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IMPACT OF NUTRITIONAL OPTIMIZATION ON FERMENTATIVE CAPACITY IN THE YEAST *Brettanomyces bruxellensis*

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ABSTRACT

Brettanomyces bruxellensis is a yeast considered as a contaminant in industrial alcoholic fermentation processes and, due to its tolerance in inhospitable environments, it has the ability to replace the initial population of *Saccharomyces cerevisiae*. *B. bruxellensis* presents the metabolic structure to carry out fermentation for ethanol production, with yields comparable to those of the main yeast. Despite being the most studied species, there are still gaps regarding its physiology. These gaps are related to their energy metabolism, which is not yet completely understood. The nitrogen source has already been reported as one of the main factors that influence the competitiveness between *S. cerevisiae* and the contaminating yeast. *B. bruxellensis* can assimilate nitrate in fermentative environments, which is not possible for the main yeast in the process. The fermentative potential of the industrial strain *B. bruxellensis* GBD248 was evaluated through a 2 x 2 full factorial design, considering three combinations of nitrogen sources and carbon concentration. Therefore, this work aimed to investigate nitrogen metabolism and, at the same time, analyze its impact on carbon concentration through factorial design. These findings provide insights into the potential for ethanol production by *Brettanomyces bruxellensis* GDB 248 and guide the development of new bioprocesses.

Keywords: Non-conventional yeast. Fermentation alcoholic. Energy metabolism. Nitrate assimilation. Factorial design.

1 INTRODUCTION

The yeast *Brettanomyces bruxellensis* is consistently observed in various segments of industrial fermentation, particularly in the production of wine, beer, kombucha, and bioethanol.[1] Due to its high stress tolerance, including the ability to withstand low pH conditions, high ethanol concentrations and efficient utilization of nutrients, *B. bruxellensis* is well adapted to the conditions present in fermentation industry.[2] Currently, *B. bruxellensis* has been identified as a significant contaminant in the sugarcane fermentation process for ethanol production in distilleries in the Northeast region of the Brazil. This contamination negatively impacts the yield and productivity of these industries.[3] Interestingly, a strain of *B. bruxellensis* was discovered as the sole producing microorganism in a continuous industrial alcohol plant based on starch, highlighting its relevant role in the alcoholic fermentation industry.[4] An additional advantage of *B. bruxellensis* is its ability to use nitrate as the sole source of nitrogen, attributed to the presence of specific genes responsible for the assimilation of this nitrogenous compound in its genome, unlike *S. cerevisiae*.[5-6]

Regarding carbon sources, *B. bruxellensis* shows a preference for the assimilation of glucose, fructose and sucrose, which are sugars capable of sustaining high growth rates.[7] These sources have industrial relevance, as they are part of the composition of substrates, such as sugarcane juice and molasses.[3] In *B. bruxellensis*, as well as in *S. cerevisiae*, the high glucose concentration results in fermentative metabolism even under aerobic conditions[8], a characteristic behavior of Crabtree positive yeasts.[9-10] The high capacity for assimilation of the different nutrients available in the fermentation substrate makes *B. bruxellensis* a potential yeast for ethanol production.[11-12] Furthermore, the assimilation of nitrate as a nitrogen source also found in industrial substrates is a clear advantage over *Saccharomyces cerevisiae*.[13]

The impact of micronutrients and macronutrients on the fermentative capacity of the industrial yeast *B. bruxellensis* can be evaluated by Design of Experiments (DOE), a widely used method to determine the influence of a set of variables on a response of interest. This technique allows you to control multiple input factors, determining their effect (individually or together) on a desired response. By controlling multiple inputs at the same time, experimental design can identify meaningful interactions that go unnoticed when experimenting one factor at a time. This tool is used in a strategic and useful way to track the most relevant variables of a given system, in which researchers can extract the maximum amount of information, carrying out a reduced number of experiments, enabling work with reduced time and cost.[14] In this sense, the present work evaluated the influence of different sources of nitrogen on the fermentative capacity of *B. bruxellensis* GDB 248.

2 MATERIAL & METHODS

B. bruxellensis GDB 248 strain, isolated from industrial processes, was used in this study. The cells were cultured in solid YPD medium containing yeast extract (10 g/L), peptone (20 g/L), dextrose (20 g /L) and agar (20 g/L).[15,3]

Before starting the fermentation assays, a complete 2^2 factorial design (DOE Model) was carried out in the Statistica 7.0 software (Table 1).[16] The variables used were carbon and nitrogen and their concentrations were defined based on previous studies.[17] The values represented in Table 1 are coded, the value +1 corresponds to the highest condition of the factors, -1 to the lowest level and the value 0 to the central point.

Table 1 Design matrix 2 x 2 for fermentation assays with sucrose concentrations and equimolar nitrogen concentrations for ammonium sulfate and sodium nitrate.

Sample	Carbon (level)	Nitrogen (level)	Sucrose (g/L)	Ammonium sulfate (g/L)	Sodium nitrate (g/L)
1	-1	-1	100	0,9	1,2
2	+1	-1	180	0,9	1,2
3	-1	+1	100	3,8	4,9
4	+1	+1	180	3,8	4,9
5	0	0	140	2,4	3,0
6	0	0	140	2,4	3,0
7	0	0	140	2,4	3,0

In order to analyze the influence of carbon and nitrogen availability on the fermentative capacity of *B. bruxellensis*, the GDB 248 strain was subjected to fermentation assays in specific YNB (Yeast Nitrogen Base) medium without addition of amino acids and ammonium sulfate, at different concentrations of carbon and nitrogen for 24h, at 30 °C and 160 rpm. Samples were collected at times 0 and 24 hours for analysis by HPLC. The factorial design was divided into two stages, using (i) ammonium sulfate or (ii) sodium nitrate. Due to the use of different nitrogen sources, Table 1 presents the appropriate concentrations of each of these sources.

The samples collected from the fermentation assays were centrifuged, diluted and filtered through a 0.20 µm sterile filter in order to quantify extracellular metabolites concentration, such as sucrose, glycerol, ethanol, and acetate by high-performance liquid chromatography. These metabolites were separated by the Aminex HPX - 87H+ column, 300mm x 7.8mm at 60°C, using 5 mM H2SO4 as mobile phase at a flow rate of 0.6 mL/min and detected by a refractive index detector. Calibration curves were constructed using standard samples of each analyte in order to calculate concentrations of each metabolite.

3 RESULTS & DISCUSSION

B. bruxellensis fermentative capacity was evaluated in response to the presence of variable concentrations of carbon and nitrogen, using experimental designs. The results obtained for sucrose consumption, ethanol production, glycerol and acetate are presented in Table 2. When the sucrose concentration goes from the lower level to the higher level (assay 1 and 2) and ammonium concentration at the lower level, the yield and ethanol production increases by 0.01 g/g and 12.38 g/L, respectively. However, when the ammonium concentration is at a higher level (assays 3 and 4), both yield and production increases by 0.12 g/g and 18.22 g/L, respectively. The results show that the use of high concentrations of ammonium sulfate is ideal for obtaining higher ethanol yields, regardless of the sucrose concentration. Despite this, the increase in carbon concentration triggered an increase in ethanol production in both environments (NO_3^- or NH_4^+). An explanation for this behavior may be associated with the initial phase of fermentation, when there was greater carbon availability, the yeast preferentially used its fermentative metabolism, a characteristic response of yeast with the Crabtree effect. Previous studies have confirmed that *B. bruxellensis* stimulates ethanol production starting immediately in response to excess glucose.[18-21]

Table 2 Factorial design responses with ammonium sulfate or sodium nitrate; SC: sucrose consumption (%); P: ethanol production (g/L); Y_{et}: ethanol yield (g/g); Y_{ace}: acetate yield (g/g); Y_{gly}: glycerol yield (g/g).

Assay	Ammonium sulfate					Sodium nitrate				
	SC	P	Y_{et}	Y_{acet}	Y_{glyce}	SC	P	Y_{et}	Y_{acet}	Y_{glyce}
1	80%	24.74	0.31	-	0.013	85%	15.20	0.18	0.04	*
2	64%	37.12	0.32	0.014	0.004	55%	26.70	0.27	0.03	*
3	82%	10.46	0.13	0.030	0.010	80%	13.70	0.17	0.05	*
4	63%	28.68	0.25	0.015	0.011	57%	26.70	0.26	0.04	*
5	74%	35.89	0.35	0.018	0.006	73%	33.70	0.33	0.05	*
6	72%	33.93	0.34	0.017	0.006	75%	32.60	0.31	0.04	*
7	76%	34.71	0.33	0.017	0.006	73%	35.00	0.34	0.05	*

(*) There was no production.

Furthermore, in *B. bruxellensis*, nitrate promotes metabolic reorientation by inducing cells to produce acetate during fermentation. Despite the production of acetate, there is also an increase in the ethanol yield under the presence of nitrate[1], probably because of a compromise in the Custer effect, as previously reported.[11] In this scenario, the reduction of nitrate to ammonium led to the reoxidation of NAD(P)H, reducing the influence of the Custer effect. When sodium nitrate as a nitrogen source, there was no production of glycerol in any of the assays. During nitrate metabolism, the high demand for the cofactor NAD(P)H responsible for the reduction of nitrate to ammonia may be the biggest detriment in preventing the production of glycerol, since it demands the same oxidation process.[22] Therefore, the presence of nitrate affects glycerol production due to competition for the same cofactor necessary for nitrate assimilation.[23]

4 CONCLUSION

In conclusion, our data reveal that carbon and nitrogen availability influence physiological parameters. *B. bruxellensis* presents a higher ethanol yield in the presence of ammonium sulfate as a nitrogen source. The nutritional condition that provided the best ethanol production results was the condition with ammonium sulfate, which can be a benefit to the process, since both sources are in the same fermentation environment and there is no way to remove contaminant yeast. On the other hand, *B. bruxellensis* showed a higher acetate yield in the condition with sodium nitrate, which indicates that the presence of nitrate promotes metabolic reorientation, reducing ethanol production during fermentation and inducing acetate production. Furthermore, *B. bruxellensis* was

not able to produce glycerol in nitrate medium because the nitrate assimilation pathway requires the same cofactor as the glycerol production pathway.

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