1 Title

2 **3-Amino-4-hydroxybenzoic acid production from sweet sorghum juice by**
3 **recombinant *Corynebacterium glutamicum***

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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 liter⁻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*.

Keywords: 3-amino-4-hydroxybenzoic acid; biomass; *Corynebacterium glutamicum*; membrane separation; sweet sorghum juice

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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50 1. Introduction

51 Sorghum (*Sorghum bicolor* (L.) Moench) is a C₄ grass with high biomass yield.
52 Compared to other biomass crops, such as corn and sugar cane, sorghum is drought-
53 tolerant, requires less fertilizer, and has a large geographical distribution (Rooney et al.,
54 2007). Juice extracted from the stalks of sweet sorghum predominantly consists of the
55 fermentable sugar sucrose (>150 g liter⁻¹), but also contains variable concentrations of
56 the reducing sugars glucose and fructose (Kawahigashi et al., 2013). The high content
57 of glucose and fructose in sorghum juice as compared to that found in sugar cane
58 extract inhibits crystallization, which limits the processing of sugar for use in foods.
59 However, sweet sorghum juice is a potentially suitable feedstock for bioethanol
60 production (Laopaiboon et al., 2009; Ratnavathi et al., 2010), although its utilization for
61 biochemical production has been poorly characterized to date.

62 3-Amino-4-hydroxybenzoic acid (3,4-AHBA) is a metabolic intermediate of
63 grixazone biosynthesis in *Streptomyces griseus* (Suzuki et al., 2006) and is synthesized
64 by the activities of the enzymes GriI and GriH, which are encoded by *griI* and *griH*,
65 respectively. GriI catalyzes an aldol condensation reaction between L-aspartate-4-
66 semialdehyde and dihydroxyacetone phosphate, and GriH converts the resulting C₇
67 metabolite into 3,4-AHBA. Through this two-step reaction, these two enzymes can
68 produce an aromatic ring from C₄ (L-aspartate-4-semialdehyde) and C₃
69 (dihydroxyacetone phosphate) primary metabolites, whereas most aromatic compounds,
70 including aromatic amino acids, are formed in a multistep reaction via the shikimate
71 pathway (Liu et al., 2013). 3,4-AHBA serves as a precursor for polybenzoxazole, a
72 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. Materials and 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 pCACgriHI, 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 & 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 µg 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 (8,000 × 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 (NH₄)₂SO₄, 1 g KH₂PO₄, 1 g K₂HPO₄, 42 g 3-morpholinopropanesulfonic acid (MOPS), 0.25 g MgSO₄·7H₂O, 10 mg CaCl₂, 10 mg FeSO₄·7H₂O, 10 mg MnSO₄·H₂O, 1 mg ZnSO₄·7H₂O, 0.31 mg CuSO₄·5H₂O, 0.02 mg NiCl₄·6H₂O, 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 CaCl₂ procedure (Sambrook & Russell, 2001).

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

The plasmids pCASE1, which was extracted from *C. 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'-*Hind*III cohesive ends. After digestion with *Eco*RI and *Hind*III, the two DNA fragments were ligated to *Eco*RI- and *Hind*III-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* gene and 5'-*Xba*I and 3'-*Sal*I cohesive ends was amplified by PCR using plasmid pKK*griH* as the template and oligonucleotide primers (5'-CTCTTCTAGAACTCCCGTTCTGGATAATGT-3' and 5'-CTCTGTCGACTTGAGATCCAGTTCGATGTA-3'). The PCR amplicon was digested with *Xba*I and *Sal*I and was then subcloned into *Xba*I- and *Sal*I-digested pCAC, yielding the plasmid pCAC*griH*. Similarly, a 1.7-kb DNA fragment containing the *tac*

promoter, *griI* gene and 5'-*Pst*I and 3'-*Xho*I cohesive ends was amplified by PCR using plasmid pKK*griI* as the template and oligonucleotide primers (5'-CTCTCTGCAGACTCCCGTTCTGGATAATGT-3' and 5'-TCTCTCGAGTTGAGATCCAGTTCGATGTA-3'). The PCR amplicon was digested with *Pst*I and *Xho*I and was then subcloned into *Pst*I- and *Sal*I-digested pCAC*griH*, yielding plasmid pCAC*griHI*, in which the cloned *P_{tac}-griH* and *P_{tac}-griI* fragments were inserted in opposite orientations.

2.4. Membrane separation of sweet sorghum juice

Three fractions of sweet sorghum juice were prepared based on molecular weight cut-off (MWCO) using a two-step membrane separation process, as described previously (Sasaki et al., 2014). Briefly, the filtrate of sweet sorghum juice was prepared from filter-sterilized sweet sorghum juice (see Section 2.1) by ultrafiltration (UF) permeation with a polyvinylidene fluoride UF membrane (RS50, MWCO 150,000 Da; Nitto Denko, Osaka, Japan). The UF-filtrated fraction was subjected to NF with a polyamide NF membrane (ESNA3, MWCO 150 Da; Nitto Denko). The resulting 1.84-fold concentrated sweet sorghum juice and its permeate were designated as the NF-concentrated and NF-filtrated fractions, respectively. In the fractionation procedure, 100 ml of UF filtrate was separated into 54 ml of NF-concentrated fraction and 46 ml of NF-filtrated fraction. All three fractions were stored at -30°C before use.

2.5. 3,4-AHBA production by recombinant *C. glutamicum*

Strain KT01 was grown aerobically to late log phase in Brain Heart Infusion medium. The pre-cultured cells were added to 20 mL CGXII medium in a 100-mL

baffled flask to give a final cell concentration corresponding to an optical density at 600 nm (OD_{600}) of 0.2. Sweet sorghum juice was added to the CGXII medium to give a sucrose concentration of 40 g liter⁻¹, unless otherwise described. As a control culture, a pure sugar solution containing sucrose, glucose, or fructose (40% [w/v]) was added to the CGXII medium to give a sugar concentration of 40 g liter⁻¹. For 3,4-AHBA production from the three fractions of sweet sorghum juice obtained by membrane separation (see *Section 2.4*), the UF-filtrated fraction (10.4 ml) or NF-concentrated fraction (5.68 ml), which was predominantly composed of sucrose, was added to the CGXII medium as a carbon source to give a final sucrose concentration of 40 g liter⁻¹. In addition, the NF-filtrated fraction (4.8 ml) was also added either alone or in combination with the NF-concentrated fraction (5.68 ml) into the inoculated CGX II medium. Sucrose was subsequently added to the NF-filtrated fraction-containing medium at a final concentration of 40 g liter⁻¹. To investigate the effects of amino acids on 3,4-AHBA production by strain KT01, amino acids (100 mg liter⁻¹ each) were individually or collectively added to CGX II medium containing sucrose as the sole carbon source. All of the 3,4-AHBA fermentation experiments were conducted at 30 °C with constant agitation at 120 rpm.

2.6. Analytical procedures

Culture samples were centrifuged (15,000 × g, 4 °C, 10 min), and the concentrations of 3,4-AHBA, fructose, glucose, and sucrose in the resulting supernatants were measured by high-performance liquid chromatography (HPLC). The concentration of 3,4-AHBA was determined by HPLC using an LC-20A apparatus (Shimadzu, Kyoto, Japan) equipped with a Cosmosil HILIC column (4.6 mm I.D. x 250

mm, Nacalai Tesque, Kyoto, Japan) and a diode array detector SPD-M20A (Shimadzu). The column oven temperature was set at 30 °C, and the mobile phase was acetonitrile/20 mM phosphate buffer (pH 7.0) (65:35 [v/v]) at a flow rate of 1.0 mL min⁻¹. The concentration of 3,4-AHBA was calculated based on standard curves obtained with an authentic sample. The concentrations of fructose, glucose and sucrose in sweet sorghum juice and culture broth were determined by HPLC using an LC-20A apparatus (Shimadzu) equipped with a Cosmosil Sugar-D column (4.6 mm I.D. x 250 mm, Nacalai Tesque) and a refractive index detector RID-10A (Shimadzu). The column oven temperature was set at 30 °C, and the mobile phase was acetonitrile/water (70:30 [v/v]) at a flow rate of 1.0 mL min⁻¹. The concentrations of fructose, glucose and sucrose were calculated based on standard curves obtained with standard substances. The composition of free amino acids in sweet sorghum juice and the three fractions obtained by membrane separation was determined using the Prominence Amino Acid Analysis System (Shimadzu), according to the manufacturer's instruction. For the analysis, each sample (10 µL) was mixed with 990 µL citrate buffer (pH 4.5), and the concentration of each amino acid in the acidified aliquot was then determined using standard curves generated using a series of dilutions of the amino acid mixtures Type AN-II and Type B (Wako Chemicals, Osaka, Japan). For the analysis of total amino acids including free amino acids, peptides, and proteins, sweet sorghum juice were hydrolyzed according to a method described previously (Eppendorfer & Bille, 1996). Briefly, 25 ml of sorghum juice was lyophilized and resulting freeze-dried samples were then hydrolyzed under reflux with 6 N HCl for 22 h at 110 °C in sealed tubes, before analyzing the contents of individual amino acids in the hydrolysates according to the methods described above. The composition of the constituent amino acids derived from

proteins or peptides was calculated based on the differences in the contents of the total and individual free amino acids. Cell mass was estimated by measuring OD₆₀₀ using a spectrophotometer (U-3010; Hitachi, Tokyo, Japan). An OD₆₀₀ of 1.0 corresponded to 0.39 mg dry weight cell per milliliter (Kawaguchi et al., 2008).

2.7 Statistical analysis

Differences in metabolite concentrations between the fermentation media were compared using the paired Student's t-test. A p value of <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Development of a 3,4-AHBA-producing strain of *C. glutamicum*

To metabolically engineer *C. glutamicum* to produce 3,4-AHBA from the glycolytic intermediate dihydroxyacetone phosphate and aspartate metabolite aspartate-semialdehyde, lysine-producing *C. glutamicum* ATCC 21799, which exhibits high metabolic flux towards aspartate-semialdehyde in lysine fermentation, was transformed by electroporation with the plasmid pCACgriHI. Transformants were selected on the basis of chloramphenicol resistance and were subsequently screened for the ability to produce 3,4-AHBA from glucose as the sole carbon source. A positive transformant, designated strain KT01, was selected for further characterization.

3.2. Sugar and amino acid composition of sweet sorghum juice

Sweet sorghum juice typically contains several fermentable sugars, including sucrose, glucose, and fructose (Kawahigashi et al., 2013). The sugar composition of the juice extracted from the stalks of sweet sorghum cultivar SIL-05 was found to predominantly consist of sucrose (87.0 ± 1.2 g liter⁻¹), but lesser amounts of fructose and glucose (30.8 ± 2.8 and 25.2 ± 3.1 g liter⁻¹, respectively; Table 1) were also present. The sugar composition and levels indicated that the juice of SIL-05 was a suitable carbon source for 3,4-AHBA production by recombinant *C. glutamicum*.

The free amino acid composition of the sweet sorghum juice was also determined. Sweet sorghum juice contained nearly all common amino acids at concentrations ranging from 85 ± 4 to 467 ± 23 mg liter⁻¹ (Table 1). Compared to nutrient-rich A medium commonly used for the culture of *C. glutamicum*, the contents of aspartate (Asp), glutamate (Glu), alanine (Ala), methionine (Met), valine (Val), leucine (Leu), and isoleucine (Ile) in the sweet sorghum juice were relatively low, whereas the concentrations of tyrosine (Tyr) and γ -amino butyric acid (GABA) were markedly higher (Table 1). The amino acid composition of culture medium has a marked impact on ethanol fermentation by yeasts (Garde-Cerdan et al., 2007). The present amino acid analysis revealed that sweet sorghum juice contains numerous amino acids at sufficient levels to support cell growth (Table 1). The composition of amino acids derived from peptides and/or proteins in the sweet sorghum juice was also determined. Sweet sorghum juice contained both free and constituent amino acids including peptides and proteins at the total concentrations of 4420 and 5662 mg liter⁻¹, respectively. Despite the presence of these peptides/proteins, they likely have limited effects on 3,4-AHBA production because *C. glutamicum* lacks extracellular proteolytic activity (Billman-Jacobe et al., 1995). Although the amino acid composition of sorghum

grain has been reported (Virupaksha & Sastry, 1968), to our knowledge, this is the first report of the amino acid profile of sweet sorghum juice.

3.3. 3,4-AHBA production from sweet sorghum juice by *C. glutamicum*

To examine the suitability of sweet sorghum juice as a substrate for 3,4-AHBA bioproduction, recombinant strain KT01 was cultivated in a minimal medium containing sweet sorghum juice as the sole carbon source. For comparison, 3,4-AHBA production by strain KT01 in a defined medium (CGX II) containing glucose, fructose, or sucrose as the sole carbon source was also measured under the same cultivation conditions. After 72 h of cultivation, strain KT01 produced 3,4-AHBA from glucose, fructose, and sucrose at a final concentration of 80 ± 1.8 , 180.3 ± 3.8 , and 166.9 ± 4.2 mg liter⁻¹, respectively (Fig. 1). In contrast, the 3,4-AHBA concentration in 72-h cultures containing sweet sorghum juice reached a final concentration of 998.8 ± 12.8 mg liter⁻¹, a level that was more than 5-fold higher than that produced from pure sucrose. At the end of the fermentation period with sweet sorghum juice, glucose, fructose, and sucrose were almost completely consumed (Fig. 1D), whereas a large proportion of glucose, fructose, and sucrose remained in the cultures containing the respective sugars as the sole carbon source (Fig. 1A-1C). It was also confirmed that the metals and biotin contained in CGX II medium were not required for 3,4-AHBA production from sweet sorghum juice (Fig. S1). Notably, however, 3,4-AHBA production from sweet sorghum juice was reduced by 57% in the absence of the added nitrogen sources urea and ammonium salts, but was increased by 58% in the presence of urea and ammonium salts compared to the levels of 3,4-AHBA in CGX II medium supplemented with metals, biotin and nitrogen (Fig. S1). Taken together, these results

suggest that sweet sorghum juice can be used as feedstock for 3,4-AHBA production without additional nutrient supplementation to support the growth of *C. glutamicum*; however, the addition of a nitrogen source(s) to fermentation cultures enhances 3,4-AHBA production by recombinant *C. glutamicum*.

3.4. Membrane separation of the components of sweet sorghum juice

To determine the components of sweet sorghum juice that promote 3,4-AHBA production by recombinant *C. glutamicum*, three fractions were prepared from sweet sorghum juice using a membrane separation approach based on molecular weight. The sugar and amino acid contents of the three fractions, which were designated UF-filtrated, NF-concentrated and NF-filtrated fractions, were compared to those in unfractionated sweet sorghum juice to investigate the distribution of these components among the three fractions (Fig. 2E). The amino acid composition of the UF-filtrated fraction was similar to that of sweet sorghum juice, which contained nearly all common amino acids at concentrations ranging from 85 ± 4 to 467 ± 23 mg liter⁻¹ (Table 1). Compared to the UF-filtrated fraction, the content of several amino acids, including arginine (Arg), Asp, Glu, Ile, Leu, Lys, proline (Pro), and Val, were higher in the NF-concentrated fraction, whereas the glycine (Gly) content was comparable between these two fractions. In addition, the concentrations of all amino acids, with the exception of Gly, were lower in the NF-filtrated fraction. These results indicate that the majority of amino acids are concentrated by NF membrane fractionation.

Fermentable sugars in sweet sorghum juice were also concentrated by NF membrane fractionation. Sucrose was exclusively contained in the NF-concentrated fraction, and the concentrations of glucose and fructose were two-fold higher in the NF-

concentrated fraction compared to the UF-filtrated fraction (Table 1). These results suggest that sugars and amino acids, which serve as organic carbon and nitrogen sources, respectively, for 3,4-AHBA production, were concentrated from the sweet sorghum juice by NF-membrane filtration.

3.5. Enhanced 3,4-AHBA production from fractionated sweet sorghum juice

To investigate the effects of the sugar and amino acid components of sweet sorghum juice separated by membrane fractionation on 3,4-AHBA production, strain KT01 was cultured in CGX II medium supplemented with either the UF-filtrated, NF-concentrated, or NF-filtrated fractions. The culture medium containing the NF-filtrated fraction was additionally supplemented with 40 g liter⁻¹ sucrose, as this fraction did not contain any detectable sucrose (Table 1). After 72 h of cultivation, the 3,4-AHBA concentration in culture medium supplemented with the UF-filtrated and NF-concentrated fractions of sweet sorghum juice reached 725.9 ± 50.7 and 828.8 ± 37.3 mg liter⁻¹, respectively (Fig. 2A and 2B), whereas 3,4-AHBA production was hardly detected in the presence of the NF-filtrated fraction (Fig. 2C). However, the 3,4-AHBA concentration in culture medium supplemented with both the NF-concentrated and NF-filtrated fractions (designated the NF-reconstituted fraction) to mimic the original composition of the UF-filtrated fraction of sweet sorghum juice, was comparable to that in medium supplemented with the NF-concentrated fraction alone (828.8 ± 37.3 vs 823.8 ± 48.1 mg liter⁻¹; Fig. 2D). The 3,4-AHBA concentration in cultures supplemented with either the UF-filtrated or NF-concentrated fractions was approximately 4-fold higher compared to cultures containing pure sucrose (Fig. 1C), suggesting that an organic component(s) of the sweet sorghum juice with a molecular

weight between 150 and 150,000 enhances 3,4-AHBA production and that a component(s) with a molecular weight <150 inhibits sucrose consumption by *C. glutamicum* for 3,4-AHBA production.

In addition to fermentable sugars and amino acids, sweet sorghum juice contains various inorganic components, including calcium, iron, copper, zinc, and manganese (Laopaiboon et al., 2009). Some of these inorganic compounds likely inhibit, rather than stimulate, 3,4-AHBA production by *C. glutamicum*. Consistent with this speculation, 3,4-AHBA production was inhibited in the presence of the NF-filtrated fraction, which contained components of the sweet sorghum juice with molecular weights of <150 (Fig. 2C). To our knowledge, the presence of a fermentation inhibitor in sweet sorghum juice has not been reported, although heavy metal stress induced by high concentrations of copper, but not manganese or zinc, has been reported in *C. glutamicum* (Schelder et al., 2011). Consistent with this finding, 3,4-AHBA production from sweet sorghum juice was reduced by 28% in CGX II medium supplemented with trace metals including copper, iron, manganese, nickel, and zinc (Fig. S1).

In contrast to the reduction of 3,4-AHBA synthesis in the presence of the NF-filtrated fraction, organic component(s) with molecular weights of >150 appeared to stimulate 3,4-AHBA production. Notably, 3,4-AHBA production by *C. glutamicum* increased with increasing volumetric ratio of the NF-concentrated fraction, which was added to the defined CGXII medium as the sole carbon source at concentrations ranging from 7.1% to 56.8% (v/v) (Fig. S2). A dose-dependent increase in 3,4-AHBA levels was also observed by the addition of a mixture of amino acids to CGX II medium containing sucrose as the sole carbon source (data not shown). In culture medium supplemented with the NF-reconstituted fraction, which was composed of NF-

concentrated and NF-filtrated fractions, the reduction of 3,4-AHBA production that was observed in cultures supplemented with the NF-filtrated fraction alone was not observed (Fig. 2C and 2D). Taken together, these results suggest that major components, such as amino acids, rather than minor components, such as vitamins and trace metals, present in sweet sorghum juice are likely responsible for enhancing 3,4-AHBA production by *C. glutamicum*. In addition, the marked reduction of 3,4-AHBA production in cultures supplemented with the NF-filtrated fraction can be minimized or masked by component(s) present in the NF-concentrated fraction.

3.6. Effects of amino acids on 3,4-AHBA production by *C. glutamicum*

To investigate the effects of amino acids on 3,4-AHBA synthesis by *C. glutamicum*, 20 amino acids were individually or collectively added to CGX II medium containing sucrose as the sole carbon source. After 72 h of cultivation, higher 3,4-AHBA concentrations were observed in cultures supplemented with cysteine (Cys), Leu, or all 20 amino acids as compared to the culture without added amino acids (Fig. 3). In addition, the 3,4-AHBA concentration was approximately two-fold higher in the culture supplemented with Leu and two-fold lower in the culture supplemented with Cys compared to the culture supplemented with all amino acids. The specific productivity of 3,4-AHBA was higher in cultures supplemented with Cys, histidine (His), or Leu compared to the non-supplemented culture, but was not significantly increased in the culture containing all 20 amino acids (Fig. 3). During 3,4-AHBA production, strain KT01 grew well in cultures supplemented with either Leu or all 20 amino acids, but only exhibited minimal growth in all other cultures (data not shown).

To investigate the effects of amino acids on 3,4-AHBA production in more detail, amino acids at the concentrations equivalent to those observed in the cultures supplemented with the UF-filtrated and NF-concentrated fractions of sweet sorghum juice (Fig. 3) were added to CGX II medium containing sucrose as the sole carbon source. After 72 h of cultivation, both the 3,4-AHBA concentration and cell growth were significantly higher in cultures supplemented with amino acids at the concentrations equivalent to those in cultures supplemented with the NF-concentrated fraction, followed by cultures supplemented with the UF-filtrated fraction, as compared to the culture without added amino acids (Fig. S3). The total concentration of amino acids in cultures supplemented with the UF-filtrated and NF-concentrated fraction was 1966 and 1418 mg liter⁻¹, respectively. The specific productivity of 3,4-AHBA was significantly higher in the culture supplemented with amino acids (1.46 ± 0.06 to 1.48 ± 0.05 mg 3,4-AHBA h⁻¹ mg DCW⁻¹) than that in the culture without added amino acids (1.11 ± 0.04 mg 3,4-AHBA h⁻¹ mg DCW⁻¹). Taken together, these results suggest that the presence of free amino acids enhanced both cell growth and the specific productivity of 3,4-AHBA.

The addition of Cys (100 mg liter⁻¹) to cultures of *C. glutamicum* strain KT01 increased the specific productivity of 3,4-AHBA, but also suppressed cell growth (Fig. 3). Cys is reported to influence stress responses of *C. glutamicum*, but also inhibits cell growth (Follmann et al., 2009). In *C. glutamicum*, Cys serves as a precursor for Met biosynthesis (Rey et al., 2003), and Cys-containing proteins plays key roles in heavy metal stress responses by altering redox signaling (Villadangos et al., 2011). Sweet sorghum juice and the three fractions obtained by membrane separation contained Cys at concentrations exceeding 100 mg liter⁻¹ (Table 1), which is considered to be

sufficiently high to increase the specific productivity of 3,4-AHBA (Fig. 3). These results suggest that the Cys present in sweet sorghum juice increases the specific productivity of 3,4-AHBA by altering protein biosynthesis and/or the stress tolerance of *C. glutamicum*.

Leu was present in the sweet sorghum juice and NF-concentrated fraction at concentrations of 85 ± 4 and 128 ± 15 mg liter⁻¹, respectively (Table 1). Notably, the addition of Asp to the culture medium did not increase the specific productivity of 3,4-AHBA by *C. glutamicum* (Fig. 3), even though Asp is a precursor for 3,4-AHBA biosynthesis (Suzuki et al., 2006). Leu and Asp are both derived from pyruvate, which serves as a source of anaplerotic carbon for lysine production (Park et al., 1997), and excess Asp inhibits pyruvate carboxylase, which is the enzyme responsible for the anaplerotic carbon supply (Koffas et al., 2002). Here, the specific productivity of 3,4-AHBA by recombinant *C. glutamicum* was higher in cultures supplemented with either Cys or Leu than in the culture containing all 20 amino acids (Fig. 3). This result suggests that Leu in sweet sorghum juice enhance 3,4-AHBA production by increasing the amount of pyruvate available for 3,4-AHBA biosynthesis in recombinant *C. glutamicum* cells.

Although the use of sweet sorghum juice significantly enhanced 3,4-AHBA production by recombinant *C. glutamicum* (Fig. 1), the final concentration (1.0 g liter⁻¹) is still too low for practical applications. The availability of aspartate semialdehyde and dihydroxyacetone phosphate during hexose metabolism may limit the productivity, as these primary metabolites are precursors for 3,4-AHBA biosynthesis (Suzuki et al., 2006). The flux toward dihydroxyacetone phosphate can be increased during the metabolism of fructose compared to glucose (Kiefer et al., 2004). This finding is

consistent with the present results that the final concentrations of 3,4-AHBA produced by *C. glutamicum* were significantly higher using fructose or sucrose as a sole carbon source compared to that generated from glucose (Fig. 1). In addition, the addition of Asp to cultures did not enhance 3,4-AHBA production (Fig. 3). Aspartate semialdehyde is biosynthesized from Asp by aspartate-semialdehyde dehydrogenase and requires NADPH as cofactor (Cremer et al., 1988). Thus, metabolic engineering strategies to enhance the pentose phosphate pathway flux to increase the production of NADPH (Becker et al., 2005) are needed to improve the productivity of 3,4-AHBA, as well as reduce the accumulation of byproducts, such as Lys, Ala, and Val, in *C. glutamicum* ATCC 21799 (Klapa et al., 2003).

4. Conclusions

The present findings demonstrate that sweet sorghum juice can serve as an alternative feedstock to sugar cane molasses, which is commonly used in industrial bioprocesses involving *C. glutamicum*, for 3,4-AHBA production by recombinant *C. glutamicum*. In addition to fermentable sugars, the stalk juice of sweet sorghum contains several amino acids that stimulate microbial growth and metabolism. In particular, Leu strongly shifts the metabolism of *C. glutamicum* towards 3,4-AHBA production. This is the first report to demonstrate that sweet sorghum juice is a potentially suitable renewable feedstock for the production of bio-based chemicals by *C. glutamicum*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://XXXX> [to be completed by the Editorial staff].

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Figure captions

Fig. 1. 3-Amino-4-hydroxybenzoic acid (3,4-AHBA) production from sweet sorghum juice by a recombinant strain of *C. glutamicum*. Glucose (A), fructose (B), sucrose (C) or sweet sorghum juice (D) was added to defined CGX II medium as the sole carbon source. The concentrations of 3,4-AHBA (circles), fructose (diamonds), glucose (squares), and sucrose (triangles) are shown. The data are presented as averages $\pm$ standard deviation calculated from the results of triplicate independent experiments.

Fig. 2. 3-Amino-4-hydroxybenzoic acid (3,4-AHBA) production from soluble fractions of sweet sorghum juice by recombinant strain KT01. Ultrafiltration (UF) permeate of sweet sorghum juice (A) and the subsequently nanofiltrated (NF)-concentrated (B) and NF-filtrated fractions (C), and an NF-reconstituted fraction composed of both the NF-concentrated and NF-filtrated fractions (D) were added to defined CGXII medium as the sole carbon source. The concentrations of 3,4-AHBA (circles), fructose (diamonds), glucose (squares), and sucrose (triangles) are indicated. The schematic flow of the membrane separation process using UF and NF membranes (E) is also indicated. The data are presented as averages $\pm$ standard deviation calculated from the results of triplicate independent experiments.

Fig. 3. Effects of amino acids on 3-amino-4-hydroxybenzoic acid (3,4-AHBA) production from sucrose by recombinant strain KT01. The concentration of 3,4-AHBA and the specific productivity based on dry cell weight (DCW) are indicated. Twenty

626 amino acids ($100 \text{ mg liter}^{-1}$ each) were individually or collectively added to defined
627 CGX II medium containing sucrose as the sole carbon source. The concentration of 3,4-
628 AHBA in each culture after 72 h of cultivation was measured, and the specific
629 productivity was calculated from the amount of 3,4-AHBA produced divided by DCW.
630

Figure 1

Figure 2

(E) Schematic flow of the membrane separation process

Figure 3

1 **Table 1.** Sugar and free amino acid compositions of sweet sorghum juice and the three
 2 fractions obtained by membrane separation

Component	Soluble fractions				A medium ^b
	Sorghum juice	UF-filtrated fraction	NF-concentrated fraction	NF-filtrated fraction	
Sugars (g liter ⁻¹) ^a					
Sucrose	87.0 ± 1.2	89.1 ± 2.3	140.9 ± 1.7 ^c	ND	NA
Glucose	25.2 ± 3.1	27.3 ± 0.9	57.8 ± 2.1 ^c	9.2 ± 1.9 ^d	40
Fructose	30.8 ± 2.8	25.0 ± 1.1	51.4 ± 0.8 ^c	6.7 ± 1.5 ^d	NA
Amino acids (mg liter ⁻¹) ^a					
Asp	348 ± 12	353 ± 8	468 ± 38 ^c	171 ± 1 ^d	426 ± 77
Thr	217 ± 3	218 ± 2	243 ± 8 ^c	175 ± 1 ^d	288 ± 39
Ser	257 ± 7	261 ± 4	284 ± 21 ^c	212 ± 4	321 ± 55
Glu	412 ± 23	423 ± 23	511 ± 32 ^c	225 ± 2 ^d	1001 ± 241
Pro	150 ± 87	142 ± 82	405 ± 49	151 ± 76 ^d	79 ± 79
Gly	106 ± 1	108 ± 1	110 ± 2	109 ± 1	157 ± 20
Ala	180 ± 5	184 ± 4	203 ± 11 ^c	158 ± 2	273 ± 48
Val	259 ± 4	261 ± 4	309 ± 17 ^c	187 ± 2 ^d	402 ± 67
Cys	187 ± 5	123 ± 61	188 ± 6	120 ± 60	123 ± 62
Met	177 ± 1	178 ± 2	184 ± 4 ^c	166 ± 1 ^d	251 ± 26
Ile	200 ± 2	202 ± 2	222 ± 6	112 ± 56	276 ± 34
Leu	85 ± 4	87 ± 3	128 ± 15 ^c	25 ± 6 ^d	279 ± 76
Tyr	265 ± 3	266 ± 2	288 ± 9 ^c	229 ± 2 ^d	234 ± 6
Phe	200 ± 3	202 ± 2	223 ± 8 ^c	170 ± 1 ^d	247 ± 27
GABA	467 ± 23	464 ± 16	505 ± 52 ^c	316 ± 9	0 ± 0
His	295 ± 9	295 ± 9	367 ± 26 ^c	171 ± 0 ^d	341 ± 54
Lys	341 ± 13	351 ± 0	400 ± 13	151 ± 76	536 ± 148
Arg	286 ± 93	287 ± 87	424 ± 65	255 ± 128	331 ± 169

^{a)} Data are presented as the average $\pm$ standard deviation calculated from the results of triplicate measurements.

^{b)} Inui et al. (2004).

^{c)} Indicates a significant difference in the concentrations of free amino acids between UF-filtrated and NF-concentrated fractions. The values were compared using the paired Student's *t*-test and P values of <0.05 were considered statistically significant.

^{d)} Indicates a significant difference in the concentrations of free amino acids between the NF-concentrated and NF-filtrated fractions. The values were compared using the paired Student's *t*-test. and P value of <0.05 was considered statistically significant.

Abbreviations: NA, not analyzed; ND, not detected.