

Altered Patterns of Maltose and Glucose Fermentation by Brewing and Wine Yeasts Influenced by the Complexity of Nitrogen Source

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ABSTRACT

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Maltose and glucose fermentations by industrial brewing and wine yeasts strains were strongly affected by the structural complexity of the nitrogen source. In this study, four *Saccharomyces cerevisiae* strains, two brewing and two wine yeasts, were grown in a medium containing maltose or glucose supplemented with a nitrogen source varying from a single ammonium salt (ammonium sulfate) to free amino acids (casamino acids) and peptides (peptone). Diauxie was observed at low sugar concentration for brewing and wine strains, independent of nitrogen supplementation, and the type of sugar. At high sugar concentrations altered patterns of sugar fermentation were observed, and biomass accumulation and ethanol production depended on the nature of the nitrogen source and were different for brewing and wine strains. In maltose, high biomass production was observed under peptone and casamino acids for the brewing and wine strains, however efficient maltose utilization and high ethanol production was only observed in the presence of casamino acids for one brewing and one wine strain studied. Conversely, peptone and casamino acids induced higher biomass and ethanol production for the two other brewing and wine strains studied. With glucose, in general, peptone induced higher fermentation performance for all strains, and one brewing and wine strain produced the same amount of ethanol with peptone and casamino acids supplementation. Ammonium salts always induced poor yeast performance. The results described in this paper suggest that the complex nitrogen composition of the cultivation medium may create conditions resembling those responsible for inducing sluggish/stuck fermentation, and indicate that the kind and concentration of sugar, the complexity of nitrogen source and the yeast genetic background influence optimal industrial yeast fermentation performance.

Key words: Amino acids, fermentation, glucose utilization, incomplete fermentation, maltose utilization, nitrogen metabolism, peptides, *Saccharomyces*, yeast.

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INTRODUCTION

In nature, carbon and nitrogen sources always occur in diverse and complex forms. Yeast is able to use a wide variety of compounds as carbon and nitrogen sources^{20,39,40}. One criterion considered in selecting industrial strains for brewing, baking, wine and the distilling industry is their capability to rapidly and completely utilize the fermentable carbohydrates available^{23,30}. However, in industrial worts and grape must, carbohydrates are not the only group of compounds in the medium. Brewer's wort is a typical example of a natural complex nutritional environment containing the sugars sucrose, fructose, glucose, maltose, maltotriose, dextrin material, as well as a complex mixture of amino acids, peptides, proteins, vitamins, ions, nucleic acids and other constituents³⁰.

In order to select the best options from the diversity of available nitrogen and carbon sources, the yeast has developed molecular mechanisms of sensing and regulation, which include induction and repression of key systems^{15,19,20,33}. Sugar catabolite repression¹² ensures an ordered sequence of sugar utilization, and during fermentation brewing yeast strains utilize sucrose, glucose, maltose and maltotriose in this approximate sequence, with some degree of overlap³⁰. However, altered patterns of sugar utilization amongst brewing, wine, baking and distilling strains have been reported^{3,23}.

Nitrogen is one of the main elements found in many macromolecules of living organisms, playing a central role in structure and function, and most organisms have elaborate control mechanisms to provide a constant supply of nitrogen^{19,20,31}. Similar to carbon catabolite repression, a mechanism known as nitrogen catabolite repression^{15,20,32} induces differential nitrogenous compound utilization. It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeast²⁰. When these primary nitrogen sources are absent, or present in concentrations low enough to limit growth, other nitrogen sources such as amides, amino acids and peptides can be used. The utilization of secondary nitrogen sources requires the synthesis of specific-catabolic enzymes and permeases, the expression of which is highly regulated by nitrogen catabolite repression. The latter is prevented in the presence of a preferred nitrogen source. During wort fermentation, patterns of amino acid assimilation are ordered and groups of amino acids have been identified on the basis of the rate of removal from the fer-

mentation medium²⁸. Wort amino acids were also classified according to the essential nature of the keto-acid analogues in yeast metabolism¹⁷. Brewer's wort contains a complex and large amount of peptides²⁶. In spite of the importance of peptide fractions on yeast growth and fermentation, studies on wort peptides and their uptake and influence on brewing fermentations are sparse. Little is known about the range of peptides present, or the order in which they are removed from wort^{5,8,14,41}. Considering that an appropriate amount and diversity of nitrogenous compounds are important in the successful completion of industrial fermentation processes and product quality^{7,30}, it is vital to select yeast strains that not only rapidly and efficiently utilize all sugars, but also are able to properly use all different kinds of nitrogen sources in order to assure final product quality^{13,21,23,30}.

In previous work we showed that, in general, the supplementation of the growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone induced higher biomass accumulation and ethanol production¹⁰. It was also described that brewer's

and baker's yeasts differ in their ability to ferment galactose, depending on the structural complexity of the nitrogen source and also on the yeast catabolite repression response to fermentable sugars⁹. In this work, we continue our study on the effects of the complexity of the nitrogen and carbon sources on the metabolism of the yeast *Saccharomyces*. Two brewer's (ale and a lager) and two wine strains were selected for further study. It will be shown that the structural complexity of the nitrogen source present in the cultivation medium, in correlation with the kind and concentration of sugar, can induce conditions which resemble those described as "stuck" fermentations^{1,4} and can greatly affect growth and ethanol production.

MATERIALS AND METHODS

Microorganisms

In this study the following yeast strains were used: brewers' ale strain LBCC A3 and lager strain LBCC L52 obtained from Labatt Breweries of Canada Culture collec-

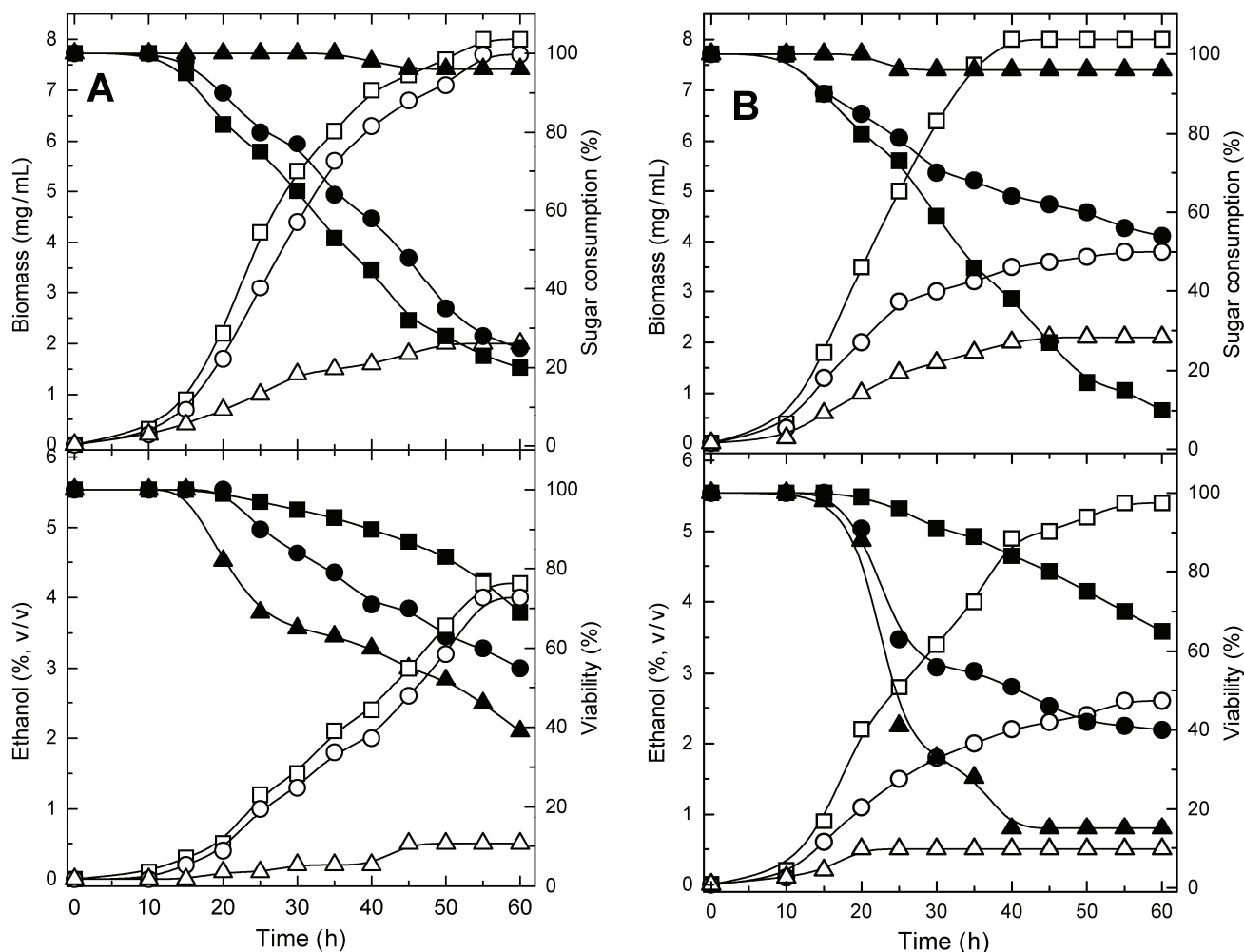


Fig. 1. Growth and ethanol production (open symbols), sugar utilization and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* L52 lager strain fermentation of YNB medium containing maltose 15% (A) or glucose 15% (B) (w/v) supplemented with 1% (w/v) peptone (squares), casamino acids (circles) and ammonium sulfate (triangles). Fermentation at 30°C, initial pH 5.0, 250 rpm.

tion; and wine strain VIN7 and VIN13 donated by Dr. I. S. Pretorius, from the Australian Wine Research Institute. Yeast strains were maintained on Peptone-Yeast Extract-Dextrose Agar slopes at 4°C, and subcultured monthly.

Chemicals and media components

Components for the growth media were from Difco Laboratories, including Yeast Nitrogen Base without amino acids and ammonium sulfate (referred to throughout this paper as Yeast Nitrogen Base), casamino acids, peptone, and yeast extract. All others media constituents were obtained from commercial sources and were of the highest available purity.

Media and growth conditions

The media for yeast fermentations contained 0.17% (w/v) Yeast Nitrogen Base, maltose or glucose at 2% or 15% (w/v), supplemented with a 1% (w/v) nitrogen source (ammonium sulfate, casamino acids or peptone). The sugar solution was autoclaved separately, at twice the concentration required, and added to the medium immediately before inoculation. An inoculum was prepared by

suspending yeast cells from slopes in sterile water and this cell suspension was inoculated into the growth medium at 0.02 g (dry weight) per liter. Growth was carried out in 125-mL Erlenmeyer flasks with 25 mL of medium. The flasks were incubated in a shaker (250 rpm) at 30°C.

Analytical methods

At specified times during the fermentation an aliquot of cell suspension was withdrawn, centrifuged and the supernatant frozen for subsequent analysis. Ethanol was analyzed by gas chromatography (Model CG-37 equipped with an integrator-processor CG-300, CG Instrumentos Científicos, São Paulo, Brazil). Cell density was measured by turbidity readings at 570 nm and correlated to a dry weight/OD calibration curve. Cell viability was determined by methylene blue staining. Carbohydrate analysis was carried out by colorimetric assay with 2-hydroxy-3,5-dinitrobenzoic acid²⁴.

Reproducibility

All results presented in this work are the average of a minimum of three independent experiments.

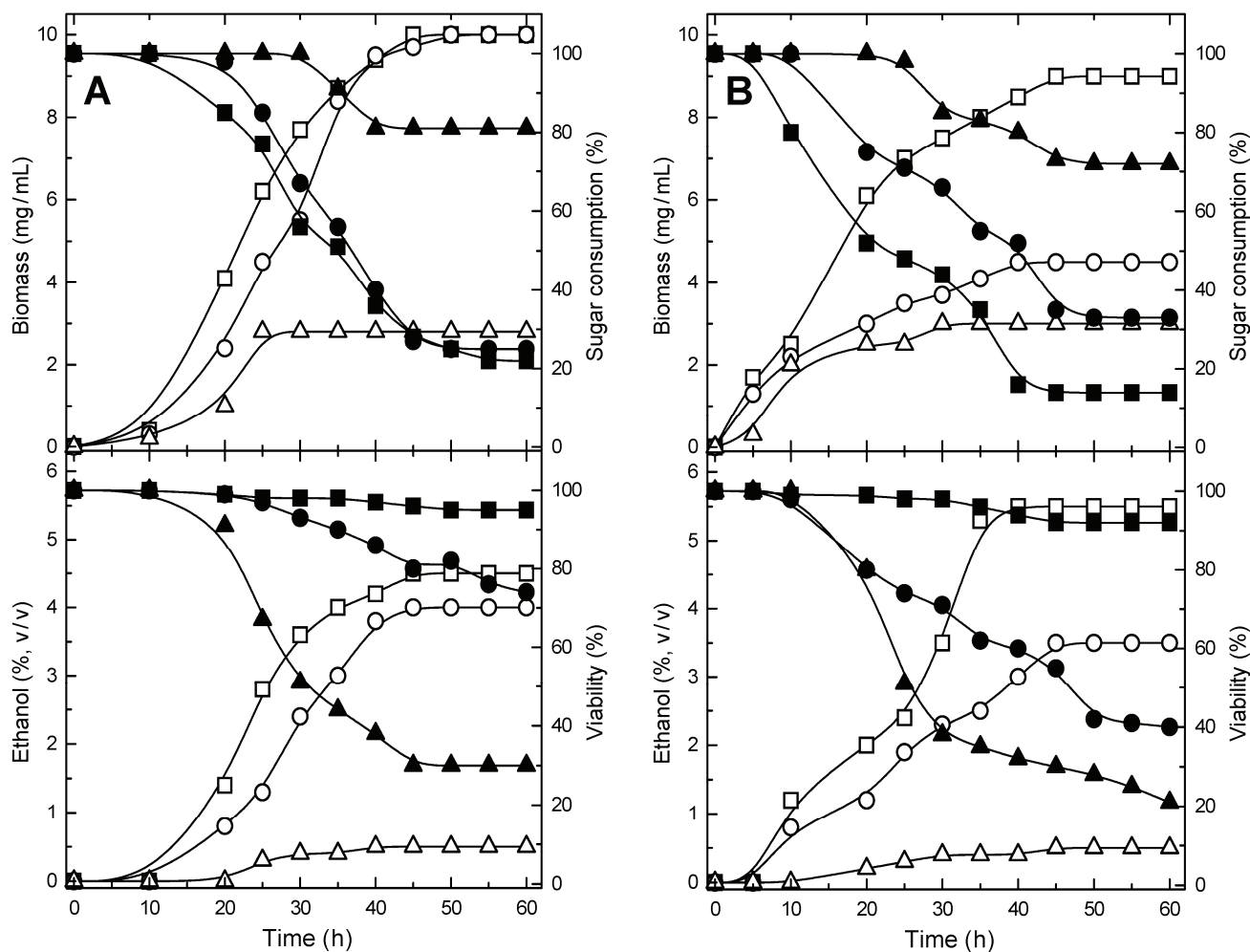


Fig. 2. Growth and ethanol production (open symbols), sugar utilization and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* VIN7 wine strain fermentation of YNB medium containing maltose 15% (A) or glucose 15% (B) (w/v) supplemented with 1% (w/v) peptone (squares), casamino acids (circles) and ammonium sulfate (triangles). Fermentation at 30°C, initial pH 5.0, 250 rpm.

RESULTS

To study the effect of the structural complexity of the nitrogen and carbon sources on the metabolism of industrial yeast strains, media containing maltose or glucose, were supplemented with nitrogen in the form of commercial enzymatic protein hydrolysates (peptone), acid hydrolysates of protein (casamino acids) or ammonium sulfate. Figs. 1 to 4 show biomass accumulation, ethanol production, sugar utilization and yeast viability during growth of *Saccharomyces cerevisiae* industrial yeasts in media containing 15% (w/v) maltose (Figs. 1A to 4A) and 15% (w/v) glucose (Figs. 1B to 4B), supplemented with different nitrogen sources. Brewing L52 lager (Fig. 1A) and wine VIN7 (Fig. 2A) strains showed similar growth and ethanol production patterns in the media under peptone and casamino acids supplementation. Improved growth and ethanol production by the ale yeast A3 (Fig. 3A) and wine VIN13 strain (Fig. 4A) was observed in the medium supplemented with casamino acids. With peptone supplementation, both strains displayed a drastic change in the pattern of maltose utilization; the yeasts used malt-

ose to grow, but were unable to efficiently ferment maltose to produce high levels of ethanol.

The effect of structural complexity of the nitrogen source on industrial brewing and wine strains was also studied in media containing glucose. Figs. 1B to 4B show biomass accumulation, ethanol production, sugar utilization and yeast viability during brewing L52 and A3 and wine VIN7 and VIN13 strains' growth in YNB medium containing glucose 15% (w/v). At higher glucose concentrations, peptone induced higher biomass accumulation and ethanol production with preservation of yeast viability. However, A3 brewing and VIN13 wine strains showed similar ethanol production in media following peptone and casamino acids supplementation. Ammonium sulfate always induced lower fermentative performance for all yeast strains in glucose and maltose.

Analysis of biomass accumulation, ethanol production and consumption, and sugar utilization during growth of *Saccharomyces cerevisiae* brewing (lager L52 and ale A3) and wine (VIN 7 and VIN 13) strains in the YNB media, containing either 2% (w/v) maltose or 2% (w/v) glucose supplemented with different nitrogen sources exhibited

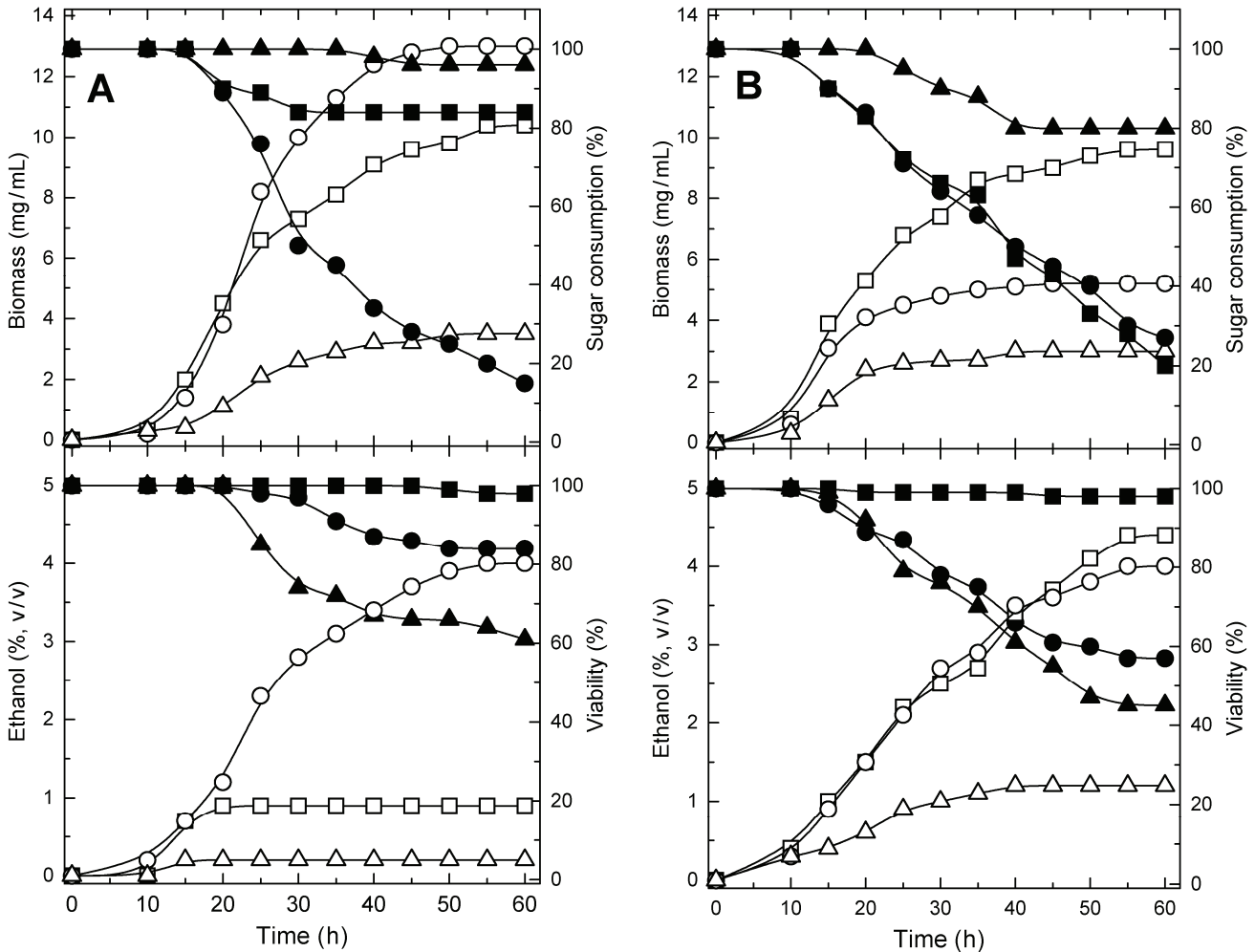


Fig. 3. Growth and ethanol production (open symbols), sugar utilization and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* A3 ale strain fermentation of YNB medium containing maltose 15% (A) or glucose 15% (B) (w/v) supplemented with 1% (w/v) peptone (squares), casamino acids (circles) and ammonium sulfate (triangles). Fermentation at 30°C, initial pH 5.0, 250 rpm.

diauxie. The yeast initially utilized sugar to produce biomass and ethanol and after sugar exhaustion, ethanol was used as a carbon source, as described previously¹⁰. Table I shows the amount of biomass produced after 20 and 60 h growth by the industrial yeasts used in this study, in YNB media containing 2% (w/v) maltose or glucose, supplemented with different nitrogen sources. Higher biomass accumulation was observed in the media with peptone and casamino acids. Ale A3 and VIN13 strain showed higher biomass accumulation and faster growth when compared with lager L52 and VIN7 strains. In addition, VIN7 strain also showed higher biomass production with casamino acids than with peptone supplementation. Ammonium sulfate always induced lower growth for all yeast strains studied.

DISCUSSION

Since most industrial worts contain sugars and nitrogen compounds of diverse structural composition^{25,30}, in our studies the effect of the nature of nitrogen source on the metabolism of industrial yeast was carried out by employ-

ing media that contained one sugar as a carbon source, and nitrogen compounds with differing levels of structural complexity^{9,10}. The sources of nitrogen varied from a single ammonium ion (ammonium sulfate) to an acid protein hydrolysis (casamino acids) consisting predominantly of free amino acids and an enzymatic hydrolysis of protein consisting predominantly of peptides (peptone)^{10,11}. The carbon source utilized in this study was glucose and maltose at low (2%, w/v) or high concentrations (15%, w/v) in order to expose yeasts cells to differing metabolic conditions i.e., low and high levels of sugar catabolite repression.

Previous results obtained in our laboratory^{9,10} showed that the structural complexity of the nitrogen source strongly affects yeast metabolism. Biomass accumulation and ethanol production, in addition to their dependence on the nature of the nitrogen supplement, were also affected by the sugar type and concentration. At low glucose and maltose concentrations (2% w/v) diauxic growth was observed. For the same strain biomass production was fairly similar, following both peptone and casamino acids supplementation. In the medium with ammonium sulfate,

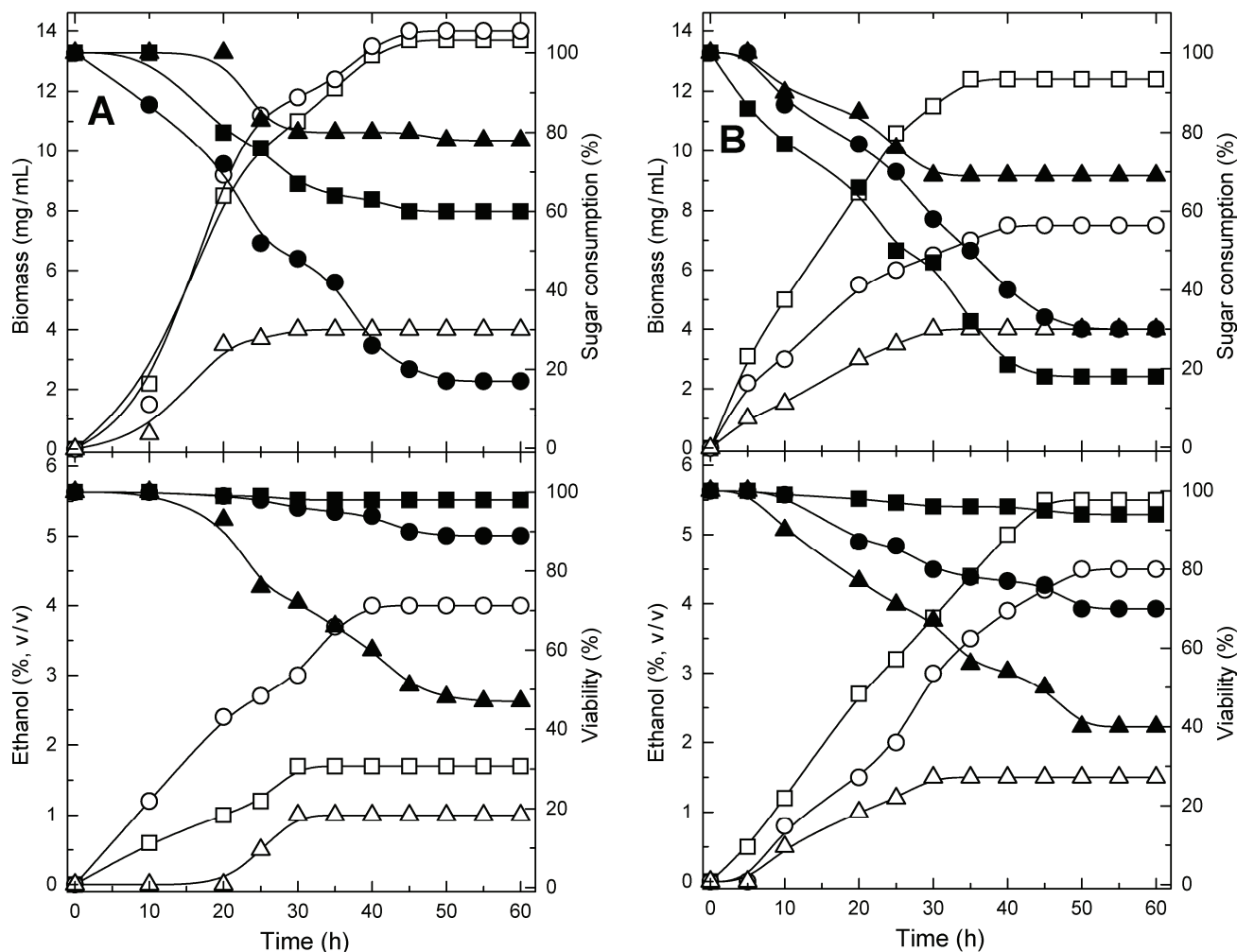


Fig. 4. Growth and ethanol production (open symbols), sugar utilization and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* VIN13 wine strain fermentation of YNB medium containing maltose 15% (A) or glucose 15% (B) (w/v) supplemented with 1% (w/v) peptone (squares), casamino acids (circles) and ammonium sulfate (triangles). Fermentation at 30°C, initial pH 5.0, 250 rpm.

TABLE I. Biomass production (mg/mL) by industrial brewing and wine yeast strains. Fermentation in YNB media containing 2% (w/v) glucose or maltose supplemented with different nitrogen sources.

Strains	Time (h)	Peptone		Casamino acids		Ammonium	
		Glucose	Maltose	Glucose	Maltose	Glucose	Maltose
A3	20	6.0	6.0	4.5	5.5	1.3	2.0
	60	8.5	8.1	9.0	10.0	2.7	4.0
VIN13	20	6.5	6.6	6.3	5.5	2.6	2.2
	60	9.0	9.0	9.4	10.0	3.3	2.8
L52	20	2.5	2.7	2.3	2.7	0.75	1.2
	60	6.2	7.0	5.5	7.7	2.0	1.8
VIN7	20	2.8	3.7	3.0	4.4	2.2	1.5
	60	4.0	6.8	6.5	9.5	2.6	2.2

Fermentation conditions: 30°C, 250 rpm, initial pH 5.0, media supplemented with 1% (w/v) nitrogen sources.

sugar was converted to ethanol and the ethanol was slowly utilized by the yeast. At higher sugar concentrations, diauxie was not as easily observed and the transition from fermentative to oxidative metabolism occurred more rapidly in the presence of peptone. Following casamino acids supplementation, a pronounced effect on yeast performance was observed. The time for metabolic shift increased with the glucose concentration, concomitantly with a decrease in biomass production, resulting in the elimination of the second growth phase. The fermentation performance of baking, ale and lager yeast strains in YNB media containing glucose and maltose supplemented with various nitrogen sources was also studied. Peptone induced a higher fermentation performance when compared to casamino acids and ammonium sulfate. It was also noted that brewer's and baker's yeasts differ in their ability to ferment galactose. The structural complexity of the nitrogen source induced altered patterns of galactose utilization at higher sugar concentration (15% w/v) which strongly affected growth and ethanol production⁹.

The results obtained in this study with brewing and wine yeast strains complement those from the previous studies, and show an altered pattern of maltose and glucose utilization by industrial strains and their dependence on the complexity of the nitrogen source. High biomass accumulation was observed for brewing and wine strains in the medium supplemented with peptone and casamino acids (Figs. 1 to 4 and Table I). At high sugar concentration, the complexity of nitrogen source induced altered patterns of maltose and glucose fermentation, strongly affecting biomass accumulation and ethanol production. Lager L52 and VIN7 strains showed similar growth and ethanol production in the media with maltose and peptone and casamino acids supplementation. Improved growth and ethanol production by the ale A3 and wine VIN13 strains were observed in the medium supplemented with casamino acids. When fermenting glucose, peptone was the nitrogen source that induced improved performance, with higher biomass and ethanol production, and preserved yeast viability. The most intriguing result observed in this study was obtained with the ale A3 and wine VIN13 strains following peptone supplementation, which displayed a drastic change in the pattern of maltose utilization. The yeast used the sugar to grow, but was unable to efficiently ferment maltose to produce high levels of ethanol. The results with A3 and VIN 13 strains in maltose media resemble those described for galactose⁹. Am-

monium sulfate always induced lower fermentative performance in all conditions used in this study.

An adequate balance of assimilable sugars and nitrogenous constituents in the fermented beverage industry is recognized as an important factor for fermentation completion and product quality^{13,21,30}. Despite the importance of the nitrogenous constituent's composition on yeast growth and fermentation, and its impact on the final quality of the beverage, not much attention has been given to the effect of the complex composition of nitrogenous constituents^{25,30}. The lack of more detailed studies on the importance of peptides in the fermentation of brewing wort and wine must is due to the difficulties involved in their isolation and characterization. Several studies have been conducted in order to elucidate the importance of the amino acid spectrum and composition on yeast fermentation and beer flavor, the amino acid and peptide profile and the removal of free amino acids and peptides^{5,8,17,26,36-38}. Recent publications have reported on the utilization and the effect of nitrogen source and concentration on the uptake of peptides by brewing lager strains in a defined medium^{26,27}. During fermentation, amino acids (free and in peptide form) are taken up from the medium by the cell. Free amino acids are incorporated directly without modification into proteins or degraded by the cell and the nitrogen is used for the synthesis of other nitrogenous cell constituents and the amino acid derivative keto-acids may be used by the cell for synthetic purposes¹⁸. The assimilation of wort amino acids is ordered and groups of amino acids have been identified on the basis of the rate of removal from the fermentation medium²⁸. Wort amino acids were also classified according to the essential nature of the keto-acid analogues in yeast metabolism¹⁷. Studies on wort peptides and their uptake and influence on brewing fermentations are sparse. Little is known about the range of peptides present and the order in which they are removed from wort^{5,8,14,41}.

Deficiency in assimilable nitrogen in industrial substrates is an important problem faced by the fermented beverage industries^{7,13,21}. Nitrogen supplementation is often used to stimulate sluggish or stuck fermentations. In the brewing industry concerns about the nutritional status of wort arose with the advent of high gravity brewing^{7,30}. During the initial attempts to ferment high gravity wort, the limiting factors implicated in many incomplete fermentations were largely attributed to ethanol toxicity, together with the inhibitory effect of high osmotic pressure

and also to induction alterations in the nutritional status of the wort⁶. Supplementation of high gravity brewing wort with complex lipidic compounds and nitrogen constituents avoided incomplete fermentations, thus allowing the production of high levels of ethanol and the preservation of yeast viability⁷. In addition to nitrogen supplementation, fermentation improvement was also reported to depend on the presence of the correct type of amino acids in the fermentation broth^{34,35}. The complete utilization of fermentable sugars from grape must is essential in the wine industry. Incomplete fermentation (stuck fermentation) or longer fermentations (sluggish fermentation)^{1,4} are a common event in the wine making process, and one procedure reported to avoid these fermentation problems is the addition of nitrogen supplements, such as ammonium salts^{2,21,22}.

The molecular mechanisms responsible for the alteration in the fermentation parameters induced by the complex composition of the nitrogen source reported in this and previous work^{9,10} are unclear. The results obtained at the low sugar concentration (2% w/v) with glucose, maltose and galactose^{9,10} and this work (Table I), in the presence of peptone and casamino acids, suggest that both nitrogenous supplements induce efficient conditions for yeast growth. Altered patterns of fermentation such as sugar utilization and ethanol production were detected at high sugar concentration (15%, w/v), where the effect of catabolite repression was stronger. For glucose, considered a repressive sugar, peptone was a better nitrogen source for yeast growth and fermentation. In the presence of maltose (this work) and galactose⁹, glucose repressed sugars, and casamino acids induced higher fermentation performance for the baking Fiso¹⁰, brewing ale A3 and VIN13 (these studies) strains. Conversely, improved maltose and galactose⁹ fermentation by lager L52 and VIN7 strains were observed following peptone supplementation.

Casamino acids are a commercial acid protein hydrolysate with predominantly free amino acids and peptone is a commercial enzyme protein hydrolysate with predominantly amino acids in the peptide form^{10,11}. Yeast fermentation performance is influenced by the ability of cells to transport the amino acids or small peptides across the plasma membrane. The uptake of amino acids by yeast involves specific permeases for certain amino acids and a general amino acid permease of broad-spectrum specificity¹⁶. The utilization of nitrogen from a complex source is due to a combination of the range of permeases present, their specificity, and feedback inhibition effects resulting from the composition of the yeast intracellular amino acids³⁰. The utilization of peptides relies on the ability of the yeast cell to transport them across the plasma membrane. Several peptide transport systems have been described²⁹, and these are distinct proteins from those involved in amino acid transport¹⁶. Results reported previously¹⁰ suggest that amino acids, free and in the peptide form, from peptone and casamino acids are utilized by yeast. This indicates that both supplements are an efficient nitrogenous source for yeast growth. The differing metabolic responses at higher sugar concentrations and the structural complexity of the nitrogen sources, in correlation with the catabolite repression intensity induced by the carbon sources accounts, in part, for the results de-

scribed in this paper and are an indication that the mutual interaction between nitrogen and carbon metabolism must be considered.

In this study we have shown that the altered patterns of maltose and glucose utilization by brewing and wine yeasts inducing strong effects on biomass and ethanol production were influenced by the structural complexity of the nitrogen source. The incomplete fermentation of sugars described here resembles conditions similar to those found in sluggish/stuck fermentations^{1,4}. These results have fundamental and industrial relevance, since they suggest that not only the structural complexity of the nitrogen source but also the yeast metabolic response to sugar catabolite repression strongly affects yeast performance. These findings, in addition to those previously reported^{9,10}, suggest that in *Saccharomyces* a complex nitrogen source is not subject to the same control mechanisms as those involved in the utilization of simpler nitrogen sources, and mutual interaction between carbon and nitrogen sources, including the mechanisms involved in sugar and nitrogen catabolite repression, may play an important role in the induction/repression processes for nitrogen and sugar utilization in yeast.

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