–94% in the 0.75% YE-supplemented worts, 75–76% in the 0.25% YE-supplemented worts, and 64–65% in the basal wort. The yield for the unsupplemented wort remained the same since YE was not present.

The effects described in this paper have been extensively noted in this laboratory when must, wort (HG or VHG), or saccharified grain mashes have been supplemented with usable nitrogen and fermented by slurries of brewers yeasts or active dry distillers' yeasts. This communication reports results from over 30 batch fermentations of VHG wort. The large yield and rate improvements caused by excess assimilable nitrogen in a batch fermentation should be examined in more detail in order to assess potential yield increases for the fuel alcohol industry. Nitrogen supplementation can be expected to lead to significant increases in ethanol productivity.

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