EFFECTS OF EXTENDED STORAGE PERIODS ON GENETICALLY MODIFIED *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Cell viability is an important parameter during development of industrial bioprocesses, and it is defined as the percentage of viable cells in a population. Cell viability is impacted by either biotic or abiotic factors and most strains applied in industrial bioprocesses suffer by long periods of storage at low temperatures and nutrient starvation. Therefore, it is necessary to analyse the cell viability of strains utilized for bioproduction over long storage periods to determine the more suitable procedure to adapt cells before storing or using them. The present study aims to investigate the effect of cell storage in a xylose-fermenting *Saccharomyces cerevisiae* strain by storing it at 4°C for 60 days. Additionally, it was examined the difference in cell viability when propagating it in different media: one containing a commercial medium with glucose and xylose as carbon sources, and another industrial medium containing molasses and sugarcane bagasse hydrolysate as the carbon sources. Fermentations were conducted in hydrolysate throughout the 60-day storage period to analyze cell viability, with the most promising results observed for the cells prepared using the commercial medium, likely due to the absence of inhibitors and solid residues as those present in the industrial medium.

Keywords: Cell Viability, *Saccharomyces cerevisiae,* Storage, Fermentation, Hydrolysate.

1 INTRODUCTION

Microbial robustness is typically evaluated for industrial phenotypes regarding levels of yield rate, gene expression, volume, and cell viability. Defined as the intrinsic capacity of microorganisms to remain constant despite disturbances[1,2,3,4,5], the present study focused on analyzing cell viability in an industrial strain constructed for second-generation ethanol production. Attention to this area should be evident in the industry, as processes constantly subject cells responsible for product generation to numerous stresses such as temperature changes, pH fluctuations, pressure, shear force, incorrect storage, and contaminants. Therefore, only robust microorganisms are capable of generating profitable productions[5].

Properly scaling up experimental processes conducted in laboratory bench settings is an essential element for developing new technological solutions, encompassing various fields and relating solutions offered by biotechnology to enable the production of optimized bioproducts[5]. Enabling well-defined processes at small scale to also perform satisfactorily at large scale makes innovation feasible and applicable, make it accessible for commercialization. Beyond enabling production in commercial level of products originating from scientific discoveries, it also plays a crucial role in cost reduction by improving efficiency and ensuring the commercial viability of new technology or product. Therefore, it is a vital commitment to ensuring that the high demand for these emerging products meets the requirement for use in different areas such as medicine, energy, agriculture, sustainability. This approach has a sustainable bias for all decision-making related to the discourse, as biofactories, for example, allow the production of various compounds on a large scale in a more sustainable way and they are critical to establish a bioeconomy.

In addition to that, the yeast *Saccharomyces cerevisiae* is one of the workhorses for many industries worldwide due to its fermentation capacity, versatility, and availability of genetic tools, enabling the implementation and scaling up of various bioprocesses[6]. *S. cerevisiae* is applied in various sectors, including food, beverages, pharmaceuticals, and biofuels, which is the focus of the present study. There is a growing perception of the importance of replacing fossil-based raw materials for renewable resources in energy and chemicals production. This not only addresses urgent challenges related to climate change and energy security but also offers a range of long-term social, economic, and environmental benefits.

It is known, therefore, that cellular robustness along with bioprocess optimization has increasingly attracted the attention of researchers. Engineered strains with excellent results in the laboratory often face challenges in dealing with disturbances on larger scales. Therefore, in addition to develop an efficient xylose-fermenting *S. cerevisiae* strain to produce ethanol from xylose and glucose present in sugarcane hydrolysate streams, our group studied the behavior of this engineered strain when subjected to a long-period of storage at 4°C for 60 days.

2 MATERIAL & METHODS

The xylose-fermenting *S. cerevisiae* strain used in the present study as developed in previous work (unpublished data) and it has the following genetic modifications: extra copies of four genes from the Pentose Phosphate Pathway, deletion of the *GRE3* gene, integration of a copy of the *XYL3* gene, and overexpression of the *xylA* gene encoding a xylose isomerase. These cells were propagated in bioreactors with a work volume of 2 L, in YP medium (1% yeast extract, 2% peptone), and varying the carbon source in commercial and industrial media, composed respectively of 70% glucose + 30% xylose and 75% molasses +

25% sugarcane hydrolysate in order to maintain the final concentration of total sugar in the cultivation medium around 2% (20 g/L). The initial optical density at 600 nm (OD_{600}) was set at 0.3, and the cells were propagated for 16 hours in batch mode in the bioreactor Labfors (Infors HT); after propagation, the cells were centrifuged at 8000 RPM, 4°C, for 15 minutes, and resuspended in 50 mL of sterile water then stored in the refrigerator at 4°C.

Every 15 days, fermentations were conducted, with a total storage time of 60 days. The experiments were carried out in shake flasks and performed in biological triplicate. The work volume was kept at 60%, consisting of 30 mL of sugar-cane hydrolysate with concentration of approximately 100 g/L and 1 mL of cells resuspended in water with an initial OD_{600nm} close to 10 (correspond to ~5 g/L). Each fermentation lasted for 72 hours, and samples were collected every 24 hours, analyzing OD_{600nm}, cell viability, based on the count of cells stained with methylene blue, and plating on Petri dishes for observation of colony morphology. The cells were plated on YPX 2% and YPD 2%, with dilution factors of 5 and 10X and incubated at 30°C for approximately 48 hours.

3 RESULTS & DISCUSSION

The results indicated in Table 1 suggest that cells cultured in the commercial medium exhibited higher cell viability, as represented by the percentage of live cells relative to total cells. It is evident that cells cultivated in the commercial medium remained alive for a longer period compared to those cultivated in the industrial medium. This could be attributed to the cultivation in a medium containing glucose and xylose, which provided a greater cellular and metabolic framework for the cells to handle the thermal stress and nutrient deprivation associated with the prolonged storage period at 4°C in water. Another possible explanation for this better performance could be the absence of inhibitors, such furan aldehydes and organic acids, in the commercial medium, since the industrial medium utilized contains both 5-(Hydroxymethyl)furfural and acetic acid.

The observed higher thermal resistance in cells cultured in the commercial medium is likely due to cryoprotection genes, possibly related to the trehalose pathway and *TPS* genes or genes related to the higher tolerance to the inhibitors, such as those listed by Cámara et al., 2022[7] Confirmation of this hypothesis would require further tests including proteomics and RNA sequencing.

Table 1 Cell count.

I (industrial) C (commercial)	N° Live Cells	N° Dead Cells	N° Total Cells	Live Cells (%)
I – 15 days: 0 hours	846	87	933	90,73
I – 15 days: 72 hours	275	454	729	38,16
C – 15 days: 0 hours	753	34	787	95,77
C – 15 days: 72 hours	619	254	873	70,79
I – 45 days: 0 hours	640	53	693	92,34
I – 45 days: 72 hours	243	604	847	28,47
C – 45 days: 0 hours	733	80	813	90,0
C – 45 days: 72 hours	690	258	690	37,56

No statistical difference was observed for the OD values measured over the fermentation (data not shown). This can be explained by the fact that, despite the significant reduction of live cells over the course of fermentations, it is not possible to distinguish live and dead cells by OD_{600}Therefore, the results of this analysis did not allow us to infer many conclusions about cell viability.

Regarding the results obtained through plating, it was observed that even after 60 days of storage, the cells still maintained their necessary characteristics to develop on plates, retaining their expected colony morphology and visual aspects. After 48 hours of incubation, growth of cells cultivated in the industrial medium was observed, while after 24 hours, significantly more growth was noted in those cultured in the commercial medium, confirming the higher thermal tolerance exhibited by the latter compared to those cultured in the industrial medium. Only at 0 hours was observed significant growth, indicating the difficulty of these cells to develop after a long period in contact with the hydrolysate. To address this issue, cellular adaptation could possibly suffice, initially performed in a rich medium for cell reactivation and subsequently in a medium similar to the reaction medium but with lower concentrations of hydrolysate, allowing for gradual acclimatization of the microorganism. Another alternative would be cell washing to ensure that no interferents in their wall negatively influence their performance. Improved treatment of the hydrolysate could also aid in the process; adding a filtration step in addition to autoclave sterilization could significantly reduce impurities that come into contact with the cells, thus facilitating fermentation. To further elucidate the hypotheses presented, these tests will be conducted.

4 CONCLUSION

The xylose-fermenting *S. cerevisiae* strain developed by our group maintained its necessary characteristics to allow for its correct development when stored at 4°C for 60 days, as observed in the plates. Therefore, it is tolerant to thermal stress,

especially those cultured in the commercial medium. However, there is a noticeable reduction in cell viability when the cells were cultivated in pure sugarcane hydrolysate, meaning a decrease in live cells, proportional to the storage time. This can be addressed with proper cell pre-adaptation along or storage in nutrient-rich solution instead of water. Further experiments will be conducted to confirm this hypothesis. Besides, transcriptomics and proteomics experiments should be performed to determine the genetic basis for the improved adaptation of cells cultivated in commercial compared to industrial medium.

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