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Dissertation Abstract

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Dissertation Title (If written in a foreign language, provide a Japanese translation as well.)

Metabolic Engineering of Yeast for Production of Lactic Acid and Lactate-containing Polymer

（酵母の代謝工学による乳酸および乳酸含有ポリマーの生産）

Academic Supervisor: Chiaki Ogino

The discourse surrounding the sustainability of plastics has emerged as a crucial challenge in contemporary times. Given the deep integration of plastics in daily life, the prospect of their complete elimination appears highly impractical. The recent transformation towards a sustainable plastic industry is marked by a shift focusing on biodegradability, the use of non-fossil-based resources, and the adoption of manufacturing processes with reduced carbon footprint. These three pivotal aspects are the focus of this research. In this context, microbial synthesis of polymers represents an ideal model for manufacturing biodegradable plastics from renewable sources in the cleanest possible way. Unlike polyhydroxyalkanoates (PHAs), which can be synthesized entirely through fermentation, polylactic acid (PLA) is typically produced industrially by combining fermentation (for monomer production) and metal-based synthesis (for polymerization). Recent studies have begun to explore the possibility of producing PLA entirely through fermentation, employing metabolic engineering of microbial hosts. However, these studies predominantly use bacterial hosts, despite eukaryotic systems offering a more sophisticated cellular architecture suitable for polymer bioproduction. This study, therefore, aims to utilize budding yeasts as microbial factories for producing lactic acid and lactate-based polymers.

Firstly, the study pursued a method to produce lactic acid, the primary building block of PLA, from renewable substrates via a simplified upstream process. This process eliminates the need for neutralizing treatments during fermentation, leading to more efficient product recovery and waste treatment. A newly isolated yeast strain, *Saccharomyces cerevisiae* BTCC3, was harnessed as a microbial host due to its robust ability to tolerate a mixture of byproducts frequently generated from lignocellulose pretreatment compared to commonly studied laboratory and industrial yeast strains, namely *S. cerevisiae* BY4741 and Ethanol-red, respectively. This prospective budding yeast strain was metabolically engineered by introducing an exogenous L-lactate dehydrogenase gene (L-*LDH*) from *Lactobacillus casei* and disrupting pyruvate decarboxylase isozymes 1 and 5 (*PDC1* and *PDC5*). The engineered strain, named BTCC3LA2, demonstrated the capability to produce lactic acid with a productivity of 3.68 $g \cdot L^{-1} \cdot h^{-1}$ under neutralizer-free conditions—only a 23% reduction compared to productivity in a semi-neutralized cultivation—whereas other studies reported a more than 50% reduction under the former settings. Additionally, catalyzed by the BTCC3LA2 strain, a hydrolysate derived from sugarcane bagasse could be converted to lactic acid at a productivity of 1.69 $g \cdot L^{-1} \cdot h^{-1}$ without prior detoxification, neutralizer addition, or pH control during fermentation. This contrasts with other studies valorizing similar biomass, which required such treatments for achieving comparable productivity levels. Therefore, using this recombinant strain under similar cultivation conditions could eliminate the need for pre-detoxification and post-acidification, significantly reducing the economic and environmental impacts of lactic acid production.

Secondly, the study explored the benefits of using a flocculant yeast strain, *S. cerevisiae* F118, for lactic acid fermentation. Its flocculation ability facilitates instant cell separation from the fermentation broth, thereby simplifying subsequent filtration, cell recycling, and product recovery. This feature in yeast has indeed been well-studied for industrial ethanol production, yet its potential application for lactic acid fermentation remains largely unexplored. Comparative metabolic profiles and transcriptome landscapes between F118L and BTCC3L—flocculating and nonflocculating strains incorporating an exogenous L-*LDH* from *L. casei* in their locus of L-lactate cytochrome-c oxidoreductase (*CYB*2) gene—revealed that the flocculation trait in the former yeast resulted in higher and more stable metabolic flows, particularly in glycolysis and pyruvate metabolism, even under varying cell densities and chemical stresses, suggesting superior performance of the flocculating strain over the non-flocculating counterpart for industrial lactic acid fermentation. Additionally, the flocculating trait appears to be linked with distinct sequences in flocculin (*FLO*) genes, namely *FLO1*, *FLO5* and *FLO9*, and mutations in the cyclin 8 (*CYC8*) and transcriptional underproduction 1 (*TUP1*) genes as the sequence of those key genes in the F118 strain were relatively distinct compared to the nonflocculating BTCC3 and S228C (reference) strains. These findings highlight the potential of flocculating yeasts in robust lactic acid production, representing an underexplored yet promising strategy for sustainable industrial applications.

Thirdly, the study proposed a one-step process to produce lactate-containing polymer, wherein the entire reaction occurs intracellularly in yeast at near-room temperature without using organic solvents. This fully fermented approach enables bypassing the conventional PLA production that still requires multi-stage monomer purification and metal-catalyzed polymerization. The employment of yeast offers several advantages over bacterial systems, including its larger cell size, the capacity for compartmentalization, distinct protein folding mechanisms, and enhanced tolerance to various stressors. The metabolic engineering technique in the studied strain, BTCC3LA2, involved introducing exogenous L-*LDH* from *L. casei*, propionate CoA-transferase (*PCT*) from *Firmicutes bacterium* and mutated polyhydroxyalkanoate synthase 1 (*PHAC1*) from *Pseudomona*s sp. 61-3 (S325T/Q481K) into its locus of the *CYB2* gene, which endowed the engineered yeast strain, named BTCC3LA2FbPLA, with the ability to synthesize a lactate-containing copolymer with higher molecular weight in comparison to other similar works. The hydrolysis reaction confirmed the incorporation of the L-enantiomer of lactate—the first achievement in this area of research—as well as 3-hydroxyburate in the structure of biopolymer obtained. Indeed, given the resilience of the BTCC3 strain against a range of byproducts associated with pretreatment processes, the microbial synthesis of L-lactate-based polymers utilizing various lignocellulosic feedstocks is feasible with this

genetically modified strain. These results are significant for advancing the effort to synthesize biopolymers using a greener technique, that is, by employing microorganisms as biocatalysts, hence diminishing the energy consumption and environmental impact of the production process.

In summary, the metabolic engineering strategies employed in this work, which includes the integration of L-*LDH*, *PCT*, and mutated *PHAC1* genes, along with the disruption of *PDC* genes, presented a feasible approach for manufacturing bio-based lactic acid and biodegradable lactate-containing polymers. This method utilizes renewable resources while aiming to minimize the carbon footprint of the overall production stages. In particular, the robust *S. cerevisiae* platforms could assist in developing optimized and streamlined upstream and downstream processes, enhancing overall efficiency compared to conventional production strategies. Undoubtedly, further enhancements are necessary to augment and optimize the production capabilities of the genetically engineered strains. Having said that, the insights derived from this study are crucial in bridging the existing knowledge gap within this nascent and emerging area of research. Finally, this work, in concert with preceding studies, paves the way to a transition towards a sustainable plastic industry, contributing to the global endeavor for environmental stewardship and a more sustainable future.