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ENHANCED ETHANOL PRODUCTION FROM LIGNOCELLULOSE USING GENETICALLY MODIFIED *Saccharomyces cerevisiae* EXPRESSING HETEROLOGOUS CELLOBIOHYDROLASES

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ABSTRACT

Agro-industrial wastes, lignocellulose-rich biomass, hold potential for second-generation ethanol production. Direct conversion of this biomass involves genetically modifying *Saccharomyces cerevisiae* for heterologous expression of cellulases, such as cellobiohydrolases. This study evaluates the synergistic activity of heterologously expressed cellobiohydrolases in a Brazilian industrial strain of *S. cerevisiae* and its cellulose-to-ethanol direct conversion capacity. The engineered strain exhibited superior enzymatic activity, reaching 860 U/L, a 12-fold increase compared to the parental strain, confirming genetic modification efficacy. It was even observed that the genetic modification did not alter the pH and temperature conditions for maximum enzymatic activity, which remained at around pH 5 and 45 °C. In ethanol production, the modified strain surpassed the parental strain in the initial six hours, yielding 9.0 g/L compared to 7.9 g/L. Beyond this period, both strains exhibited similar ethanol yields, recording at 24 hours. Thus, genetically modified *S. cerevisiae* shows promise for second-generation ethanol production, requiring further research to optimize operational conditions and explore the integration with other cellulose-degrading enzymes.

Keywords: Biofuel. Genetic engineering. Cellulose degradation. CRISPR/Cas9. *Trichoderma reesei*.

1 INTRODUCTION

The growing global demand for biofuels, driven by the need to reduce reliance on fossil fuels due to climate changes, underscores the importance of renewable energy sources[1]. Ethanol is a prominent biofuel, already used in the automotive sector in numerous countries[2], produced through a Consolidated Bioprocess involving fermentation by microorganisms, predominantly *Saccharomyces cerevisiae*[3]. However, *S. cerevisiae* faces inherent limitations in degrading complex polysaccharides like lignocellulose in agro-industrial wastes[4]. To utilize this abundant biomass for ethanol production in the second-generation process, it must be converted into fermentable sugars via enzymatic saccharification[5], a step typically separate from fermentation, thereby increasing costs[6]. Direct Microbial Conversion (DMC) aims to integrate saccharification and fermentation within a single microorganism[7]. Since *S. cerevisiae* cannot naturally perform lignocellulose saccharification, it requires genetic modification to express cellulolytic enzymes. The challenge lies in optimizing conditions that combine enzyme action with cell growth and fermentation for effective yield and productivity[8].

This study utilized a thermotolerant *S. cerevisiae* strain from the Santa Adélia biorefinery, engineered to express cellobiohydrolases 1 and 2 (CBHs) from *Trichoderma reesei* by Lopes (2020)[9] via CRISPR/Cas9. CBHs, crucial for cellulose degradation, release cellobiose units at the cellulose chain's reducing and non-reducing ends through the actions of CBH1 (EC 3.2.1.176) and CBH2 (EC 3.2.1.91)[10]. This study aimed to assess the synergistic activity of heterologously expressed CBHs in genetically modified *S. cerevisiae* and their ability to directly convert cellulose into ethanol.

2 MATERIAL & METHODS

The genetically modified strain and its parental counterpart were cultivated at 35 °C in modified liquid YPD medium (1% yeast extract, 2% peptone, and 6% glucose; m/v) with 0.5% carboxymethylcellulose (m/v). For enzyme production, cultivation lasted 48 hours with increased agitation and larger headspace, while for ethanol production, it lasted up to 24 hours under more anaerobic conditions. The fermented broth was centrifuged at 10,000 rpm for 10 min to separate the cells. The liquid part consists of the crude fermentation extract. Glucose and ethanol concentrations were determined by high-performance liquid chromatography (Accela™, Thermo Fisher Scientific, Waltham, MA) using a refractive index detector with a HyperREZTM XP (Thermo Fisher Scientific) Organic Acids column at 35 °C, with 0.6 mL/min eluent of 5 mM H_2SO_4 solution at pH 2.6[11].

The synergistic activity of CBHs in cellobiose release was determined via the hydrolysis reaction of ρ-nitrophenyl-β-D-cellobioside (pNPC)[12]. Enzymes produced were concentrated from the crude fermentation extract using Vivaspin centrifugal concentrators (3-kDa molecular size cutoff; Sartorius AG, Göttingen, Germany). The concentrated enzyme extract was incubated with 4 mmol/L pNPC at pH 5.0 (0.1 mol/L sodium acetate buffer) in a ratio of 1:1 (sample:substrate; v/v) at 50 °C for 90 min. The reaction was stopped with 0.5 mol/L sodium carbonate (Na_2CO_3) in a 1:1 ratio (Na_2CO_3:mixture; v/v). The amount of ρ-nitrophenol (pNP) released was determined by measuring absorbance at 410 nm. One unit of activity (U) was defined as the enzyme extract amount required to release 1 nmol of p-nitrophenol per min. This measure was subsequently expressed on a volumetric basis, denoted as units per liter of enzyme extract (U/L).

A central composite rotational design (CCRD) with 11 assays (Table 1) was used to examine the effects of pH and temperature on CBHs activity. The central point was based on the conditions of the activity determination protocol. pH values for the pNPC hydrolysis reaction were adjusted with the 0.1 mol/L citrate-phosphate buffer solution.

Table 1 Independent Variables and Coded Levels in CCRD for CBHs activity.

Independent variables	Levels				
	–1.41	–1	0	+1	+1.41
pH (x_1)	3.6	4	5	6	6.4
Temperature (x_2, °C)	30.9	35	45	55	59.1

The coefficient of determination (R^2) and Fisher's test (ANOVA) were used to verify the statistical adequacy of the models coded to the real points. Protimiza Experimental Design (Brazil) was utilized for experimental design, data analysis, and model building.

3 RESULTS & DISCUSSION

The genetically modified strain exhibited a peak enzyme activity of approximately 860 U/L, a 12-fold increase compared to the parental strain (Figure 1), confirming the success of the genetic modification. This peak occurred on the third day, suggesting adaptability in metabolic resources allocation to produce cellobiohydrolases in response to a possible substrate limitation. However, a significant drop on the fourth day indicated limitations, possibly due to thermal stability issues of CBHs and inhibitory metabolite production. For example, cellobiose can associate with the tryptophan residue located close to the active site of cellobiohydrolase and then form a steric obstacle that prevents the chains of the cellulose molecule from entering the active site of cellulase[13].

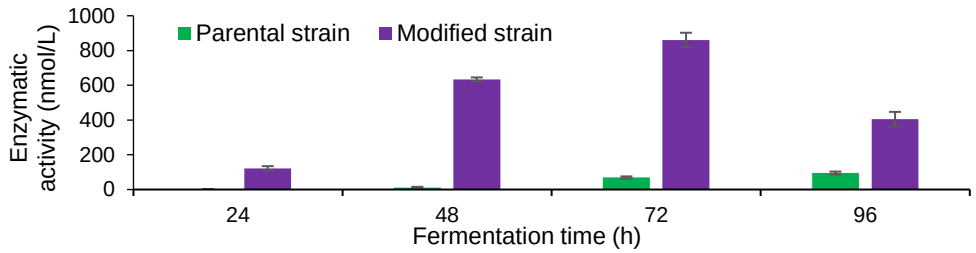

Figure 1 Enzymatic activity of CBHs of concentrated enzyme extract produced by the modified strain compared to its parental counterpart as a function of fermentation time.

A CCRD assessed the effects of pH and temperature on CBHs activity, revealing that only the linear coefficient for temperature (x_2) did not significantly affect CBHs activity (Figure 2A; $p < 0.05$). ANOVA showed the model explained 89.4% of the total variation, indicating appropriateness for a bioprocess. The regression model generated a response surface (Figure 2B), where the region of action around the central point is evident, which underscores that CBHs produced by genetically modified strain preserved the optimal conditions.

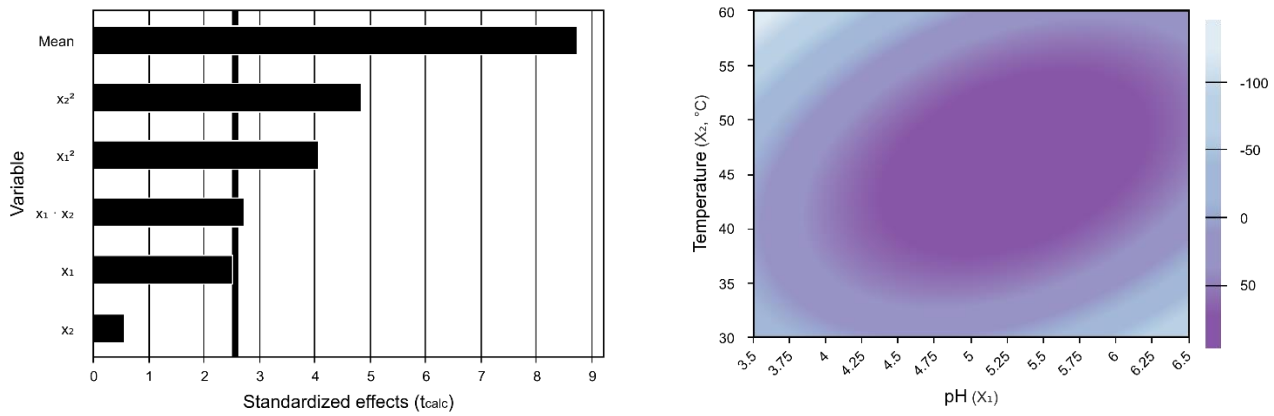

Figure 2 Pareto chart (A) and response surface (B) for the relative activity of heterologous CBHs as a function of pH and temperature (°C).

The proficient growth of the genetically modified strain, capable not only of producing heterologous enzymes but also of sustaining their optimal performance conditions, serves as evidence that the genetic modification process effectively promoted the integration of genes responsible for CBHs expression. This aligns with what was expected from a technique as minimally invasive and precise as CRISPR/Cas9. However, the necessity to concentrate the fermented broth implies that the current enzyme expression levels are relatively modest, highlighting the imperative to optimize cultivation practices for enhanced production stimulation. The limited quantity of enzyme produced is reflected in ethanol production (Figure 3). The modified strain demonstrated a noteworthy initial superiority, producing 9.0 g/L within the first six hours compared to 7.9 g/L of the parental strain. However, both strains converged to similar ethanol yields after 24 h, with the modified strain reaching 0.408 g/g and the parental strain 0.399 g/g.

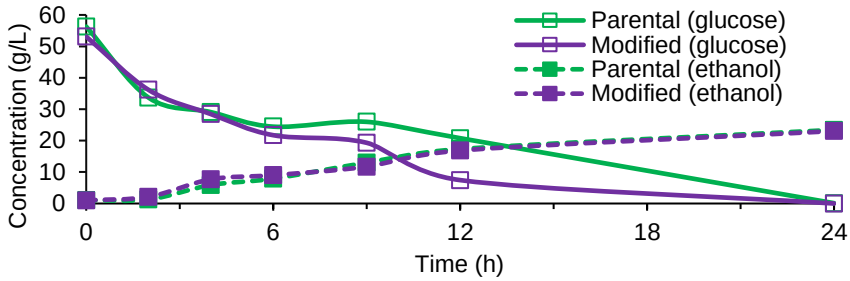

Figure 3 Concentration of ethanol produced by the modified strain and its parental counterpart over time.

Enhancing the performance of the genetically modified strain involves operational adjustments, such as increased aeration and agitation initially, to stimulate enzyme production. In essence, achieving a balance between the essential conditions for enzyme production and activity and ethanol production is fundamental and challenging. One strategy involves a phased approach: first, implementing conditions to stimulate enzyme production; subsequently, implementing conditions to stimulate ethanol production. Alternatively, additional approaches include exploring further metabolic engineering possibilities[14].

Thus, this genetically modified *S. cerevisiae* strain is promising for second-generation ethanol production by direct conversion, requiring further research on other enzymes involved in cellulose degradation. Furthermore, given that this study utilized carboxymethylcellulose, the subsequent phase entails applying this modified strain in processes involving agricultural waste, providing insights into its feasibility and sustainability for large-scale ethanol production from lignocellulosic biomass, aligning with the broader goal of establishing eco-friendly biofuel production methods.

While genetic modification procedures may initially incur expenses associated with research, development, and implementation, the direct conversion of lignocellulosic biomass to ethanol offers promising advantages in terms of cost reduction over traditional methods. By integrating saccharification and fermentation into a single microorganism, significant reductions in process complexity and operational costs can be achieved. Future industrial-scale research and trials should include comprehensive economic evaluations to provide a clearer understanding of the overall cost-effectiveness of this approach.

4 CONCLUSION

The genetically modified strain of *S. cerevisiae* demonstrates potential for second-generation ethanol production from lignocellulosic waste. The expressing heterologous cellobiohydrolases led to an enzymatic activity of 860 U/L while maintaining optimal enzyme conditions around pH 5 and 45 °C. Thus, the modified strain exhibited an initial advantage in ethanol production during the first six hours, reaching 9.0 g/L. However, both strains reached similar yields in 24 hours. Thus, the expression of heterologous cellobiohydrolases by this strain of *S. cerevisiae* is a breakthrough for the direct conversion of cellulose to ethanol, laying the groundwork for future optimizations.

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