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Polyhydroxyalkanoates production from cellulose hydrolysate in *Escherichia coli* LS5218 with superior resistance against 5-hydroxymethylfurfural

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Abstract

Poly[3-hydroxybutyrate-*co*-3-hydroxyvalerate (3HV)] was produced in recombinant *Escherichia coli* LS5218 from the ruthenium-catalyzed cellulose hydrolysate and propionate. The strain was found to be resistant against 5-hydroxymethylfurfural (5-HMF), which is a major inhibitory byproduct generated in the cellulose hydrolysis reaction. The 3HV fraction was successfully regulated in the range of 5.6 - 40 mol%.

Keywords; Biobased plastic; Renewable biomass; 5-HMF tolerance; Polyhydroxybutyrate; P(3HB-*co*-3HV); Cellulose

Introduction

Polyhydroxyalkanoates (PHAs) are a class of storage polymers synthesized by various microorganisms (1). These polymers have been considered as alternatives to petroleum-derived materials. However, commercialization efforts of these polymers have been hampered by high production cost, significantly contributed by the carbon sources used for their production (1, 2). Therefore, it is essential to use inexpensive carbon substrates for the production of PHAs so as to render them cost competitive. One such inexpensive carbon-rich source is cellulose, which can be easily obtained from plant resources such as wood, grasses, straw and so on. Furthermore, cellulosic biomass is inedible therefore its usage is not in direct competition with food or animal feeds production (2, 3).

Cellulose is a high molecular weight polymer of glucose molecules that cannot be utilized directly by most bacteria. Therefore, its hydrolysis into glucose is essential to be metabolized for bacterial PHAs production. For example, Yu and Stahl

used sulfuric acid to hydrolyze bagasse to obtain glucose and xylose that were used to produce up to 65 wt% poly(3-hydroxybutyrate) [P(3HB)] in *Ralstonia eutropha* (2). Though acid catalysts have been used for hydrolysis of cellulosic biomass, they have several limitations such as; they are not reusable, generate large amounts of neutralization waste and corrode equipments (2). The use of the more mild and environmentally friendly cellulase-catalyzed cellulose hydrolysis has been investigated, but the reaction is slow and the cost of enzymes is high thereby presenting obstructs in industrial applications (3).

To overcome those obstructs, Kobayashi et al. utilized ruthenium (Ru) as an alternative catalyst for hydrolyzing cellulose (4). It is advantageous in that the solid Ru-catalyst can be easily filtered from the reaction mixture and reused in repeated reactions. In our previous study, Ru-catalyzed cellulose hydrolyzation provided glucose as the major product and the hydrolysate was directly fed to recombinant *Escherichia coli* cells that accumulated up to 42 wt% P(3HB). *E. coli* is a useful platform because it utilizes cellulosic biomass-derived sugars, and can be easily genetically modified to produce a variety of PHAs. However, when the hydrolysate was fed to give higher glucose concentrations, there was no cell growth. It was then demonstrated that 5-hydroxymethylfurfural (5-HMF) was the main byproduct contributing to the cell growth inhibition of the hydrolysate to the cells (5). The use of 5-HMF resistant strains is one of the approaches of overcoming hydrolysate toxicity for further improved PHAs production (5, 6). Thus, the aim of this study was to use 5-HMF resistant *E. coli* strains for the improved production of PHAs from Ru-catalyzed cellulose hydrolysate.

The cellulose hydrolysis procedure was as previously described (5). Briefly, 2.59 g of cellulose was mixed with Ru/ γ -Al₂O₃ catalyst and 20 mL of water then

heated in a reactor at 215°C. The hydrolysate was cooled down and subjected to component analysis by HPLC (5). The major products of the hydrolysate were glucose (10.8% (mol-carbon) yield corresponding to concentration of 1.39 wt%) and water-soluble oligosaccharides (total 5% yield: cellobiose 2.7%, cellotriose 1.4% and others 0.9%), and minor ones were fructose (1.9%), mannose (0.8%), levoglucosan (0.5%), 5-HMF (1%) and furfural (0.6%). The pH of the cellulose hydrolysate was adjusted to pH 7 by 1 N NaOH then the hydrolysate was used to prepare the culture medium containing 1 wt% glucose for polymer production.

In order to screen for 5-HMF resistance, five *E. coli* strains were investigated. The strains were; *E. coli* DH5 α and *E. coli* JM109 (Takara, Japan) which are laboratory strains widely used in genetic and molecular studies. Others were; *E. coli* LS5218 (7), a *fadR* and *atoC(con)* mutant that constitutively expresses the enzymes involved in the utilization of fatty acids and used in the production of PHA copolymers (8, 9, 10) and *E. coli* JW2978, Keio collection mutant ($\Delta yqhD$) that was shown to have improved resistance against 5-HMF (11, 12). *E. coli* BL21 (13) representing *E. coli* B strain was also investigated. All the strains were grown at 37°C overnight in LB medium. Aliquots of the overnight cultures were then transferred (1:100 dilution) into fresh LB medium supplemented with variable concentrations of 5-HMF (0, 2.0, 2.5, 3.0 and 4.0 g/L) and cultured at 37°C. The 5-HMF resistance of each strain was spectrophotometrically measured from the ratios of optical densities (OD₆₀₀) at 600 nm.

The cell growth of the *E. coli* strains after 22 hours of culturing at different concentrations of 5-HMF is shown in Fig. 1A. At 2.0 g/L of 5-HMF, all the strains had a less than 5% growth inhibition compared to respective cells growing in the absence of 5-HMF. When the concentration of 5-HMF was increased to 2.5 g/L, all

the strains grew but with some inhibition. At 3.0 g/L of 5-HMF, *E. coli* LS5218 and JW2978 had a less than 20% growth inhibition, whereas strains BL21, DH5 α and JM109 had over 80% growth inhibition (Fig. 1A). The time course of the *E. coli* strains grown in the presence of 3.0 g/L 5-HMF is shown in Fig. 1B. Although *E. coli* LS5218 and JW2978 had lag phases, they had a less than 20% growth inhibition, demonstrating their superior 5-HMF resistance compared with the other strains. Therefore, we selected *E. coli* LS5218 for polyester production from Ru-catalyzed cellulose hydrolysate, because in addition to its superior 5-HMF resistance, this strain has been shown to be capable of synthesizing various useful 3HB-based (15) and lactate(LA)-based (15, 16) copolymers such as poly[3HB-co-3-hydroxyvalerate(3HV)] (8), P(3HB-co-3-hydroxyalkanoates) (10) and P(LA-co-3HB-co-3-hydroxyhexanoate) (9). Here, we attempted to produce P(3HB-co-3HV) from cellulose hydrolysate as a typical example to demonstrate the potential application of *E. coli* LS5218. P(3HB-co-3HV) is an attractive polymer as incorporation of the 3HV fraction to approximately 20% improves the strength and flexibility of the polymer compared to P(3HB) homopolymer which is highly crystalline and brittle (1, 8).

<Fig. 1 here>

The production of P(3HB-co-3HV) was carried out in *E. coli* LS5218 harboring pGEM*phaCAB* (17) which bears *phaC*, *phaA* and *phaB* genes from *R. eutropha* encoding the three P(3HB) biosynthetic enzymes (PHA synthase, β -ketothiolase, acetoacetyl-CoA reductase, respectively). Cells were grown on the cellulose hydrolysate and propionate which is a precursor for supplying the 3HV monomer. *PhaA* and *PhaB* also act as a 3HV-CoA supplying enzymes from propionate as follows; propionate is activated into propionyl-CoA which is then condensed with

acetyl-CoA by PhaA forming 3-ketovaleryl-CoA. Subsequently, 3-ketovaleryl-CoA is converted by PhaB into 3HV-CoA that could be finally incorporated into the growing polymer by PhaC to form P(3HB-*co*-3HV) (8, 18).

The cells were cultivated in 2 mL LB medium supplemented with 100 $\mu\text{g/L}$ of ampicillin at 37°C for 12 h and harvested by centrifugation then resuspended in fresh LB medium containing 100 $\mu\text{g/L}$ of ampicillin, cellulose hydrolysate giving glucose concentration of 1.0% (w/v) and variable concentrations of propionate (0, 5, 10, 20, and 40 mM) in a total volume of 2 mL each. Cultivation of the cells without propionate was done for the production of P(3HB). Cultures having the same concentration of analytical grade glucose prepared likewise were used as a control. Subsequently, the cells were cultivated at 30°C for 60 h, harvested by centrifugation and lyophilized. Polymer accumulated in the cells was extracted with chloroform and applied to gas chromatography/mass spectrometry (GC/MS) analysis as described by Arai et al. (19).

Table 1 summarizes the cell dry weight, P(3HB-*co*-3HV) and P(3HB) yields from both cellulose hydrolysate and analytical grade glucose in *E. coli* LS5218. Growth and polymer production from cellulose hydrolysate (at 1 wt% glucose concentration) indicates that *E. coli* LS5218 was resistant against inhibitors including 5-HMF present in the hydrolysate. In the absence of propionate, the P(3HB) content and polymer yield from the cellulose hydrolysate were 59 wt% and 3.3 g/L, respectively. These results were almost the same with those obtained from analytical grade glucose; 58 wt% and 3.4 g/L for P(3HB) content and yield, respectively. The P(3HB) yield was improved from our previous report (42 wt%) using *E. coli* JM109 (5). When propionate was added, the 3HV fraction in the polymer ranged between 5.6 - 40 mol% depending on the propionate concentration in the culture medium

(Table1). These 3HV fractions provide copolymers with altered thermal and physical properties with potential as materials for a wide range of applications compared to P(3HB) homopolymer (8). In all cases, analytical grade glucose had slightly higher 3HV fraction than cellulose hydrolysate. In contrast, cellulose hydrolysate had higher polymer yield (g/L) than analytical grade glucose probably due to the presence of other sugars such as mannose and fructose that could be metabolized for polymer production. There was a general trend where cell weight (g/L) and polymer yield (g/L) decreased with increase in propionate concentrations due to toxicity of propionate to the cells. This phenomenon had been previously reported by Slater et al. (8).

<Table 1 here>

In conclusion, *E. coli* LS5218 was shown to be relatively 5-HMF resistant and was used for the cost-effective production of P(3HB-co-3HV) and P(3HB) polyesters from the Ru-catalyzed cellulose hydrolysate. The yields for cell growth and polymers were almost the same with those performed for the case of analytical grade glucose. It was reported that *E. coli* LS5218 was capable of degrading furans including furfuryl alcohol (20). Because of structural similarity between furfuryl alcohol and 5-HMF, 5-HMF might be similarly degraded thereby conferring *E. coli* LS5218 with 5-HMF resistance. The *E. coli* LS5218 strain could be applicable to the production of the other types of copolyesters with different material properties by using the Ru-catalyzed hydrolysate of cellulose as well as hemicellulose.

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Figure legends

Fig. 1 (A) OD₆₀₀ of *E. coli* strains grown on LB medium containing different concentrations of 5-HMF after 22 hours. Symbols: white, absence of 5-HMF; diagonal, 2 g/L; checked, 2.5 g/L; black, 3.0 g/L; dotted, 4 g/L. **(B)** Time course of growth of *E. coli* strains on LB medium in the presence of 3 g/L of 5-HMF. Closed diamonds, JM109 cells growing in the absence of 5-HMF (positive control); closed circles, DH5 α ; closed triangles, JM 109; open squares, LS5218; closed squares, JW2978; open triangles, BL21

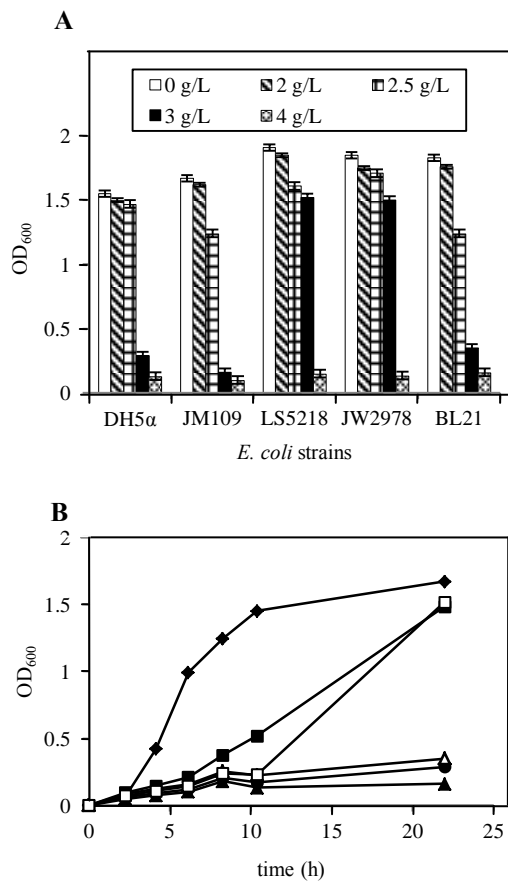


FIG. 1

TABLE 1. The growth and biosynthesis of P(3HB-*co*-3HV) and P(3HB) by *E. coli* LS5218 from cellulose hydrolysate and propionate.

propionate concentration (mM)	cellulose hydrolysate				analytical grade glucose			
	cell dry weight (g/L)	polymer yield (g/L)	polymer content (wt%)	3HV fraction ^a (mol%)	cell dry weight (g/L)	polymer yield (g/L)	polymer content (wt%)	3HV fraction (mol%)
0	5.6±0.4	3.3±0.2	59	0	5.9±0.3	3.4±0.3	58	0
5	4.2±0.6	2.1±0.4	50	5.6±1.6	3.7±0.6	1.7±0.3	46	6.8±2.5
10	3.3±0.2	1.5±0.4	45	9.8±3.8	3.1±0.5	1.2±0.2	39	13±2
20	2.7±0.3	0.4±0.1	15	28±3	2.5±0.5	0.2±0	8	30±3
40	2.7±0.1	0.2±0	7	40±4	2.5±0.4	0.2±0	8	42±2

^a3HV, 3-hydroxyvalerate. The standard deviations are from duplicate measurements.