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Biosynthesis of polyhydroxyalkanoates from sugarcane molasses by recombinant *Ralstonia eutropha* strains

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Abstract−Sugarcane molasses was examined for the production of poly(3-hydroxybutyrate) [P(3HB)] and poly(3-hydroxybutyrate-*co*-lactate) [P(3HB-*co*-LA)] in recombinant *Ralstonia eutropha* strains expressing *Mannheimia succiniciproducens sacC* gene encoding *β*-fructofuranosidase, which can hydrolyze sucrose into glucose and fructose in the culture medium. When crude sugarcane molasses was added to the culture medium to support 20 g/L of sucrose in flask cultivation, the growth of *R. eutropha* NCIMB11599 expressing the *sacC* gene was significantly inhibited, which resulted in OD_{600} of 1.2 with P(3HB) content of 0.1 wt%. The inhibition of cell growth due to the usage of the crude sugarcane molasses was relieved by pretreatment of sugarcane molasses with activated charcoal. Sugarcane molasses pretreated with activated charcoal could support the growth of *R. eutropha NCIMB11599* expressing the *sacC* gene to OD_{600} of 87.2 with P(3HB) content of 82.5 wt% in batch fermentation when it was added to culture medium to support 20 g/L of sucrose. Also, *R. eutropha* 437-540 expressing *Escherichia coli ldhA* gene encoding lactate dehydrogenase along with the *sacC* gene produced P(3HB-*co*-6.2 mol%LA) with 29.1 wt% polymer content from sugarcane molasses in batch fermentation.

Keywords: *Ralstonia eutropha*, Sugarcane Molasses, Polyhydroxyalkanoates

INTRODUCTION

Along with the increasing concerns on environmental pollution, climate change and fossil fuel depletion, there have been several studies to reduce the use of petroleum-based materials by adopting biorefinery processes for the production of desired products from inexpensive resources [1-4]. More recently, plastic pollution including plastic islands and microplastics caused by an excessive use of non-degradable plastics derived from fossil fuels has emerged as one of the most pressing environmental issues [3,4]. Along with this rising concern to reduce hard-to-degrade plastics, environment-friendly polyhydroxyalkanoates (PHAs) have gained much attention as promising substitutes for petroleum-based plastics, of which around 80% are abandoned and accumulated in the environment [3-5]. PHAs are natural polyesters synthesized and accumulated as the carbon and energy storage materials in the cytoplasm of microorganisms [5-8]. PHAs as bio-competitive alternatives of general plastics find their applications in biomedical, electronics, construction, automotive, packaging and agricultural areas, with their complete biodegradability along with comparable material properties to conventional plastic materials [9,10].

One of the challenging problems for commercialization of PHAs is their production cost, much higher than petroleum-based polymers. Several factors such as PHA productivity, content, yield, recovery efficiency and the cost of the carbon substrate have been reported to highly affect the production cost of PHAs. Among these factors, the cost of carbon source has been the dominant bottleneck for the large scale fermentation and has most significantly affected general PHA production cost accounting almost 50% for the total cost [11,12]. For several decades, microbial fermentation processes have extensively been developed considering these factors for the cost-

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competitive production of PHAs. Besides the economic advantage, the carbon source also affects productivity and carbon yield of PHAs, cell growth, redox potential of cell metabolism and byproduct formation, all of which are closely related to the general production cost of PHAs. Thus, biorefinery processes for the efficient production of PHAs have extensively been developed based on the cheap carbon sources such as sucrose, glycerol, xylose, and starch to reduce total production cost of PHAs by employing natural and recombinant microbial host strains able to efficiently utilize these carbon sources [13-15].

Inexpensive and nutrient-rich sugarcane molasses can be obtained in large amount during sugar extraction from sugarcane. About 5 tons of molasses, containing around 50% sugars such as sucrose, glucose, and fructose, are generated from 100 tons of sugarcane process. Since the total sugar content of molasses is much higher than that of other lignocellulosic biomasses ranging from 40% to over 50%, sugarcane molasses has been suggested as one of the promising biomass-based carbon sources that can support higher carbon yield and productivity per unit mass of biomasses [16-22]. Also, higher sugar content is more beneficial to develop an economically feasible biomass pretreatment process to make cheaper sugars available for microbial fermentation processes [23,24]. The price of raw sugarcane molasses is relatively cheap, less than $20 per ton in average, thanks to its large quantity as a byproduct in the sugar industry based on about 200 million tons of crystalline sugar global market [25]. Therefore, sugarcane molasses has widely been examined as carbon source for microbial fermentative production of value-added chemicals such as amino acids (e.g. L-ornithine, L-threonine), mannitol, ethanol, 2,3-butanediol, succinic acid and polymers (e.g. poly(3-hydroxybutyrate) [P(3HB)]) by a variety of natural and recombinant bacteria such as *Lactobacillus reuteri* CRL 1101, *Saccharomyces cerevisiae*, *Enterobacter aerogenes*, *Escherichia coli* and *Bacillus cereus* SPV [18-22,26-31].

In spite of its advantages as a carbon source, one of the possible limiting factors of crude sugarcane molasses is its organic and inorganic inhibitors that would cause retarded cell growth [18]. Light and heavy metal ions, including potassium, sodium, calcium, iron, magnesium, and copper, are present in sugar cane molasses with relatively high content around 8.9% (w/w), which might result in osmotic inhibition on cell growth when sugarcane molasses is used as a feedstock without pretreatment [32]. Stronger inhibition on growth rate of host strains and on production of target products may be caused by the use of much higher concentration of sugarcane molasses, which is needed to support the same concentration of defined sugar. Therefore, several strategies to reduce the concentration of the inhibitory compounds in sugarcane molasses have been reported, in which sulfuric acid, potassium ferrocyanide, ion-exchange resin and activated carbon have been employed for the pretreatment of sugarcane molasses [24,32,33].

The other limitation lies in the utilization of sucrose. Even though sugarcane molasses contains a substantial amount of glucose and fructose as well, its main sugar component is sucrose. Thus, it is important to engineer a microbial host capable of utilizing sucrose along with glucose and fructose for the efficient PHA production from sugarcane molasses. *Ralstonia eutropha* (recently renamed as *Cupriavidus necator*) has widely been used for the synthesis of PHAs, such as poly(3-hydroxybutyrate) [P(3HB)] and its copolymers, because it can produce large amount of PHAs with high polymer content (i.e., up to 90% of cell dry weight) from glucose; however, wild type *R. eutropha*, which is known as one of the most effective host strains of PHAs, cannot utilize sucrose [34-36]. Broadening the range of the carbon sources, which are one of the main cost drivers in microbial fermentative processes, is important. Accordingly, there has been considerable effort to develop the metabolically engineered *R. eutropha* and *E. coli* strains to utilize defined sucrose for the production of PHAs through the expression of *Mannheimmia succiniciproducens* MBEL55E *sacC* gene encoding β-fructofuranosidase mediating the hydrolysis of sucrose to glucose and fructose [37,38].

In this study, we demonstrate the utilization of sugarcane molasses for the production of P(3HB) and poly(3-hydroxybutyrate-*co*-lactate) [P(3HB-*co*-LA)] by recombinant *R. eutropha* strains. Two approaches were examined for the efficient utilization of sugarcane molasses as a carbon source to enhance the cell growth and polymer accumulation during flask and batch cultivation of recombinant *R. eutropha* strains. First, crude sugarcane molasses was intermittently fed for cultivation to increase the total amount of the fed sucrose with reduced inhibition of cell growth. Secondly, sugarcane molasses pretreated by activated charcoal was used as a carbon source to support the enough sucrose concentration for high cell mass with high polymer content.

MATERIALS AND METHODS

1. Bacterial Strains and Plasmid

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (Stratagene Cloning Systems, La Jolla, CA, USA) was used for plasmid maintenance and *E. coli* S17-1 was used as a plasmid donor for conjugation experiments (Table 1). *R. eutropha* NCIMB11599 was used as a host strain for the production of P(3HB) from sugarcane molasses. *R. eutropha* 437-540 that expresses *Pseudomonas* sp. 6-19 PhaC1437 and *Clostridium propionicum* Pct540 [7] was used as a host strain for the production of P(3HB-*co*-LA) from sugarcane molasses. Plasmid pKM212-SacC for the expression of the *M. succiniciproducens sacC* gene under *tac* promoter and pKM212-SacCLdhA for the expression of the *M. succiniciproducens sacC* gene and *E. coli ldhA* gene under *tac* promoter have previously been described [37] (Table 1).

2. Culture Conditions

E. coli XL1-Blue and *E. coli* S17-1 were routinely cultured in Luria-Bertani (LB) medium, containing 10 g tryptone, 5 g yeast extract and 5 g NaCl per liter, at 37 °C. Chemically defined MR medium was used to examine the production of P(3HB) and P(3HB-*co*-LA) of *R. eutropha* strains from sugarcane molasses in flask cultures. MR medium (pH 7.0) contains (per liter) 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.8 g citric acid and 5 mL trace metal solution. The trace metal solution contains (per liter of 0.5 M HCl) 10 g $FeSO_4 \cdot 7H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 1 g $CuSO_4 \cdot 5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 0.02 g $Na_2B_4O_7 \cdot 10H_2O$. Seed cultures for flask cultures were prepared in 14 mL round tubes containing 2 mL of LB medium and were incubated at 30 °C overnight in a rotary shaker at 200 rpm. Then,

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference of source
Strains		
E. coli XL1-Blue	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F′ *proAB lacI*^{q}*ZΔM*15 Tn10 (Tet^{R})]	Stratagene
E. coli S17-1	Conjugative transfer of pK19mobSacB derivative plasmids into *R. eutropha* for gene replacement	ATCC
R. eutropha NCIMB11599	PHA producing bacteria	NCIMB
R. eutropha 437-540	*R. eutropha* NCIMB11599 derivative; the $phaCAB_{Re}$ genes are replaced with the $phaC1437_{Ps6\text{-}19}$ gene and the $pct540_{Cp}$ gene	[7]
Plasmids		
pKM212-MCS	pBBR1MCS2 derivative; *tac* promoter, *R. eutropha* PHA biosynthesis genes transcription terminator; Km^{R}	[7]
pKM212-SacC	pKM212-MCS derivative; *tac* promoter, *M. succiniciproducens sacC* gene, *R. eutropha* PHA biosynthesis genes transcription terminator; Km^{R}	[36]
pKM212-SacCLdhA	pKM212-MCS derivative; *tac* promoter, *M. succiniciproducens sacC* gene and *E. coli ldhA* gene, *R. eutropha* PHA biosynthesis genes transcription terminator; Km^{R}	[36]

1 mL of the overnight culture was used to inoculate 100 mL of MR medium supplemented with sugarcane molasses to support desired concentration of sucrose. Cells were further cultured at 30 °C in a rotary shaker at 250 rpm for 96 h.

Batch fermentation of recombinant *R. eutropha* strains was carried out in a 2.5-L jar fermenter (CNS Co. Ltd., Korea) containing 1 L of MR medium supplemented with pretreated sugarcane molasses with activated charcoal supporting desired concentration of sucrose at 100-200 rpm and 30 °C and pH was maintained as pH 6.9 by the addition of 28% (v/v) ammonia solution. Foam formation was suppressed by the addition of Antifoam 289 during fed-batch fermentation. Seed cultures were prepared in 250 mL flasks containing 100 mL LB medium at 200 rpm and 30 °C. The culture pH during fermentation was maintained at pH 7 by automatic addition of 28% (v/v) NH_4OH. Kanamycin (300 μg/ml) and gentamycin (10 μg/mL) were added in the culture medium in flask and batch cultures for recombinant *R. eutropha* strains. Kanamycin (30 μg/ml) was used for *E. coli* strains. Sugarcane molasses was sterilized by autoclaving at 121 °C for 15 min. During the flask cultivation, cell growth was monitored with $OD_{600\,nm}$ using UV-Visible spectrophotometer (UV-PharmaSpec 1700, Shimadzu) and the final DCW was measured. In batch fermentation, cell growth was measured by DCW in 6 h term after 24 h.

3. Analytical Methods

The sugar composition of sugarcane molasses was measured by high performance liquid chromatography (HPLC; 1260 infinity series; Agilent Technologies). Organic elements such C, H, O and N and ion were determined by elemental analyzer (EA, Flash 2000 CHNS/O Analyzers; Thermo Fisher Scientific Inc., Waltham, MA, USA) and energy dispersive X-ray fluorescence (ED-XRF) spectrometer (ARL Quant'X ED-XRF spectrometer; Thermo Fisher Scientific Inc.), respectively. The content and monomer composition of polymers accumulated in the cells were determined by gas chromatography (GC) using Agilent 6890N GC system (Agilent Technologies, CA, USA) equipped with Agilent 7683 automatic injector, flame ionization detector and a fused silica capillary column (SPB^{TM}-5, 30 m×0.32 mm ID, 0.25 μm film; Supelco, Bellefonte, PA, USA) [39]. All the samples used for the analytical standard were purchased from Sigma-Aldrich (St. Louis, MO).

4. Preparation of Sugarcane Molasses

Sugarcane molasses (Paik Kwang industrial Co., LTD., Seoul, Republic of Korea) was found to contain 390 g/L sucrose, 138 g/L glucose and 227 g/L fructose and some metal ions (Table 2). It was diluted with deionized water to meet the sucrose concentrations of the experimental design. For pretreatment, sugarcane molasses was initially diluted with deionized water (1 : 4) and 20 wt% of activated charcoal was added. After stirring the solution for 1 h at room temperature, activated charcoal was removed by centrifugation. The supernatant was filtered to remove debris and was autoclaved for sterilization in prior to use as substrate for the experiment. After

Table 2. Composition of Sugarcane molasses

Composition	Before treatment	After treatment
Sugar (g/L)		
Sucrose	390 g/L	343 g/L
Glucose	138 g/L	128 g/L
Fructose	227 g/L	200 g/L
Elements (wt%)		
C H O	96.884	97.98
Si	0.48	ND
P	0.05	ND
S	0.23	0.19
Cl	0.57	0.63
K	0.96	0.97
Ca	0.77	0.21
Ti	0.01	ND
Fe	0.04	0.01

ND: Not detected

the pretreatment, it was found to contain 343 g/L sucrose, 128 g/L glucose and 200 g/L fructose and less metal ions (Table 2). The pH of each sugarcane molasses solution was adjusted to neutral before it was autoclaved.

RESULTS AND DISCUSSION

1. PHA Production from Sugarcane Molasses by *R. eutropha* Strains in Flask Cultivation

Since sugarcane molasses was found to contain 390 g/L sucrose, 138 g/L glucose and 227 g/L fructose (Table 2), *R. eutropha* strains previously engineered to utilize sucrose by introduction of *M. succiniciproducens* SacC [37] were used as host strains for the production of P(3HB) and P(3HB-*co*-LA) from the renewable resource, sugarcane molasses. When sugarcane molasses was added to the culture medium as a sole carbon source to support 20 g/L sucrose as much amount as in previous study [37], the recombinant *R. eutropha* NCIMB11599 harboring pKM212-SacC did not grow or produce P(3HB) at all in flask cultivation (Table 3; Fig. 1(a)). Even though the strain hydrolyzed 14.5 g/L sucrose into glucose and fructose, 4.3 g/L of sucrose still remained at 96 h and the concentration of glucose and fructose kept increasing through the whole cultivation time from 5.8 and 8.0 g/L to 11.9 and 15.6 g/L (Fig. 1(a)), respectively, indicating that some inhibitors in sugarcane molasses may seriously inhibit both the cell growth and the production of P(3HB) in the recombinant *R. eutropha*.

Therefore, a reduced amount of sugarcane molasses was then fed to the recombinant *R. eutropha* to support 10 g/L sucrose. As shown in Fig. 1, the strain could grow from OD_{600} of 1.3 to 17.3 after 96 h, and produce 2.0 g/L P(3HB) with a content of 46.9 wt% in flask cultivation (Table 3; Fig. 1(b)). 9.4 g/L of sucrose was completely hydrolyzed after 48 h, and glucose and fructose increased from 3.3 and 4.6 g/L to 5.4 and 7.8 g/L along with the decrease of sucrose, and then decreased to 0.6 and 6.6 g/L (Fig. 1(b)). As β-fructofuranosidase was reported to be secreted into the medium and have high activity toward sucrose, the recombinant strain could hydrolyze 20 g/L of defined sucrose into glucose and fructose in very early stage of cultivation in the previous report [37]. However, when sugarcane molasses was added to support 10 g/L sucrose, it took 48 h to be completely hydrolyzed even though the concentration of sucrose was less than the one in the previous study [37]. And even after 96 h, 6.6 g/L of residual fructose remained, 2.0 g/L more than the starting concentration, 4.6 g/L.

Thus, a strategy of intermittent feeding was employed in order to increase the total amount of sugarcane molasses with less negative effect on the cell growth and P(3HB) production derived from the high concentration of sugarcane molasses. Sugarcane molasses corresponding to 5 g/L of sucrose in the culture medium was added four times at specific time intervals of 24 h, which provided the total 20 g/L sucrose in flask cultivation. This intermittent feeding significantly relieved the cell growth inhibition and as a result, *R. eutropha* NCIMB11599 harboring pKM212-SacC grew to an OD_{600} of 50.6 at 96 h with PHA content and concentration of 53.0 wt% and 4.6 g/L, respectively (Table 4; Fig. 2(a)). Each 5 g/L sucrose was completely hydrolyzed at every 24 h after addition; however, the concentration of fructose still kept increasing for 96 h from 2.1 to 12.5 g/L, and 2.6 g/L glucose still remained in the culture medium at the end of cultivation (Fig. 2(a)). The intermittent feeding to *R.*

Table 3. Biosynthesis of P(3HB) by flask-cultures of recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC) in sugarcane molasses based MR medium initially containing 10 g/L of sucrose and 20 g/L of sucrose for the synthesis of P(3HB)

Strain	Plasmids	Initial sucrose concentration (g/L)	DCW (g/L)	PHA conc. (g/L)	PHA content (wt%)
R. eutropha NCIMB11599	pKM212-SacC	20 g/L	1.43	0.003	0.11
		10 g/L	4.38	2.05	46.87

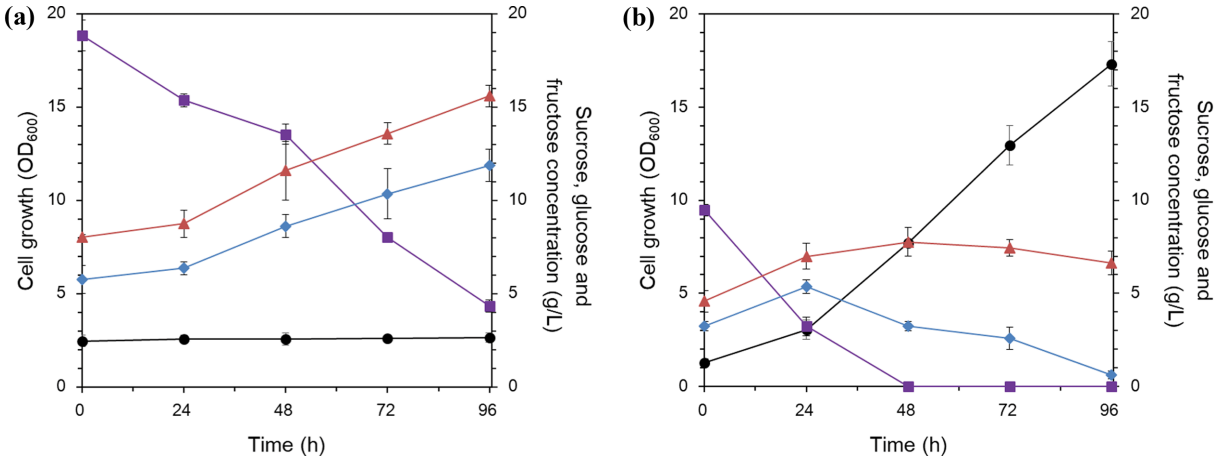

Fig. 1. Time profiles of flask-cultures of recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC) in sugarcane molasses based MR medium initially containing (a) 20 g/L of sucrose and (b) 10 g/L of sucrose for the synthesis of P(3HB) (Symbols: purple Square, sucrose concentration; blue diamond, glucose concentration; red triangle, fructose concentration; closed circle, cell growth).

Table 4. Biosynthesis of P(3HB) and P(3HB-co-LA) by flask-cultures of recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC) and *R. eutropha* 437-540 (pKM212-SacCLdhA) in sugarcane molasses based MR medium initially containing 5 g/L of sucrose. During the cultures, an appropriate amount of molasses solution was supplemented to the broth at every 24, 48, and 72 h

Strain	Plasmids	Polymer	DCW (g/L)	PHA conc. (g/L)	LA fraction (mol%)	PHA content (wt%)
R. eutropha NCIMB11599	pKM212-SacC	P(3HB)	8.68	4.60	0	53.0
R. eutropha 437-540	pKM212- SacCLdhA	P(3HB-*co*-LA)	3.10	0.58	5.79	18.7

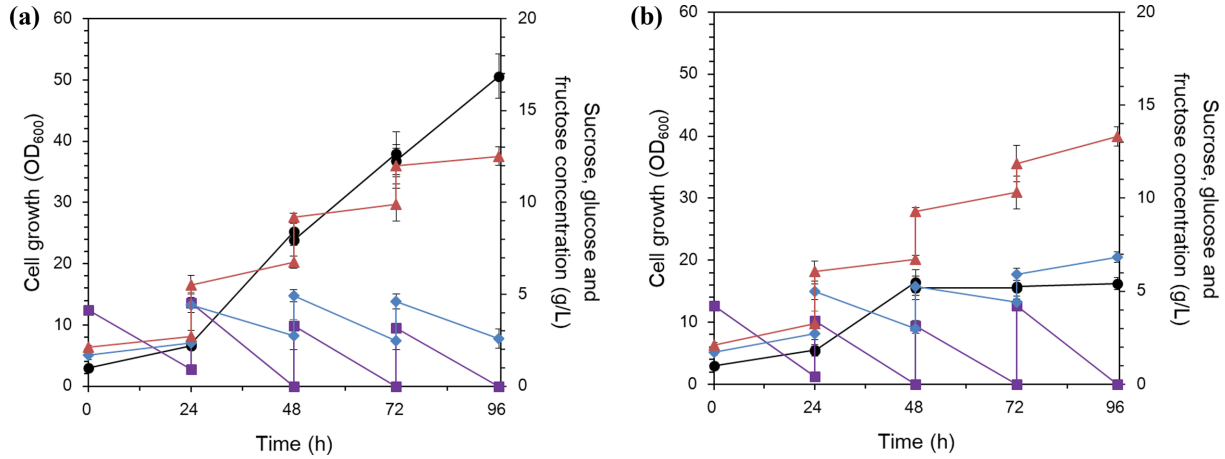

Fig. 2. Time profiles of flask-cultures of (a) recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC), (b) *R. eutropha* 437-540 (pKM212-EcLdhA-SacC) in sugarcane molasses based MR medium initially containing 5 g/L of sucrose for the synthesis of P(3HB). During the cultures, an appropriate amount of molasses solution was supplemented to the broth at every 24, 48, and 72 h (Symbols: purple Square, sucrose concentration; blue diamond, glucose concentration; red triangle, fructose concentration; closed circle, cell growth).

eutropha 437-540 harboring pKM212-SacCLdhA strain, which was previously developed to produce P(3HB-*co*-LA), showed similar trend in sucrose consumption with *R. eutropha* NCIMB11599 harboring pKM212-SacC, but with more remnant monosaccharide, 6.8 g/L glucose and 13.3 g/L fructose (Fig. 2(b)). The strain grew to OD_{600} of 16.2 and produced 18.7 wt% P(3HB-*co*-5.8 mol% LA) (Table 4).

The intermittent addition of sugarcane molasses to support 5 g/L sucrose into the culture medium led to more than doubled amount of PHB production in the recombinant *R. eutropha* NCIMB11599 harboring pKM212-SacC in flask scale. However, such strategy was not as effective in fermenter scale, with no cell growth nor PHA production (data not shown). Also, a certain amount of glucose and fructose still remained in both recombinant strains, indicating that it is not a suitable solution enough for the efficient utilization of total sugar. The potential inhibitory compounds still present in sugarcane molasses and the intermittent feeding may cause the accumulation of the inhibitors, which is fatal in fermenter scale cultivation. This might lead to some interruption in the complete sugar consumption of the strains, such as glucose and fructose, requiring further treatment to resolve the inhibition.

2. PHA Production from Activated Charcoal Treated Sugarcane Molasses by *R. eutropha* Strains in Flask Cultivations

As the crude sugarcane molasses may contain the undesirable substances which may affect the growth of the host strain and the production of PHAs, several pretreatment methods of sugarcane molasses have been developed by employing sulfuric acid, activated carbon, calcium hydroxide, and potassium ferrocyanide [24,32,33]. The effectiveness of the pretreatment for sugarcane molasses has been examined for the production of several chemical compounds in *Actinobacillus succinogenes* [32] and *Corynebacterium glutamicum* [33]. Among those various substances for pretreatment, activated charcoal is known as a standard reagent for removal of light/heavy metals, dissolved organic matters and pigments [40,41]. Since activated charcoal has low cost and does not significantly affect the total sugar quantity, it has been preferred for the pretreatment of agricultural waste carbon sources over other methods such as the use of ion-exchange resins [40,42,43]. Therefore, in this study, pretreatment of sugarcane molasses by activated charcoal was applied for more efficient utilization of sugarcane molasses. In advance of microbial cultivation, the composition of organic and inorganic matters was analyzed before and after treatment (Table 2). Through the pretreatment with activated charcoal, only 12.1% of sucrose, 7.2% of glucose, and 11.9% of fructose were eliminated while silicon, phosphorus, calcium, and titanium significantly decreased (Table 2).

To verify the effect of the pretreatment with activated charcoal on the cell growth and PHA production, cultivation of the recombinant *R. eutropha* strains was carried out in flask scale. In the flask scale with the pretreated sugarcane molasses to support 20 g/L of sucrose, *R. eutropha* NCIMB11599 harboring pKM212-SacC grew to final OD_{600} of 49.0 at 96 h. The result is much higher than the one obtained with sugarcane molasses to support 20 g/L of sucrose without treatment (OD_{600} of 2.6) (Fig. 1(a); Fig. 3(a)). In addition, the recombinant *R. eutropha* NCIMB11599 harboring pKM212-

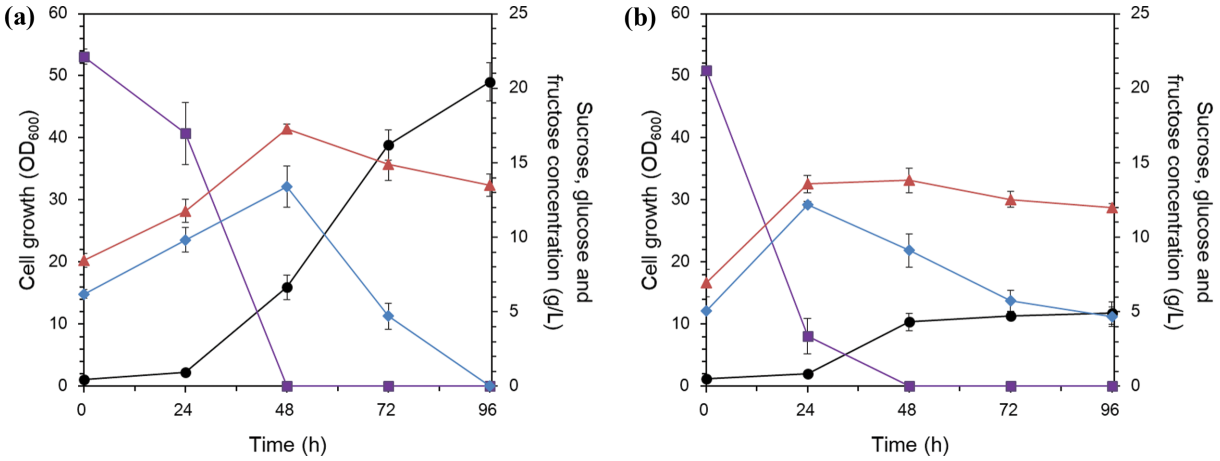

Fig. 3. Time profiles of flask-cultures of (a) recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC), (b) *R. eutropha* 437-540 (pKM212-EcLdhA-SacC) in charcoal-treated sugarcane molasses based MR medium initially containing 20 g/L of sucrose for the synthesis of P(3HB) (Symbols: purple Square, sucrose concentration; blue diamond, glucose concentration; red triangle, fructose concentration; closed circle, cell growth).

Table 5. Biosynthesis of P(3HB) and P(3HB-co-LA) by flask-cultures of recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC) and *R. eutropha* 437-540 (pKM212- SacCLdhA) in charcoal-treated sugarcane molasses based MR medium initially containing 20 g/L of sucrose

Strain	Plasmids	Polymer	DCW (g/L)	PHA conc. (g/L)	LA fraction (mol%)	PHA content (wt%)
R. eutropha NCIMB11599	pKM212-SacC	P(3HB)	5.57	3.02	0	54.1
R. eutropha 437-540	pKM212- SacCLdhA	P(3HB-*co*-LA)	2.89	0.53	9.32	18.3

SacC could produce high amount of P(3HB) as high as 3.0 g/L from activated charcoal treated sugarcane molasses with 54.1 wt% P(3HB) content, whereas only 0.003 g/L P(3HB) with 0.1 wt% was produced from crude sugarcane molasses (Table 3; Table 5). The sugarcane molasses pretreated with activated charcoal also led to more efficient utilization of total sugar with less amount of remnant fructose, 13.5 g/L and no remnant glucose (Fig. 3(a)). Even though the pretreatment method caused 7 to 12% decrease in the sugar concentration of sugarcane molasses, it augmented PHA production and consumption of glucose and fructose by *R. eutropha* NCIMB11599 harboring pKM212-SacC. *R. eutropha* 437-540 harboring pKM212-SacCLdhA produced P(3HB-*co*-LA) with more lactic acid fraction when sugarcane molasses treated with activated charcoal was used. P(3HB-*co*-9.3 mol%LA) with a polymer concentration and content of 0.5 g/L and 18.3 wt% was produced from sugarcane molasses treated with activated charcoal (Table 5).

In both recombinant strains, 20 g/L sucrose was completely hydrolyzed after 48 h. The reaction was much slower than the one in previous study with 20 g/L defined sucrose [37], but almost twice faster than the one with crude sugarcane molasses supporting 10 g/L sucrose (Fig. 1(b)). Since it is known that there are some organic and inorganic matters which have negative effect on cell growth, enzyme activity, etc. [44-46], the activity of SacC might be inhibited with crude sugarcane molasses compared to the one with pure sucrose [37]. The activated charcoal pretreatment would reduce both organic and inorganic inhibitors [40,41], and this might have increased the ability of the strain to hydrolyze sucrose, as well as the utilization of sugarcane molasses, cell growth and productivity [47,48].

Although the production of PHA and the growth of *R. eutropha* strains were improved by activated charcoal pretreatment in the flask cultivation of sugarcane molasses, still high portion of initial fructose in the sugarcane molasses remained not utilized until the end of batch (13.5 g/L by *R. eutropha* NCIMB11599 harboring pKM212-SacC and 12 g/L by *R. eutropha* 437-540 harboring pKM212-SacCLdhA) (Fig. 3). Fructose was not efficiently utilized as carbon source in flask cultivation by recombinant *R. eutropha* strains [37].

3. Batch Fermentation of Sugarcane Molasses by *R. eutropha* Strains in 2.5L Reactors

R. eutropha NCIMB11599 harboring pKM212-SacC could completely consume sugars derived from sugarcane molasses with an initial sucrose concentration of 20 g/L within 36 h in batch fermentation, whereas it took 96 h for glucose with no complete consumption for fructose in flask cultivation (Fig. 3(a); Fig. 4(a)). *R. eutropha* 437-540 harboring pKM212-SacCLdhA is known to have lower cell growth and PHA accumulation than *R. eutropha* NCIMB11599 strain [7], but compared to its flask cultivation, it showed better performance in sugar consumption as it completely consumed glucose within 60 h while 4.7 g/L glucose were left in flask scale. 6.9 g/L fructose, which was less than 12.0 g/L in flask scale, however, still remained in the batch fermentation (Fig. 4(b)), indicating that its productivity or yield of total PHA production may be improved with metabolic engineering for enhanced fructose metabolism, such as introduction of the gene encoding fructokinase [49].

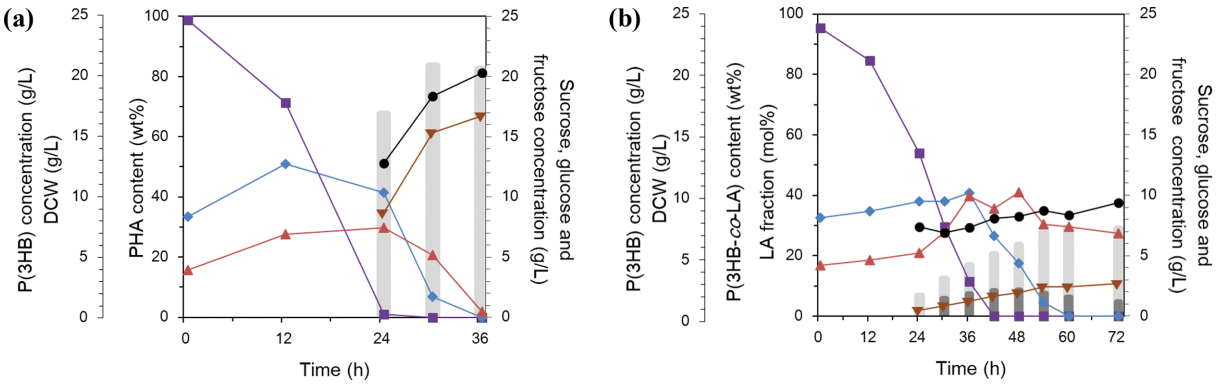

Fig. 4. Time profiles of batch fermentation of (a) recombinant *R. eutropha* NCIMB 11599 (pKM212-SacC), (b) *R. eutropha* 437-540 (pKM212-EcLdhA-SacC) in charcoal-treated sugarcane molasses based MR medium initially containing 20 g/L of sucrose for the synthesis of P(3HB) (Symbols: purple Square, sucrose concentration; blue diamond, glucose concentration; red triangle, fructose concentration; closed circle, dry cell weight; brown inverted triangle, PHA concentration; light grey bar, PHA content; dark grey bar, lactate fraction).

Batch fermentation with maintaining constant pH and aeration enhanced the hydrolysis of sucrose and the utilization of glucose than flask cultivation, which makes cells consume more fructose after using glucose. Unlike the flask cultivation of sugarcane molasses, *R. eutropha* NCIMB11599 harboring pKM212-SacC completely consumed fructose and only half amount of fructose remained in *R. eutropha* 437-540 harboring pKM212-SacCLdhA in batch fermentation. These results indicate that the batch fermentation at reactor scale promotes the cell to utilize sugar more rapidly and more efficiently than flask cultivation. This result is in accordance with the previous studies that *R. eutropha* strains consumed more fructose derived from sucrose and rice bran under the batch fermentation systems controlled with constant pH and aeration [15,37].

The recombinant *R. eutropha* NCIMB11599 harboring pKM212-SacC grew up to 20.3 g/L DCW and produced P(3HB) to 16.8 g/L, which was 5.6-fold higher than 3.0 g/L in flask scale with P(3HB) content of 82.5 wt% in 36 h (Fig. 4(a)). *R. eutropha* 437-540 harboring pKM212-SacCLdhA grew to 8.4 g/L DCW and produced P(3HB-*co*-LA) with 6.2 mol% LA and a polymer concentration of 2.4 g/L, which was 4.8 times higher than 0.5 g/L in flask scale with 29.1 wt% content in 60 h. Both recombinant strains produced much more PHAs in batch fermentation than in flask scale, with faster hydrolysis of sucrose and more consumed sugars.

Generally, sugarcane molasses has been utilized as supplement or co-utilized with other substrate such as acetate for production of PHA because the high concentration of sugarcane molasses could provoke toxicity in cell viability [50]. When sugarcane molasses was utilized as a sole carbon source, diverse microorganisms were usually co-cultivated or fermented in advance [14,17,20,40,51]. The results of this study demonstrate that the pretreated sugarcane molasses to support a concentration of sucrose as high as 20 g/L could be feasible as a feedstock for P(3HB) and P(3HB-*co*-LA) production via aerobic batch fermentation with pure culture of recombinant *R. eutropha* strain.

CONCLUSION

Sugarcane molasses is a cheap substrate with rich sugar content that can be employed as an alternative to other expensive carbon sources. Several strategies, including intermittent substrate feeding and pretreatment using activated charcoal, were conducted for more efficient utilization of sugarcane molasses. These strategies reduced the negative effect of sugarcane molasses on the cell growth and PHA production during the cultivation of the recombinant *R. eutropha* strains. Thus, the strains could successfully produce P(3HB) and P(3HB-*co*-LA) from the pretreated sugarcane molasses as a sole carbon source. Even though there still remains the possibility of other strategies, such as adaptive evolution for crude sugarcane molasses or synthetic biological tools for better utilization of sugarcane molasses, this study should be very helpful in broadening the availability of sugarcane molasses for biorefinery processes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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