Enhancing tolerance of *Heyndrickxia coagulans* through adaptive laboratory evolution for efficient conversion of lignocellulosic municipal forest wastes

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Municipal forest waste (MFW) is a lignocellulosic raw material representing a sustainable and abundant feedstock with significant potential for developing biorefinery systems. Despite their availability, MFWs are predominantly utilized for compost production, generating products with relatively low added value and limited commercial applications. Exploring alternative valorization strategies, such as bioconversion into high-value biochemicals and bio-based materials, could enhance their economic and environmental benefits within a circular bioeconomy framework. The sugars present in MFW can serve as a valuable carbon source for the conversion into polylactic acid (PLA)-based bioplastics, which have promising applications in packaging for the biomedical and food industries. The development and integration of this alternative bioprocess not only promote the efficient utilization of lignocellulosic resources within a sugar platform but also contribute to advancing the comprehensive implementation of a circular bioeconomy model, fostering sustainability and reducing reliance on fossil fuel-based materials.

Fermentation of lignocellulosic sugars is a challenging process, primarily due to recalcitrant effect of the biomass and the inhibitory compounds, such as furans, weak acids, and aromatic compounds, which are generated during the biomass pretreatment phase. This pretreatment process is essential for disrupting the complex structural matrix of lignocellulose, thereby enhancing the accessibility of hydrolytic enzymes to the specific carbohydrates during the subsequent enzymatic hydrolysis step. Addressing these inhibitory effects is crucial for improving fermentation efficiency and overall yields in converting lignocellulosic biomass to valuable bioproducts.

To optimize the fermentation process and minimize associated costs, it is essential to implement a high-gravity simultaneous saccharification and fermentation (SSF) strategy, where the solid and liquid fraction of the raw material can be hydrolysate and ferment in same bioreactor. This approach requires the employment of robust and thermotolerant microorganisms capable of efficiently converting lignocellulosic substrates into desired products under challenging conditions. By combining these methodologies, it is possible to enhance process efficiency and economic viability, facilitating the sustainable production of biofuels and bio-products from lignocellulosic biomass.

The thermotolerant bacteria *Heyndricksia coagulans* is a promising candidate for lactic acid production from lignocellulosic hydrolysates. This bacteria is capable of growing at temperatures between 45-55 °C, being able to ferment different carbon sources such as xylose and glucose, and is recognized as a GRAS (generally regarded as safe) microorganism. This bacteria produces lactic acid from fermenting sugars, which can be subsequently used for PLA production. However, the presence of lignocellulosic-derived inhibitors alters the fermentation performance, and usually, a detoxification step is required, what increases the process costs.

In this work, the lactic acid bacteria *H. coagulans* DSM 2314 strain was submitted to an adaptative laboratory evolution (ALE) strategy to increase its robustness to lignocellulosic-derived inhibitors. The ALE consists of two phases. The first phase was performed in batch culture, where the concentration of the liquid fraction obtained after steam explosion pretreatment of MFW was added to the media from 50% to 75% v/v concentration. In total, 25 passages were performed in flasks, increasing hydrolysate concentration by 5% (v/v) every 5 passages. When 75% (v/v) concentration was reached, the bacteria was not able to grow and a fed-batch strategy with continous hydrolysate feeding was carried out using a 250-mL bioreactor. This second phase started using 70% (v/v) of the liquid fraction of pretreated MFW. This hydrolysate concentration was gradually increased until no dilution. After 43 days of continuous fermentation, the bacteria were capable of completely consuming both glucose and xylose and producing lactic acid in the non-diluted liquid fraction with conversion yields higher than 90% of the theoretical.

Several clones were isolated from the evolved population using plates with 50-75% (v/v) hydrolasate concentration. Isolated clones were subjected to a spot test with serial dilution in plates of MRS with 50 and 75% prehydrolysate. The clones that presented improved cell growth in terms of best growth with minimun dilution were inoculated in MRS with 50% v/v of a lignocellulosic derived inhibitors cocktail (Table 1).

The clones A1, A2, C6 and C10 which presented a better glucose consumption and lactic acid production, were selected for further ferementation tests to select the best clone. From this test, clone A2 was selected and further subjected to genome sequencing. This genomic analysis will allow the identification of potential gene mutations or genomic modifications responsible for the improvement of fermentation capacity of this bacteria in

presence of lignocellulosic-derived sugars, thus contributing to the understanding of the mechanisms involved in cell tolerance under such challenging conditions.

This work will set the basis for the establishment of a robust *H. coagulans* strain to produce lactic acid from MFW in the presence of lignocellulosic-derived inhibitors, and the understanding of molecular routes and genome targets that are implied in cell robustness of lignocellulosic feedstock fermentation.

Table 1. Candidate's evaluation

Candidate	Glucose Consumption (%)	Lactic acid Production (g/L)	Fermentation yield (g/g)
Control	19.7	1.2	0.40
A1	42.5	6.1	0.95
A2	41.0	5.6	0.95
C1	17.5	0.9	0.35
C2	38.9	5.1	0.88
C4	39.0	5.5	0.93
C6	49.2	5.9	0.80
C10	41.6	5.9	0.94
C11	37.3	5.7	0.93
C12	18.2	0.5	0.19
C13	32.7	4.2	0.86

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Fermentation was performed in batches. Samples were measured at the end of the fermentation (t = 48h) when the acidification of the media stopped the cell growth.

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