Sucrose Fermentation by Brazilian Ethanol Production Yeasts in Media Containing Structurally Complex Nitrogen Sources

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ABSTRACT

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Four *Saccharomyces cerevisiae* Brazilian industrial ethanol production strains were grown, under shaken and static conditions, in media containing 22% (w/v) sucrose supplemented with nitrogen sources varying from a single ammonium salt (ammonium sulfate) to free amino acids (casamino acids) and peptides (peptone). Sucrose fermentations by Brazilian industrial ethanol production yeasts strains were strongly affected by both the structural complexity of the nitrogen source and the availability of oxygen. Data suggest that yeast strains vary in their response to the nitrogen source's complex structure and to oxygen availability. In addition, the amount of trehalose produced could be correlated with the fermentation performance of the different yeasts, suggesting that efficient fuel ethanol production depends on finding conditions which are appropriate for a particular strain, considering demand and dependence on available nitrogen sources in the fermentation medium.

Key words: amino acids, anaerobiosis, ethanol production yeasts, nitrogen metabolism, peptides, *Saccharomyces*, sucrose fermentation, trehalose, yeast.

INTRODUCTION

Industrial worts are examples of a nutritional environment that contains a complex mixture of nutrients including carbon and nitrogen sources[34,36]. 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[14,17,22,31,35]. Sugar catabolite repression[14,35] 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[11]. However, altered patterns of sugar utilization among brewing, wine, baking and distilling strains have been reported[5,25]. In fuel ethanol production from the sugar-cane syrup industry, sucrose is the main fermentable carbohydrate. *S. cerevisiae* is known to be glucophilic[13] despite being used concomitantly with glucose, fructose is usually the sugar present in the later stages of must fermentation[16]. Discrepancy between glucose and fructose consumption is not a fixed parameter, and it has been shown that the preference for glucose over fructose varies among strains and is dependent on the yeast's genetic background and on external conditions[4,26]. Selection of strains for the brewing, baking, wine and distilling industry considers their capability to rapidly and completely utilize the fermentable carbohydrates present[25,34]. The efficient utilization of sucrose is also a concern in Brazilian alcohol plants, where a high-cell-density process with cell reuse is carried out over long successive fed-batch fermentation cycles[2,33]. An abundant sugar concentration at the beginning of the fermentation process and high amounts of ethanol at the end, subject yeast cells to varying degrees of osmotic and ethanol stress. In addition, nutritional deficiencies induced by varying the compositions of industrial raw material, as well as oxygen limitation, are common phenomena in industrial fermentations, all contributing negatively to yeast performance.

In industrial fermentations, carbohydrates are not the only important group of compounds in the medium. 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[8,34]. Nitrogen deficiencies have been identified as one of the main causes of stuck or sluggish fermentations[1,6]. Considering the differential sugar utilization among industrial yeast strains, their sensibility to osmotic pressure and ethanol, and also their nitrogen demand, it is important to select yeast strains that not only rapidly and efficiently utilize all the sugars present, but that they are also able to properly use all of the different nitrogen sources in order to ensure an efficient fermentation. In the case of fuel ethanol production with cell reuse, it is important for yeast viability and vitality maintenance between cycles.

Similar to carbon catabolite repression, a mechanism known as nitrogen catabolite repression[17,22] induces differential nitrogenous compound utilization. It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeast[22]. When these primary nitrogen sources are absent, or present in concentrations

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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 media, this would suggest that the mutual interaction of these nutrients may play an important role in yeast metabolism as suggested by Peter et al.[29], who describe the regulation of amino acid permeases by carbon catabolite repression.

In previous studies we have shown, that in general, the supplementation of the yeast growth media, containing maltose, glucose or fructose, with a more complex structural nitrogen source such as peptone, induced higher biomass accumulation and ethanol production[3,9,10,26]. Studies with wine yeasts also showed the effects of oxygen availability on yeast fermentation performance[26]. It has also been reported that brewer's and baker's yeast differ in their ability to ferment galactose, depending on the structural complexity of the nitrogen source and the yeast catabolite repression response to the fermentable sugar[9]. We have continued our studies on the effect of the complexity of the nitrogen source on the metabolism of industrial yeasts by studying four commercial fuel ethanol production strains and the effect of the nitrogen source on sucrose fermentation.

MATERIALS AND METHODS

Microorganisms

Strains BG, SA and CAT are commercial *S. cerevisiae* ethanol production strains, used as fermentations starters, in Brazilian ethanol plants. RED STAR is an industrial strain kindly donated by Lasaffre, Lille, France. The yeast strains were maintained on Yeast Extract-Peptone-Dextrose (YEPD) 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 the fermentations contained 0.17% (w/v) Yeast Nitrogen Base W/O. Carbohydrate was added as sucrose at 22% (w/v). The medium was supplemented with the 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 containing 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) and

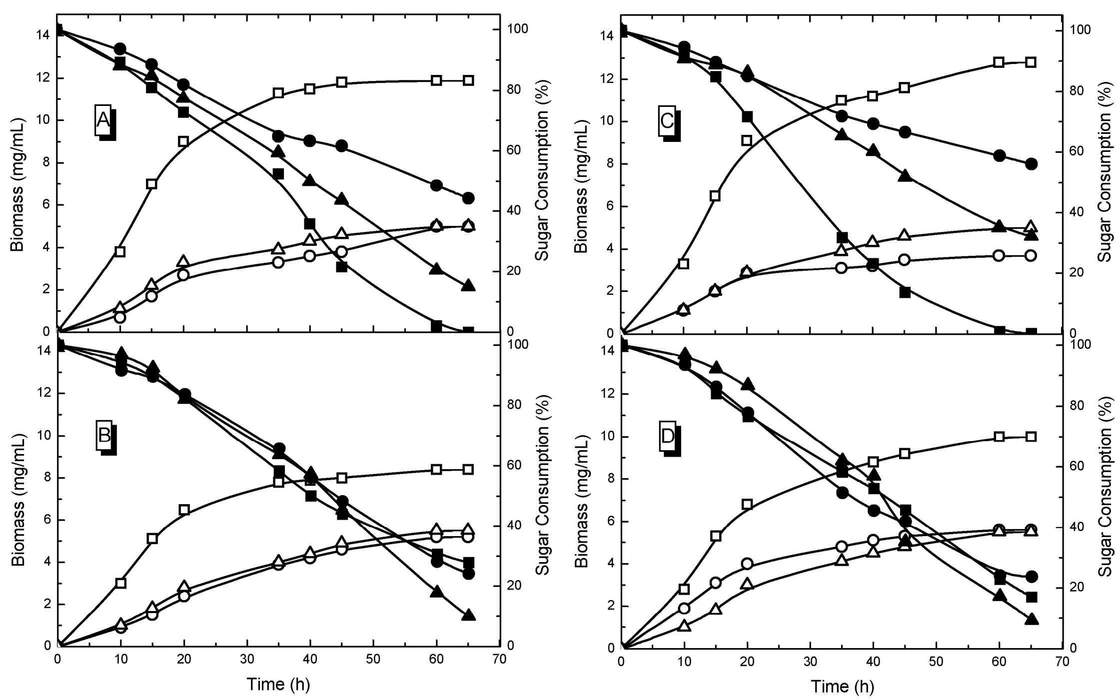

Fig. 1. Growth (open symbols) and sugar utilization (closed symbols) with shaken fermentations. Graph A, strain RED Star; graph B, strain BG (B); graph C, strain CAT; graph D, strain SA. YNB medium containing sucrose 22% (w/v) at 30°C, 200 rpm, supplemented with 1% (w/v) peptone (square), casamino acids (circle) and ammonium sulfate (triangle). The media containing ammonium sulfate had the pH adjusted to 5.0 with NaOH 1M.

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 occurred during the sampling process.

Analytical methods

At specified times during the fermentation, an aliquot of cell suspension was withdrawn, centrifuged and the supernatant frozen for subsequent analysis. 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[20]. Carbohydrate analysis was carried out using a colorimetric assay with 2-hydroxy-3,5-dinitrobenzoic acid[27].

Trehalose assay

For the determination of trehalose, 1 mL of cell suspension was centrifuged and the cells were washed 3 times with saline solution and the cell samples were resuspended in 1 mL 0.25 M Na_2CO_3 and incubated for 20 min in a boiling water bath. After centrifugation, an aliquot of 25 μL was withdrawn and used for the enzymatic determination of trehalose, with a trehalase preparation obtained from *Humicola grisea*[40]. The assay was carried out in a total volume of 100 μL, 25 μL of sample, 12.5 μL 300 mM acetate buffer pH 5.5 containing $CaCl_2$ 15 mM, 12.5 μL of 1M acetic acid and 50 μL of *Humicola* trehalase suspension. Incubation was overnight at 50°C and the reaction was stopped by incubating the samples at 100°C for 10 min. The tubes were cooled in an ice bath and the glucose released during trehalose hydrolysis was determined using a commercial kit (Labtest, Brazil), based on the glucose oxidase-peroxidase reaction. Trehalose was expressed as nmols of trehalose/mg cells (dry weight).

Reproducibility

The results presented in this study were the average of a minimum of three independent experiments.

RESULTS

Media containing 22% (w/v) sucrose 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 and sugar utilization during the growth of *S. cerevisiae* industrial yeasts in media containing 22% (w/v) sucrose, supplemented with different nitrogen sources, under shaken and static conditions. In general under peptone supplementation, all strains, shaken and static conditions (Figs. 1 and 2), showed higher biomass accumulation, efficient sugar utilization and yeast viability was preserved (Table I). Casamino acids and ammonium sulfate always induced poorer fermentation performances, with lower biomass and sugar consumption, and maintenance of yeast viability differed among strains (Figs. 1 and 2, Table I). Despite considerable loss of cell viability, when compared with peptone, ammonium sulfate supplementation induced improved fermentations compared to casamino acid supplementation. Static cultivation, as expected, produced lower biomass accumulation, but induced efficient consumption of sugar with peptone and ammonium sulfate, along with the maintenance of higher yeast viability. In addition to the

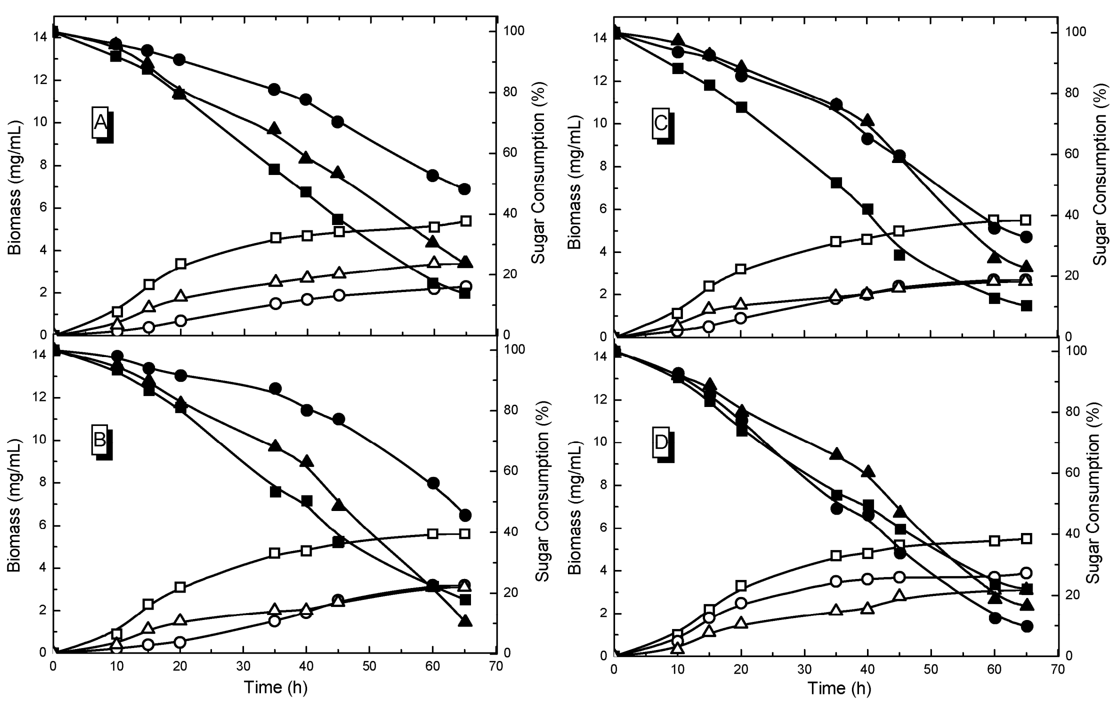

Fig. 2. Growth (open symbols) and sugar utilization (closed symbols) with static fermentations. Graph A, strain RED Star; graph B, strain BG; graph C, strain CAT; graph D, strain SA. YNB medium containing sucrose 22% (w/v) at 30°C, supplemented with 1% (w/v) peptone (square), casamino acids (circle) and ammonium sulfate (triangle). The media containing ammonium sulfate had the pH adjusted to 5.0 with NaOH 1M.

Table I. The viability profiles (methylene blue) of different ethanol production yeast strains in YNB W/O medium containing 22% (w/v) sucrose supplemented with 1% (w/v) of the various nitrogen sources.[a]

Strains	Time (h)	Peptone		Casamino acids		Ammonium sulfate[b]	
		Agitation[c]	Static	Agitation	Static	Agitation	Static
RED STAR	40	98.9	99.2	61.3	83.1	83.8	95.6
	65	92.5	96.9	58.0	89.3	79.2	95.4
BG	40	92.3	98.5	69.0	85.1	67.6	91.8
	65	49.0	93.0	62.2	84.3	63.2	87.7
CAT	40	97.3	97.1	52.5	75.0	47.3	86.2
	65	82.3	82.7	40.2	71.4	28.0	87.0
SA	40	100.0	100.0	66.9	93.1	63.3	97.4
	65	97.8	96.8	46.2	85.4	67.4	98.3

[a] Fermentation condition: 30°C and initial pH 5.0.
[b] The media containing ammonium sulfate had the pH adjusted to 5.0 with NaOH 1M.
[c] Agitation refers to shaking at 200 rpm.

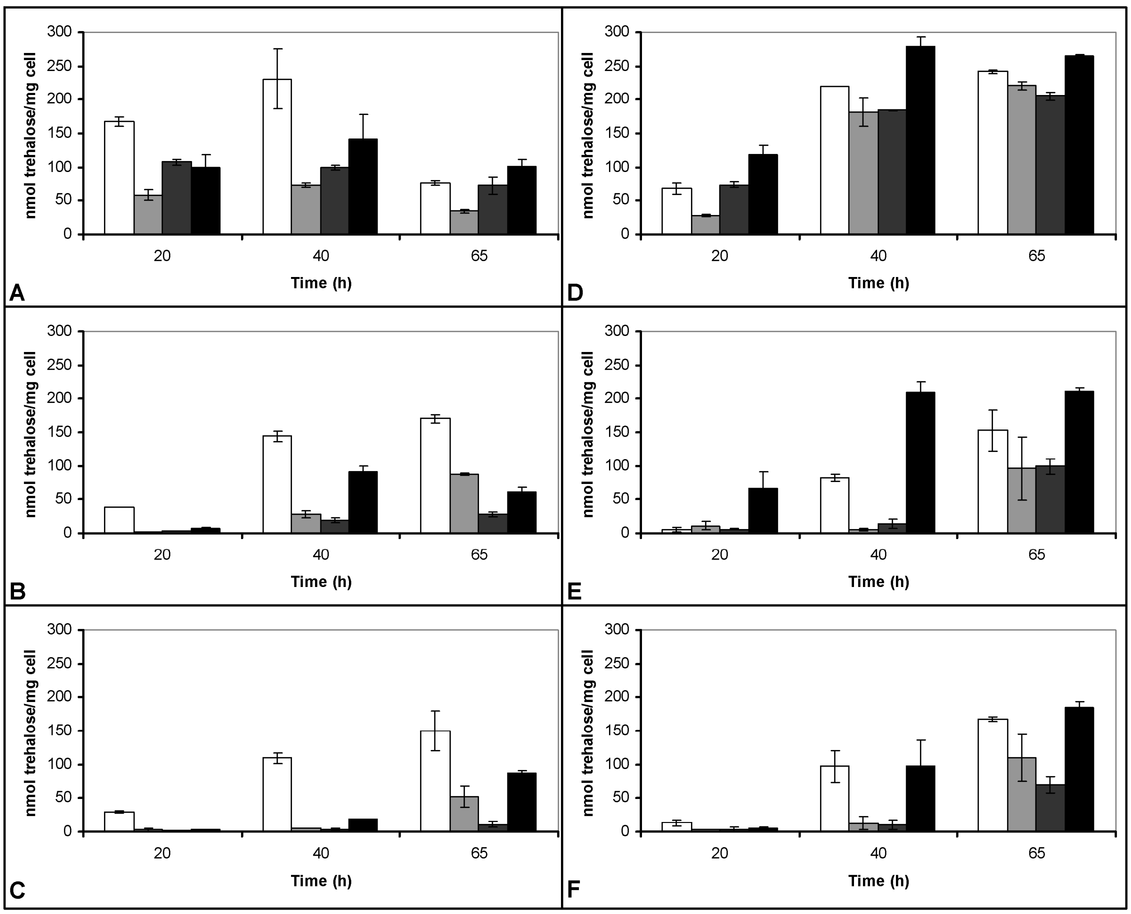

Fig. 3. Trehalose production by *S. cerevisiae* strain RED Star (□), strain BG (▨), strain CAT (▩) and strain SA (■). YNB medium contained sucrose 22% (w/v) at 30°C, supplemented with 1% (w/v) peptone (A and D), casamino acids (B and E) and ammonium sulfate (C and F) in experiments with agitation (A, B and C) and without agitation (D, E and F). The media containing ammonium sulfate had the pH adjusted to 5.0 with NaOH 1M.

effect of nitrogen demand among strains, oxygen availability also has a strong effect on yeast metabolism, affecting growth, sugar consumption and cell viability.

The effect of the complex nature of the nitrogen source on trehalose accumulation during sucrose fermentation was studied. Figure 3 shows the amount of trehalose accumulated during fermentation with supplementation of peptone (Figs. 3A and 3D), casamino acids (Figs. 3B and 3E) and ammonium sulfate (Figs. 3C and 3F), under shaken and static conditions respectively. Peptone induced higher accumulation of trehalose for all strains, when compared with casamino acids and ammonium sulfate. RED STAR and strain SA accumulated more trehalose than strains BG and CAT, which could explain the higher levels of viability observed with peptone supplementation. Oxygen was an important parameter, not only for defining the level of growth and sugar consumption, but its presence is also known to have a strong impact on trehalose accumulation. In general, static fermentations produced higher levels of trehalose than shaken fermentations. The effect of oxygen availability, in fermentations with complex nitrogen source supplementation, has already been described in our previous study with wine strains[26].

DISCUSSION

In this work, studies were conducted on sucrose fermentations with fuel ethanol production yeasts used as starter cultures in Brazilian ethanol plants, in media supplemented with nitrogen sources of differing structural complexity. One of the main problems faced by the wine industry is the undesirable occurrence of stuck or sluggish fermentations[1,6], whereas in the Brazilian fermentation process for fuel ethanol production, the major biological concern, besides bacterial contamination, is the decreased level of industrial and selected starter yeast competitiveness during the production season and their substitution by indigenous yeasts. In the Brazilian fuel ethanol production process, yeast cells are recycled, with the process operating at a very high cell density fermentation and with a very short fermentation time[2,33]. Osmotic stress, high ethanol concentration, bacterial contamination and acidity are some of the conditions imposed on the yeast during fermentation, and the cell recycle process is recognized as a main reason why starter strains are unable to survive during the industrial process of fuel ethanol production[2]. In the wine industry, troubled fermentations have been always attributed to the nutritional status of grape musts due to suboptimal yeast nutrients, especially assimilable nitrogen[4,18,23,36]. A common practice in the wine industry, for nitrogen limited fermentations, is the addition of nitrogen supplements using inorganic salts such as diammonium phosphate[24,30]. Only a few publications are available concerned with yeast performance in the Brazilian fuel ethanol fermentation process. Similar to the wine industry, the problems faced by yeast in the Brazilian ethanol fuel industry could be in part attributed to media nutritional deficiencies, mainly nitrogen, leading to a degree of nutritional stress inducing viability loss of the starter industrial yeast, and allowing prevailing and persistent indigenous yeasts, with undesirable fermentation features, to flourish.

This work was carried out to study the nitrogen demand of typical fuel ethanol production yeast strains and their response to the structural complexity of the nitrogen source. The metabolism of industrial ethanol production yeasts was examined by employing media that contained sucrose as a carbon source, along with nitrogen compounds with differing levels of structural complexity, varying from a single ammonium salt (ammonium sulfate) to an acid protein hydrolysate (casamino acids) consisting predominantly of free amino acids, and an enzymatic hydrolysate of protein consisting predominantly of peptides (peptone)[10,12]. The carbon source employed was sucrose at 22% (w/v), in order to subject the yeast cells to similar metabolic osmotic conditions as those found in Brazilian ethanol producing plants.

Previous results from this laboratory[2,9,10,26] 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. With casamino acid 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[3,10]. 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 induces altered patterns of galactose utilization at higher sugar concentrations, strongly affecting both growth and ethanol production[9]. Additional studies with brewing and wine strains have shown altered patterns of maltose and glucose utilization by industrial strains and their dependence on the complexity of the nitrogen source[3]. In addition to the complexity of the nitrogen source, oxygen availability affects glucose and fructose fermentations by wine yeasts[26].

Results obtained in this study with fuel ethanol production strains, showed that the presence of oxygen, in addition to the complexity of the nitrogen source, strongly affected sucrose fermentation. The complexity of the nitrogen source, and the form of cultivation (shaken and static) induced altered patterns of sucrose fermentation, affecting biomass production, sugar consumption and intracellular trehalose accumulation. In the medium containing sucrose, under agitation, the strains showed high biomass accumulation and efficient sugar consumption in the media supplemented with peptone, as well as the preservation of yeast viability. In the medium supplemented with casamino acids or ammonium sulfate, all of the strains produced lower biomass. Sugar consumption rates varied among strains, and there was a considerable loss of cell viability (Fig. 1, Table I). In the static fermentations, with a low concentration of oxygen, despite the low biomass accumulation, strains with all of the nitrogen supplementations displayed efficient sugar consumption and yeast viability was kept high throughout the fermentation (Fig. 2, Table I).

In order to further characterize the effect of the complexity of nitrogen source on ethanol production strain metabolism, the accumulation of trehalose, considered a stress protectant metabolite, was studied under differing fermentation conditions. The concentration of trehalose was higher under peptone supplementation and this may be a reflection of improved fermentation performance in the presence of peptone. The presence of oxygen also affected the production of trehalose and static fermentation induced higher accumulation levels of the disaccharide. Strains Red Star and SA accumulated higher amounts of trehalose, suggesting that fermentation performance could be directly correlated with intracellular trehalose content and strains producing higher levels of trehalose would confer improved yeast vitality under the stress conditions found in the Brazilian fermentation process.

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[7,15,18,34,36]. It has been reported that nitrogen deficiency induces lower biomass yields, which slows fermentation rates, increasing the risk of sluggish or stuck fermentations[1,6]. The molecular mechanisms responsible for the alteration in the fermentation parameters induced by the complex composition of the nitrogen source are unclear and have been discussed in this and previous publications[2,8,9,26]. The results obtained with glucose, maltose and galactose during this study, in the presence of peptone and casamino acids, suggest that nitrogenous supplements induce efficient conditions for yeast growth and fermentation. In general, supplementation with peptone, an enzymatic hydrolysate of protein consisting predominantly of peptides, induced improved fermentation performance. The role of peptides in yeast fermentation is poorly understood[28] and recent studies have addressed to the importance of small peptides in a brewing fermentation[19,21]. 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[22,29]. It is also worth noting the strong effect of oxygen availability on yeast metabolism and its effect on fermentation parameters (Figs. 1 and 2, Table I). Recent studies report on the implications in the central carbon metabolism of *S. cerevisiae* induced by aerobic and anaerobic conditions and address the complexity of the role of oxygen in controlling cellular physiology in yeasts[32,37,38,39], considering its multiple role in yeast metabolism, such as during the biosynthesis of unsaturated fatty acids, ergosterol, heme group, mitochondrial development and others[37]. It has been reported that an optimized oxygen supply is critical, because too much oxygen could cause yeast degeneration, due to the toxic effect of reactive oxygen species[37]. Similar to oxygen, depending on the yeast strain's nitrogen demand, an improved fermentation performance could be attained by finding an optimized supply of the appropriate nitrogen source.

In this work we have shown the influence of the structural complexity of the nitrogen source and the availability of oxygen on sucrose fermentation by ethanol producing strains. Altered patterns of sucrose utilization by yeasts induced strong effects on biomass production and cell viability, and also on the accumulation of the disaccharide trehalose. These results have academic and industrial relevance, since they suggest that not only the structural complexity of the nitrogen source, but also the yeast metabolic response to the presence of oxygen strongly affects yeast performance. The results suggest that yeast strains vary in their response to nitrogen source complex structure and to oxygen availability. The amount of trehalose production correlated with the fermentation performance by differing yeast, suggesting that efficient fuel ethanol production depends on finding the appropriate nutritional conditions for a particular strain, considering the strain's demand and dependence on the available nitrogen source in the fermentation medium.

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