HOKKAIDO UNIVERSITY

Title	Chemo-microbial conversion of cellulose into polyhydroxybutyrate through ruthenium-catalyzed hydrolysis of cellulose into glucose
Author(s)	Matsumoto, Ken'ichiro; Kobayashi, Hirokazu; Ikeda, Koji; Komanoya, Tasuku; Fukuoka, Atsushi; Taguchi, Seiichi
Citation	Bioresource Technology, 102(3), 3564-3567 https://doi.org/10.1016/j.biortech.2010.09.098
Issue Date	2011-02
Doc URL	http://hdl.handle.net/2115/45033
Type	article (author version)
File Information	BT102-3_3564-3567.pdf

Instructions for use

Chemo-microbial conversion of cellulose into polyhydroxybutyrate through ruthenium-catalyzed hydrolysis of cellulose into glucose

Ken'ichiro Matsumoto [a,c], Hirokazu Kobayashi [b,c], Koji Ikeda [a], Tasuku Komanoya [b,c], Atsushi Fukuoka [b,c], and Seiichi Taguchi [a,c,*]

[a] *Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo, 060-8628, Japan.* [b] *Catalysis Research Center, Hokkaido University, N21W10, Kita-ku, Sapporo, 001-0021, Japan.* [c] *Division of Chemical Sciences and Engineering, Graduate School of Chemical Sciences and Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo 060-8628, Japan.*

* Corresponding author. Tel./fax: +81 11 706 6610. *E-mail*: staguchi@eng.hokudai.ac.jp

Keywords: polyhydroxyalkanoate; cellulose hydrolysate; supported ruthenium catalyst;

Abstract

Cellulose-derived glucose generated using the supported ruthenium catalyst was applied to poly(3-hydroxybutyrate) [P(3HB)] production in recombinant *Escherichia coli*. By the reaction with the catalyst at 220 °C, 15 to 20 carbon mol% of cellulose was converted into glucose. The hydrolysate also contained byproducts such as fructose, mannose, levoglucosan, oligomeric cellulose, 5-hydroxymethylfurfural (5-HMF), and furfural together with unidentified compounds. Setting the reaction temperature lower (215 °C) improved the ratio of glucose to 5-HMF, which was a main inhibiting factor for the cell growth. Indeed, the recombinant *E. coli* exhibited better performance on the hydrolysate generated at 215 °C and accumulated P(3HB) up to 42 wt%, which was the same as the case of the same concentration of analytical grade glucose. The result indicated that the

ruthenium-mediated cellulose hydrolysis has a potency as a useful biorefinery process for production of bio-based plastic from cellulosic biomass.

1. Introduction

Polyhydroxyalkanoates (PHAs) are representative bio-based and biodegradable plastics produced by extensive strains of bacteria, and have been considered as an alternative to petroleum-derived materials (Chen, 2009). To date, a large variety of PHAs have been produced to cover a wide range of applications. These polyesters, however, are not currently widespread. A major obstacle to the practical use of PHAs is their high cost of production. Thus, utilization of inexpensive feedstock for PHA production has attracted research interests to reduce the cost of carbon sources, which contributes to a large portion of the entire cost (Keenan et al., 2006; Zhu et al., 2010). In particular, cellulose is an attractive target because large amount of cellulose can be obtained from inexpensive resources, such as wood, paper, cloth, straw, grass and so on (Octave & Thomas, 2009). Additionally, use of the inedible cellulosic biomass is preferable because of no competition with food production.

Because most bacteria are not capable of utilizing cellulose directly, cellulose hydrolysis is an essential step for the conversion of cellulose into PHAs. For example, bagasse hydrolysate generated by sulfuric acid containing xylose and glucose was used for PHA production in *Burkholderia* strains (Silva et al., 2004). Sulfuric acid is a common acidic catalyst for hydrolysis of cellulosic biomass (Rinaldi & Schuth, 2010; Rodrigues et al., 2010), but has several disadvantages in that it is not reusable, generates large amount of neutralization waste, and causes corrosion of the process. The more mild and environmentally friendly treatment, cellulase-catalyzed hydrolysis of cellulose, has also been investigated, but cost of enzyme and time-consuming reaction have been considered as obstacles for industrial applications (Zhang et al., 2006). Therefore, alternative cellulose hydrolyzing process was needed as an interface that can give efficient linkage with bacterial PHA production

system.

In this study, we investigated the use of solid metal catalyst for cellulose hydrolysis. We recently reported the supported ruthenium (Ru) catalysts rapidly converting cellulose into glucose (Fukuoka & Dhepe, 2006; Kobayashi et al., 2010). The reaction occurs under the aqueous solution that is preferable for feeding to microbes. In addition, solid catalysts are easily separated from yielded sugar by filteration, facilitating the recycle of the catalyst. The aim of this study is to examine the potential of the cellulose hydrolysate generated by Ru catalyst as a carbon source for bacterial PHA production.

For this purpose, we attempted to produce P(3-hydroxybutyrate) [P(3HB)], a most typical member of PHAs, in recombinant *Escherichia coli*, which is not only a useful model system, but also a versatile platform applicable to production of various PHAs (Matsumoto & Taguchi, 2010). Hence, if we could produce P(3HB) in *E. coli* from cellulose hydrolysate, this suggests the potential of wide range of applications. P(3HB) is synthesized from acetyl-coenzyme A (CoA) via three successive reactions catalyzed by β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC). The starting substance acetyl-CoA is a product of glycolysis pathway, thus P(3HB) could be efficiently produced from glucose. Here, P(3HB) production from cellulose hydrolysate and improvement in hydrolysis conditions towards optimizing the polymer productivity are reported.

2. Materials and Methods

2.1. Catalyst preparation

Ru/γ-Al$_2$O$_3$ catalyst (Ru metal loading 2 wt%) was prepared by a conventional impregnation method as follows: RuCl$_3$ aq. (0.202 mmol in 5 mL of water) was dropped into a mixture of γ-Al$_2$O$_3$ (1.00 g, JRC-ALO-2, the Catalysis Society of Japan) and water (20 mL), and the mixture was stirred for 16 h. After drying *in vacuo*, the solid was calcined with O$_2$ (30 mL min^{-1}) and reduced with H$_2$ (30 mL min^{-1}) at 400 °C for 2 h in a fixed-bed flow reactor. The mean particle size of Ru was 10 nm,

which was determined with XRD and TEM.

2.2. Pretreatment and hydrolysis of cellulose

Cellulose (Merck, Avicel) was milled using ZrO_2 balls at 60 rpm for 4 days. Hydrolysis of cellulose was carried out in a high-pressure reactor (OM Lab-Tech MMJ-100, 100 mL). The milled cellulose (0.162~2.59 g, 1.9~14.9 mmol glucose units, containing 7.0 wt% physisorbed water), Ru/γ-Al_2O_3 (100 mg) and water (20 mL) were charged in the reactor, and the mixture was heated from 25 °C to certain temperature (210~220 °C) in 15 min with stirring at 600 rpm and then rapidly cooled down to 25 °C.

2.3 Analysis of cellulose hydrolysate

Composition of the cellulose hydrolysates was determined as follows. The reaction mixtures were separated by centrifugation and decantation, and water-soluble products were analyzed using high-performance liquid chromatography (HPLC; Shimadzu LC10-AT*VP*, refractive index detector). The columns used in this work were a Phenomenex Rezex RPM-Monosaccharide Pb++ column (ø7.8×300 mm, mobile phase: water 0.6 mL min^{-1}, 80 °C), and a Shodex Sugar SH-1011 column (ø8×300 mm, mobile phase: water 0.5 mL min^{-1}, 50 °C). Product yields were calculated based on the mol of carbon in charged cellulose.

2.4. Culture media and P(3HB) production

Culture media were prepared by combining the cellulose hydrolysates with 3-fold condensed LB medium along with sterile water to contain certain concentration of glucose indicated in Table 2. LB media containing various concentrations of analytical grade glucose were prepared likewise. *E. coli* JM109 harboring pGEM-CAB, which bears *phaC, phaA* and *phaB* genes from *Ralstonia eutropha* encoding PHB biosynthetic enzymes (β-ketothiolase, acetoacetyl-CoA reductase, and PHA

synthase, respectively), was cultured for 72 h at 30°C. For molecular weight analysis, P(3HB) produced using diluted the hydrolysate of Run5 (Table 1) containing 0.3 wt% glucose was used.

2.5. Polymer analysis

P(3HB) content in the cells was determined using HPLC equipped with Aminex HPX-87H column (Biorad) as described previously (Karr et al., 1983). In brief, lyophilized cells were treated with concentrated sulfuric acid at 120°C for 45 min in a micro tube with screw cap to convert intracellular PHB into crotonic acid. The solution was diluted with 0.014N H_2SO_4 ten times and applied to HPLC with isocratic mode. Molecular weight of polymer, which was extracted with chloroform from lyophilized cells, was determined using gel permeation chromatography equipped with tandem two K-806L columns (Shodex, Japan) as described (Kusaka et al., 1997).

3. Results and Discussion

3.1. Cellulose hydrolysis

Table 1 summarizes the results of the hydrolysis of cellulose using 2 wt% Ru/γ-Al_2O_3 catalyst. When 0.8 wt% cellulose was loaded, the major products were glucose [17% (mol-carbon%) yield, corresponding to the concentration of 0.14 wt%] and water-soluble oligosaccharides (total 2.8% yield: dimer 1.0%, trimer 1.0%, and others 0.8%), and minor ones were fructose (3.6%), mannose (1.0%), levoglucosan (0.6%), 5-hydroxymethylfurfural (5-HMF, 5.8%), and furfural (0.6%) (Run1). The conversion of the cellulose was 69%. Thereby, the selectivity of glucose based on the conversion was as low as 25%, and large amount of byproducts formed during the hydrolysis reaction, which suggested the competition between the hydrolysis and side reactions. Furthermore, the concentration of glucose (0.14 wt%) was insufficient for the microbial fermentation. Thus, a higher concentration of cellulose would be favorable for the selective hydrolysis and for obtaining a higher concentration of glucose. By increasing the cellulose concentration from 0.8% to 13% (Run1-5), the yield of

by-products significantly lowered with maintaining the yield of glucose (15-20%), and therefore, the concentration of glucose reached 2.0 wt% with 54% selectivity at the highest concentration of cellulose (Run5). The hydrolysate of Run5 had sufficient glucose concentration to prepare the media for *E. coli*, and thus, was used for further experiments.

3.2. Application of cellulose hydrolysate to P(3HB) production

We next applied the cellulose hydrolysates to P(3HB) production in *E. coli*. To evaluate potency of the hydrolysate, cell growth of *E. coli* and cellular P(3HB) content are important factors, because it is known that the production of P(3HB) takes place in association with cell growth. The recombinant *E. coli* harboring pGEM-CAB was grown on LB media containing the hydrolysate of Run5 in various fractions to be the concentration of glucose from 0.1 to 1.0 wt%. The hydrolysate was added to the medium without any pretreatment and/or purification steps. When the hydrolysate of Run5 was diluted to contain less than 0.4 wt% glucose, the cells grew and accumulated P(3HB) up to 21 wt% in concentration-dependent manner (Table 2), indicating that the hydrolysate of Run5 could be a carbon source for P(3HB) production. The dry cell weight and polymer content were almost the same as control cells grown on the medium containing the same concentration of analytical grade glucose. However, addition of higher concentration of the hydrolysate inhibited cell growth. This result suggested that the hydrolysate of Run5 contained toxic byproducts.

Additionally, effect of the hydrolysate on molecular weight of the polymer, which critically affected strength of the material, was investigated, since it has been known that chain transfer reagents, typically alcoholic compounds, could reduce the molecular weight of polymer (Kawaguchi & Doi, 1992; Madden et al., 1999). As the result, the molecular weight of P(3HB) produced from the hydrolysate of Run5 (M_n, 3.1×10^5 and M_w, 16×10^5) was slightly lower than that of produced from analytical grade glucose (M_n, 4.3×10^5 and M_w, 24×10^5). The difference would not lead to significant impact on material properties. Thus, the compounds in hydrolysate of Run5 had little

effect on molecular weight of polymer.

3.3. Analysis of the effects of byproducts on cell growth and polymer synthesis

To estimate the toxic effect of the hydrolysate on cell growth, the recombinant *E. coli* was grown on the media containing a mixture of the identified compounds in the hydrolysate of Run5. The cell growth was inhibited at dilution rates containing more than 0.8 wt% glucose (Table 2), indicating that a part of these compounds caused the toxicity (see below). In addition, the maximum concentration for growth (0.8 wt% glucose) was greater than the highest concentration for the actual hydrolysate of Run5 (0.3 wt% glucose). This result suggested that the unidentified fraction in the hydrolysate may also contain toxic byproducts.

Among the identified compounds in the hydrolysate of Run5, 5-HMF and furfural were thought to be toxic to cell growth (Palmqvist & Hahn-Hagerdal, 2000). Thus contributions of the two compounds to cell toxicity of the hydrolysate were estimated by culturing the recombinant *E. coli* on the LB media containing 2.0 wt% glucose together with 0.3 wt% 5-HMF or 0.04 wt% furfural, which are equal to those in the hydrolysate of Run5. As the result, addition of 5-HMF inhibited growth of *E. coli*, while that of furfural did not (data not shown), indicating that 5-HMF mainly contributed to the toxicity of the hydrolysate.

3.4. Improvement of hydrolysis condition and application to polymer production

Because 5-HMF in the hydrolysate was shown to be an inhibitor for cell growth, we attempted to improve the hydrolyzing reaction to reduce the generation of 5-HMF, but retaining the efficient generation of glucose. 5-HMF is generated from glucose under hydrothermal conditions via isomerization of glucose into fructose and successive dehydration to 5-HMF and three water molecules (Kuster, 1990). Because these reactions are accelerated at higher temperatures, we expected the reduction of 5-HMF by lowering the reaction temperature in the Ru-catalyzed

hydrolysis of cellulose. As shown in Table 1, concentration of 5-HMF was reduced by one-third at 215 °C without largely reducing glucose concentration (Run6) compared to Run5 (220 °C), and therefore, the ratio of glucose to 5-HMF was markedly raised up from 4.9 to 10, although cellulose conversion was decreased to 20%. Furthermore, unidentified fraction, which was shown to possess inhibitory effect, was also reduced by decreasing temperature. In the case of the reaction at 210 °C, a higher ratio of glucose to 5-HMF (14) was obtained; however, the concentration of glucose significantly decreased to 0.75% (Run7). Thus the hydrolysate of Run6 was applied to P(3HB) production.

As expected, the hydrolysate of Run6 allowed cells to grow up to the dilution rate to contain the higher concentration of glucose (0.7 wt%) than the hydrolysate of Run5 (0.3 wt%) (Table 2), indicative of the remarkable reduction in toxicity of the hydrolysate of Run6. The cells grown on the hydrolysate of Run6 accumulated up to 42 wt% P(3HB), which was twofold higher than the highest polymer content using the hydrolysate of Run5. Therefore, the strategy for reducing 5-HMF in the hydrolysis was effective to improve productivity of P(3HB). The dry cell weight and polymer content for the hydrolysate of Run6 were almost the same as the case conducted using analytical grade glucose. The same phenomenon was observed for the hydrolysate of Run5. These results suggested that the byproducts in hydrolysate inhibited cell growth but did not affect P(3HB) biosynthesis at the applicable concentrations.

4. Conclusions

The cellulose hydrolysate generated by Ru catalysts was shown to be a carbon source for P(3HB) production in recombinant *E. coli* without any pretreatments. The hydrolysate exhibited no apparent effect on molecular weight of polymer. Among byproducts in the hydrolysate, 5-HMF was identified as a main contributor to inhibitory effect for cell growth, and its reduction in the hydrolysate successfully improved the efficiency of P(3HB) production. The result of this study

demonstrated that the Ru-catalyst has a potency as a useful biorefinery process of cellulosic biomass, which can be conjugated with the microbial process for biobased plastic production.

References

Chen, G.Q. 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem. Soc. Rev., **38**(8), 2434-2446.

Fukuoka, A., Dhepe, P.L. 2006. Catalytic conversion of cellulose into sugar alcohols. Angew. Chem. Int. Edit., **45**(31), 5161-5163.

Karr, D.B., Waters, J.K., Emerich, D.W. 1983. Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid-chromatography and UV detection. Appl. Environ. Microbiol., **46**(6), 1339-1344.

Kawaguchi, Y., Doi, Y. 1992. Kinetics and mechanism of synthesis and degradation of Poly(3-hydroxyburyrate) in *Alcaligenes eutrophus*. Macromolecules, **25**, 2324-2329.

Keenan, T.M., Nakas, J.P., Tanenbaum, S.W. 2006. Polyhydroxyalkanoate copolymers from forest biomass. J. Ind. Microbiol. Biot., **33**(7), 616-626.

Kobayashi, H., Komanoya, T., Hara, K., Fukuoka, A. 2010. Water-tolerant mesoporous-carbon-supported ruthenium catalysts for the hydrolysis of cellulose to glucose. ChemSusChem, **3**(4), 440-443.

Kusaka, S., Abe, H., Lee, S.Y., Doi, Y. 1997. Molecular mass of poly[(*R*)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol., **47**(2), 140-143.

Kuster, B.F.M. 1990. 5-Hydroxymethylfurfural (Hmf) - a Review Focusing on Its Manufacture. Starch-Starke, **42**(8), 314-321.

Madden, L.A., Anderson, A.J., Shah, D.T., Asrar, J. 1999. Chain termination in polyhydroxyalkanoate synthesis: involvement of exogenous hydroxy-compounds as chain

transfer agents. Int. J. Biol. Macromol., **25**(1-3), 43-53.

Matsumoto, K., Taguchi, S. 2010. Enzymatic and whole-cell synthesis of lactate-containing polyesters: toward the complete biological production of polylactate. Appl. Microbiol. Biotechnol., **85**(4), 921-932.

Octave, S., Thomas, D. 2009. Biorefinery: Toward an industrial metabolism. Biochimie, **91**(6), 659-664.

Palmqvist, E., Hahn-Hagerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol., **74**(1), 25-33.

Rinaldi, R., Schuth, F. 2010. Acid hydrolysis of cellulose as the entry point into biorefinery schemes. ChemSusChem, **3**(3), 296-296.

Rodrigues, R.D.L.B., Rocha, G.J.M., Rodrigues, D., Filho, H.J.I., Felipe, M.D.A., Pessoa, A. 2010. Scale-up of diluted sulfuric acid hydrolysis for producing sugarcane bagasse hemicellulosic hydrolysate (SBHH). Bioresour. Technol., **101**(4), 1247-1253.

Silva, L.F., Taciro, M.K., Ramos, M.E.M., Carter, J.M., Pradella, J.G.C., Gomez, J.G.C. 2004. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. J. Ind. Microbiol. Biot., **31**(6), 245-254.

Zhang, Y.H.P., Himmel, M.E., Mielenz, J.R. 2006. Outlook for cellulase improvement: Screening and selection strategies. Biotechnol. Adv., **24**(5), 452-481.

Zhu, C.J., Nomura, C.T., Perrotta, J.A., Stipanovic, A.J., Nakas, J.P. 2010. Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC 17759. Biotechnol. Prog., **26**(2), 424-430.

Table 1. Composition of cellulose hydrolysates generated by Ru-catalyst at various conditions

Run	Conc. of cellulose (wt%)	Temp. (°C)	Yield of products based on mol-carbon /%								Conv. of cellulose (%)
			Glucose	Oligosaccharides[a]	Fructose	Mannose	Levoglucosan	5-HMF	Furfural	Unidentified	
1	0.81	220	17.3 (0.14)[b]	2.8	3.6	1.0	0.56	5.8 (3.0)[c]	0.58	37.3	69
2	1.62	220	18.8 (0.31)[b]	3.0	4.1	1.3	1.1	7.6 (2.5)[c]	0.72	17.8	54
3	3.24	220	18.7 (0.63)[b]	3.5	4.4	1.1	1.0	7.1 (2.6)[c]	1.2	12.5	49
4	6.48	220	19.7 (1.3)[b]	3.7	4.0	0.33	1.0	4.6 (4.3)[c]	0.87	2.3	36
5	13.0	220	15.2 (2.0)[b]	3.2	2.9	0.23	0.70	3.1 (4.9)[c]	0.65	2.6	28
6	13.0	215	11.6 (1.6)[b]	5.3	2.1	0.91	0.58	1.1 (10)[c]	0.62	<0.5	20
7	13.0	210	5.6 (0.75)[b]	5.5	0.78	0.46	0.18	0.40 (14)[c]	0.26	<0.5	12

[a] Oligomer from dimer to octamer. [b] The concentration of glucose (wt%) in each hydrolysate is shown in parenthesis. [c] A ratio of glucose (mol) against 5-HMF (mol).

Table 2. Dry cell weight of *E. coli* grown on cellulose hydrolysates and P(3HB) content

Glucose Conc. (wt%)	Cellulose hydrolysate				Mixture of identified products[b]		Analytical grade glucose	
	Hydrolysate Run5 (220 ºC)		Hydrolysate Run6 (215 ºC)					
	DCW[a] (g/L)	P(3HB) content (wt%)	DCW (g/L)	P(3HB) content (wt%)	DCW (g/L)	P(3HB) content (wt%)	DCW (g/L)	P(3HB) content (wt%)
2.0							8.9 ± 0.2	68 ± 2
1.5							7.7 ± 0.2	65 ± 1
1.0	0	-	0	-	0	-	5.7 ± 0.2	51 ± 2
0.8			0	-	4.9 ± 0.1	42 ± 1	4.7 ± 0.2	46 ± 2
0.7			3.9 ± 0.1	42 ± 0	4.4 ± 0.1	41 ± 0	4.2 ± 0.2	41 ± 2
0.6	0	-	3.9 ± 0.1	31 ± 2	4.0 ± 0.1	39 ± 1	3.6 ± 0.5	38 ± 1
0.5	0	-	3.4 ± 0.1	26 ± 1	3.5 ± 0.1	33 ± 1	3.5 ± 0.1	33 ± 1
0.4	0	-	3.1 ± 0.1	23 ± 3	3.1 ± 0.1	26 ± 0	3.1 ± 0.1	28 ± 0
0.3	2.8 ± 0.2	21 ± 2	2.3 ± 0.1	24 ± 2	2.8 ± 0.1	19 ± 0	2.7 ± 0.1	19 ± 2
0.2	2.3 ± 0.2	11 ± 2	2.1 ± 0.1	7 ± 2			2.4 ± 0.1	12 ± 1
0.1	3.0 ± 0	n.d.[c]					2.9 ± 0	n.d.
0.0							2.5 ± 0	

[a] DCW, dry cell weight. [b] The mixture of the identified products in Run5 (2.0% glucose, 0.38% fructose, 0.03% mannose, 0.25% cellobiose, 0.1% levoglucosan, 0.08% furfural and 0.41% 5-HMF) was used instead of the actual hydrolysate of Run5. [c] n.d., not determined.