Glucose and Fructose Fermentation by Wine Yeasts in Media Containing Structurally Complex Nitrogen Sources

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ABSTRACT

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Glucose and fructose fermentations by industrial yeasts strains are strongly affected by both the structural complexity of the nitrogen source and the availability of oxygen. In this study two *Saccharomyces cerevisiae* industrial wine strains were grown, under shaken and static conditions, in a media containing either a) 20% (w/v) glucose, or b) 10% (w/v) fructose and 10% (w/v) glucose or c) 20% (w/v) fructose, all supplemented with nitrogen sources varying from a single ammonium salt (ammonium sulfate) to free amino acids (casamino acids) and peptides (peptone). Data suggest that a complex structured nitrogen source is not submitted to the same control mechanisms as those involved in the utilization of simpler structured nitrogen sources, and mutual interaction between carbon and nitrogen sources, including the mechanisms involved in the regulation of aerobic/anaerobic metabolism, may play an important role in defining yeast fermentation performance and the differing response to the structural complexity of the nitrogen source, with a strong impact on fermentation performance.

Key words: Amino acids, anaerobiosis, fermentation, fructose utilization, glucose utilization, nitrogen metabolism, peptides, Saccharomyces, stuck fermentation, wine yeasts.

INTRODUCTION

Wine must and brewer's wort are examples of natural nutritional environments that contain a complex mixture of carbon and nitrogen sources[30,32]. During industrial fermentations, 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 the induction and repression of key regulatory systems[13,16,19,20,28,31]. Sugar catabolite repression[13,31] 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 overlap[10]. However, altered patterns of sugar utilization among brewing, wine, baking and distilling strains have been reported[4,23]. Glucose and fructose are the main fermentable sugars in a wine must, and *Saccharomyces cerevisiae* is known to be glucophilic[12], and despite being concomitantly used with glucose, fructose is the main sugar present in the later stages of alcoholic fermentation[15]. It has been shown that the preference for glucose over fructose is strain dependent, and the discrepancy between glucose and fructose consumption is not a fixed parameter, but is dependent on the yeast's genetic background and on external conditions[4]. For these reasons, one criterion considered in selecting industrial strains for the brewing, baking, wine and distilling industry is their capability to rapidly and completely utilize the fermentable carbohydrates present[23,30]. However, in industrial brewing worts and wine musts, carbohydrates are not the only important group of compounds in the medium.

Nitrogen, together with carbon, 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]. Similarly to carbon catabolite repression, a mechanism known as nitrogen catabolite repression[16,19] induces differential nitrogenous compound utilization. It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeast[19]. When these primary nitrogen sources are absent, or present in concentrations low enough to limit growth, other nitrogen sources such as 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. Considering that carbon and nitrogen are the main nutrients in industrial fermentation substrates, it would imply that the mutual interaction of these nutrients may play an important role in yeast metabolism, as suggested by a recent study which describes the regulation of amino acid permeases by carbon catabolite repression[26].

It has been suggested that an appropriate amount and diversity of nitrogenous compounds are important for the successful completion of industrial fermentation processes and product quality[7,30]. Nitrogen deficiencies in grape must are described as being one of the main causes of stuck or sluggish fermentations[1,6]. Considering the differential sugar utilization among industrial yeast strains, and also their nitrogen demand, it is vital to select yeast strains that not only rapidly and efficiently utilize all sugars, but that they are also able to properly use all

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different kinds of nitrogen sources in order to ensure an efficient fermentation and good final product quality[14,21,23].

In previous work, we have shown that in general, the supplementation of the yeast growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone, induced higher biomass accumulation and ethanol production[2,8,9]. It has also been reported 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 due to the yeast catabolite repression response to fermentable sugars[8]. In this work, we continue our study on the effect of the complexity of the nitrogen and carbon sources on the metabolism of industrial yeasts, studying two wine strains, and their fermentation of the sugars glucose and fructose.

MATERIALS AND METHODS

Microorganisms. VIN7 and VIN13 are *S. cerevisiae* wine strains kindly donated by Dr. I. S. Pretorius, from the Australian Wine Research Institute. The yeast strains were maintained on peptone yeast extract dextrose slopes at 4°C, and subcultured monthly.

Chemicals and media components. Components for the growth media were from Difco Laboratories (Detroit MI), including Yeast Nitrogen Base without amino acids and ammonium sulfate (referred to throughout this paper as Yeast Nitrogen Base W/O), casamino acids, peptone, and yeast extract. 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 W/O. Carbohydrate was added as glucose at 20% (w/v), fructose at 20% (w/v), or glucose and fructose at 10% (w/v) each. The medium was 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 fresh slopes in sterile water and this cell suspension was inoculated into the growth medium at 0.02 mg/mL (dry weight).

Growth was carried out in 125-mL Erlenmeyer flasks with 25 mL of medium. The flasks were incubated with shaking (200 rpm), or left unshaken in an incubator chamber in 125-mL Erlenmeyer flasks with 35 mL of medium, at 30°C. The medium pH varied from 5.0 (initial pH) to 4.0 (in the medium supplemented with peptone and casamino acids) and dropped to values below 3.0 (with ammonium sulfate supplementation). Thus with ammonium sulfate supplementation, the medium pH was adjusted to 5.0 throughout fermentation. The unshaken cultivations were not grown under totally anaerobic conditions, since limited aeration was necessary during sampling.

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). Biomass production 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[18]. Carbohydrate analysis was carried out by colorimetric assay using 2-hydroxy-3, 5-dinitrobenzoic acid[23,24].

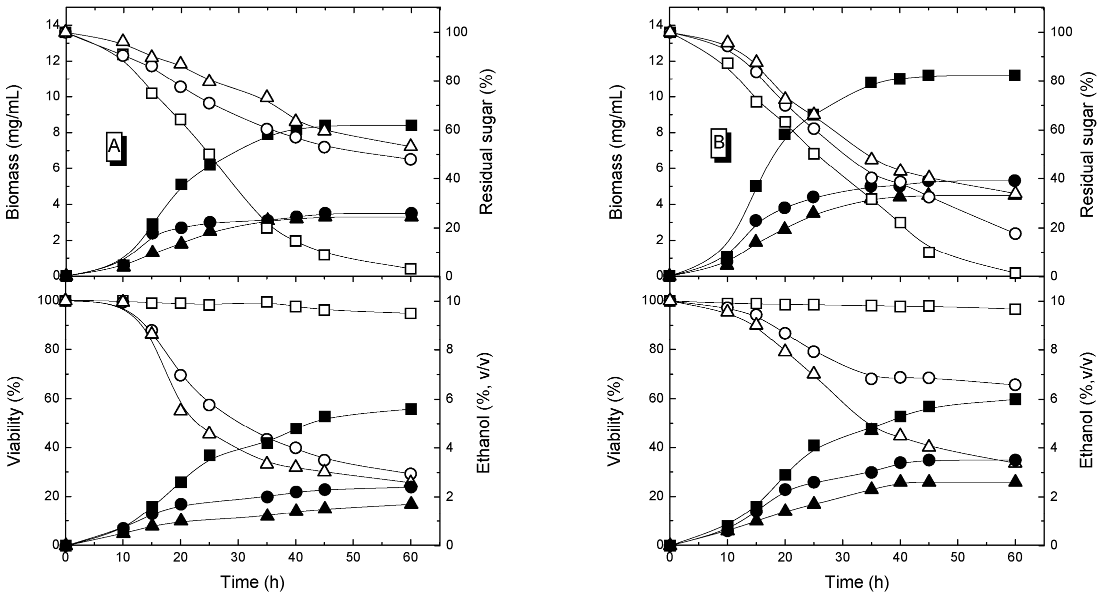

Fig. 1. Growth and ethanol production (closed symbols), sugar utilization and viability (open symbols). Strain VIN7 (A) and VIN13 (B), YNB W/O medium containing glucose 10% (w/v) and fructose 10% (w/v) at 30°C, with shaking at 200 rpm, supplemented with 1% (w/v) peptone (square), casamino acids (circle) and ammonium sulfate (triangle). The media containing ammonium sulfate had the pH adjusted to ~ pH 5.0.

Reproducibility. All results presented in this work 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 wine yeasts, media containing 10% (w/v) glucose plus 10% (w/v) fructose, 20% (w/v) glucose or 20% (w/v) fructose were supplemented with nitrogen in the form of commercial enzymatic protein hydrolysates (peptone), acid hydrolysates of protein (casamino acids) or ammonium sulfate. Figures 1 and 2 show biomass accumulation, ethanol production, sugar utilization and yeast viability during the growth of *S. cerevisiae* industrial yeasts in a media containing 10% (w/v) glucose plus 10% fructose (Fig. 1 - shaken cultures, and Fig. 2 - unshaken culture), supplemented with different nitrogen sources. Under peptone supplementation, strains VIN7 (Fig. 1A) and VIN13 (Fig. 1B) showed high biomass accumulation and ethanol production and yeast viability was preserved. Casamino acids and ammonium sulfate always induced poorer fermentation performance, with lower biomass and ethanol production, and loss of yeast viability. Unshaken cultivation produced lower biomass accumulation, but induced efficient consumption of sugar, with the production of higher concentrations of ethanol. Yeast viability was preserved (Fig 2A and 2B) with all three nitrogen supplements. These results suggest that oxygen availability during fermentation has a strong effect on wine yeast metabolism, affecting growth, ethanol production and cell viability.

To further characterize the effect of the nature of the nitrogen source on sugar fermentations, the additional alternative carbon sources, glucose and fructose, were evaluated. Table I shows biomass accumulation, sugar utilization and yeast viability, after 60 h of growth of wine yeasts, after the fermentation of 20% (w/v) glucose or 20% (w/v) fructose under shaken and static cultivation conditions. The results obtained for biomass production, sugar consumption and yeast viability for the fermentation of 20% (w/v) glucose, were similar to those described for 10% (w/v) glucose plus 10% (w/v) fructose (Figs. 1 and 2). *S. cerevisiae* VIN7 and VIN13 wine strains did not ferment fructose as efficiently as glucose, since with fructose, in general, biomass production, sugar consumption and yeast viability were lower than with glucose. In addition, the glucose to fructose discrepancy was stronger in strain VIN7, and this discrepancy was accentuated by the structural complexity of the nitrogen source (Table I).

DISCUSSION

One of the main problems faced by the wine industry is the undesirable possibility of the occurrence of stuck or sluggish fermentations[1,6]. Troubled fermentations have been attributed to the nutritional status of grape musts, once they contain suboptimal yeast nutrients, especially assimilable nitrogen[3,17,21,32]. A common practice in the wine industry for nitrogen limited fermentation is the addition of nitrogen supplements, using inorganic salts such as diammonium phosphate[22,27]. In addition to nutritional deficiencies, grape must contains approximately equimolar amounts of glucose and fructose, which are cofermented to ethanol, with a preference for glucose[12]. *Saccharomyces* strains prefer glucose, but to varying degrees, inducing an increased discrepancy between glucose and fructose utilization among strains during the course of fermentation[4]. Consequently, fructose becomes the main

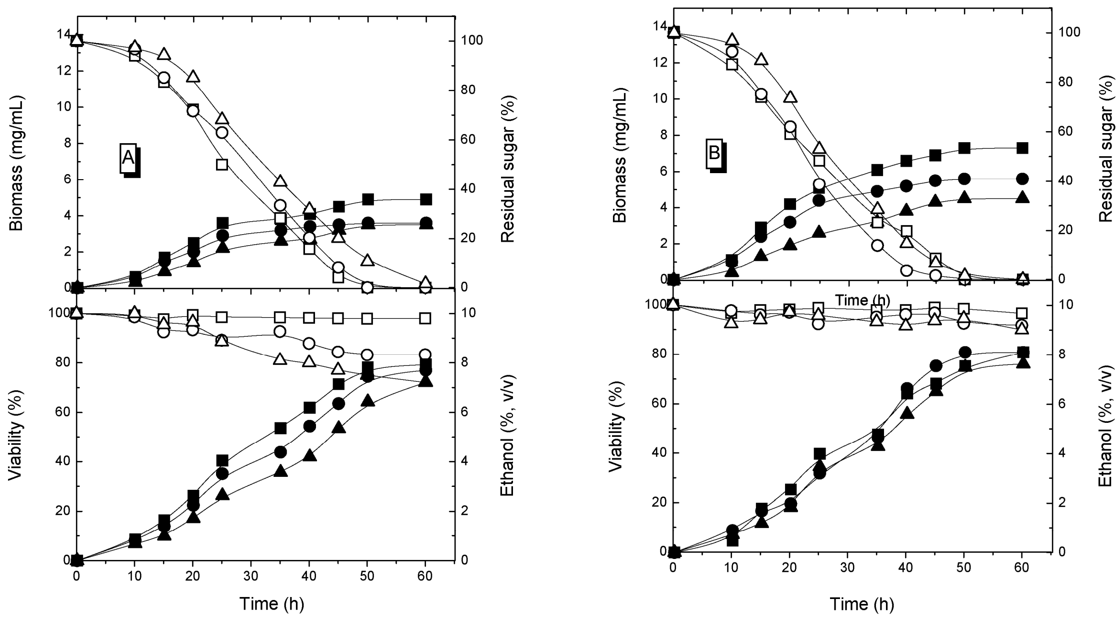

Fig. 2. Growth and ethanol production (closed symbols), sugar utilization and viability (open symbols). Strain VIN7 (A) and VIN13 (B), YNB W/O medium containing glucose 10% (w/v) and fructose 10% (w/v) at 30°C, incubated without shaking, and supplemented with 1% (w/v) peptone (square), casamino acids (circle) and ammonium sulfate (triangle). The media containing ammonium sulfate had the pH adjusted to ~ pH 5.0.

Table I. The growth and fermentation profiles of different wine yeast strains in YNB W/O medium containing either 20% (w/v) glucose or 20% (w/v) fructose supplemented with 1% (w/v) of various nitrogen sources.

				Peptone			Casamino acids			Ammonium sulfate		
Fermentation condition	Strains	Carbon source	Time (h)	Biomass (mg/mL)	Viability (%)	Residual sugar (%)	Biomass (mg/mL)	Viability (%)	Residual sugar (%)	Biomass (mg/mL)	Viability (%)	Residual sugar (%)
With agitation (200 rpm)	*VIN7*	*Glucose*	60	8.8	94.0	2.0	3.9	30.0	42.4	35	15.0	53.7
		Fructose	60	8.7	99.8	1.8	2.9	22.0	57.7	2.3	20.0	58.9
	VIN13	*Glucose*	60	12.1	98.2	3.1	5.5	73.0	12.0	5.3	58.2	15.5
		Fructose	60	12.2	98.2	1.2	5.5	69.8	12.6	3.6	44.3	37.5
Without agitation	*VIN7*	*Glucose*	60	4.1	94.2	0	3.4	84.2	0.60	3.3	83.6	0
		Fructose	60	3.5	87.6	1.1	1.9	56.8	30.3	1.8	28.2	51.0
	VIN13	*Glucose*	60	6.8	97.0	0	5.6	93.0	0	3.5	95.5	0
		Fructose	60	6.0	95.2	2.9	4.0	85.9	7.2	2.8	79.9	23.7

Fermentation conditions were 30°C, initial pH 5.0 and media with ammonium sulfate had pH adjustment.

carbohydrate during the late stages of alcoholic fermentation, and the yeast has to ferment this sugar under conditions of high ethanol concentration and nitrogen limitation, which may lead to stuck or sluggish fermentations[1,4]. Under these stressful conditions, a yeast strain able to efficiently ferment fructose is essential for completion of alcoholic fermentation. The reasons for the preference for glucose are still unclear, but studies have recently been carried out to try to elucidate the physiological and molecular basis of the glucose to fructose discrepancy[5,15].

Since in grape must, glucose and fructose are the main soluble sugars, in our studies the effect of the nature of nitrogen source on the metabolism of industrial wine yeasts was examined by employing media that contained glucose and fructose as a carbon source, along with nitrogen compounds with differing levels of structural complexity. The sources of nitrogen varied from a single ammonium salt (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)[9,11]. The carbon sources utilized were glucose or fructose at 20% (w/v) and glucose plus fructose at equal amounts of 10% (w/v) each, in order to subject the yeast cells to similar metabolic conditions as those found in grape must.

Previous results from this laboratory[2,8,9] have shown that the structural complexity of the nitrogen source strongly affected yeast metabolism. Biomass accumulation and ethanol production, in addition to their dependence on the nature of the nitrogen supplement, were also affected by the type and concentration of sugar. At low glucose and maltose concentrations, diauxic growth was observed. For the same strain, biomass production was approximately similar, with both peptone and casamino acids supplementation. In the medium with ammonium sulfate, sugar was converted to ethanol and the ethanol was slowly utilized by the yeast. 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. Under casamino acids supplementation, a drastic effect on yeast performance was observed and the time for metabolic shift increased with the glucose concentration, concomitantly with a decrease in biomass production, resulting in the extinction of the second growth phase. The fermentation performance of baking, and ale and lager brewing strains in YNB W/O media, containing glucose and maltose supplemented with various nitrogen sources, was also studied. Peptone induced improved fermentation performance when compared to the casamino acids and ammonium sulfate supplementation. It has also been shown that brewer's and baker's yeasts differ in their ability to ferment galactose, and that the structural complexity of the nitrogen source induced altered patterns of galactose utilization at higher sugar concentrations, strongly affecting both growth and ethanol production[9]. Additional studies with brewing and wine strains showed altered patterns of maltose and glucose utilization by industrial strains and the dependence on the complexity of the nitrogen source[2].

Results obtained in this study with wine strains complements those from previous work, and show that the presence of oxygen, in addition to the complexity of the nitrogen source, strongly affects glucose and fructose fermentation. The complexity of the nitrogen source, in addition to the form of cultivation (shaken and unshaken) induced altered patterns of sugar fermentation, affecting both biomass accumulation and ethanol production. In the medium containing glucose and fructose as the carbon sources, under agitation, the VIN7 and VIN13 strains showed high biomass accumulation and ethanol production in the media supplemented with peptone, in addition to preserving yeast viability. In the medium supplemented with casamino acids or ammonium sulfate, both strains produced low biomass and ethanol, and there was a considerable loss of cell viability (Figs. 1 and 2). Surprisingly, in the unshaken fermentations, with a low concentration of oxygen, despite the low biomass accumulation, strains VIN7 and VIN13 under all nitrogen supplementations, displayed efficient sugar consumption, produced high amounts of ethanol, and yeast viability was kept high throughout the fermentation. In the medium containing only glucose, profiles for biomass production, yeast viability and sugar consumption were similar to those obtained with glucose plus fructose, for both strains, with all three nitrogen supplementations, under both shaken and unshaken conditions (Table I). Studies with fructose showed that the yeast strains did not ferment fructose as efficiently as glucose, and also the differing structural complexity of the nitrogen sources accentuated this difficulty (Table I).

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[14,17,30,32]. The main sources of assimilable nitrogen in grape must are ammonium compounds, α-amino acids and to a lesser extent peptides[31]. During fermentation, amino acids free and in peptide form are taken up, and amino acids are accumulated by yeasts in the early stages of fermentation. Their metabolism provides the amino acid pools needed for protein synthesis and growth, with the surplus stored in the vacuoles[25,30]. It has been reported that nitrogen deficiency induces lower biomass yields, which slows the fermentation rate and thereby increases the risk of sluggish or stuck fermentations[1,4]. In this work it has been shown that an efficient utilization of glucose and fructose was observed with higher biomass in shaken cultivation, with peptone supplementation, and with lower biomass in unshaken cultivation, with ammonium, casamino acids and peptone supplementation. This suggests that in addition to nitrogen, the presence of air affects yeast growth and nitrogen utilization and this in turn affects the ability of yeast cells to ferment glucose and fructose (Figs. 1 and 2). Our results also suggest that the availability of oxygen, in correlation with the structural complexity of the nitrogen source, also affects the discrepancy of glucose to fructose utilization. In general, strain VIN13 showed improved fermentation performance in comparison with strain VIN7, probably reflecting the lower nitrogen requirement and/or a decreased glucose to fructose discrepancy.

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 works[2,8,9] are unclear. The previous results obtained with glucose, maltose and galactose, and this work, in the presence of peptone and casamino acids, suggest that both nitrogenous supplements induced efficient conditions for yeast growth and fermentation. Altered patterns of fermentation parameters, such as sugar utilization and ethanol production, were better detected at higher sugar concentrations, where the effect of catabolite repression is stronger. Differing fermentation performance was dependent on the structural complexity of the nitrogen source and was different for baking, brewing and wine strains, perhaps a reflection of yeast response to the mutual interaction between carbon and nitrogen regulation pathways, which include carbon and nitrogen catabolite repression[19,26]. It is also worth noting, the strong effect of oxygen availability on yeast metabolism, affecting growth and ethanol production (Figs. 1 and 2). Recent studies report on implications in the central carbon metabolism of *Saccharomyces cerevisiae* induced by aerobic and anaerobic conditions, and address the complexity of the role of oxygen in controlling cellular physiology in yeasts[29,33].

In this work we have shown altered patterns of glucose and fructose utilization by wine yeasts inducing strong effects on biomass and ethanol production, and the influence of the structural complexity of the nitrogen source and the availability of oxygen. These results have academic and industrial relevance, since they suggest that not only the structural complexity of nitrogen source but also the yeast metabolic response to the presence of oxygen strongly affects yeast performance. These findings, in addition to those previously reported[2,8,9], suggest that in *S. cerevisiae* a complex structured nitrogen source is not submitted to the same control mechanisms as those involved in the utilization of simpler structured nitrogen sources. Mutual interaction between carbon and nitrogen sources, including the mechanisms involved in the regulation of aerobic/anaerobic metabolism, may play an important role in defining yeast fermentation performance and its differing response to the structural complexity of nitrogen source, with a strong impact on fermentation performance.

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