

Effect of Sugar Catabolite Repression in Correlation with the Structural Complexity of the Nitrogen Source on Yeast Growth and Fermentation

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ABSTRACT

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Biomass and ethanol production by industrial *Saccharomyces cerevisiae* strains were strongly affected by the structural complexity of the nitrogen source during fermentation in media containing galactose, and 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 galactose concentrations independent of nitrogen supplementation. At high sugar concentrations altered patterns of galactose utilisation were observed. Biomass accumulation and ethanol production depended on the nature of the nitrogen source and were different for baking and brewing ale and lager strains. Baking yeast showed improved galactose fermentation performance in the medium supplemented with casamino acids. High biomass production was observed with peptone and casamino acids for the ale brewing strain, however high ethanol production was observed only in the presence of casamino acids. Conversely, peptone was the nitrogen supplement that induced higher biomass and ethanol production for the lager brewing strain. Ammonium salts always induced poor yeast performance. The results with galactose differed from those obtained with glucose and maltose which indicated that supplementation with a nitrogen source in the peptide form (peptone) was more positive for yeast metabolism, suggesting that sugar catabolite repression has a central role in yeast performance in a medium containing nitrogen sources with differing levels of structural complexity.

Key words: Amino acids, fermentation, nitrogen metabolism, peptides, *Saccharomyces*, sugar catabolite repression, yeast.

INTRODUCTION

In nature, carbon and nitrogen sources always occur in diverse and complex compositions. Yeast are able to use a wide variety of compounds as a carbon and nitrogen source^{6,41,42}. Brewer's wort is a typical example of a natu-

ral 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 constituents³². In order to select the best options out of the large 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^{6,18,34}. In wort, nitrogen and carbon are the main nutrients and this implies that the mutual interaction of these nutrients may play an important role in the metabolism of yeasts³³. Sugar catabolite repression ensures an ordered sequence of sugar utilisation, and during fermentation brewing yeast strains utilise sucrose, glucose, maltose and maltotriose in this approximate sequence, with some degree of overlap³². Altered patterns of sugar utilisation among strains have been reported²¹. Vital to industrial fermentation is the selection of yeast strains that rapidly and efficiently utilise all wort sugars.

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^{6,18,19,34,42}. It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeast^{18,34}, which is attributed to a mechanism known as nitrogen catabolite repression¹⁸. When these primary nitrogen sources are absent, or present in concentrations low enough to limit growth, other nitrogen sources such as nitrite, nitrate, amides, amino acids and peptides can be used. The utilisation 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^{18,42}. In wort, the assimilation of amino acids is ordered and groups of amino acids have been identified on the basis of their rate of removal from the fermentation broth²⁶. Wort amino acids have also been classified according to the essential nature of the keto-acid analogues in yeast metabolism¹⁵. In contrast, 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^{2,5,13,43}. In previous studies we have shown that in general, the supplementa-

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tion of the growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone induced higher biomass accumulation and ethanol production⁷. In this study we continued to study the effect of the complexity of the nitrogen and carbon sources on the metabolism of the yeast *Saccharomyces*, except that the sugar was the non catabolite repressing sugar, galactose. It will be shown that the structural complexity of the nitrogen source, in correlation with the effect of sugar catabolite repression, greatly affects the yeast fermentation performance, and differs from the results observed with glucose and maltose.

MATERIALS AND METHODS

Microorganisms

In this study single colony isolates from a commercial baking yeast purchased from “Fleischmann e Royal Produtos Alimentícios Ltda do Brasil” (strain Fiso), and brewer’s ale strain A3 and brewer’s lager strain L52, from Labatt Breweries of Canada were used. Yeast strains were maintained on peptone–yeast extract–glucose 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 (Cat. no. 0335-15-9) (referred to throughout this paper as Yeast Nitrogen Base), casamino acids (Cat. no. 0230-01-1), peptone (Cat. no. 0118-01-1), and yeast extract (Cat. no. 0127-01-7). All other 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, galactose at various concentrations, supplemented with a 1% (w/v) nitrogen source (ammonium sulfate, casamino acids or peptone). The sugar solution was autoclaved separately, at twice the concentration of the experiment, and added 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 250-mL Erlenmeyer flasks, containing 50 mL of medium, or in 125-mL Erlenmeyer flasks with 20 mL of medium. The flasks were incubated in a shaking incubator (250 rpm) at 30°C.

Analytical methods

At specified times during the fermentation an aliquot of the cell suspension was withdrawn, centrifuged and the supernatant frozen for subsequent analysis. Ethanol was analysed 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 staining⁴¹. Carbohydrate analysis was carried out by colorimetric assay with 2-hydroxy-3,5-dinitrobenzoic acid²².

Reproducibility

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

RESULTS

To study the effect of the structural complexity of the nitrogen and carbon sources on the metabolism of industrial yeast strains, media containing galactose, were supplemented with nitrogen in the form of commercial enzymatic protein hydrolysates (peptone), acid hydrolysates of protein (casamino acids) or ammonium sulfate. Fig. 1A shows biomass accumulation, ethanol production and consumption, sugar utilisation and yeast viability during growth of *Saccharomyces cerevisiae* baking strain Fiso in the YNB media, containing 2% (w/v) galactose supplemented with different nitrogen sources. Higher biomass accumulation was observed in the media with peptone and casamino acids. Analysis of biomass accumulation, ethanol production and consumption, and sugar utilisation clearly showed diauxie. The yeast initially utilised galactose to produce ethanol and biomass and after galactose exhaustion, ethanol was used as a carbon source.

Fig. 1B shows the same parameters and fermentations conditions of Fig. 1A, except that the media contained galactose at the 15% (w/v) level. Improved ethanol production by the baking yeast strain Fiso was observed in the medium supplemented with casamino acids when compared with peptone and ammonium sulfate, with higher biomass accumulation and faster fermentation. The results with galactose media differ from those with glucose and maltose media, where high sugar concentrations were found to induce higher biomass and ethanol production in the media supplemented with peptone⁷.

In order to further characterize the effect of catabolite repression and the structural complexity of the nitrogen source on yeast metabolism, the effect of different nitrogen sources on the fermentation performance of industrial brewing strains was examined. Figs. 2 and 3 show biomass accumulation, ethanol production, sugar utilisation and yeast viability during the growth of ale strain A3 and lager strain L52 in YNB media containing either 2% (w/v) galactose or 15% (w/v) galactose. At low sugar concentrations diauxie was observed for both ale and lager strains, but ale strain A3 showed higher biomass accumulation and faster growth when compared with the lager strain (Figs. 2A and 3A). However, an altered pattern of galactose utilisation was observed at higher sugar concentration for both ale and lager strains. Figure 2B shows that peptone induces higher fermentative performance for lager strain L52, with increased amounts of biomass and ethanol production, when compared with casamino acids. Conversely, ale strain A3 showed higher fermentative performance with casamino acids supplementation (Fig. 3B). In addition, the results obtained with ale strain A3 showed that the yeast utilises galactose to accumulate biomass but it is not able to efficiently ferment the sugar to produce ethanol. Ammonium sulfate always induced lower fermentative performance for all yeasts strains used in this study.

The fermentation parameters of the yeast *Saccharomyces cerevisiae* are strongly dependent on the sugar and the

concentration employed. Also there is a correlation with the structural complexity of the nitrogen source. In glucose, a repressive carbon source, improved fermentation performance was observed in the medium supplemented with peptone⁷. In galactose, a non-repressive sugar, fermentation performance was different for baking and brewing strains and altered patterns of galactose utilisation, depending on the nitrogen supplement, were observed. The baking yeast Fiso showed improved fermentation performance following casamino acids supplementation (Fig. 1B). Higher biomass accumulation and ethanol production for the lager strain L52 were detected in the presence of peptone (Fig. 2B). Ale strain A3 showed improved fermentation performance with casamino acids supplementation, and despite the accumulation of a high amount of biomass with the ale strain, there was very low ethanol production in the presence of peptone (Fig. 3B). The results of this study not only reflect the yeast catabolite repression response to fermentable sugars but also indicates the influence of the structural complexity of nitrogen source on yeast metabolism.

DISCUSSION

Despite the importance of the nitrogenous constituents on the composition of brewer's wort on yeast growth and fermentation, and the impact on the final quality of the beer, little attention has been given to the effect of the complex composition of nitrogenous constituents in brewing fermentation. Brewer's wort contains the sugars sucrose, fructose, glucose, maltose, maltotriose, dextrin material, and a complex mixture of amino acids, peptides, proteins, vitamins, ions, nucleic acids and others constituents³². In the majority of industrial yeast fermentations a key requirement is the ability of the yeast strain to rapidly and efficiently utilise all sugars contained in the medium²¹. In order to elucidate the importance of amino acid spectrum and composition on yeast fermentation and beer flavor, the amino acid and peptide content and composition of wort and the removal of amino acids in a free and peptide form have been studied for many years^{2,5,15,26,37-39}. Recent publications have studied peptide utilisation and the effect of nitrogen source and concentration on the up-

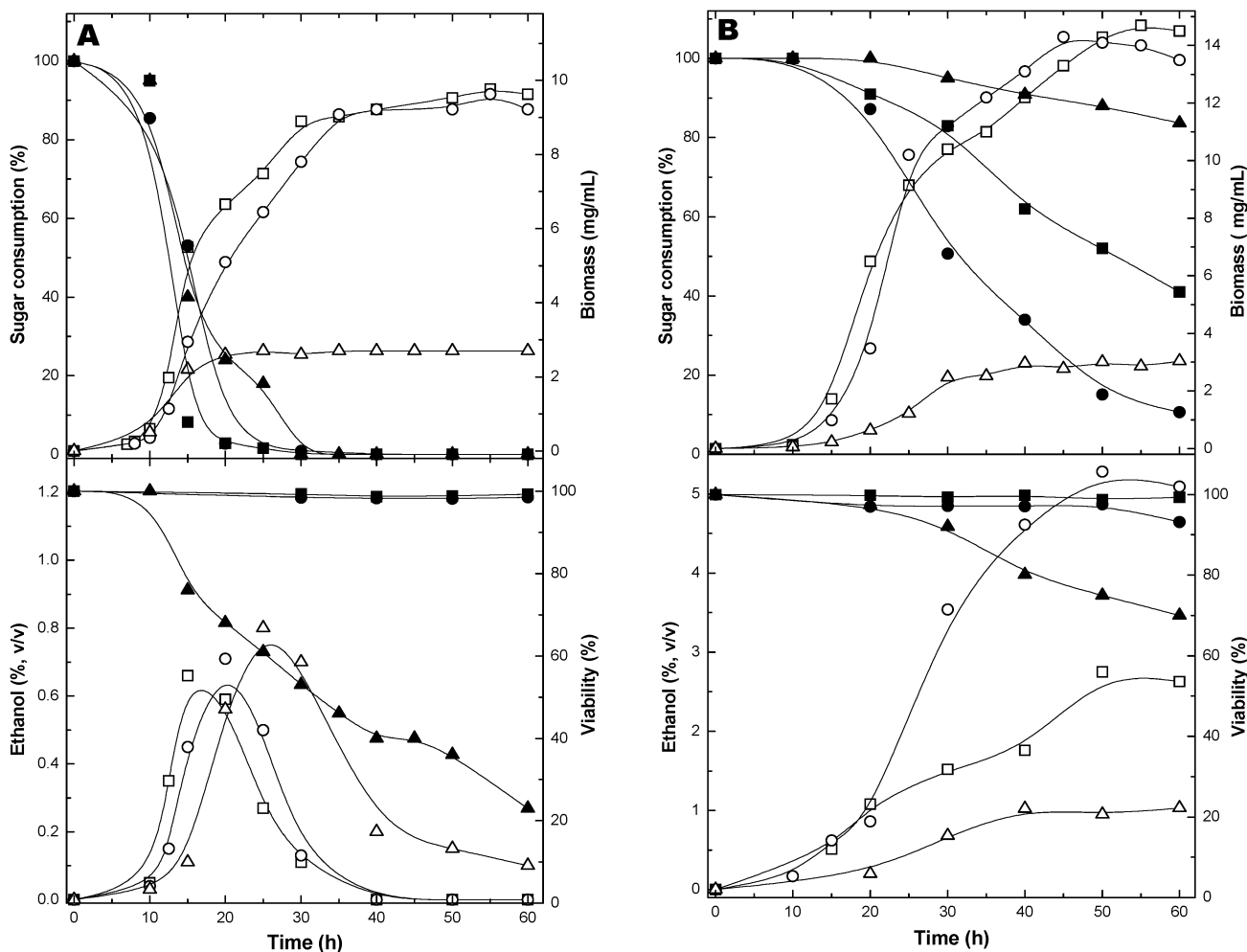


Fig. 1. Growth and ethanol production (open symbols), sugar utilisation and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* baking strain Fiso fermentation of YNB medium containing 2% (w/v) galactose (A) or 15% (w/v) galactose (B) supplemented with 1% (w/v) peptone (squares),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

take of peptides by brewing lager strains in a defined medium^{23,24}. 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 purposes¹⁶. 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 broth²⁶. Wort amino acids have also been classified according to the essential nature of the keto-acid analogues in yeast metabolism¹⁵. In contrast, 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^{2,5,13,43}.

Additional concerns about the nutritional status of brewer's wort arose with the advent of high gravity brewing. In the initial attempts to ferment high gravity wort, the limiting factors implicated in many incomplete fermentations were attributed largely to ethanol toxicity, to-

gether with the inhibitory effect of high osmotic pressure and also to induction alterations in the nutritional status of the wort³. 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 viability⁴. In addition to nitrogen supplementation, fermentation improvement was also dependent on the presence of the correct type of amino acids in the fermentation broth^{35,36}.

Since industrial worts contain sugars and nitrogen compounds of diverse structural compositions³², in this study the effect of the nature of nitrogen source on the metabolism of industrial baking and brewing strains was studied by employing media that contained galactose as a carbon source, and nitrogen compounds with differing levels of structural complexity. The sources of nitrogen varied from a single ammonium salt (ammonium sulfate), an acid protein hydrolysate (casamino acids) consisting predominantly of free amino acids to an enzymatic protein hydrolysate consisting predominantly of peptides (peptone)^{7,10}.

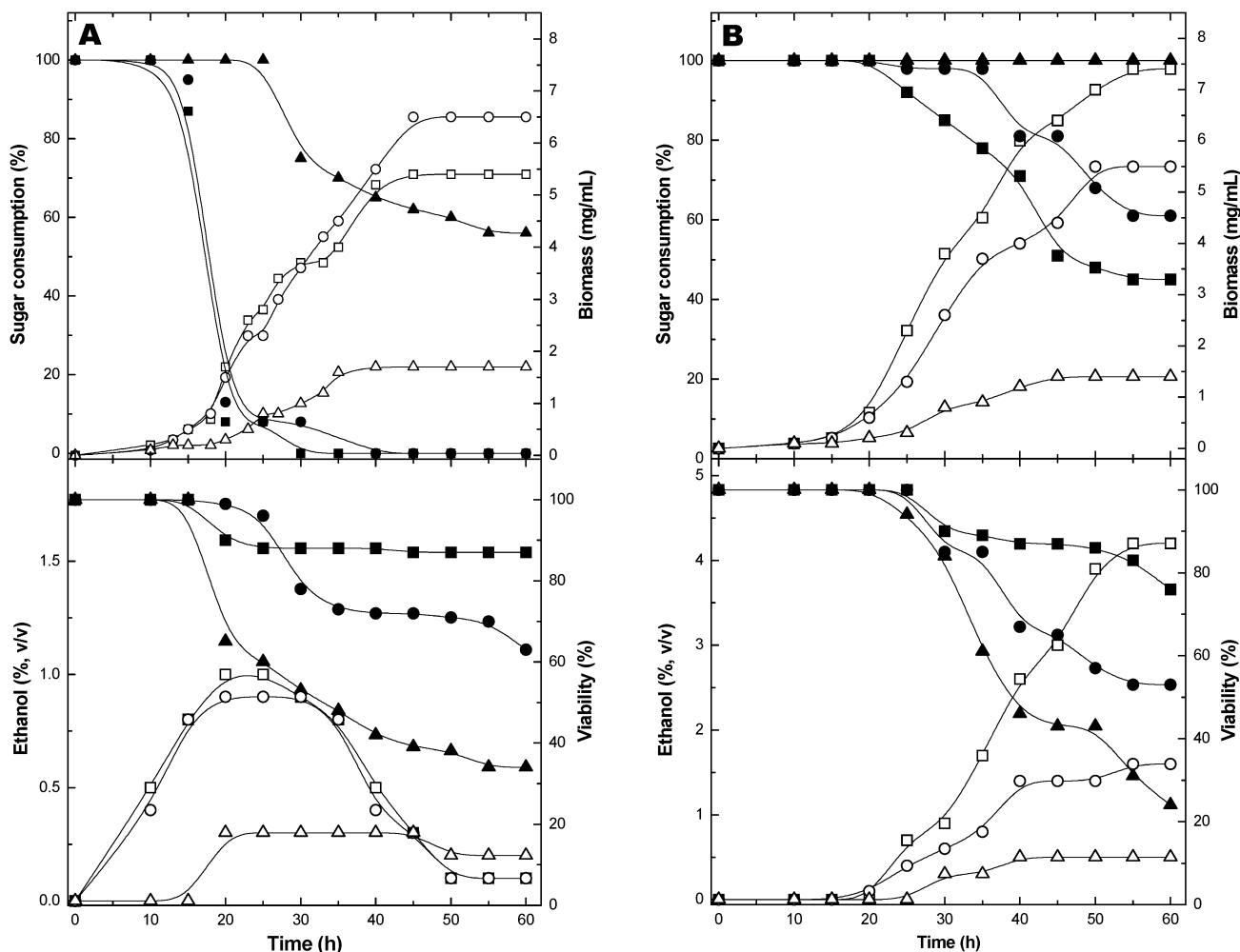


Fig. 2. Growth and ethanol production (open symbols), sugar utilisation and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* lager strain L52 fermentation of YNB medium containing 2% (w/v) galactose (A) or 15% (w/v) galactose (B) supplemented with 1% (w/v) peptone (squares), casamino acids (circles) and ammonium sulfate (triangles). Fermentation was at 30°C, initial pH 5.0 and 250 rpm.

Previous results obtained in our laboratory⁷ 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 sugar concentration. At low glucose and maltose concentrations (2% w/v) diauxic growth was observed. Biomass production was similar with both peptone and casamino acids supplementation. In the medium with ammonium sulfate, sugar was converted to ethanol and the ethanol was slowly utilised by the yeast. It was observed that at higher sugar concentrations, diauxie was not easily observed and the transition from fermentative to oxidative metabolism occurred more rapidly in the presence of peptone. When glucose concentration levels with casamino acids supplementation were examined, it was found that the time for metabolic shift increased with the glucose concentration and concomitantly a decrease in biomass production, inducing a strong effect on yeast performance and resulting in the extinction of the second growth phase, probably due to the loss of yeast viability. The fermentation performances of ale and lager brewing strains in YNB

media containing glucose and maltose supplemented with various nitrogen sources were also studied. For the baking strain and all ale and lager strains tested, peptone induced higher fermentation performance when compared with casamino acids and ammonium sulfate.

This study has shown an altered pattern of galactose utilisation at higher sugar concentration (15% w/v) for both baking and brewing ale and lager yeast strains, strongly affecting growth and ethanol production, and this physiological phenomenon depends on the structural complexity of the nitrogen source. Biomass and ethanol production for the baking strain Fiso were higher and faster in the medium supplemented with casamino acids (Fig. 1B). The increased accumulation of biomass and ethanol production for the lager L52 strain was with peptone supplementation (Fig. 2B). Ale strain A3 showed higher fermentative performance with casamino acids supplementation (Fig. 3B), however, the results obtained with peptone showed that the yeast utilises galactose to form biomass, but it is not able to efficiently ferment the sugar to produce ethanol. Ammonium sulfate always induced lower fermentative performance in all conditions used in this study.

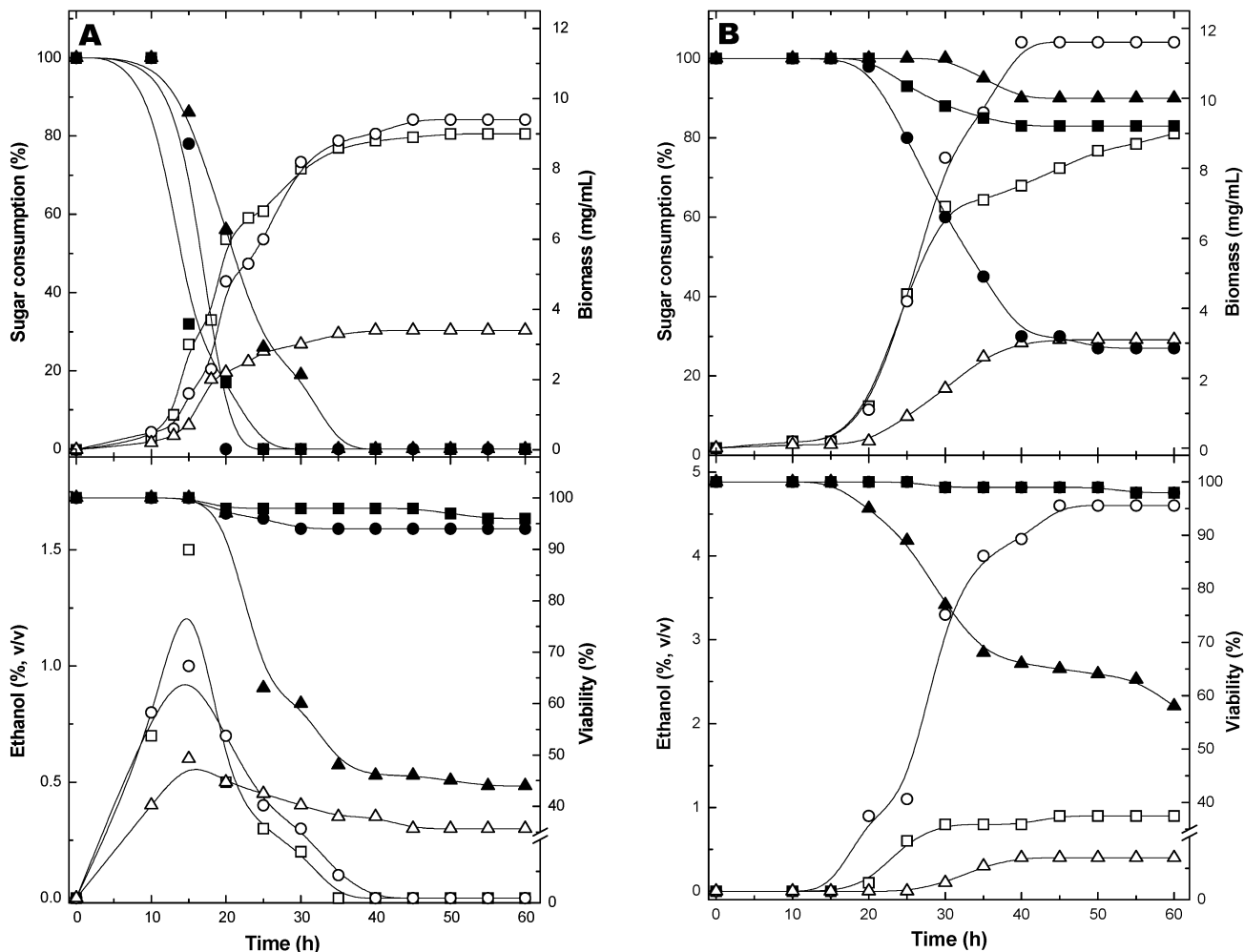


Fig. 3. Growth and ethanol production (open symbols), sugar utilisation and yeast viability (closed symbols) measured during *Saccharomyces cerevisiae* ale strain A3 fermentation of YNB medium containing 2% (w/v) galactose (A) or 15% (w/v) galactose (B) supplemented with 1% (w/v) peptone (squares), casamino acids (circles) and ammonium sulfate (triangles). Fermentation was at 30°C, initial pH 5.0 and 250 rpm.

These results differ from the previously reported study in media containing glucose and maltose, which showed that peptone, a nitrogen source where amino acids predominate in the peptide form, was more efficient for yeast growth and fermentation⁷. Lower biomass accumulation in the media supplemented with ammonium sulfate was detected in both studies and this resulted from the yeast using the carbohydrate as a carbon and an energy source. Additional energy and carbon are required by the cell to carry out the synthesis of its amino acids derived from both the carbon source and the ammonium sulfate in the media. The proton excess generated during amino acid synthesis must be pumped out of the cell to maintain the internal pH, resulting in acidification of the medium, which affects ammonium uptake and growth⁴².

The results obtained at the low sugar concentration (2% w/v) with glucose and maltose⁷ and galactose (this study), in the presence of peptone and casamino acids, suggests that both nitrogenous supplements induce efficient conditions for yeast growth. At 15% (w/v) sugar where the effect of catabolite repression is stronger, supplementation with structurally more complex nitrogen sources induces a differing yeast fermentation response. For glucose, considered a repressive sugar, peptone is a better nitrogen source for yeast growth and fermentation. In the presence of galactose, a non-repressive sugar, casamino acids induce higher fermentation performance for the baking strain Fiso and A3 ale strains. Conversely, improved galactose fermentation by lager strain L52 was with peptone supplementation. These results suggest that the genetic and biochemical differences among industrial yeast strains may have a strong effect on fermentation performance in the presence of a nitrogen source with a differing level of structural complexity. 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^{7,10}. 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 yeasts involves specific permeases for certain amino acids and a general amino acid permease of broad-spectrum specificity¹⁴. The utilisation 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 acids³².

Since yeast cells do not secrete significant quantities of proteolytic enzymes, amino acids in the peptide form present in peptone and casamino acids enter the cell mainly as peptides, mediated by peptide permeases. Thus, 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 described³¹, and these are distinct proteins from those involved in amino acid transport¹⁴. Results shown previously⁷ suggested that most amino acids, free and in the peptide form, from peptone and casamino acids are utilised 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 described in this study and are an indication that the mutual interaction between nitrogen and carbon metabolism must be considered.

In addition to the studies already discussed in this paper, the complexity of the medium supplementation has been reported to interfere with specific metabolic pathways, such as control of glycogen metabolism in yeast¹¹, secretion of enzymatic activities^{9,17,25,28-30,40,42}, primary¹ and secondary⁸ metabolites. The effect induced by specific kinds of peptides on metabolic routes of nitrogen and carbon metabolism have also been considered. It is worth noting that there is an increasing interest in biologically active peptides released from proteins by enzymatic hydrolysis such as those obtained from milk proteins^{20,27}. Recent studies have reported on the effect of specific peptides of casein pancreatic digestion on the production of tetanus toxin²⁸ and on the increased growth effect on lactic acid bacteria promoted by yeast extract filtrates¹².

We have previously shown that the structural complexity of the nitrogen source interferes with the fermentation performance of industrial baking and brewing ale and lager yeast strains⁷. Biomass accumulation, ethanol production and yeast viability were strain dependent. In general, it was observed that upon supplementation of the growth media, containing glucose or maltose, with a more complex structural nitrogen source such as peptone, that this induced higher biomass accumulation and ethanol production. In this study, we have shown altered patterns of galactose utilisation by industrial yeast and these were influenced by the structural complexity of the nitrogen source. These results have 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 fermentation. These findings, in addition to those previously reported⁷, suggest that in *Saccharomyces*, a complex structural nitrogen source is not submitted to the same control mechanisms as those involved in the utilisation of simpler structured nitrogen sources, and that sugar catabolite repression may play an important role in the of induction/repression processes for nitrogen and sugar utilisation in yeast.

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