Increasing fermentation efficiency at high sugar concentrations by supplementing an additional source of nitrogen during the exponential phase of the tequila fermentation process

Javier Arrizon and Anne Gschaedler

Abstract: In the tequila industry, fermentation is traditionally achieved at sugar concentrations ranging from 50 to 100 $g \cdot L^{-1}$. In this work, the behaviour of the *Saccharomyces cerevisiae* yeast (isolated from the juices of the *Agave tequilana* Weber blue variety) during the agave juice fermentation is compared at different sugar concentrations to determine if it is feasible for the industry to run fermentation at higher sugar concentrations. Fermentation efficiency is shown to be higher (above 90%) at a high concentration of initial sugar (170 $g \cdot L^{-1}$) when an additional source of nitrogen (a mixture of amino acids and ammonium sulphate, different than a grape must nitrogen composition) is added during the exponential growth phase.

Key words: *Saccharomyces cerevisiae*, fermentation efficiency, nitrogen source, tequila.

Résumé : Au cours du procédé d'élaboration de la tequila, la fermentation est traditionnellement faite à des concentrations en sucre allant de 50 à 100 $g \cdot L^{-1}$. Ce travail présente une comparaison du comportement d'une souche de *Saccharomyces cerevisiae* (isolée de jus d'*Agave tequilana* Weber variété bleue) au cours de la fermentation faite sur des moûts présentant diverses concentrations de sucre. Le but était de vérifier la faisabilité pour l'industrie d'effectuer la fermentation à des concentrations plus élevées en sucre. L'efficacité de la fermentation la plus élevée (supérieure à 90 %) est obtenue à une concentration initiale forte en sucre soit 170 $g \cdot L^{-1}$ lorsqu'une source additionnelle d'azote (un mélange d'acides aminés et de sulfate d'ammonium dont le contenu en azote est différent de celui d'un moût de raisin) est ajoutée durant la phase exponentielle de croissance.

Mots clés : *Saccharomyces cerevisiae*, efficacité de la fermentation, source d'azote, tequila.

Introduction

Tequila is a Mexican alcoholic beverage obtained by distilling and rectifying fermented agave juice and is produced in a territory protected by a guarantee of origin. There are two main types of tequila: tequila 100% obtained exclusively from sugars of the *Agave tequilana* Weber blue variety and tequila produced using 49% sugars from a source other than the agave. Sugar cane, "sugar-loaf", molasses, or hydrolysed maize syrup may be used.

The process is divided into four main phases: cooking, milling, fermenting, and distilling. The agave is harvested 8 years after planting and contains an average of 27% reducing sugars (Cedeño 1995). The agaves are cooked in autoclaves or brick ovens. Most of the assimilable nitrogen is degraded by heat, making the juice low in nitrogen for fermentation (an initial nitrogen content of 40 $mg \cdot L^{-1}$ was determined in our laboratory). For many years, tequila fermentations were carried out without nitrogen supplementation. Normally, in the tequila fermentation, the agave juice is diluted and, in some cases, an inorganic nitrogen source is added at the beginning of fermentation. Nitrogen addition during fermentation of grape must increases fermentation rate (Ribereau-Gayon et al. 1975; Ingledew and Kunkee 1985). Therefore, in the case of agave juice, which is low in nitrogen, nitrogen addition during fermentation would be expected to increase fermentation rate and efficiency. Most companies use native yeasts under no control, which leads to a long fermentation time and considerable variability in the quality of the fermentation. Lachance (1995) identified 14 different yeasts in a spontaneous fermentation process, where *Saccharomyces cerevisiae* was the

Received 17 June 2002. Revision received 7 October 2002. Accepted 9 October 2002. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 26 November 2002.

Javier Arrizon. Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Avenida Normalistas 800, Sector Hidalgo, Apto. postal 2-191, 44270, Guadalajara, México, and Universidad de Guadalajara, Departamento de Ingeniería Química, Blvd. Marcelino García Barragán y Calzada Olímpica, S.R. C.P. 44460, Guadalajara, México.
Anne Gschaedler.[1] Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Avenida Normalistas 800, Sector hidalgo, Apto. postal 2-191, 44270 Guadalajara, México.

[1]Corresponding author (e-mail: agschaedler@ciatej.net.mx).

 DOI: 10.1139/W02-093 © 2002 NRC Canada

predominant yeast. Few companies use yeasts isolated and selected from musts.

The sugar content in raw agave is at higher concentrations than are used in the fermentation process. Nevertheless, most companies dilute the juice to less than 100 g sugar/L (Cedeño 1995).

Many studies of fermentation report that as sugar concentrations increase from 120 to 180 $g \cdot L^{-1}$, decreases in growth and viability of yeasts occur (Bafrnacová et al. 1999; Casey and Ingledew 1986; Ivorra et al. 1999; Xu et al. 1996), likely because of yeast osmotic stress (Ivorra et al. 1999). Thomas et al. (1996) studied anabolic and catabolic pathway regulation under osmotic stress to understand its effect on cell metabolism, and they established evidence that the synthesis of glycolytic enzymes, as well as enzymes of the hexose monophosphate pathway, are regulated by sugar concentration and nitrogen limitation. Another phenomenon observed, especially regarding œnologic fermentation, is sluggish or stuck fermentation related to an inhibition of fermentative metabolism. There are several explanations for this phenomenon, including the toxicity of some yeast subproducts, such as hexanoic, octanoic, and decanoic acids (Muñoz and Ingledew 1990). Moreover, it has been shown that the addition of suitable nitrogen eliminates sluggish fermentations (Ingledew and Kunkee 1985). However, Salmon (1989), Mauricio and Salmon (1992), and Salmon et al. (1993) proved that the principal factor limiting fermentative metabolism is an inhibition of sugar transport. Sugar transport in yeasts has been widely studied; it is characterized by the presence of several transporters that have a distinct affinity to glucose (Ciriacy and Reifenberger 1997). Those presenting high affinity to the substrate are subject to catabolic repression and are not detected in fermentation at high sugar concentrations (Bisson et al. 1987). In addition, when protein synthesis is inhibited, the sugar transport system is inactivated, even though sugars are still present in the medium (Busturia and Lagunas 1986). A key element in limiting protein synthesis is the availability of nitrogen in the medium. In the specific case of wine, it is well known that the lack of assimilable nitrogen in must is the most common cause of fermentation problems (Ingledew and Kunkee 1985). There are several studies on the use of nitrogen in the form of amino acids and ammonia and their impact on alcoholic fermentation. The main conclusion is that if an adequate source of nitrogen is added, fermentation can be considerably activated by activating sugar transport (Bely et al. 1990; Jiranek et al. 1995; Thomas and Ingledew 1990). In the case of grape juice, must contains more amino acids than ammonia, and an addition of organic nitrogen is more effective in fermentation activation or elimination of stuck fermentation (Albers et al. 1996; Bafrnacová et al. 1999; Manginot et al. 1997; Salmon and Barre 1998; Thomas and Ingledew 1990). It is also important to point out that the nitrogen requirements of *S. cerevisiae* strains can be different (Jiranek et al. 1995; Manginot et al. 1998). In the specific case of tequila, little research on the fermentation phase has been published (Lachance 1995; Pinal et al. 1997). Lachance (1995) determined the yeast species present in the tequila process, and Pinal et al. (1997) worked on the relation between the C:N ratio and higher alcohol production. In this work, a strategy for improving the tequila fermentation process is presented, including fermenting at a higher sugar concentration. Additionally, the effect of an adequate supply of a suitable nitrogen source on the acceleration of fermentation was studied. A two-factor factorial ANOVA experimental design was utilized to determine the influence of the carbon source concentration and supplementation of an additional source of nitrogen during the exponential phase of yeast growth in the fermentation process.

Materials and methods

Yeast strain

The strain of *Saccharomyces cerevisiae* (MG) used in this study was isolated from the tequila industry and conserved in our laboratory collection (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C.).

Culture media

The must used is an *Agave tequilana* Weber blue variety juice. It was filtered, sterilized (121°C, 15 min), and diluted to reach sugar concentrations of 170, 80, and 30 $g \cdot L^{-1}$. The sugar average composition of agave juice is 94% fructose, 5% glucose, and 1% sucrose (determination made in our laboratory by high-pressure liquid chromatography). In all cases, 1 $g \cdot L^{-1}$ of ammonium sulphate was added at the beginning of the fermentation. Nitrogen addition in exponential growth, as performed at sugar concentrations of 170 and 30 $g \cdot L^{-1}$, was compared with fermentations without nitrogen addition (170, 80, and 30 g of sugar/L). Nitrogen addition during the exponential growth phase was carried out at a final concentration of 437.12 milligrams of nitrogen source per litre of must. The composition of the nitrogen source was ammonium sulphate (74%, w/w), alanine (1.8%, w/w), arginine (4.57%, w/w), aspartate (0.5%, w/w), glutamate (1.5%, w/w), glutamine (6.3%, w/w), leucine (0.57%, w/w), phenylalanine (0.45%, w/w), proline (7.5%, w/w), tryptophan (2.3%, w/w), and valine (0.5%, w/w).

Inoculation conditions

Cells were grown for 12 h at 30°C with shaking (250 rpm) in 500-mL Erlenmeyer flasks with 200 mL of must, reaching cell populations of 200×10^6 cells/mL. The must consisted of *A. tequilana* Weber blue variety juice (60 g of sugar/L) that was filtered and sterilized (121°C, 15 min). The reactor was inoculated with 200 mL, which provided an initial population in the reactor of 20×10^6 cells/mL.

Fermentation conditions

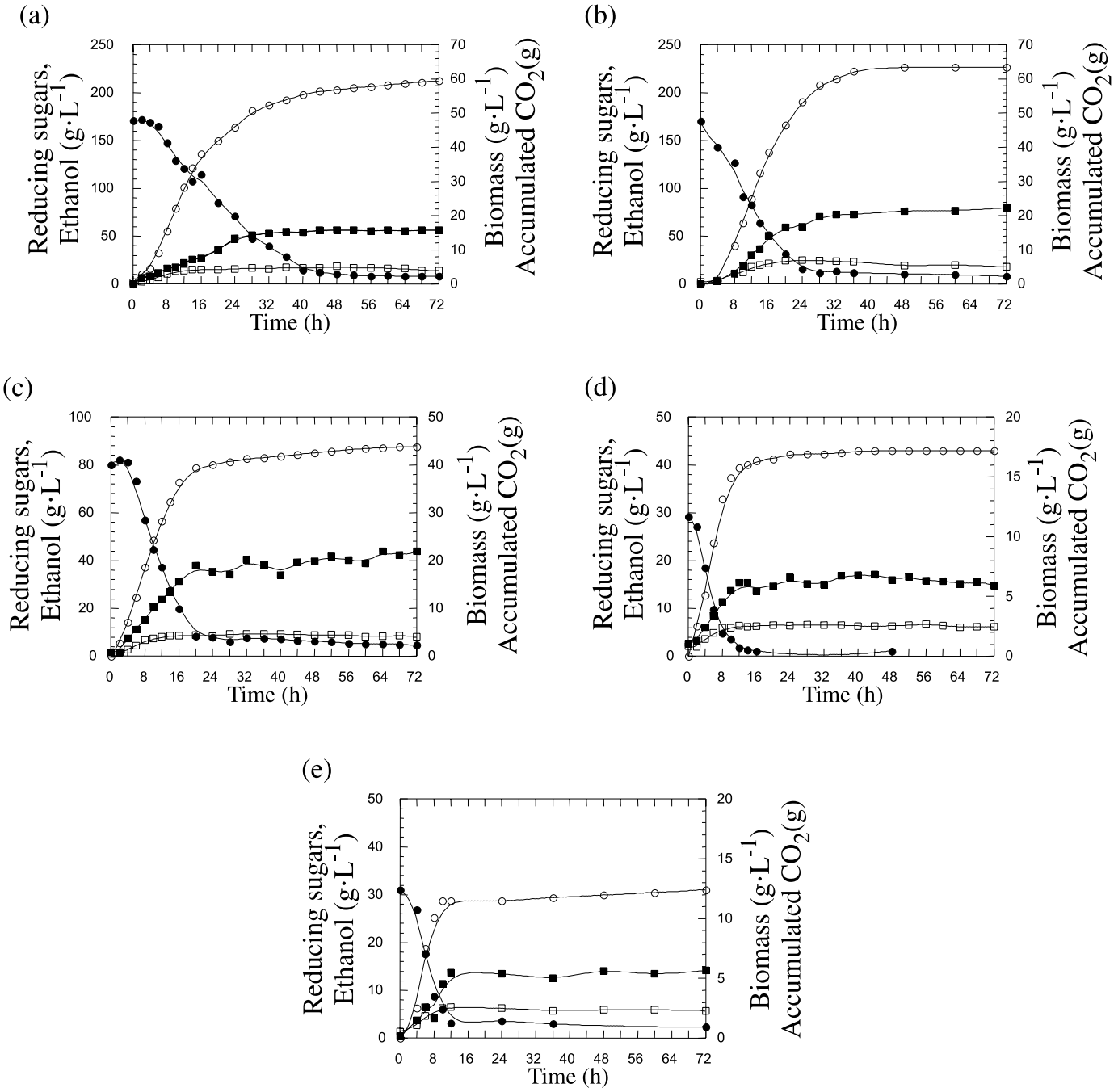
Batch cultures were carried out under semi-anaerobic conditions in 3-L fermentors containing 2 L of must. Fermentation was run with constant low stirring (200 rpm) at 35°C. The medium was saturated with air before inoculation. The nitrogen addition was carried out after 6 and 4 h of culture at sugar concentrations of 170 and 30 $g \cdot L^{-1}$, respectively. Each fermentation was run in triplicate to statistically analyze the fermentation parameters.

Monitoring of fermentation

The amount of CO_2 released was determined by measurements of fermentation weight loss at the time of sampling (Bezenger et al. 1985). Samples were taken every 2 h during

© 2002 NRC Canada

Fig. 1. Concentrations of biomass dry weight (□), reducing sugar (●), ethanol (■), and accumulated CO_2 (○) during fermentation of agave must containing 170 g·L^{-1} of initial sugar concentration without additional nitrogen (*a*) and with additional nitrogen (*b*), 80 g·L^{-1} of initial sugar concentration without additional nitrogen (*c*), and 30 g·L^{-1} of initial sugar concentration without additional nitrogen (*d*) and with additional nitrogen (*e*).



the first 16 h of fermentation and every 4 h until 72 h of culture to determine yeast biomass and the concentrations of sugar, nitrogen, and ethanol.

Analysis

Biomass

Yeast population was determined by measuring dry weight. The cellular dry weight was obtained by collecting the cells from 12 mL of the culture medium by centrifugation (3000 × *g*, 20°C, 20 min), rinsed with the same amount of distilled water, and desiccated at 108°C until a constant weight was obtained.

Nitrogen

The ammonium sulfate concentration was measured according to the method of Chaney and Marbach (1962).

Reducing sugars

The reducing sugar concentration in the medium was de-

© 2002 NRC Canada

Table 1. Principal kinetic parameters of the fermentations at different sugar concentrations with or without nitrogen addition.

	Kinetics parameter			
Sugar concn. ($g \cdot L^{-1}$)	r_{smax} ($g \cdot h^{-1}$)	μ_{max} (h^{-1})	r_{pmax} ($g \cdot h^{-1}$)	Efficiency (%)
170 without additional N	7.3	0.34	2.0	73.0
170 with additional N	8.0	0.45	4.2	94.5
80 without additional N	6.3	0.36	2.5	80.0
30 without additional N	4.4	0.45	1.6	88.0
30 with additional N	5.1	0.47	1.8	92.6

Note: r_{smax}, maximum rate of sugar consumption; μ_{max}, maximum specific growth rate; r_{pmax}, maximum rate of ethanol formation; efficiency, ethanol yield of theoretical.

termined by using 3,5-dinitrosalicylic acid reagent (Miller 1959).

Ethanol

Samples were first distilled, and the resultant ethanol concentration was measured using dichromate reagent (Bohringer and Jacob 1964).

Parameters calculation

Efficiency was calculated from the ratio between the average produced ethanol at the end of fermentation and the ethanol theoretically produced in the biochemical conversion of the sugar consumed (initial sugar – final sugar). For maximum ethanol and biomass formation or sugar consumption rates, experimental data of production of sugar and ethanol concentrations were adjusted to a mathematical model using the program Curve Expert 1.3 (EBT Comm, Columbus, Miss.). We then interpolated 100 points with this model, and the maximum rates and maximum specific rates were obtained from the maximum slopes (sugar, biomass, or ethanol concentration divided by time).

Statistical analysis

To see if the sugar concentration, the nitrogen addition, or the interaction of both factors have a statistically significant influence on kinetic parameters, an experimental design two-factor factorial ANOVA was used. The first factor was sugar concentration, with 170 and 30 $g \cdot L^{-1}$, and the second factor was nitrogen addition with or without supplementation at high and low levels, respectively. Statistical analysis was performed with the Statgraphics (Manugistics Inc., Rockville, Md.) software according to Montgomery (1991).

Results

During the first phase of the work, an *S. cerevisiae* yeast strain was selected from our collection of yeasts isolated from the tequila production. The main selection criterion was production of a high ethanol concentration and maintenance of high viability throughout fermentation. The behavior of four different strains were compared. The MG strain was selected (data not shown).

The progress of the different fermentations can be observed in Fig. 1. At low sugar concentrations (Figs. 1*d* and 1*e*), adding nitrogen did not seem to significantly affect sugar consumption nor ethanol production. The sugar was consumed within 12 h, and the ethanol concentration reached 15 $g \cdot L^{-1}$. On the contrary, at high sugar concentrations (Figs. 1*a* and 1*b*), important differences in the rates of the sugar consumption, biomass production, and ethanol production were observed when comparing the runs with or without nitrogen. By adding nitrogen, the sugars were consumed in 24 h compared with 48 h without nitrogen addition. A higher production of biomass, and above all, of ethanol, was obtained. When nitrogen was added, 80 $g \cdot L^{-1}$ of ethanol production was observed, compared with 50 $g \cdot L^{-1}$ with no addition of nitrogen. Compared with fermentation at a sugar concentration of 80 $g \cdot L^{-1}$ (Fig. 1*c*), which are conditions widely used in the industry, fermentation times were quite similar. However, at higher sugar concentrations, adding nitrogen led to production of twice the amount of ethanol. The CO_2 produced during fermentation was also measured; it was observed that CO_2 production increased when the sugar concentration increased and when nitrogen addition was performed at high sugar concentration. This reflects the metabolic activity of the cells.

The main kinetic parameters of maximum rate of sugar consumption, maximum rate of ethanol production, and maximum specific rate of growth, as well as the fermentation efficiencies are presented in Table 1. The statistical analysis shows that sugar concentration, nitrogen addition, and the interaction of the two have a statistically significant influence on parameters, especially on fermentation efficiency.

Fermentation efficiency is a key parameter for the industry (Table 1). A very high efficiency was obtained (94.5%) at high sugar concentrations by adding nitrogen compared with 73% efficiency without nitrogen. The efficiency obtained was even higher than under the traditional conditions of the industry, where efficiency reaches 80%. At low sugar concentrations, the nitrogen effect is not as significant. However, a slight increase in fermentation efficiency was observed.

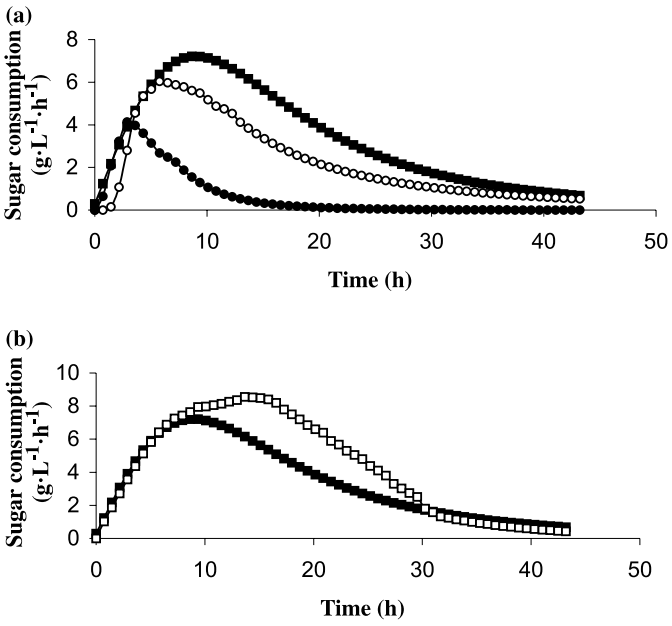
Finally, in Fig. 2, sugar consumption rates at different sugar concentrations are presented, with or without nitrogen additions. The lower the sugar concentration, the faster the maximum consumption rate is reached (Fig. 2*a*). Adding nitrogen has two main effects: increasing the maximum sugar consumption rate and maintaining a high consumption over a longer period of time (Fig. 2*b*).

Discussion

By supplementing an additional nitrogen source (a mixture of ammonium sulphate and amino acids) at 6 h of cul-

© 2002 NRC Canada

Fig. 2. Sugar consumption rates: (*a*) with initial sugar concentrations of 170 (■), 80 (○), and 30 g·L^{-1} (●); (*b*) with initial sugar concentration of 170 g·L^{-1} with (□) and without (■) nitrogen addition.



ture, an important increase in fermentation efficiency was achieved, as well as a reduction in fermentation time. The activation of transport is higher when this transport is still active, which is an important element when the nitrogen is still assimilable in the medium (Salmon et al. 1993). Similarly, it has been shown that nitrogen addition is more efficient at the early fermentation phase (Bely et al. 1990). In our case, the nitrogen present in the medium at the beginning was consumed during the first 8 h; additional nitrogen should therefore be injected at 6 h of fermentation.

The second key factor is the composition of the additional source of nitrogen. Based on research stressing the importance of the composition of the source of nitrogen (Albers et al. 1996; Bafrnacová et al. 1999; Jiranek et al. 1995; Thomas and Ingledew 1990), and considering that agave juice is poor in organic nitrogen, a mixture of organic (30%) and inorganic (70%) nitrogen was chosen. In wine, when only ammonium sulphate is added, sugar transport is activated; however, part of the ammonium salt is deviated for amino acid synthesis, which consequently causes an increase in the cellular NADH concentration, and thereby promotes glycerol synthesis (Albers et al. 1996). This means that part of the sugars consumed are deviated to the synthesis of glycerol instead of being used to produce ethanol. Thus, amino acids were added to reduce amino acid synthesis and, therefore, glycerol synthesis. In grape must fermentation, the nitrogen mass composition is higher in organic nitrogen, and it has been shown that addition of amino acids is more effective and also that a higher ammonium concentration inhibits efficient nitrogen utilization (Bisson 1999). Nevertheless, the yeast strains isolated from tequila must fermentation fermented over a long time in must with a poor organic nitrogen content (such as amino acids). Although it has been shown that yeasts with low nitrogen requirements are more effective for fermentation (Manginot et al. 1998), the nitrogen concentration used in this study was lower than other reports (Albers et al. 1996; Bafrnacová et al. 1999; Thomas and Ingledew 1990; Mauricio and Salmon 1992; Salmon and Mauricio 1994; Manginot et al. 1998), even considering the sugar concentration. Other reports on transport activation do not show any significant fermentation efficiency increase. For example, Bafrnacová et al. (1999) achieved increased productivity and an important fermentation time reduction but not an increased efficiency. In other work, Thomas and Ingledew (1990) reduced fermentation time from 8 to 3 days.

The effect of adding nitrogen on sugar transport is clearly observed in the evolution of the sugar consumption rate (Fig. 2). The maximum rate is higher, but above all, a high rate is maintained for a longer time. The general activation of protein synthesis includes sugar transport synthesis, which maintains a large number of active transporters in spite of their high degradation rate.

Other factors that could affect sugar transport are alcohol concentration and temperature. Thomas et al. (1994) showed that amino acids such as proline protect the cells against ethanol; the addition of nitrogen in this study was performed with a mix of amino acids rich in proline (29% w/w) to decrease the effect of ethanol concentration. This may have contributed to a higher rate of fermentation and increased efficiency. High temperatures are considered to affect sugar transport. Most studies in grape juice fermentation have been performed with temperatures between 20 and 30°C degrees. In this study, the fermentation temperature used was 35°C because it is a common temperature reached in most of the tequila industry. Thus, when comparing these results with the grape juice fermentation temperatures, no affect on activation of fermentation was seen by nitrogen addition. This factor has to be better studied in future work.

Conclusion

This work shows that an adequate strategy of adding nitrogen during fermentation makes an important sugar transport activation possible, thereby improving fermentation efficiency, even at high sugar concentrations. In this research, we aim at fermentation efficiency to obtain tequila; however it would be interesting to try to apply this strategy to other alcoholic fermentations, as well as with higher sugar concentrations. Another aspect, based on recent studies, has shown that the nitrogen requirements for different yeast strains can be different (Jiranek et al. 1995; Manginot et al. 1998; Salmon and Mauricio 1994). There is a need to characterize the strains that we have isolated from the tequila industry with regard to their nitrogen requirements, so that the least demanding strain can be selected and supplied to the industry within the scope of optimizing the agave fermentation.

Acknowledgement

This research was supported by the Consejo Nacional de la Ciencia y Tecnología (CONACYT) (project 32719-B).

© 2002 NRC Canada

References

Albers, E., Larsson, C., Lidén, G., Niklasson, C., and Gustafsson L. 1996. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Appl. Environ. Microbiol. **62**(9): 3187–3195.

Bafrnacová, P., Smogrovicova, D., Slavikova, I., Patkova, J., and Domeny, Z. 1999. Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. Biotechnol. Lett. **21**: 337–341.

Bely, M., Salmon, J.M., and Barre, P. 1990. Assimilable nitrogen addition and hexose transport system activity during enological fermentations. J. Inst. Brew. **100**: 279–282.

Bezenger, M.C., Navarro, J.M., Abbal, P., and Sablayrolles, J.M. 1985. Suivi de fermentation à l'aide d'un microordinateur personnel, application à la fermentation alcoolique en oenologie. Ind. Agric. Aliment. **102**: 1283–1291.

Bisson, L.F. 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. **50**(1): 107–119.

Bisson, L.F., Neigeborn, L., Carlson, M., and Fraenkel, D.G. 1987. The SNF3 gene is required for high-affinity glucose transport in *Saccharomyces cerevisiae*. J. Bacteriol. **169**: 1656–1662.

Bohringer, P., and Jacob, A. 1964. The determination of alcohol using chromic acid. Zeitschr. Flussiges Abst. **31**: 233–236.

Busturia, A., and Lagunas, R. 1986. Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **132**: 379–385.

Casey, G.P., and Ingledew, W.M. 1986. Ethanol tolerance in yeast. Crit. Rev. Microbiol. **13**: 219–280.

Cedeño, M. 1995. Tequila production. Crit. Rev. Biotechnol. **15**(1): 1–11.

Chaney, A.L., and Marbach, E.P. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. **8**: 130–134.

Ciriacy, M., and Reifenberger, M. 1997. Hexose transport. *In* Yeast sugar metabolism. *Edited by* F.K. Zimmermann and K. Entian. Technomic Publishing Company, Lancaster, Pennsylvania.

Ingledew, W.M., and Kunkee, R. 1985. Factors influencing sluggish fermentation in grape juice. Am. J. Enol. Vitic. **36**: 65–76.

Ivorra, C., Pérez-Ortin, J., and Del Olmo, M. 1999. An inverse correlation between stress resistance and stuck fermentation in wine yeast. A molecular study. Biotechnol. Bioeng. **64**(6): 698–708.

Jiranek, V., Langridge, P., and Henschke, A. 1995. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. Am. J. Enol. Vitic. **46**(1): 75–83.

Lachance, M.A. 1995. Yeast communities in a natural tequila fermentation. Antonie van Leeuwenhoek **68**: 151–160.

Manginot, C., Sablayrolles, J.M., Roustan, J.L., and Barre, P. 1997. Use of constant rate alcoholic fermentations to compare the effectiveness of different nitrogen sources added during the stationary phase. Enzyme Microb. Technol. **20**: 373–380.

Manginot, C., Roustan, J.L., and Sablayrolles, J.M. 1998. Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. Enzyme Microb. Technol. **23**: 511–517.

Mauricio, J.C., and Salmon, J.M. 1992. Apparent loss of sugar transport activity in *Saccharomyces cerevisiae* may mainly acount for maximum ethanol production during alcoholic fermentation. Biotechnol. Lett. **14**(7): 577–582.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**(3): 426–428.

Montgomery, D.C. 1991. Design and analysis experiments. John Wiley and Sons, New York.

Muñoz, E., and Ingledew, W.M. 1990. Yeast hulls in wine fermentations. A review. J. Wine Res. **1**(3):197–209.

Navarro, J.M., and Durand, G. 1978. Fermentation alcoolique. Influence de la température sur l'accumulation d'alcool dans les cellules de levure. Ann. Microbiol. Inst. Pasteur **129**: 215–221.

Pinal, L., Cedeño, M., Gutiérrez, H., and Alvarez-Jacobs J. 1997. Fermentation parameters influencing higher alcohol production in the tequila process. Biotechnol. Lett. **19**(1): 45–47.

Ribereau-Gayon, J., Peynaud, E., Ribereau-Gayon, P., and Sudraud, P. 1975. Les agents de la fermentation: les levures. Traité d'oenologie — sciences et techniques du vin. 2nd ed. Vol. 2. Dunod, Paris.

Salmon, J.M. 1989. Effect of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. Appl. Environ. Microbiol. **55**(4): 953–958.

Salmon, J.M., and Barre, P. 1998. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. Appl. Environ. Microbiol. **64**(10): 3831–3837.

Salmon, J.M., and Mauricio, J.C. 1994. Relationship between sugar uptake kinetics and total sugar consumption in different industrial *Saccharomyces cerevisiae* strains during alcoholic fermentation. Biotechnol. Lett. **16**(1): 89–94.

Salmon, J.M., Vincent, O., Mauricio, J.M., Bely, M., and Barre, P. 1993. Sugar transport inhibition and apparent loss of activity in *Saccharomyces cerevisiae* as a major limiting factor of enological fermentations. Am. J. Enol. Vitic. **44**(1): 56–64.

Thomas, K., and Ingledew, W.M. 1990. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity mashes. Appl. Environ. Microbiol. **56**: 2046–2050.

Thomas, K., Hynes, S.H., and Ingledew, W.M. 1994. Effects of particulate materials and osmoprotectants on very-high-gravity ethanolic fermentation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **60**: 1519–1524.

Thomas, K., Hynes, S.H., and Ingledew, W.M. 1996. Effect of nitrogen limitation on synthesis of enzymes in *Saccharomyces cerevisiae* during fermentation of high concentration of carbohydrates. Biotechnol. Lett. **18**(10): 1165–1168.

Xu, P., Thomas, A., and Gilson, C.D. 1996. Combined use of three methods for high concentration ethanol production by *Saccharomyces cerevisiae*. Biotechnol. Lett. **18**(12): 1439–1440.

© 2002 NRC Canada