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Genetic engineering to improve 1,3-propanediol production in an isolated *Citrobacter freundii* strain

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ABSTRACT

In this study, the isolated strain, *Citrobacter freundii* AD970, was genetically modified to improve production of 1,3-propanediol (1,3-PD). The strain was modified through overexpression of 1,3-PD oxidoreductase (PDOR from *Shimwellia blattae*), catalyzing the final step of glycerol-to-1,3-PD conversion. The aim of such approach was to decrease accumulation of toxic 3-hydroxypropionaldehyde (3-HPA) and to increase 1,3-PD synthesis. To this end, a genetic construct suitable for the modification of the strain was developed. Our results showed that PDOR was successfully overexpressed in the novel expression system. Flask cultivations in a glycerol-based medium demonstrated that the modified strain produced nearly two-fold higher concentrations of 1,3-PD (8.6 vs. 4.75 g/L). During bioreactor fed-batch cultivations in a glycerol-based medium, accumulation of 3-HPA was successfully decreased, leading to enhanced 1,3-PD synthesis (35.6 vs. 25.5 g/L), at higher yield (0.49 vs. 0.44 mol/mol at 48 h) and productivity (12.41 vs. 8.06 $g_{1,3\text{-PD}}/g_{DCW}$ and 0.32 vs. 0.23 $g_{1,3\text{-PD}}/(L^*h)$). Considering the final volume of the fed-batch cultures, the V+I strain synthesized 20 g more of 1,3-PD (49.9 g vs. 29.9 g). To the best of our knowledge, this is the first report on genetic engineering of a wild-type *Citrobacter* strain to modulate the 1,3-PD synthesis pathway.

1. Introduction

1,3-Propanediol (1,3-PD) is a platform chemical with numerous industrial applications, as reported in a number of papers [1–4]. A considerable international effort has been devoted to developing an economically feasible process of 1,3-PD production from residual glycerol, a by-product of biodiesel production plants. These efforts have involved all aspects of biotechnology, from successful cultivation approaches [5–9], inventive genome shuffling [10], and directed evolution of proteins [11,12] to sophisticated genetic engineering strategies (thoroughly reviewed in Ref. [13]). Recently, several interesting and novel directions in the biotechnological production of 1,3-PD have been demonstrated, such as competitive production of 1,3-PD by *Lactobacillus diolivorans* [14,15], which allows high-titer 1,3-PD production without compromising the process safety issues, or efficient production of 1,3-PD under non-sterile conditions [8,16,17], which greatly reduces the investment costs. Similarly, studies on utilization of various types of crude glycerol in microbial fermentations are of particular importance in the pursuit of an economically feasible process for biotechnological 1,3-PD production [18–22].

For many years, 1,3-PD production has relied primarily on two genera of microorganisms: *Clostridium* spp. [23] and *Klebsiella* spp. [24], along with genetically engineered *Escherichia coli*, which is operating in an industrial process [25]. Several recent papers have demonstrated that *Citrobacter* species are worth considering as 1,3-PD producers [8,18,26,27]. In particular, an outstanding result obtained using the *Citrobacter freundii* FMCC-B 294 (VK-19) strain (66 g/L of 1,3-PD at high yield and productivity from raw glycerol and under non-sterile conditions, [8]) places this species among the major players in microbial 1,3-PD production.

1,3-PD is an end product of a reductive branch of a glycerol fermentation pathway [Fig. 1]. 3-HPA (3-hydroxypropionaldehyde) is a direct intermediate of 1,3-PD synthesis and is known to have antimicrobial properties, exerting its toxic effect toward 1,3-PD producers [28]. It has been reported in many papers that 3-HPA accumulation triggers irreversible cessation of cellular growth and metabolic activity of the 1,3-PD producers, especially the enterobacterial strains [29], although the exact mechanism of its action is not clear [30,31]. It was shown by mathematical modeling [32] and experimentally confirmed [33] that in *Klebsiella pneumoniae*,

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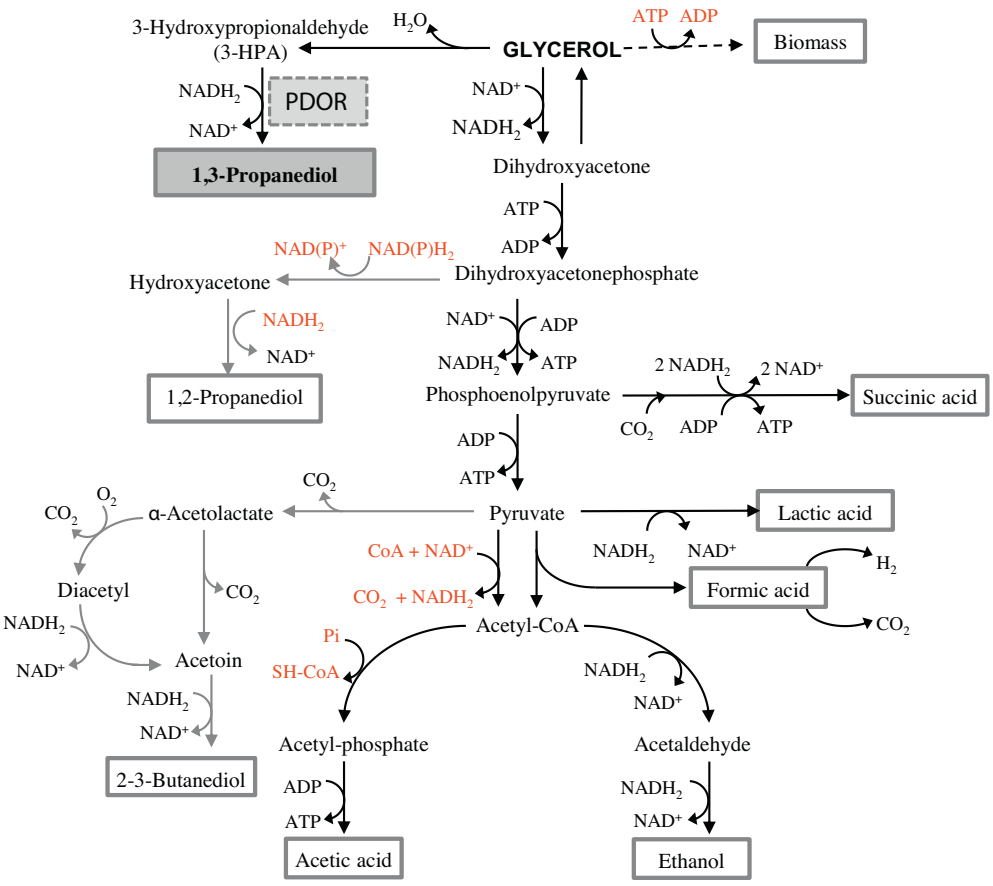

Fig. 1. The glycerol catabolism pathways in enterobacterial 1,3-PD producers (modified, according to Ref. [46]). PDOR (dashed frame) is an enzymatic activity of 1,3-propanediol oxidoreductase, cloned and expressed in this study. Gray lines: pathways not active in *Citrobacter freundii*. Dashed line: multi-step anabolic pathways leading to biomass production.

3-HPA exerts an inhibitory effect on the catalytic activities of the two enzymes directly involved in 1,3-PD synthesis, glycerol dehydratase GDHt (in which 3-HPA acts as an inhibitory product) and 1,3-PD oxidoreductase PDOR (in which 3-HPA acts as an inhibitory substrate). 3-HPA accumulation is driven by a positive feedback mechanism with respect to PDOR activity, as an increase in the 3-HPA concentration triggers a decrease in PDOR activity [29], which results in further increases in 3-HPA. As previously described [34], the reason for 3-HPA accumulation is an unbalanced ratio of GDHt and PDOR activities. Hence, it can be assumed that a reduction in 3-HPA accumulation could be achieved by either reduction of its synthesis (reduction of GDHt activity) or enhancement of its consumption (induction of higher PDOR activity). However, as noted by Chen et al. [35], the former mechanism would cause the reduction of the overall production of 1,3-PD, and therefore, the latter strategy is preferable. Earlier studies suggested that GDHt is the rate-limiting enzyme [36] in this pathway. However, the omnipresent problem of 3-HPA accumulation contradicts the idea that GDHt is the rate-limiting step catalyst [32]. Taking into consideration the strong inhibitory effect of 3-HPA toward PDOR, it was concluded that PDOR, and not GDHt, may be the rate-limiting enzyme in 1,3-PD production under conditions of excessive glycerol feeding. Indeed, enhancing PDOR activity by overexpression of an additional copy of the *dhaT* gene or the homologous *yqhD* gene was shown to improve 1,3-PD yield from glycerol [37–40] (discussed hereafter).

In this paper, we report the genetic engineering of a wild-type environmental isolate strain, *C. freundii* AD970, to reduce 3-HPA accumulation and improve 1,3-PD synthesis. Our approach relied on overexpression of PDOR from the other enterobacterial 1,3-PD producer, *Shimwellia blattae*, expressed under the control of a Lac promoter. The *dhaT* gene from *S. blattae* was chosen to supply the DNA sequence coding for additional PDOR activity. *S. blattae* is another 1,3-PD producer belonging to *Enterobacteriaceae* family. Our recent findings demonstrate that this organism might be another interesting species for biotechnological 1,3-PD production. However, its potential for this purpose has not been thoroughly explored to date. According to the BRENDA database, the PDOR enzyme is a broad-range oxidoreductase with specificity toward small molecular weight aldehydes and alcohols. The nucleotide sequence comparison, using the BLASTN tool, showed on average 83% sequence similarity to the *dhaT* genes from *K. pneumoniae* and *Citrobacter* spp. On the amino acid sequence level, PDOR from *S. blattae* is highly similar (90–95%) to the enzymes from *Citrobacter* species and *Klebsiella oxytoca* (less than 90% similarity with *K. pneumoniae*). Close phylogenetic proximity of *Citrobacter* and *Shimwellia*, highly similar amino acid sequences of the PDORs, and high, but still not thoroughly explored, potential of *Shimwellia* to efficiently produce 1,3-PD were the rationale behind selection of this *dhaT* gene. We assumed that since PDOR serves as a safeguard to maintain 3-HPA concentration at low level, its overexpression would provide two benefits: reduced 3-HPA accumulation and increased 1,3-PD synthesis. The obtained strain was characterized with respect to its performance in flask and bioreactor cultivations, and showed superior properties over the parental strain.