Increasing the Protein Content in *Saccharomyces cerevisiae* during Ethanol Production from Sugarcane Molasses with Nitrogen Supplementation

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ABSTRACT

Brazil stands out as the largest producer of sugar cane, with expected growth in the years to come. The Brazilian ethanol industry, derived from sugarcane, distinguishes itself in competitiveness due to its superior yield and lower greenhouse gas emissions compared to other raw materials such as corn or beet. The predominant fermentation process, known as Melle-Boinot, involves yeast addition to the must, continuing fermentation until substrate depletion. The resulting yeast cream, a byproduct, is sought after by animal nutrition companies as a low-cost protein source. This study aims to assess the impact of sugarcane molasses supplemented with urea in fed-batch fermentations with cell recycle and acid treatment for high-protein biomass production using the yeast strain Saccharomyces cerevisiae PE-2. Trials with different molasses sources and urea proportions revealed significant variations in protein content. Specifically, the addition of 0.375% (w/v) urea to molasses showed a notable increase in protein compared to the control (29.5% on wet basis). Although positive effects on viability and protein content were observed, higher urea concentrations exhibited lower ethanol titers in view of high cell biomass formation. These findings underscore the importance of precise must control to optimize high-protein biomass production.

Keywords: *Saccharomyces cerevisiae*. Urea Supplementation. Fed-Batch Fermentation. Protein Content.

1 INTRODUCTION

Brazil is the world largest producer of sugar cane, with significant growth observed in recent years. From 2005 to 2021, sugar cane production surged by approximately 41%, reaching 657 million tons, with over 50% of this production concentrated in São Paulo state, the leading sugar cane producer in Brazil[1]. Correspondingly, ethanol production in the country also witnessed a substantial increase, rising from 15.4 billion liters in 2005 to 32.5 billion liters in 2021[2]. According to the National Supply Company, 55.6% of Brazil's sugar cane is directed towards ethanol production, solidifying Brazil's position as one of the world's largest ethanol producers derived from sugar cane. In a world increasingly seeking sustainable, renewable, and cost-effective energy sources, ethanol emerges as the most utilized biofuel.

The Brazilian ethanol production industry from sugar cane stands out for its high competitiveness compared to other raw materials such as corn or beet, owing not only to its superior yield but also to its lower greenhouse gas emissions. The predominant fermentation process utilized in Brazil is batch fermentation, also known as the Melle-Boinot method, wherein yeast is added to the must, and fermentation proceeds until all substrate is consumed by the yeast. Subsequently, the fermented must is centrifuged, and the yeast is recovered and treated for reuse. The yeast obtained after centrifugation, also known as yeast cream, can be directed to animal nutrition companies, making it an attractive source of low-cost protein[3].

Enhancing the protein content in *S. cerevisiae* yeast as a by-product of ethanol production is a strategy to obtain a high added value product for the animal nutrition market[4, 5]. Thus, this study aims to evaluate the influence of nitrogen supplementation during molasses fermentation using the cell recycle system employed in Brazil, as well as the underlying impacts on yeast physiology of such strategy.

2 MATERIAL & METHODS

The yeast strain employed for the fermentative assays in this study was *S. cerevisiae* PE-2. To assess protein content, an experiment was conducted comparing a cultivation medium consisting solely of sugar cane molasses with a concentration of 20% total reducing sugars, commonly used in sugar cane fermentation mills, with another medium containing the same sugar concentration supplemented with 0.375% (w/v) urea (referred to as MU03).

The experiment started with the centrifugation of the cell suspension obtained from propagation, yielding approximately 5 g of wet cells. Subsequently, 2 ml of the wine obtained from centrifugation and 6 ml of distilled water were added, simulating the conditions of the yeast slurry which is industrially used as the inoculum for the beginning of each fermentation cycle.

Fermentations were conducted in triplicate and incubated at 30°C without agitation for 4 fermentation cycles. Three feedings of 9.25 ml of the must were added during the process at 0, 2 and 4 h (to mimic the fed-batch process. The tubes (flasks) were weighed hourly for 8 h to monitor CO_2 loss. After the final weighing, the tubes were left on the bench at room temperature overnight. In the following day, the tubes were weighed and homogenized, and a 1 ml sample was collected from each tube for cell viability analysis. Subsequently, the tubes were centrifuged to collect the cells, and the supernatant was collected for HPLC analysis. The same process was repeated on the same day with pH correction at 2.5 using sulfuric acid (to mimic the industrial acid wash step). This procedure was repeated four times, totaling 4 fermentation cycles. Wet cell biomass determination was performed by gravimetric analysis after centrifugation. Total nitrogen content in biomass and wine was quantified using the Leco equipment (FP-528 model). Ethanol production was assessed by HPLC.

3 RESULTS & DISCUSSION

Based on the results obtained, we could observe that biomass growth was slightly higher under condition with urea supplementation (**Figure 1**), which was followed by glycerol titers (6.14 vs. 8.42 g/L). These differences increased along the fermentation cycles, which highlights the importance of studying this process mimicking as far as possible the industrial scenario. Urea supplementation in molasses revealed significant variations in protein content (**Table 1**). Specifically, the addition of 0.375% (w/v) urea to molasses showed a notable increase in protein content when compared to the control condition (29.5% on wet basis). Although positive effects on viability (data not shown) and protein content were observed, higher urea concentrations exhibited lower ethanol titers, probably in view of high cell biomass formation.

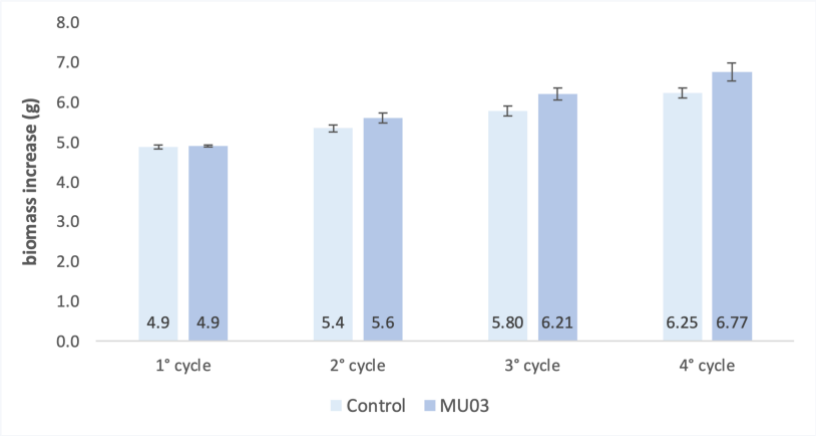

Figure 1. Biomass (g per flask) at the end of each fermentation cycle.

Table 1. Protein content (% of wet biomass weight) at the end of the 4th fermentation cycle, and average ethanol titers (%, in v/v) at all cycles.

Condition	Protein (% in w/w)	Ethanol (% in v/v)
Control	7.38	11.61
MU03 (0,3% urea)	9.56	10.81

Nutrient availability is a critical factor for the efficient development of fermentation. Previous studies corroborates that the addition of urea can act as an additional nitrogen source, promoting protein synthesis without compromising cell viability[5]. The present results contributes to the understanding of the role of urea in optimizing fermentative processes, suggesting that its supplementation in molasses during the cell recycling process can be a viable strategy to increase protein content in microbial cultures with minor effects on ethanol titers.

4 CONCLUSION

We observed that the addition of urea in molasses during yeast fermentation with cell recycle resulted in a significant increase in protein production compared to the control (non-supplemented). However, since cell biomass values showed an increase, ethanol production was reduced. Urea supplementation proved to be beneficial for protein enhancement without exhibiting toxic effects on the cells, indicating promising potential for the continuation of the study.

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