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Phenyllactic acid production by simultaneous saccharification and fermentation of pretreated sorghum bagasse

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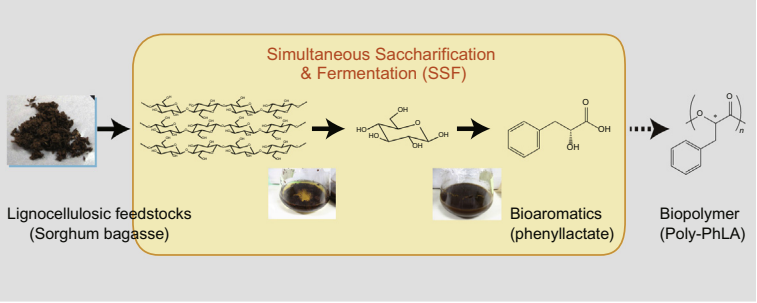
HIGHLIGHTS

- Phenyllactate was produced from dilute acid-pretreated sorghum bagasse by SSF.
- Sorghum bagasse yielded fermentation inhibitors of aldehyde and phenolic acid.
- Sorghum bagasse altered metabolic profiles during phenyllactate fermentation.
- SSF yielded 4.8-fold more phenyllactate than separate hydrolysis and fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

Dilute acid-pretreated sorghum bagasse, which was predominantly composed of glucan (59%) and xylose (7.2%), was used as a lignocellulosic feedstock for D-phenyllactic acid (PhLA) production by a recombinant *Escherichia coli* strain expressing phenylpyruvate reductase from *Wickerhamia fluorescens*. During fermentation with enzymatic hydrolysate of sorghum bagasse as a carbon source, the PhLA yield was reduced by 35% compared to filter paper hydrolysate, and metabolomics analysis revealed that NAD(P)H regeneration and intracellular levels of erythrose-4-phosphate and phosphoenolpyruvate for PhLA biosynthesis markedly reduced. Compared to separate hydrolysis and fermentation (SHF) with sorghum bagasse hydrolysate, simultaneous saccharification and fermentation (SSF) of sorghum bagasse under glucose limitation conditions yielded 4.8-fold more PhLA with less accumulation of eluted components, including

Abbreviations: AcCoA, acetyl-CoA; Aco, aconitate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AKG, 2-oxoglutarate; ATP, adenosine triphosphate; Cit, citrate; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; EH, enzymatic hydrolysis; F6P, fructose-6-phosphate; FBP, frucotose-1,6-bisphosphate; FPU, filter paper unit; Fum, fumarate; G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; GC–MS, gas chromatography–mass spectrometry; 4-HBA, 4-hydroxybenzaldehyde; 5-HMF, 5-hydroxymethylfurfural; HPLC, high-performance liquid chromatography; LC–MC/MS, liquid chromatography–tandem quadrupole mass spectrometry; Mal, malate; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; OXA, oxaloacetate; PDH, pyruvate dehydrogenase; 6PG, 6-phosphoglycerate; PEP, phosphoenolpyruvate; PGA, 2- or 3-phosphoglycerate; Phe, phenylalanine; PhLA, phenyllactic acid; PPP, pentose phosphate pathway; Pyr, pyruvate; R5P, ribose-5-phosphate; Rib5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; SHF, separated hydrolysis and fermentation; Shk, shikimate; SSF, simultaneous saccharification and fermentation; Suc, succinate; TCA, tricarboxylic acid; Trp, tryptophan; Tyr, tyrosine; X5P, xylulose-5-phosphate.

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Fermentation inhibitor

p-coumaric acid and aldehydes, which inhibited PhLA fermentation. These results suggest that gradual enzymatic hydrolysis during SSF enhances PhLA production under glucose limitation and reduces the accumulation of fermentation inhibitors, collectively leading to increased PhLA yield.

1. Introduction

Lignocellulosic biomass is an abundant, domestic and renewable feedstock that can be converted into biofuels and other chemicals by fermentation. Sorghum (*Sorghum bicolor* L. Moench) is a drought-tolerant C4 grass that has high photosynthetic efficiency and is widely cultivated in warm climates (Rooney et al., 2007). Compared to other energy crops, such as sugar cane, sorghum requires less fertilizer and water, and has similar annual productivity per acre (Kim and Day, 2011). In addition, molecular genetic engineering techniques for the breeding of sorghum have also been established (Ordonio et al., 2014). For these reasons, the utilization of sorghum for biofuel production has been intensively investigated (Matsakas and Christakopoulos, 2013; Wang et al., 2013). However, few studies have examined the utilization of sorghum biomass for the production of valuable chemicals.

Sorghum biomass typically contains a mixture of structural (53–59%) and non-structural (27–36%) carbohydrates (Rooney et al., 2007), and its bagasse contains a large proportion of cellulose (>40%), with smaller amounts of hemicellulose (27%) and lignin (21%) (Kim and Day, 2011). Thus, sorghum bagasse, which is the fibrous plant material remaining after juice extraction, can also be used as feedstock for biofuel production, as the cellulose in bagasse fibers can be converted into fermentable sugars. Among hybrid forage sorghum cultivars, Tentaka (F1 hybrid) is the top yielding cultivar per unit of land (40.3 t ha^{-1} y^{-1}) (Venuto and Kindiger, 2008). Therefore, the bagasse of Tentaka may be a suitable lignocellulosic feedstock for biofuel and biochemical production.

3-Phenyllactic acid (PhLA) is a promising building block for bio-based materials, as PhLA can be polymerized into the biopolymer poly-PhLA (Fujita et al., 2013). Unlike polylactic acid, poly-PhLA exhibits high ultraviolet-absorbing properties due to the bulky aromatic side chain (Fujita et al., 2013). In lactic acid bacteria, PhLA is naturally produced from glucose by the non-specific reduction of phenylpyruvate (Mu et al., 2009). A recombinant *Escherichia coli* strain expressing the *Wickerhamia fluorescens pprA* gene, which encodes phenylpyruvate reductase, was also able to produce optically pure D-PhLA from glucose (Fujita et al., 2013). This finding suggests that D-PhLA can be produced from the enzymatic hydrolysate of cellulosic feedstocks.

Recently, D-PhLA was produced by a recombinant *E. coli* strain from cellulosic feedstocks of kraft pulp by simultaneous saccharification and fermentation (SSF) (Kawaguchi et al., 2014); however, reduced PhLA yields were obtained when kraft pulp hydrolysate was used as a feedstock. Pretreated lignocellulosic feedstocks such as kraft pulp typically contain a variety of organic compounds, such as acetate and furfural, that can inhibit fermentation (Larsson et al., 1999). Although we previously reported the metabolic profiles of ethanologenic *Saccharomyces cerevisiae* in the presence of acetate, showing the reduced flux of the non-oxidative pentose phosphate pathway (PPP) (Hasunuma et al., 2011), metabolomic analysis to investigate synergistically inhibitory effects of the enzymatic hydrolysis of pretreated lignocellulosic biomass on microbial fermentation including PhLA production is still limited. Therefore, a better understanding of the underlying inhibitory mechanisms may aid in the development of an effective bioprocess for PhLA production from pretreated biomass.

In the present study, we examined PhLA production from a lignocellulosic feedstock consisting of the washed fiber fraction of dilute acid-treated sorghum bagasse using SSF. A PhLA-producing *E. coli* strain was used for the direct bioconversion of sorghum bagasse by SSF and was also cultivated on filter paper treated with commercial cellulase. The SSF yield of PhLA from the lignocellulosic feedstocks was compared to that obtained by separate saccharification and fermentation (SHF). In addition, the possible reasons for enhanced PhLA yield by SSF compared to SHF were also examined by characterizing potential fermentation inhibitors released from sorghum bagasse during enzymatic hydrolysis, evaluating the effects of the hydrolysate on the metabolic profile, particularly for the central carbohydrate metabolic and related amino acid biosynthesis pathways, of *E. coli* cells during PhLA production, and investigating the effects of potential fermentation inhibitors on PhLA fermentation from glucose.

2. Methods

2.1. Materials, bacterial strains and media

PhLA-producing *E. coli* strain GK1 (Kawaguchi et al., 2014), which expresses the phenylpyruvate reductase gene (*pprA*) derived from *W. fluorescens* TK1 (Fujii et al., 2011), was aerobically cultured at 37 °C in Luria–Bertani medium (Sambrook and Russell, 2001) containing 20 μg mL^{-1} kanamycin.

The hybrid sorghum cultivar Tentaka (Venuto and Kindiger, 2008) was grown in 2013 at an experimental field in Okinawa, Japan. Whole plants at the heading stage were harvested and fully dried in a greenhouse. After removal of the panicles, culms were ground into a fine powder using a blender (WB-1; TGK, Hachioji, Japan) fitted with a 2-mm screen. Dilute acid-pretreated sorghum bagasse was prepared by suspending culm powder (6 g) in 80 mL of 1% (v/v) sulfuric acid and incubating the resulting mixture at 180 °C for 45 min with agitation at 200 rpm (Teramura et al., 2013). After the acid pretreatment, acid-insoluble residue was separated from the hydrolysate by membrane filtration. The obtained fiber residue, which was designated as dilute-acid pretreated sorghum bagasse, was washed twice with distilled water to achieve neutral pH. Prior to use as substrate for both enzymatic hydrolysis and fermentation, the dilute acid-pretreated sorghum bagasse was dried at 80 °C for more than 12 h, and the dry weight of the sample was measured.

A control cellulosic feedstock was also prepared by autoclaving Whatman Qualitative Grade 1 Filter Paper (GE Healthcare Life Sciences, Little Chalfont, UK), which was then dried using the same conditions as described above. A commercially available cellulase cocktail, Cellic CTec2 (Novozymes, Bagsværd, Denmark), was used for both enzymatic hydrolysis and SSF for PhLA production. The activity of CTec2 cellulase was estimated to be 106 filter paper units (FPU) mL^{-1} using a standard method (Ghose, 1987). One FPU was defined as the amount of enzyme that released 1 μmol of glucose equivalents from Whatman Qualitative Grade 1 Filter Paper per minute. Both enzymatic hydrolysis and microbial