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3-Amino-4-hydroxybenzoic acid production from sweet sorghum juice by recombinant *Corynebacterium glutamicum*

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HIGHLIGHTS

- The stalk juice of sweet sorghum contained fermentable sugars and amino acids.
- Recombinant *Corynebacterium glutamicum* produced 3,4-AHBA from sorghum juice.
- Components of sweet sorghum juice were fractionated by membrane separation.
- Amino acids of Leu or Cys in sweet sorghum juice enhanced 3,4-AHBA production.

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ABSTRACT

The production of the bioplastic precursor 3-amino-4-hydroxybenzoic acid (3,4-AHBA) from sweet sorghum juice, which contains amino acids and the fermentable sugars sucrose, glucose and fructose, was assessed to address the limitations of producing bio-based chemicals from renewable feedstocks. Recombinant *Corynebacterium glutamicum* strain KT01 expressing *griH* and *griI* derived from *Streptomyces griseus* produced 3,4-AHBA from the sweet sorghum juice of cultivar SIL-05 at a final concentration ($1.0\ g\ l^{-1}$) that was 5-fold higher than that from pure sucrose. Fractionation of sweet sorghum juice by nanofiltration (NF) membrane separation (molecular weight cut-off 150) revealed that the NF-concentrated fraction, which contained the highest concentrations of amino acids, increased 3,4-AHBA production, whereas the NF-filtrated fraction inhibited 3,4-AHBA biosynthesis. Amino acid supplementation experiments revealed that leucine specifically enhanced 3,4-AHBA production by strain KT01. Taken together, these results suggest that sweet sorghum juice is a potentially suitable feedstock for 3,4-AHBA production by recombinant *C. glutamicum*.

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a C_4 grass with high biomass yield. Compared to other biomass crops, such as corn and sugar cane, sorghum is drought-tolerant, requires less fertilizer, and has a large geographical distribution (Rooney et al., 2007). Juice extracted from the stalks of sweet sorghum predominantly consists of the fermentable sugar sucrose (>$150\ g\ l^{-1}$), but also contains variable concentrations of the reducing sugars glucose and fructose (Kawahigashi et al., 2013). The high content of glucose and fructose in sorghum juice as compared to that found in sugar cane extract inhibits crystallization, which limits the processing of sugar for use in foods. However, sweet sorghum juice is a potentially suitable feedstock for bioethanol production (Laopaiboon et al., 2009; Ratnavathi et al., 2010), although its

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Abbreviations: 3,4-AHBA, 3-amino-4-hydroxybenzoic acid; GABA, γ-amino butyric acid; MWCO, molecular weight cut-off; NF, nanofiltration; UF, ultrafiltration.

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utilization for biochemical production has been poorly characterized to date.

3-Amino-4-hydroxybenzoic acid (3,4-AHBA) is a metabolic intermediate of grixazone biosynthesis in *Streptomyces griseus* (Suzuki et al., 2006) and is synthesized by the activities of the enzymes GriI and GriH, which are encoded by *griI* and *griH*, respectively. GriI catalyzes an aldol condensation reaction between L-aspartate-4-semialdehyde and dihydroxyacetone phosphate, and GriH converts the resulting C_7 metabolite into 3,4-AHBA. Through this two-step reaction, these two enzymes can produce an aromatic ring from C_4 (L-aspartate-4-semialdehyde) and C_3 (dihydroxyacetone phosphate) primary metabolites, whereas most aromatic compounds, including aromatic amino acids, are formed in a multistep reaction via the shikimate pathway (Liu et al., 2013). 3,4-AHBA serves as a precursor for polybenzoxazole, a thermostable bioplastic, and can be biosynthesized from fermentable sugars (Suzuki et al., 2006). To date, however, the production of 3,4-AHBA from renewable feedstocks has not been investigated.

Corynebacterium glutamicum, a nonpathogenic Gram-positive bacterium, is widely used for the industrial production of various amino acids and nucleic acids (Nakayama et al., 1961; Wittmann et al., 2004). In contrast to *Escherichia coli*, which lacks invertase activity to allow for sucrose utilization, *C. glutamicum* possesses inherent invertase activity and is therefore able to ferment the sucrose contained in sweet sorghum juice (Wittmann et al., 2004) and can also simultaneously consume glucose and fructose (Dominguez et al., 1997). Due to these fermentative properties, *C. glutamicum* may be a suitable host for sweet sorghum juice-based bioproduction. In current industrial bioprocesses, such as amino acid fermentation, using *C. glutamicum*, sugar cane molasses is mainly used as a feedstock (Xu et al., 2013). Although sweet sorghum juice is a suitable feedstock for ethanol fermentation by yeast (Laopaiboon et al., 2009), its compatibility for fermentation by *C. glutamicum* remains to be evaluated.

In the present study, we examined 3,4-AHBA production from sweet sorghum juice containing the fermentable sugars sucrose, glucose, and fructose as carbon sources, and amino acids as a source of nitrogen. Because *C. glutamicum* lacks the capability for 3,4-AHBA synthesis, a strain of *C. glutamicum* was metabolically engineered to produce 3,4-AHBA from sweet sorghum juice. In addition, the sorghum component(s) that promoted 3,4-AHBA production by the recombinant strain was examined by fractionation of the sweet sorghum juice using nanofiltration (NF) membrane separation.

2. Methods

2.1. Materials, bacterial strains, and media

A recombinant 3,4-AHBA-producing strain of *C. glutamicum* (KT01) was derived from lysine-producing *C. glutamicum* ATCC 21799 (Kubota et al., 1973) by transformation of ATCC 21799 with the plasmid pCAC*griHI*, which harbors the *griH* and *griI* genes derived from *S. griseus* for 3,4-AHBA biosynthesis (Suzuki et al., 2006). *E. coli* HST02 (Takara, Shiga, Japan) was used as a host for plasmid construction and was grown aerobically at 37 °C in Luria–Bertani medium (Sambrook and Russell, 2001). For the aerobic growth of *C. glutamicum*, the wild-type and recombinant strains were grown at 30 °C to late log phase in Brain Heart Infusion medium (BD Biosciences, NJ, USA), unless indicated otherwise. When appropriate, media were supplemented with 5 and 50 $\mu g\ ml^{-1}$ chloramphenicol for *C. glutamicum* and *E. coli*, respectively.

Japanese sweet sorghum cultivar SIL-05 was grown at Togo Field of the Science and Education Center of Nagoya University (Aichi, Japan) in 2013. Whole plants at the heading stage were harvested and the juice was immediately extracted from stalks using a juice extractor (Okuhara Tekko, Okinawa, Japan). After removal of suspended solids by filtration with non-woven fabric (AS ONE, Osaka, Japan), the filtrate was centrifuged (8000*g*, 4 °C, 30 min) and the obtained supernatant was then filtered aseptically through a filter membrane (500 ml Rapid-flow filter unit, 0.2-μm pore size; Thermo Fischer Scientific, Waltham, MA). The filter-sterilized sweet sorghum juice was stored at −30 °C before use.

For 3,4-AHBA production from either sweet sorghum juice or pure sugar, recombinant strain KT01 was aerobically cultured in a defined medium, which was based on CGXII medium (Keilhauer et al., 1993) and was composed of (per liter) 5 g urea, 20 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 1 g K_2HPO_4, 42 g 3-morpholinopropanesulfonic acid (MOPS), 0.25 g $MgSO_4{\cdot}7H_2O$, 10 mg $CaCl_2$, 10 mg $FeSO_4{\cdot}7H_2O$, 10 mg $MnSO_4{\cdot}H_2O$, 1 mg $ZnSO_4{\cdot}7H_2O$, 0.31 mg $CuSO_4{\cdot}5H_2O$, 0.02 mg $NiCl_4{\cdot}6H_2O$, 0.2 mg biotin, 0.03 mg protocatechuic acid, and 0.5 g L-homoserine, and was additionally supplemented with carbon sources.

2.2. DNA manipulations

Plasmid DNA was isolated from *E. coli* and *C. glutamicum* as previously described (Kawaguchi et al., 2008). Chromosomal restriction endonucleases were purchased from New England Biolabs (MA, USA) and used according to the manufacturer's instructions. The PCR conditions were described previously (Kawaguchi et al., 2008), unless indicated otherwise. PCR fragments were purified using a QIAquick PCR Purification kit (Qiagen, CA, USA). Corynebacteria were transformed by electroporation as previously described (Vertès et al., 1993), whereas *E. coli* was transformed using the $CaCl_2$ procedure (Sambrook and Russell, 2001).

2.3. Construction of a recombinant plasmid for 3,4-AHBA production

The plasmids pCASE1, which was extracted from *Corynebacterium casei* JCM12072 and has been used to construct an *E. coli*–*C. glutamicum* shuttle vector (Tsuchida et al., 2009), and pHSG398 (Takara) were used to construct an *E. coli*–*C. glutamicum* shuttle vector to express genes for 3,4-AHBA biosynthesis. The *lac* promoter, multi-cloning site, and *cat* gene encoding chloramphenicol acetyltransferase for chloramphenicol resistance were divergently amplified by PCR using plasmid pHSG398 as the template and oligonucleotide primers (5-CCCAGATCTATTCAGCTTGGCCCAGTG-3′ and 5′-CCCAGATCTTTTCTGCCATTCATCCGC-3′) to generate a 2227-kb DNA fragment with *Bgl*II cohesive ends. The purified PCR amplicon was digested with *Bgl*II and was then ligated in a unimolecular reaction, yielding plasmid pYTK23. In addition, a 1436-bp DNA fragment with *Bgl*II cohesive ends and containing the pCASE1 *ori* region was PCR amplified using pCASE1 plasmid DNA as the template and oligonucleotide primers (5′-CCCAGATCTCCTAGAACGTCCGTAGGAGC-3′ and 5′-CCCAGATCTCTGACTTGGTTACGATGGAC-3′). The obtained PCR amplicon was digested with *Bgl*II and then ligated to *Bgl*II-digested pYTK23 DNA, yielding the *E. coli*–*C. glutamicum* shuttle vector pCAC.

The genes *griH* (1203 bp) and *griI* (837 bp) were individually synthesized by Eurofins Genomics (Bayern, Germany) based on the sequence of the grixazone biosynthesis gene cluster in the *S. griseus* chromosome (Suzuki et al., 2006). The synthesized genes were optimized for codon usage for expression in *C. glutamicum* and contained 5′-*Eco*RI and 3′-*Hin*dIII cohesive ends. After digestion with *Eco*RI and *Hin*dIII, the two DNA fragments were ligated to *Eco*RI- and *Hin*dIII-digested pKK223-3 (GE Healthcare, Buckinghamshire, UK) to obtain plasmids pKK*griH* and pKK*griI*, respectively. A 2.0-kb DNA fragment containing the *tac* promoter, *griH*