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Regulation of ρ-coumaric acid tolerance in *Clostridium beijerinckii* by disturbing the intracellular electron transport chain

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ABSTRACT

ρ-coumaric acid with strong antibacterial activity is produced during the pretreatment and hydrolysis of lignocellulosic biomass to monomeric sugars. The cell growth and metabolism of *C. beijerinckii* NCIMB 8052 is significantly inhibited by ρ-coumaric acid. In this study, adaptation of *C. beijerinckii* NCIMB 8052 to ρ-coumaric acid was remarkably enhanced after disruption of the intracellular electron transport chain, and the antibacterial activity assay showed a 2-fold increase in cell viability in the presence of 0.5 g/L ρ-coumaric acid 24 h after inoculation. Additionally, electricity generation in the wild-type and recombinant strains was measured using microbial fuel cell devices, and the intracellular levels of co-factor NAD(P)H were determined by the enzyme cycling method to show disturbed intracellular electron transfer after disruption of gene Cbei_2996. Furthermore, the metabolism of ρ-coumaric acid by *C. beijerinckii* was analyzed. These data indicated that gene Cbei_2996 plays a significant role in regulating ρ-coumaric acid tolerance in *C. beijerinckii*; the metabolism of ρ-coumaric acid mainly coupled with co-factor NADH is catalyzed by reductase. The strategy used in this study provides a potential approach for producing dominant microorganisms with high inhibitor tolerance for butanol production using renewable lignocellulosic materials.

1. Introduction

Since butanol is an important chemical and solvent with wide industrial applications and a potential substitute for gasoline, Acetone-Butanol-Ethanol (ABE) fermentation by *Clostridia* has been widely studied. The yield of total solvents in traditional ABE fermentation using glucose is 0.35–0.43 g/g (butanol: acetone: ethanol = 6:3:1), and the yield of butanol is 0.21–0.26 g/g. Thus, consumption of 3.85–4.76 tons of glucose would be required to produce one ton of butanol by ABE fermentation. Based on this calculation, the cost of producing one ton of butanol by ABE fermentation in China using glucose ($319.6/ton, industrial-grade), corn ($79.9–$111.86/ton, in China), and straw ($31.96–$47.94/ton, hydrolysis rate obtained on using diluted solution of sulfuric acid was calculated as 30–40% of glucose and xylose) would be over $1054.68, $958.8, and $474.61, respectively. For cost-efficiency, the use of renewable lignocellulosic materials, such as wheat straw, corn fiber, and bagasse fiber, as substrates has been significantly investigated. In the last five years, data from the National Bureau of Statistics, China, showed that the annual Output of Grain Crops was almost 60 million tons, where corn and rice production accounted for around half of the total grain output (Fig. 1), and the amount of crop straw produced was up to one billion tones. Regrettably, 40%–50% of crop straw is incinerated, especially in villages and towns, which results in significant resource wastage. Bio-ethanol production has been widely studied using biomass feedstocks or lignocellulosic materials; efficient conversion of crop straw to ethanol would result in an annual output of $79.9 billion in China. With respect to butanol production, the output would be greater [1]. However, several lignocellulose-derived microbial inhibitory compounds (LDMICs) are generated along with sugars during pretreatment [2]. LDMICs, especially, phenolic compounds, significantly inhibit growth and solvent production by solventogenic *Clostridium* species, even at low concentrations [3].

The antimicrobial activity of phenolic compounds is determined by their chemical structure. The six classic lignocellulosic material-derived phenolic acid compounds are classified into three types: I. cinnamic acid (ferulic acid and ρ-coumaric acid), II. benzoic acid (vanillic acid

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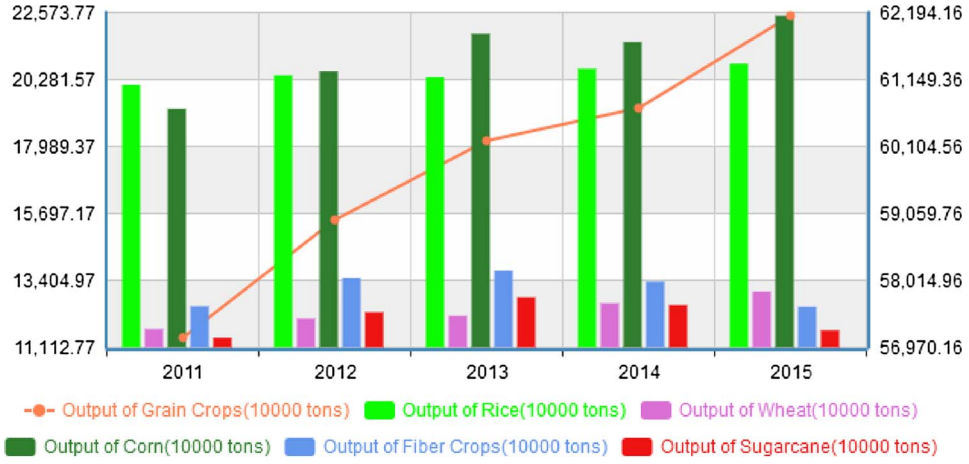

Fig. 1. Output of major farm products in china from 2011 to 2015.

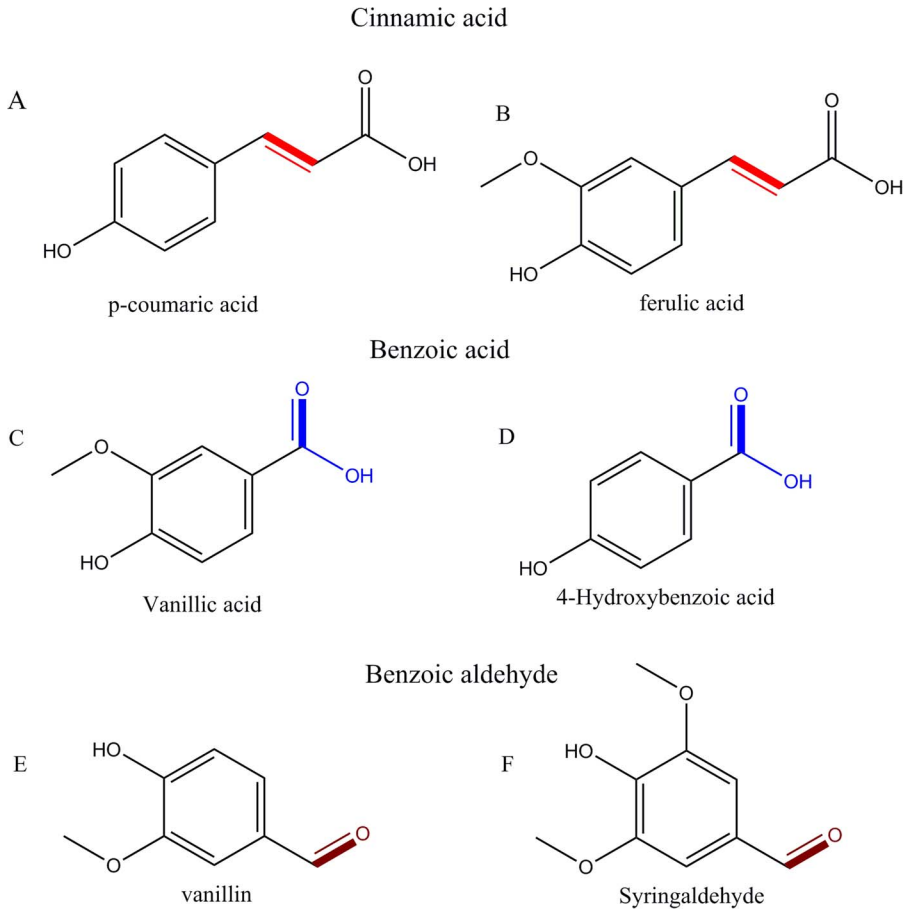

Fig. 2. Chemical structure of the six kinds of model phenolic compounds. The six classic lignocellulosic material phenolic acid compounds were divided into cinnamic acid (ferulic acid and ρ-coumaric acid), benzoic acid (Vanillic acid and 4-Hydroxybenzoic acid), and benzoic aldehyde (vanillin and Syringaldehyde).

and 4-hydroxybenzoic acid), and III. benzoic aldehyde (vanillin and syringaldehyde) (Fig. 2). One of these compounds, ρ-coumaric acid (Fig. 2A; ρCA), comprises a benzene ring, hydroxyl groups, and a double carbon bond in the side chain. We previously reported that 0.5 g/L phenolic compounds, including ρCA, remarkably reduced *C. beijerinckii* NCIMB 8052 cell growth by more than 76% with little butanol production (less than 1.1 g/L) [4]. A mutant strain, *C. beijerinckii* M11 with high ferulic acid tolerance, produced 7.24 g/L butanol in the presence of 0.5 g/L ferulic acid, but little or no butanol in the presence of 0.5 g/L ρCA [5]. Besides the absence of methoxy group on the benzene ring, the chemical structure of ρ-coumaric acid is same as that of ferulic acid (Fig. 2A and B), which indicates that its antimicrobial activity against *C. beijerinckii* could be reduced by targeting the methoxy group. Additionally, Cho et al. found that ρCA was the most toxic among the six model phenolic compounds, inhibiting cell growth by 74% at a concentration of 1 g/L [6]. These data suggest that ρCA significantly inhibits cell growth and butanol production in *C. beijerinckii*, but the exact mechanism underlying ρCA toxicity and tolerance is still not known.

Energy and reducing power (NADH/NADPH) significantly influence reduction of furfural and 5-hydroxymethyl-furfural (HMF) toxicity in bacterial strains [7–9]. The reduction of furfural and HMF to alcohol is dependent on NADH and NADPH, respectively. In addition, our