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Integrated process development for grass biomass utilization through enzymatic saccharification and upgrading hydroxycinnamic acids via microbial funneling

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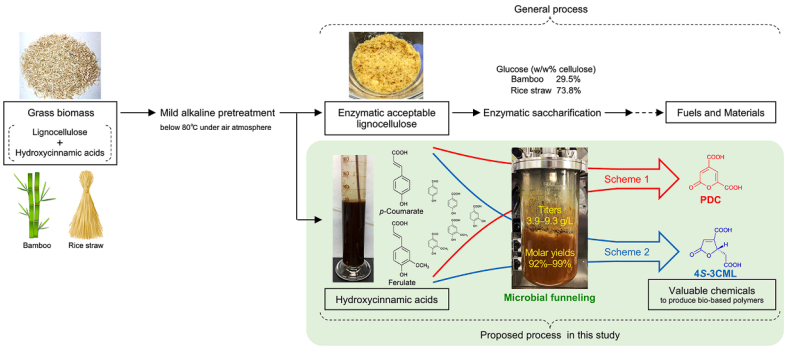
HIGHLIGHTS:

- Hydroxycinnamic acids can be easily extracted from grass biomass.
- Hydroxycinnamic acids were converted to single intermediate via microbial funneling.
- PDC is a promising building block for bio-based polymers.
- 4*S*-3CML is a promising chiral building block for bio-based polymers.
- Hydroxycinnamic acids extraction improved enzymatic accessibility of lignocellulose.

GRAPHICAL ABSTRACT



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ABSTRACT

In grass biomass, hydroxycinnamic acids (HCAs) play crucial roles in the crosslinking of lignin and polysaccharides and can be easily extracted by mild alkaline pretreatment, albeit heterogeneously. Here, HCAs were extracted from bamboo and rice straw as model grass biomass with different HCAs composition, and microbial funneling was then conducted to produce 2-pyrone-4,6-dicarboxylic acid (PDC) and (4*S*)-3-carboxymuconolactone (4*S*-3CML), promising building blocks for bio-based polymers, respectively. *Pseudomonas putida* PpY1100 engineered for efficient microbial funneling completely converted HCAs to PDC and 4*S*-3CML with high titers of 3.9–9.3 g/L and molar yields of 92–99%, respectively. The enzymatic saccharification efficiencies of lignocellulose after HCAs extraction were 29.5% in bamboo and 73.8% in rice straw, which are 8.9 and 6.8 times higher than in alkaline-untreated media, respectively. These results provide a green-like process for total valorization of grass biomass through enzymatic saccharification integrated with upgrading heterogeneous HCAs to a valuable single chemical via microbial funneling.

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1. Introduction

Lignocellulosic biomass is a recalcitrant complex mixture primarily composed of cellulose, hemicellulose, and lignin at varying composition depending on the plant species. Cellulose can be used as a raw material not only for pulp, but also for bioethanol and polylactic acid production through hydrolysis into monosaccharides via enzymatic saccharification (Sharma et al., 2020; Svetlitchnyi et al., 2022). To break down lignocellulose recalcitrance and enhance enzymatic saccharification, green-like and cost-effective pretreatment development that does not generate toxic byproducts has been encouraged (Alam et al., 2020; Gao et al., 2021; Li et al., 2018; Wang et al., 2021). These pretreatment technologies can also recover lignin, which is potentially a valuable precursor for bio-based material. Recently, lignin valorization using microbial funneling processes has attracted increasing research attention (Gómez-Álvarez et al., 2022; Johnson et al., 2019; Lee et al., 2022; Perez et al., 2019, 2022). The technical developments for production of a single intermediate from lignin-derived aromatic compounds by microbial funneling, and synthesis of a variety of bio-based polymers from it as a building block have been reported (Shikinaka et al., 2018; Suzuki et al., 2021, 2020). The protocatechuate (PCA) 4,5-cleavage pathway (Kamimura & Masai, 2014) and the PCA 3,4-cleavage pathway (Kondo et al., 2016; Okamura-Abe et al., 2016) are beneficial metabolic pathways to produce a single intermediate from lignin-derived aromatic compounds.

The application of microbial funneling for woody biomass utilization has been previously reported. Specifically, *Pseudomonas putida* PpY1100 was engineered by introducing the PCA 4,5-cleavage pathway enzyme genes *ligAB* and *ligC* of *Sphingobium* sp. SYK-6 to produce 2-pyrone-4,6-dicarboxylic acid (PDC) from vanillin (VL) and vanillate (VA) prepared from industrial lignin, which is a byproduct of wood pulp production (Suzuki et al., 2020). PDC is a promising bioprivileged molecule (Huo & Shanks, 2020) because its chemical synthesis is difficult and has high functionality that is unprecedented for chemicals derived from fossil resources. A series of PDC-based polyesters synthesized by direct dehydrated polycondensation with various diol co-monomers were demonstrated to have excellent biodegradability and mechanical and adhesive properties (Bito et al., 2008; Hasegawa et al., 2009; Hishida et al., 2009; Michinobu et al., 2011, 2010a, 2010b, 2009a, 2009b, 2008, 2007). Recently, PDC was found to selectively bind and precipitate cesium ion in aqueous solution, indicating its potential for application in the remediation of radioactive cesium contamination (Bito et al., 2019).

To encourage the various applications of bio-based polymers, technical development of the production of intermediates using the PCA 3,4-cleavage enzyme genes *pcaHG* of prokaryotic *P. putida* KT2440 and 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme gene (*CMLE*) of eukaryotic *Neurospora crassa* N150 has been described. The PCA 3,4-cleavage pathway has been mainly studied in prokaryotes. However, the dicarboxylic acid 4-carboxymuconolactone (4CML) could not be stably produced via the PCA 3,4-cleavage pathway of prokaryotes nor purified from the culture medium because of its chemical instability (Kondo et al., 2016). Meanwhile, the PCA 3,4-cleavage pathway of eukaryotes was studied in *N. crassa*, finding that it proceeds via a different intermediate to that observed in prokaryotes, i.e., (4*S*)-3-carboxymuconolactone (4*S*-3CML) (Gross et al., 1956; Martins et al., 2015), which is produced in optically pure form by the stereoselective CMLE of *N. crassa* (Mazur et al., 1994). Therefore, 4*S*-3CML could be an unprecedented chiral building block for lignin derivatives with potential applications in functional materials with optical activity, such as liquid crystalline polymers. Stable production and purification of 4*S*-3CML from PCA using CMLE of *N. crassa* N150 has been reported (Kondo et al., 2016). However, 4*S*-3CML production from lignocellulosic biomass-derived aromatic compounds has not been achieved.

The present study focused on hydroxycinnamic acids (HCAs), which are abundant in grass biomass, to diversify the plant species used as a source of lignocellulosic biomass. In grass biomass, HCAs play crucial roles in the crosslinking of lignin and polysaccharides and can be easily extracted by mild alkaline pretreatment, albeit heterogeneously (Johnston et al., 2020; Karlen et al., 2020). In addition, HCAs extraction may improve the efficiency of lignocellulose utilization in processes such as bioethanol and polylactic acid production because it releases polysaccharides from lignin and increases the enzymatic accessibility of lignocellulose. HCAs extracted from grass biomass is a complex mixture of *p*-coumarate (CA) and ferulate (FA) (Johnston et al., 2020; Karlen et al., 2020). Upgrading the extracted heterogeneous HCAs to a valuable single intermediate while improving the efficiency of lignocellulose utilization is a promising approach for total valorization of grass biomass.

Here, HCAs were extracted by mild alkaline pretreatment from bamboo and rice straw as model grass biomass, which differ in their content ratios of CA and FA. Subsequently, microbial funneling was conducted to produce PDC and 4*S*-3CML. The key to achieve efficient production of these products from HCAs was the use of feruloyl-CoA synthetase (FerA) and feruloyl-CoA hydratase/lyase (FerB) from *Sphingobium* sp. SYK-6, which show broad substrate specificities (Masai et al., 2002), and an adequate supply of cofactor CoA (Fig. 1). Furthermore, to evaluate the enzymatic accessibility of lignocellulose after HCAs extraction, its enzymatic saccharification efficiency was investigated.

2. Materials and methods

2.1. Preparation of grass biomass extracts

2.1.1. Optimization of the conditions for HCAs extraction

One-year-old bamboo (*Phyllostachys edulis*) with height similar to that of mature plants was obtained from an experimental bamboo forest at Forestry and Forest Products Research Institute (Ibaraki, Japan). Mature rice straw (derived from *Oryza sativa* L.) was obtained from an experimental paddy field at Tokyo University of Agriculture & Technology (Tokyo, Japan). The grass biomass samples were dried at 60 °C until the moisture contents were reduced to less than 10% (6.9% for the bamboo sample and 5.3% for the rice straw sample). The dried biomass was milled to a particle size of 6 mm or less using a P-15 cutting mill (Fritsch Japan Co., ltd., Kanagawa, Japan). The optimal alkaline pretreatment conditions were investigated at a reaction temperature of 40–100 °C and an NaOH concentration of 1–6% w/v. The milled biomass (1 g) and 9 mL of NaOH solution of a given concentration were put into a 100 mL volume eggplant flask. The alkaline pretreatment was performed under air atmosphere in an oil bath for 2 h while stirring at 500 rpm. A Dimroth condenser with running tap water was placed above the heated eggplant flask to prevent experimental errors caused by changes in the volume of the reaction solution due to vaporization. After the reaction, the lignocellulose was removed by filtration. The filtrate was acidified to less than pH 2.0 with 10 N HCl and extracted three times with 5 mL of ethyl acetate. The ethyl acetate extracts were rotary-evaporated and dissolved in 50 mL of water, and the pH was adjusted to 8.2 with 28% w/v ammonia solution. HCAs and OAs in the resulting extracts were quantified by high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) analyses, respectively (see the section “Analytical methods” for details). The model aromatic compounds, CA, FA, VA, and PCA were purchased from Sigma-Aldrich Japan (Tokyo, Japan). VL was purchased from Tokyo Chemical Industry Co., ltd. (Tokyo, Japan). *p*-hydroxybenzaldehyde (HBAL) and *p*-hydroxybenzoate (HBA) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). The yields (w/w) of aromatic compounds were calculated on the basis of Klason lignin (bamboo, 22.8% w/w; rice straw, 14.84% w/w).

2.1.2. Scaling up for fed-batch production

Biomass (five batches of 100 g, accounting for a total of 500 g) was subjected to mild alkaline pretreatment and used as a feedstock for fed-batch production with an initial culture volume of 1 L. Briefly, each

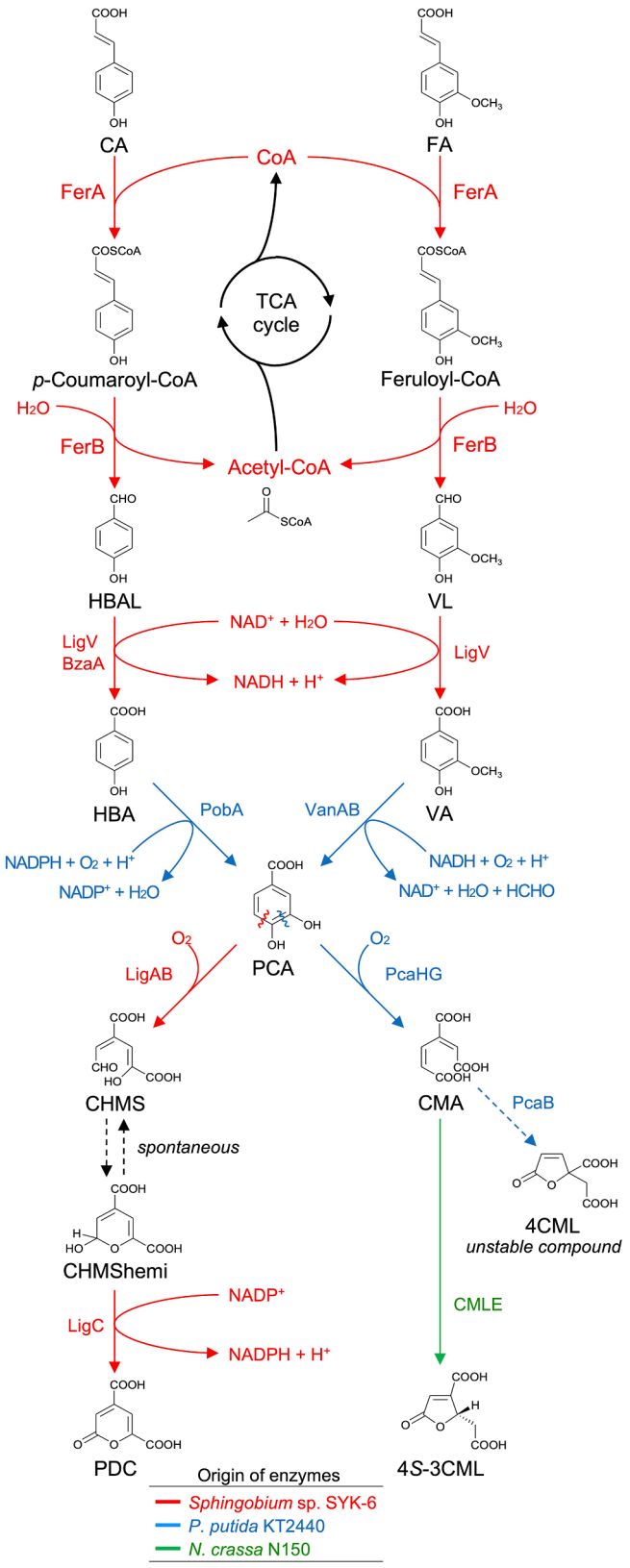

Fig. 1. Metabolic pathways of CA and FA in engineered *P. putida* PpY1100 in this study. The continuous arrows represent pathways introduced into *P. putida* PpY1100. Abbreviations: CHMS, 4-carboxy-2-hydroxymuconate-6-semialdehyde; CHMShemi, hemiacetal form of CHMS; CMA, 3-carboxy-*cis*,*cis*-muconate; PcaB, 3-carboxy-*cis*,*cis*-muconate cycloisomerase of *P. putida* KT2440.

batch of milled biomass (100 g) and 900 mL of NaOH solution were put into a 1 L eggplant flask and subjected to alkaline pretreatment under the optimal conditions of 80 °C and 4% NaOH concentration for the bamboo biomass and 60 °C and 4% NaOH concentration for the rice straw biomass (Fig. 2). A filtrate of the reaction solution was acidified to less than pH 2.0 with 10 N HCl and extracted three times with 500 mL of ethyl acetate. The ethyl acetate extract was rotary-evaporated and dissolved in 24 mL of water containing 8 g of glucose. The pH of the resulting solution was adjusted to 8.2 with 28% w/v ammonia solution. A total of five batches of the resulting extracts were mixed to a total volume of 120 mL and used for a subsequent fed-batch production test. For a preliminary test of the fed-batch production, feedstock solutions containing 10 g of CA or FA and 40 g of glucose with a total volume of 120 mL adjusted to pH 8.2 were also prepared.

2.2. Plasmid constructions and bacterial strains

The plasmids and bacterial strains used in this study are listed in Table 1. To produce efficiently the target products from CA and FA, the *ferA*, *ferB*, VL dehydrogenase gene (*ligV*), and the benzaldehyde derivatives dehydrogenase gene (*bzaA*) of *Sphingobium* sp. SYK-6 and HBA hydroxylase gene (*pobA*) of *P. putida* KT2440 were cloned into pJB866, and the resulting construct was named pJFVV2AB. In addition, the *ligAB* and *ligC* genes of *Sphingobium* sp. SYK-6 and VA demethylase gene (*vanAB*) of *P. putida* KT2440 were cloned into pKT230MC for PDC production, and the resulting construct was named pDVZ21X. The *CMLE* gene derived from cDNA of *N. crassa* N150 and the *vanAB* and *pcaHG* genes of *P. putida* KT2440 were cloned into pKT230MC for 4*S*-3CML production, and the resulting construct was named pDVZ21HB. Isolation of total RNA and cDNA synthesis from *N. crassa* N150 was performed according to a previously reported method (Kondo et al., 2016). pJFVV2AB and pDVZ21X were introduced into *P. putida* PpY1100-dHG, a *pcaHG*-inactivated mutant described in a previous study (Qian et al., 2016), for PDC production, which was named PpY1100-dHG/pJF-X. For 4*S*-3CML production, pJFVV2AB and pDVZ21HB were introduced into *P. putida* PpY1100, which was named PpY1100/pJF-HB.

2.3. Fed-batch production in a jar fermenter

2.3.1. Strain preparation

To revive a glycerol stock (200 μL) stored at −80 °C, it was melted on ice and then inoculated into L-type test tubes containing 10 mL of culture medium with 1.8% w/v glucose as a growth source for the host strain. The culture medium was supplemented with 5.6 g/L of $(NH_4)_2SO_4$, 3.6 g/L of KH_2PO_4, 8.2 g/L of Na_2HPO_4, 4.2 g/L of yeast extract, 12.5 mg/L of tetracycline, 25 mg/L of kanamycin, and a 1% v/v metal solution composed of 10.75 g/L of MgO, 2.00 g/L of $CaCO_3$, 4.50 g/L of $FeSO_4{\cdot}7H_2O$, 1.44 g/L of $ZnSO_4{\cdot}7H_2O$, 1.12 g/L of $MnSO4{\cdot}4H_2O$, 0.25 g/L of $CuSO_4{\cdot}5H_2O$, 0.28 g/L of $CoSO_4{\cdot}5H_2O$, 0.06 g/L of H_3BO_3, and 5.13% v/v 12 N HCl. The yeast extract was purchased from Thermo Fisher Scientific Inc. (Tokyo, Japan), and the other reactants were provided by Wako Pure Chemical Industries, ltd. (Osaka, Japan). The strain was cultured at 28 °C and 160 rpm for 36–60 h in an incubator, and the culture growth was determined by monitoring an optical density at 660 nm (OD_{660}) using a spectrophotometer (V-630BIO; JASCO Co., ltd., Tokyo, Japan). The revived strain culture (100 μL) was inoculated again into an L-type test tube containing 10 mL of the same culture medium described above and incubated at 28 °C and 160 rpm for 6–9 h until the OD_{660} value reached approximately 0.8. The resulting strain was washed with culture medium of the above composition without glucose and resuspended in 10 mL of the same culture medium.

2.3.2. Final growth culture preparation and bioreactor control

A strain suspension was inoculated into a jar fermenter (BMS-10NP3; ABLE Corporation, Shinjuku, Japan) containing 1 L of final culture medium with an initial OD_{660} of 0.001. The final culture medium was

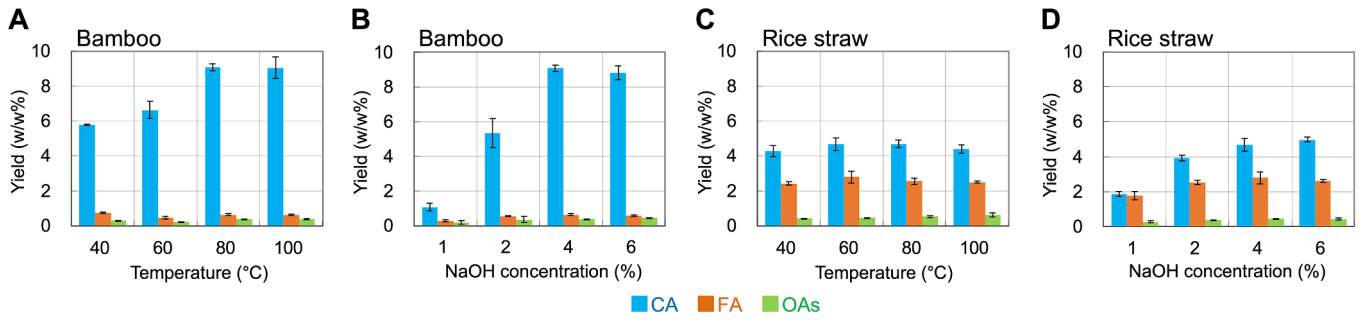

Fig. 2. Investigation of the optimal conditions for hydroxycinnamic acids (HCAs) extraction from bamboo and rice straw as model grass biomass. (A, C) Aromatic compound extractions at different temperatures (40–100 °C) and 4% NaOH concentration. (B, D) Aromatic compound extractions at different NaOH concentrations (1–6%) and 80 °C for bamboo and 60 °C for rice straw. The total yields of OAs are shown. The error bars represent the standard deviation of two replicates.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Reference
Strains		
Sphingobium sp. SYK-6	Nal^r Sm^r	(Suzuki et al., 2020)
N. crassa N150		(Kondo et al., 2016)
P. putida KT2440	Cm^r	(Suzuki et al., 2021)
P. putida PpY1100	Nal^r	(Suzuki et al., 2020)
P. putida PpY1100-dHG	Mutant derivative of *P. putida* PpY1100; Gm^r gene insertion mutant of *pcaHG*; Nal^r Gm^r	(Qian et al., 2016)
PpY1100-dHG/pJF-X	*P. putida* PpY1100-dHG mutant strain bearing the pJFVV2AB and pDVZ21X plasmids; Nal^r Gm^r Km^r Tc^r	This study
PpY1100/pJF-HB	*P. putida* PpY1100 strain bearing the pJFVV2AB and pDVZ21HB plasmids; Nal^r Km^r Tc^r	This study
Plasmids		
pJB866	RK2 broad-host-range cloning vector; Tc^r	(Suzuki et al., 2021)
pJFVV2AB	*ferA, ferB, ligV, bzaA* and *pobA* cloned in pJB866 under *lac* promoter; Tc^r	This study
pKT230MC	IncQ broad-host-range cloning vector; Km^r	(Suzuki et al., 2021)
pDVZ21X	*vanAB*, *ligAB* and *ligC* cloned in pKT230MC under *lac* promoters; Km^r	This study
pDVZ21HB	*vanAB*, *pcaHG* and *CMLE* cloned in pKT230MC under *lac* promoters; Km^r	This study

Abbreviations: Nal^r, Sm^r, Cm^r, Gm^r, Km^r, and Tc^r resistance to nalidixic acid, streptomycin, chloramphenicol, gentamicin, kanamycin, and tetracycline, respectively.

composed of 3.6% w/v glucose, 6.7 g/L of $(NH_4)_2SO_4$, 4.3 g/L of KH_2PO_4, 9.1 g/L of $(NH_4)_2HPO_4$, 6.5 g/L of yeast extract, 12.5 mg/L of tetracycline, 25 mg/L of kanamycin, and 8% v/v metal solution. The final growth culture was performed at 28 °C and an aeration of 3.5 L/min while controlling the pH at 6.5 with 2 M H_3PO_4 and 14% w/v ammonia solution. Agitation was initially operated at 700 rpm and manually increased to 1000 rpm when oxygen saturation fell below 20%. Antifoam A concentrate (Sigma-Aldrich Japan) was added as a defoamer in a timely manner when bubbles were significantly generated.

2.3.3. Feeding of raw material solutions

When the OD_{660} value of the culture medium reached approximately 5.0–8.0, a feed of glucose solution (500 mg/mL) was introduced at a flow rate of 9.6 mL/h using a Perista pump (4.8 g/h of glucose). When the OD_{660} value of the culture medium reached approximately 30, the feed of glucose was stopped and replaced with four kinds of feedstock solutions, i.e., CA, FA, bamboo extract, and rice straw extract, respectively. For detailed compositions of the feedstock solutions, see the section "Preparation of grass biomass extracts." In all tests, the flow rate of the feedstock solution was 14.4 mL/h while feeding glucose at an unchanged rate of 4.8 g/h. Fed-batch productions were performed in duplicate. Samples (3 mL) were taken periodically and analyzed for host growth (OD_{660}) and intermediates.

The values provided for the titers (g/L) of PDC and 4*S*-3CML correspond to the concentration at the end of cultivation. The molar yields (%) were calculated by comparing the molar quantities between the aromatic compounds used as feedstocks and the products. Authentic purified PDC and 4*S*-3CML samples of known optical purity were used (Kondo et al., 2016; Suzuki et al., 2020).

2.4. Enzymatic saccharification of lignocellulose

Under the optimal conditions for HCAs extraction, i.e., 80 °C and 4% NaOH concentration for bamboo and 60 °C and 4% NaOH concentration for rice straw, 1 g of each milled biomass was subjected to mild alkaline pretreatment. Subsequently, lignocellulose was obtained by filtration using a glass fiber filter and washed thoroughly with ultrapure water. The washed lignocellulose and untreated biomass (1 g) were suspended in 100 mM phosphate buffer (pH 5.0) to a total weight of 20 g containing cellulase. Enzymatic saccharifications were performed using GODO cellulase F with an enzyme loading of 30 filter paper units (FPU)/g glucan at 50 °C for 48 h while stirring at 500 rpm. The glucose produced by enzymatic saccharification was quantified by HPLC analysis (see the section "Analytical methods" for details). The yields (w/w) of glucose were calculated on the basis of α-cellulose (47.0% w/w in bamboo and 35.5% w/w in rice straw).

2.5. Analytical methods

To determine the HCAs, PDC, and 4*S*-3CML content, the grass biomass extracts and culture media were analyzed using an HPLC system (JASCO Corp., Tokyo, Japan) equipped with a C-18 reverse-phase column (Inertsil ODS-3, 4.6 × 250 mm, 5 μm column, GL Sciences Inc, Japan) and a UV/VIS detector (UV970, JASCO Corp., Tokyo, Japan) set to 290 nm for HCAs and PDC and to 220 nm for 4*S*-3CML. The analyses were performed at 40 °C with an isocratic mobile phase (10% of acetonitrile and 90% of 10 mM phosphoric acid) flowing at 1 mL/min.

Glucose in the enzymatic saccharide solutions was analyzed using an HPLC system (LC-20AD, Shimadzu Corporation, Kyoto, Japan) equipped with a combined ligand exchange and size exclusion chromatography

column (Sugar KS-802, 8.0 × 300 mm, 6 μm column, Shodex Co., ltd., Japan) and an RI detector (RI-201H, Shodex Co., ltd., Japan). The analyses were performed at 80 °C with flowing water at 1 mL/min as an eluent. All sample solutions were filtered through a syringe filter with a nylon membrane (porosity 0.45 μm) before injection into the HPLC column.

OAs in grass biomass extracts and culture media were analyzed by GC–MS using a JMS-Q1000 GC instrument (JEOL, ltd., Tokyo, Japan) with a DB-5MS UI capillary column (30 m × 0.25 mm; Agilent Technologies, Inc., Calif., USA). A sample solution (200 μL) was acidified to less than pH 2.0 with 10 N HCl and extracted with 200 μL of ethyl acetate. Next, 100 μL of the organic solvent phase was collected and volatilized using argon gas for the subsequent analyses. *p*-Anisic acid (Tokyo Chemical Industry Co., ltd.) was added as an internal standard to the sample solutions when necessary. The samples were derivatized using N,O-bis(trimethylsilyl)trifluoroacetamide (Tokyo Chemical Industry Co., ltd.) prior to the GC–MS analyses. The injection and detection temperatures were 250 °C and 200 °C, respectively. The resulting samples were subjected to chromatography using a temperature program consisting of holding at 50 °C for 10 min and then increasing the temperature to 300 °C at a rate of 10 °C/min.

The analyte concentrations were calculated by comparing the peak areas obtained using the calibration curves prepared with commercially available authentic compounds. All analyses were performed in duplicate and average values are reported.

3. Results and discussion

3.1. Optimization of the conditions for HCAs extraction

The optimal conditions to extract HCAs efficiently via mild alkaline pretreatments at 100 °C or less under air atmosphere without pressure were investigated using bamboo (*Phyllostachys edulis*; one-year-old stems, 6.9% w/w moisture content, 22.8% w/w Klason lignin, 47.0% w/w α-cellulose) and rice straw (derived from *Oryza sativa* L.; mature straw, 5.3% w/w moisture content, 14.8% w/w Klason lignin, 35.5% w/w α-cellulose) as model grass biomass. Specifically, 1 g of biomass was subjected to mild alkaline pretreatment under different conditions of reaction temperature (40–100 °C) and NaOH concentration (1–6% w/v). The aromatic compounds in the reaction solutions were quantified by HPLC and GC–MS analyses, and the yields obtained in each condition are shown in Fig. 2. According to the HPLC analyses, the optimal conditions to extract HCAs from bamboo and rice straw were 80 °C of reaction temperature and 4% NaOH concentration and 60 °C and 4% NaOH concentration, respectively. The main component of both aromatic compounds was CA, but the FA content increased significantly in the rice straw extract (see Supplementary Material). The GC–MS analyses revealed that in addition to CA and FA, the reaction solutions contained trace amounts of HBAL, HBA, VL, and VA (henceforth named OAs for "other aromatic compounds"), which are feedstocks for the production of PDC and 4*S*-3CML. The total yields of OAs are shown in Fig. 2.

Next, scaling up was performed to prepare the extracts used as feedstocks in the fed-batch production of PDC and 4*S*-3CML. Briefly, equal amounts of bamboo and rice straw biomass (500 g) were subjected to mild alkaline pretreatment under the optimal conditions described above, respectively. Ethyl acetate extracts of the pretreated solutions were rotary-evaporated and then dissolved in water containing glucose as a source of strain growth and the pH was adjusted to 8.2 with ammonia solution. Henceforth, the resulting extracts are called "bamboo extract" and "rice straw extract." The content in HCAs and OAs of these extracts are shown in Tables 2, 3.

3.2. PDC production from grass biomass extracts

3.2.1. Strain construction for the efficient production of PDC from HCAs

To efficiently produce the target products from HCAs derived from various grass biomass sources with different CA and FA content ratios, it is important to select enzymes, which catalyze side-chain cleavage in the first steps (Fig. 1). Enzymes associated with the side-chain cleavage of HCAs have been identified in a few bacteria (Achterholt et al., 2000; Gasson et al., 1998; Masai et al., 2002; Otani et al., 2014; Overhage et al., 1999; Venturi et al., 1998). Since FerA and FerB of *Sphingobium* sp. SYK-6 were shown to have broad substrate specificities for a variety of HCAs, these enzymes were adopted in the present study. FerA catalyzes the transfer of the cofactor CoA to the carboxyl group of HCAs for the formation of CoA derivatives *p*-coumaroyl-CoA and feruloyl-CoA, which are then hydrated by FerB and converted to acetyl-CoA and the benzaldehyde derivatives HBAL and VL (Masai et al., 2002). In this study, it was envisaged that the target products could be efficiently produced from HCAs providing that these first steps catalyzed by FerA and FerB proceeded well and integrated with the subsequent process for the

Table 2
PDC titers and yields mediated by PpY1100-dHG/pJF-X with an initial culture volume of 1 L.

	Feedstock Name	Aromatic compound (g)		Produced PDC (mM)	Produced PDC (g)	PDC titer (g/L)	Molar yield (%)
Trial 1	Commercial product	CA	10.0	54.9 ± 0.6	11.0 ± 0.1	10.1 ± 0.1	98.3 ± 0.9
Trial 2	Commercial product	FA	10.0	43.5 ± 0.6	9.1 ± 0.1	8.0 ± 0.1	96.2 ± 1.1
Trial 3	Bamboo extract	HCAs		47.5 ± 2.3	10.7 ± 0.2	8.7 ± 0.4	93.6 ± 3.8
		CA	9.103 ± 0.717				
		FA	0.813 ± 0.097				
		OAs					
		HBAL	0.149 ± 0.003				
		HBA	0.056 ± 0.002				
		VL	0.086 ± 0.005				
		VA	0.066 ± 0.006				
Trial 4	Rice straw extract	HCAs		21.3 ± 0.8	4.6 ± 0.1	3.9 ± 0.1	92.4 ± 0.9
		CA	2.926 ± 0.006				
		FA	1.602 ± 0.002				
		OAs					
		HBAL	0.031 ± 0.001				
		HBA	0.025 ± 0.001				
		VL	0.042 ± 0.001				
		VA	0.057 ± 0.001				

Table 3
4*S*-3CML titers and yields mediated by PpY1100/pJF-HB with an initial culture volume of 1 L.

	Feedstock Name	Aromatic compound (g)		Produced 4*S*-3CML (mM)	Produced 4*S*-3CML (g)	4*S*-3CML titer (g/L)	Molar yield (%)
Trial 5	Commercial product	CA	10.0	56.7 ± 0.0	11.2 ± 0.1	10.5 ± 0.0	99.3 ± 0.1
Trial 6	Commercial product	FA	10.0	44.3 ± 1.4	9.4 ± 0.1	8.2 ± 0.3	97.7 ± 1.5
Trial 7	Bamboo extract	HCAs		49.8 ± 2.4	10.6 ± 0.5	9.3 ± 0.5	99.1 ± 0.3
		CA	8.409 ± 0.432				
		FA	0.679 ± 0.048				
		OAs					
		HBAL	0.147 ± 0.002				
		HBA	0.058 ± 0.003				
		VL	0.078 ± 0.007				
		VA	0.071 ± 0.002				
Trial 8	Rice straw extract	HCAs		21.3 ± 0.5	4.5 ± 0.1	4.0 ± 0.1	94.6 ± 1.4
		CA	2.761 ± 0.143				
		FA	1.471 ± 0.094				
		OAs					
		HBAL	0.034 ± 0.006				
		HBA	0.029 ± 0.003				
		VL	0.047 ± 0.008				
		VA	0.059 ± 0.001				

already reported microbial conversion of benzaldehyde derivatives as feedstocks (Suzuki et al., 2020).

A *pcaHG*-inactivated mutant of *P. putida* PpY1100-dHG was adopted as a host strain for PDC production. This host mutant was engineered to produce PDC from CA and FA efficiently by introducing plasmids pJFVV2AB carrying the genes encoding FerA, FerB, LigV, BzaA and PobA, and pDVZ21X carrying the genes encoding VanAB and Lig enzymes (LigAB and LigC) of the PCA 4,5-cleavage pathway. The resulting strain was termed PpY1100-dHG/pJF-X (Table 1). All genes adopted in this study, including *ferA* and *ferB*, were transcribed from *lac* promoters and expressed at high level without inducers in a host strain.

3.2.2. Optimization of PDC production using commercial products

CoA is expected to be unilaterally consumed in the cells due to high level expression of *ferA* and *ferB*. Previously, Otsuka et al. (2006) reported the efficient production of PDC from PCA using glucose as a growth source for the host strain to activate the intracellular glycolytic pathway and a subsequent tricarboxylic acid (TCA) cycle for promoting the recycling use of cofactors such as $NADP^+$, which is necessary to maintain the enzymatic activity of LigC. In this study, glucose was expected to promote the recycling use of CoA. Hence, all tests were validated using an initial culture volume of 1 L with glucose as the growth source.

As a preliminary step to verify the production of PDC from the grass biomass extracts, jar fermenter tests were performed to examine the ability of PpY1100-dHG/pJF-X to produce PDC from commercially available CA and FA. PpY1100-dHG/pJF-X was grown until an OD_{660} of approximately 30 was obtained, and 10 g of CA and 10 g of FA were then continuously fed into the culture media for approximately 8.3 h, respectively (Fig. 3A, B). Glucose was also fed continuously during the CA or FA feeding. The feeding rates were as follows: CA, 1.2 g/h; FA, 1.2 g/h; glucose, 4.8 g/h. CA and FA were quickly metabolized and completely converted to PDC with molar yields of 96% or higher. The PDC titers were 10.1 ± 0.1 g/L for CA and 8.0 ± 0.1 g/L for FA as feedstocks (Table 2). The PDC content increased linearly during addition of the feedstock, while CA and FA were rapidly metabolized (Fig. 3A, B). These results suggest that the enzymatic activity of FerA and FerB were sufficiently maintained and CoA was recycled efficiently.

3.2.3. PDC productivity with grass biomass extracts as feedstocks

The production of PDC from the bamboo and rice straw extracts was investigated. PpY1100-dHG/pJF-X was cultured under the same conditions as in the tests using the commercial products, and the extracts were continuously fed into the culture media for approximately 8.3 h, respectively (Fig. 3C, D). The feeding rates were as follows: CA, 1.07 ± 0.12 g/h; FA, 0.09 ± 0.01 g/h; glucose, 4.80 g/h in the bamboo extract and CA, 0.35 ± 0.01 g/h; FA, 0.19 ± 0.01 g/h, glucose, 4.80 g/h in the rice straw extract. The aromatic compounds contained in each extract were quickly metabolized and completely converted to PDC with molar yields of 92% or higher. The PDC titers were 8.7 ± 0.4 g/L for the bamboo extract and 3.9 ± 0.1 g/L for the rice straw extract (Table 2), which are high values comparable to that in the previous report (Suzuki

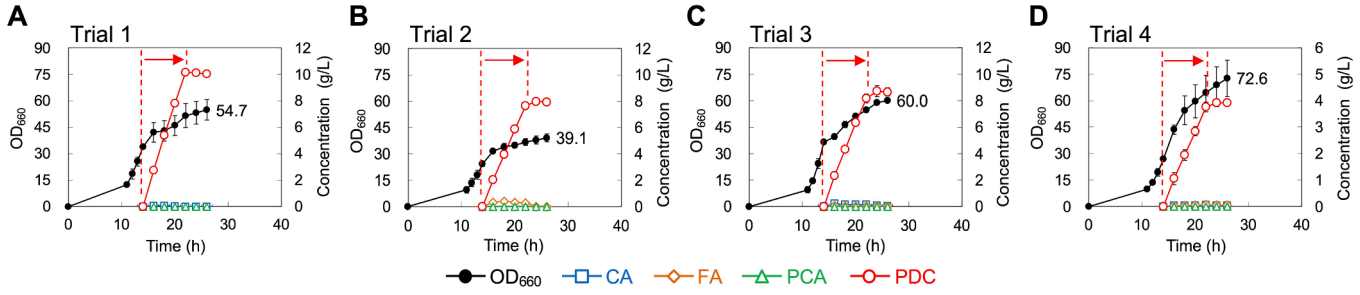

Fig. 3. PDC production mediated by PpY1100-dHG/pJF-X from (A) CA, (B) FA, (C) bamboo extract, and (D) rice straw extract. The red arrows indicate the time points of feeding raw material solutions. The maximum OD_{660} values are shown in the graphs, respectively. The error bars represent the standard deviation of two replicates.

et al., 2020) for the production from biomass derivative as feedstock (see Supplementary Material).

Interestingly, although the same feeding rate of glucose was used in all tests (4.8 g/h), the strain growth in the tests using the grass biomass extracts (maximum $OD_{660} = 66.3 \pm 6.3$) was higher than that observed using the commercial products (maximum $OD_{660} = 46.9 \pm 7.8$) (Fig. 3). This result indicates the absence of toxic byproducts for microbial growth and the presence of other growth sources such as organic acids in the grass biomass extracts obtained by mild alkaline pretreatment. In previous study, a correlation was found between the microbial growth and the metabolism activity (Suzuki et al., 2021). This may be also related to the recycling use of cofactors mentioned above. Higher titers might be obtained if grass biomass extracts could be prepared on a larger pilot-plant scale.

3.3. 4S-3CML production from grass biomass extracts

P. putida PpY1100, whose genomic DNA encodes the *pcaHG* gene, was adopted as a host strain for 4*S*-3CML production. This host strain was engineered to produce 4*S*-3CML from CA and FA efficiently by introducing plasmids pJFVV2AB carrying the genes encoding FerA, FerB, LigV, BzaA and PobA, and pDVZ21HB carrying the genes encoding VanAB, PcaHG and CMLE of the PCA 3,4-cleavage pathway. The resulting strain was termed PpY1100/pJF-HB (Table 1).

Under similar conditions to those used for the PDC production, jar fermenter tests were conducted to evaluate the ability of PpY1100/pJF-HB to produce 4*S*-3CML from commercially available CA and FA (Fig. 4A, B). CA and FA were quickly metabolized and completely converted to 4*S*-3CML with molar yields of 98% or higher. The 4*S*-3CML titers were 10.5 ± 0.0 g/L for CA and 8.2 ± 0.3 g/L for FA as feedstocks (Table 3). Using the bamboo and rice straw extracts as a feedstock, the aromatic compounds contained in each extract were quickly metabolized and completely converted to 4*S*-3CML with molar yields of 95% or higher (Fig. 4C, D). The feeding rates were 0.99 ± 0.03 g/h of CA, 0.08 ± 0.01 g/h of FA, and 4.80 g/h of glucose in the bamboo extract and 0.33 ± 0.02 g/h of CA, 0.18 ± 0.01 g/h of FA, and 4.80 g/h of glucose in the rice straw extract. The 4*S*-3CML titers were 9.3 ± 0.5 g/L for the bamboo extract and 4.0 ± 0.1 g/L for the rice straw extract as feedstocks (Table 3). The strain growth in the tests of 4*S*-3CML productions using the grass biomass extracts (maximum $OD_{660} = 73.8 \pm 0.6$) was also higher than that observed using the commercial products (maximum $OD_{660} = 61.4 \pm 0.1$) similarly to PDC productions (Fig. 4).

This study demonstrated the production of 4*S*-3CML, which has been identified as an intermediate in PCA degradation by eukaryotes (Gross et al., 1956; Kondo et al., 2016; Martins et al., 2015; Mazur et al., 1994), from biomass derivatives for the first time (see Supplementary Material). The microbial funneling developed in this study can target the PDC or 4*S*-3CML products according to the applications.

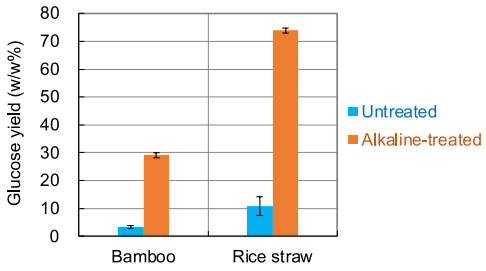

Fig. 5. Comparison of the saccharification efficiency of untreated and alkaline-treated lignocellulose in bamboo and rice straw. The error bars represent the standard deviation of two replicates.

3.4. Enzymatic accessibility of lignocellulose after HCAs extraction

To evaluate the enzymatic accessibility of lignocellulose after HCAs extraction, the enzymatic saccharification of lignocellulose was investigated. Briefly, lignocellulose was hydrolyzed using GODO cellulase F (Oenon Holdings, Inc., Japan) at 50 °C for 48 h with an enzyme loading of 30 FPU/g glucan. The results showed that the enzymatic saccharification efficiency was 8.9-fold higher in bamboo and 6.8-fold higher in rice straw than in untreated media (Fig. 5). These results indicate that the mild alkaline pretreatment afforded HCAs capable of being upgraded to a valuable single intermediate via microbial funneling, while improving the enzymatic accessibility of lignocellulose.

The enzymatic saccharification efficiency in rice straw was $73.8\% \pm 0.8\%$ while that in bamboo was lower at $29.5\% \pm 0.8\%$ (Fig. 5), which is mainly due to the fact that bamboo lignocellulose is composed of highly crystalline cellulose and lignin (Gao et al., 2021). The mild alkaline pretreatments adopted in this study at 80 °C or less under air atmosphere without pressure has the advantage of not generating toxic byproduct that inhibit the microbial funneling process. On the other hand, Gao et al. (2021) achieved a complete enzymatic saccharification of bamboo biomass with a yield of 100% w/w (respect to cellulose) by optimizing the steam explosion and subsequent green liquor treatment (see Supplementary Material). It will be necessary to employ such advanced methods to develop a more cost-effective and green-like process.

4. Conclusions

A green-like process was developed for grass biomass utilization consisting of enzymatic saccharification integrated with upgrading heterogeneous HCAs to a valuable single intermediate via microbial funneling. Notably, the *P. putida* PpY1100 engineered for efficient microbial funneling completely converted heterogeneous HCAs extracted from grass biomass to PDC and 4*S*-3CML with high titers of 3.9–9.3 g/L and molar yields of 92–99%, respectively. Since the process can be applied to various grass biomass sources with different HCAs composition, it can be expected to be applicable not only to bamboo and rice

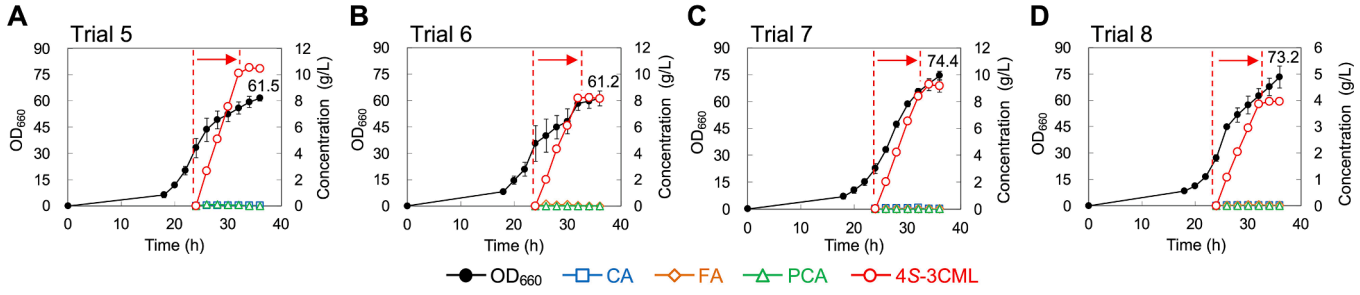

Fig. 4. 4*S*-3CML production mediated by PpY1100/pJF-HB from (A) CA, (B) FA, (C) bamboo extract, and (D) rice straw extract. The red arrows indicate the time points of feeding raw material solutions. The maximum OD_{660} values are shown in the graphs, respectively. The error bars represent the standard deviation of two replicates.

straw but also to other biomass sources.

CRediT authorship contribution statement

Yuzo Suzuki: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Yuriko Okamura-Abe:** Methodology, Investigation, Resources, Writing – review & editing. **Yuichiro Otsuka:** Methodology, Investigation, Resources, Writing – review & editing. **Takuma Araki:** Methodology, Investigation, Writing – review & editing. **Masanobu Nojiri:** Investigation, Writing – review & editing. **Naofumi Kamimura:** Resources, Writing – review & editing. **Eiji Masai:** Resources, Writing – review & editing. **Masaya Nakamura:** Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127836.

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