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ORIGINAL PAPER

Production of Polyhydroxybutyrate from *Ralstonia eutropha* H-16 Using Makgeolli Lees Enzymatic Hydrolysate

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Abstract
The objective of this study was to investigate the potential use of waste Makgeolli lees (ML), a by-product of Makgeolli production, as a feedstock for biorefinery production of polyhydroxybutyrate (PHB). ML enzymatic hydrolysate (MLEH) was prepared from ML subjected to liquefaction with amylase and subsequent saccharification with glucoamylase. Since MLEH contains glucose (81 g/L) and protein (39 g/L) among other elements, it was used as the carbon source for PHB production by *Ralstonia eutropha* H-16. After flask incubation with MLEH, the obtained biomass and PHB content were 10.03 g/L and 34%, respectively, which were higher than the contents obtained when using glucose as the sole carbon source. However, batch fermentation resulted in low PHB accumulation despite suitable cell growth being observed, and these results were thought to be due to the unbalanced C/N ratio. To control the C/N ratio to 20:1, sterile glucose solution was added twice in the fed-batch fermentation with MLEH, and we could obtained dry cell weight and PHB of 36.9 g/L and 24.1 g/L, respectively. Therefore, this study demonstrated the potential use of waste ML as a feedstock for industrial PHB production, which has the important advantage of reduced production cost.

Keywords Biorefinery · Feedstock · Hydrolysis · Polyhydroxybutyrate · *Ralstonia eutropha* · Makgeolli lees

Introduction

Polyhydroxyalkanoates (PHAs) are synthesized by various microorganisms under of the availability of limited nutrients, such as nitrogen, phosphorous, oxygen, sulfur, or magnesium, in the presence of excess carbon source [1]. Bacteria are capable of utilizing intracellular PHA as carbon and energy sources and under conditions of limited storage materials [2]. PHAs have been classified into the following two major groups according to the number of carbon atoms in the PHA monomer. First, short-chin-length PHAs (scl-PHAs), which contain 3 to 5 carbon atoms in their monomers [e.g. 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV)]; secondly, medium-chin-length PHAs (mcl-PHAs), which contain 6 to 14 carbon atoms in their monomer units [e.g. 3-hydroxyhexanoate (3HHx) or 3-hydroxyoctanoate (3HO)]. The variation in monomer composition in PHA polymers is a function of contents in polymer, primarily depends on the microbial species, substrates, and fermentation conditions [3].

Poly(3-hydroxybutyrate) (PHB) is naturally produced by various prokaryotic microorganisms, including archaea and bacteria (Gram-positive or Gram-negative), as well as some phototrophic cyanobacteria. The chemical and physical properties of PHB are similar to those of synthetic polymers. Thus, PHB has been produced for use as bulk commodity plastics and fishing lines, as well as for medical applications, such as pharmacy and drug delivery systems.

PHB has received significant attention as biodegradable polymers [4]. However, the commercialization of PHB is limited by its high production costs [5], low PHB content, and efficiency of product recovery in downstream processes. Substrate costs can comprise up to 40% of the total PHB production expenses. Therefore, the use of inexpensive raw materials that are abundant and locally available can significantly reduce the costs of PHB production. According to previous study, PHB was produced using renewable resources carbon containing cellulose such as bagasse [6], cassava [7], office paper [8], wheat straw [9], brown seaweed [10], and rice straw [11]. Cellulosic biomass such as agricultural residues are potentially inexpensive renewable

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feedstock for the biorefineries of alternative fuels, chemicals and materials [12]. The recalcitrant composite structure of lignin, hemicellulose and cellulose in plant cell wall, however, blocks the hydrolysis of polysaccharides into fermentative sugars. Moreover, cellulose is difficult to decompose and lignin inhibits the growth of microorganisms [13]. Starch more degradable than cellulose and can be used as a more effective carbon source. Starchy materials derived from crops or wastewater from starch manufacturing processes are suitable for PHB production and are available at low cost [7].

Makgeolli is a traditional Korea alcoholic beverage. In the food industry, Makgeolli lees (ML) is a byproduct of Makgeolli production. The production and consumption of Makgeolli has dramatically increased in recent years, leading to higher ML production and increased ML [14]. However, the majority of ML is considered as industrial waste and is routinely discarded.

ML is a complex mixture that can comprise up to 20% amino acids, peptides, carbohydrates, in addition to various nutrients, such as inorganic salts and nucleic acids from koji and yeast [14]. ML contains higher starch or protein contents relative to those of raw rice or wheat and therefore exhibits great potential as a renewable resource.

Investigations on ML are being conducted in various fields of scientific research. Several studies on human physiology have demonstrated that ML regulates blood sugar levels in diabetic patients [15] and exhibits a blood pressure-lowering effect in hypertensive patients [16]. ML extracts have been demonstrated to exert anti-allergic effects, reduce nitrite scavenging, and inhibit the enzymatic activities of tyrosinase, xanthine oxidase, and angiotensin converting enzyme [17]. ML extracts also exhibit antimicrobial or antioxidant activity [18]. ML extracts have been shown to exert anti-obesity, anti-inflammatory, and anti-cancer activities [19]. Recently, ML has been demonstrated to exert wrinkle-reducing effects because it can promote collagen synthesis [20]. However, no studies have evaluated the potential of ML as a carbon source for PHB production.

In this study, enzymatic hydrolysis methods were employed for the preparation of ML enzymatic hydrolysate (MLEH) and the potential use of MLEH as a carbon source for PHB production in *Ralstonia eutropha* H-16 was evaluated.

Materials and Methods

Microorganism and Maintenance

The *R. eutropha* H-16 strain was purchased from the Korean Collection for Type Cultures (number 22469). *Ralstonia eutropha* H-16 was stored on nutrient agar slants at 4 °C and transferred every month. In addition, *R. eutropha* H-16 cultures ware maintained as glycerol stock at − 80 °C.

Media and Culture Conditions

A mineral salt medium containing 20 g/L glucose, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 11.1 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, and 1 mL of trace metal solution was used as the basal medium for the initial cultivation studies. The trace metal solution contained the following ingredients (per liter): 9.7 g of $FeCl_3$, 10.35 g of $CaCl_2{\cdot}2H_2O$, 0.156 g of $CuSO_4{\cdot}5H_2O$, 0.248 g of $CoCl_2{\cdot}6H_2O$, 0.216 g of $NiCl_2{\cdot}6H_2O$, and 0.139 g of $CrCl{\cdot}6H_2O$. For PHB production, glucose was replaced by MLEH as the carbon source. These two carbon sources were autoclaved separately to avoid precipitation during medium preparation. All components were mixed, and the pH of the culture medium was adjusted to pH 7 using 1 N NaOH/HCl.

Preparation of MLEH

ML was obtained from KOOKSOONDANG (Korea). Crude ML was diluted with distilled water to a final concentration of 50% (w/w, wet basis). For liquefaction treatment, ML slurry was adjusted to pH 5 with 3 N NaOH, after which 20 U of amylase (Megazyme, USA) was added per gram of wet ML; the resulting slurry were incubated at 50 °C for 2 h in a water bath. After liquefaction, the incubation temperature of the slurry was adjusted to 60 °C. Afterwards, 10 U of glucoamylase (Sigma Aldrich, USA) were added per gram of wet ML for saccharification over 24 h. The resulting mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was designated as MLEH and used as a medium supplement.

Growth Conditions

Flask cultivations were performed in 250-mL Erlenmeyer flasks containing 50 mL of culture medium (pH 7). *Ralstonia eutropha* H-16 cells were incubated at 30 °C and 200 rpm and allowed to grow for 96 h. The 3-L jar (Biofors, Korea) containing MLEH or glucose in 1.25 L of MS medium was fermented for 96 h, with an inoculum size of 10% (v/v). The temperature was set to 30 °C, and the pH was maintained at 7 by the addition of 1 N NaOH or 1 N HCl. The airflow rate and stirring speed were set to 1.5 vvm and 200 rpm, respectively. Fed-batch culture condition was same as the fermentation condition, and sterile glucose solution was added after 24 and 48 h in fermentation.

ML Moisture, Total Nitrogen (T-N), and Glucose Content

The moisture content of the ML was determined by drying at 105 °C overnight [21]. The T-N content in sample was measured following the Kjeldahl method (AOAC, 1999). Glucose levels were analyzed by the Lane-Eynone method, and organic acid contents were analyzed by high performance liquid chromatography (HPLC). Samples were collected at 12-h intervals and then centrifuged at 10,000 rpm for 10 min. The cell pellet was dried at 60 °C for determination of dry cell weight (DCW).

Gas Chromatography (GC)

The PHB contents of the dried cells were analyzed by GC [8]. Briefly, 50 mg of dried cells were added to 2 mL of chloroform and 2 mL of acidic methanol (2.8 M H_2SO_4 in methanol) containing benzoic acid (10 mL/L) as an internal standard and subsequently heated at 100 °C for 4 h. After cooling, 1 mL of distilled water was added to the mixture, vortexed vigorously for 3 min, and then centrifuged at 2000×*g* for 1 min. Afterwards, the organic phase (methylated monomers dissolved in chloroform) obtained from each sample was filtered and analyzed via GC. The gas chromatographic separation was performed on an Agilent J &W HP-5 capillary column (30 × 0.32 mm × 0.25 mm). The PHB contents were estimated using calibration with commercial PHB (Sigma Aldrich, USA), and expressed as the percentage of dry cell weight (%). Benzoic acid (Sigma Aldrich, USA) was used as an internal standard.

Analysis of Thermal Properties

The thermal degradation of the samples was investigated using a TA Instruments (Q2000, UK) differential scanning calorimeter (DSC) under nitrogen. The DSC was used to characterize the melting temperature (T_m) and glass transition temperature (T_g) for PHB samples. The samples were heated with heating rate of 10 °C/min in the temperature interval from 20 to 260 °C. Data were collected during the first heating run and transition temperatures were taken as peak maxima.

The thermal decomposition studies were carried out in a TG–DTA 8122 (Rigaku, Japan). Approximately 10 mg of sample was weighed into an aluminium pan and analyzed by thermogravimetric analysis (TGA). Heating was at a rate of 10 °C/min and was performed in the temperature interval from 25 to 900 °C. The test was performed in an atmosphere of nitrogen, which was injected at a flow rate of 15 mL/min. A curve of weight loss against temperature was constructed from the data obtained.

Gel Permeable Chromatography (GPC)

Molecular weights (weight average, Mw, and number average, Mn) and polydispersity (PDI = Mw/Mn) of PHB were determined using GPC Alliance e2695 (Waters, USA). Chloroform was used as the mobile phase.

Results and Discussion

Effect of Enzymatic Treatment of ML

The compositions of ML and MLEH are provided in Table 1. ML is a by-product of the process of making Makeolli, which is prepared from rice and nuruk. Nuruk, which is used as a Korean fermentation starter, is a dry and molded cereal cake that can be used to produce hydrolysable enzymes and to decompose macromolecules into monomers to promote yeast growth. Therefore, yeast can be fed with sugars produced by saccharification of rice starch to produce alcohol [22]. The protein or starch contents were found to be relatively higher in ML, which is a by-product of rice wine [23]. To be able to utilize the carbohydrates in the ML for PHB fermentation, liquefaction and saccharification were performed to convert starch present in the ML into glucose. The glucose and total protein concentrations in MLEH were influenced by liquefaction and saccharification using amylase and glucoamylase, respectively. The glucose concentrations in MLEH were almost 2-fold higher compared to those in ML. However, the protein content in MLEH was approximately 35% lower than that in ML. Consequentially, 81 g/L of glucose and 39 g/L of protein in MLEH were used as the carbon and nitrogen sources for PHB production, respectively. Similar results were obtained using Japan Sake lees (SL) enzymatic hydrolysate [21]. After enzymatic liquefaction and saccharification, starch was not detected in the enzymatic

Table 1 Components of untreated and pretreated ML

	ML[a] (g/L)	MLEH (g/L)
Moisture	814 ± 2	–
Protein	60 ± 5	39 ± 4
Glucose	40 ± 6	81 ± 4
Succinate	2 ± 0	1 ± 0
Starch and others	106 ± 6	ND[b]

Results are in form average ± standard deviation

[a]Crude ML

[b]Not detected

hydrolysate of SL, and glucose, protein, and succinic acid levels were significantly different from those of SL.

PHB Production Using Glucose or MLEH Used as the Sole Carbon Source

To evaluate the potential use of MLEH as a feedstocks in a biorefinery, MLEH was used as the sole carbon source for PHB synthesis (Table 2). Glucose was used as the control carbon source for PHB production. *Ralstonia eutropha* H-16 strain was cultivated for 96 h in MS medium containing 2% glucose. MLEH was added to the medium to obtain the desired glucose concentration. After cultivation, residual glucose was dramatically decreased when MLEH was used as the sole carbon source. The MLEH treatment produced 3-fold higher DCW or PHB levels relative to those of the control when glucose was used as the carbon source. However, as shown in Table 2, the PHB content was only 1.3-fold higher when MLEH was used as the sole carbon source. In fact, MLEH contains both glucose and proteins, which could explain the higher nitrogen content of the medium containing MLEH when compared to that of the control. The above results suggested that MLEH is a suitable carbon source for supporting bacterial growth. However, results indicated that the C/N ratio in the MS medium prepared using MLEH was not suitable for PHB production.

Table 2 Flask cultivation of *R. eutropha* H-16 using glucose and MLEH as a carbon source

Carbon source	DCW (g/L)	PHB yield (g/L)	PHB content (%)
Glucose	3.6 ± 0.1	1.0 ± 0.2	26.5 ± 5.8
MLEH	10.3 ± 0.7	3.6 ± 1.0	34.5 ± 6.1

Batch Fermentation Using Glucose or MLEH

Batch fermentation was performed by incubating *R. eutropha* H-16 using 3% glucose or MLEH as the sole carbon source for 96 h at 30 °C at pH 7. As shown in Fig. 1a, glucose was consumed steadily until the end of cultivation, and the nitrogen source was completely exhausted after 36 h. Afterwards, PHB accumulation was observed and 9.5 g/L of PHB was obtained finally. The above findings indicated that glucose was consumed during cell growth until the nitrogen source in the culture medium was exhausted, after which the residual carbon started to accumulate as PHB granules.

Fermentation results are shown in Fig. 1b using MLEH as the sole carbon source. MLEH was added aseptically to the medium to obtain as desired glucose concentration of 3% (wt/v). Consistent with the expected results, cell growth was considerably higher in the MLEH medium than that in the glucose medium, which could be explained by the complex components present in MLEH. The total nitrogen content was almost 2-fold higher in the MLEH medium; in addition, cell growth was 2-fold higher than that observed in the control. However, the PHB content in the cell was dramatically lower (< 4 wt%), similar to the results obtained from the flask test at a C/N ratio of 9:1. A higher C/N ratio is known to increase PHB accumulation [8]. A previous study showed that a higher C/N ratio leads to higher PHB production and concluded that the nitrogen limiting condition favors PHB accumulation while minimizing cell growth. These results suggested that MLEH is a suitable sole carbon source for supporting cell growth, although MLEH could require further optimization for PHB production.

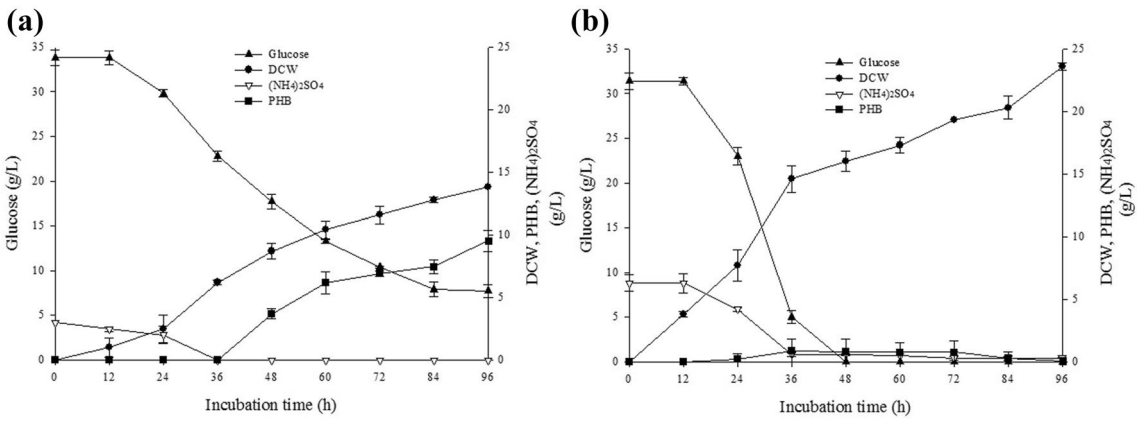

Fig. 1 Cell biomass, PHB production, residual glucose, and nitrogen levels during batch fermentation of *R. eutropha* H-16 using **a** glucose and **b** MLEH

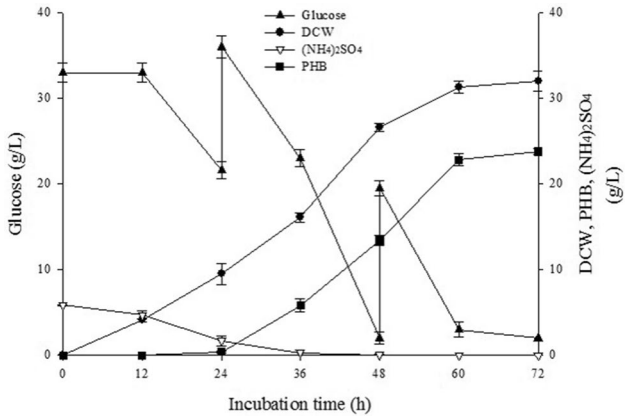

Fig. 2 Time course analysis of biomass, PHB production, residual glucose, and nitrogen levels during fed-batch fermentation. Sterile glucose solution was added after 24 and 48 h of fermentation

Fed-Batch Fermentation Using MLEH Containing Glucose

Figure 2 shows the results of fed-batch fermentation. *Ralstonia eutropha* H-16 was cultured using MLEH, which was added aseptically to the medium to achieve the desired glucose concentration of 3% (wt/v). To adjust the C/N ratio, 15 g/L sterile glucose was added twice (24 and 48 h). Rapid consumption of the carbon source was observed from 24 to 48 h, and the nitrogen source was exhausted after 48 h of cultivation (Fig. 2). *Ralstonia eutropha* H-16 reached the stationary phase at 60 h of cultivation, with a corresponding dry cell mass of 36.9 g/L. PHB accumulation was induced after the nitrogen source was exhausted, corresponding to a PHB yield of about 10.6 g/L, which eventually reached 24.1 g/L at 72 h, and a PHB content of 79.3% (wt/wt).

The PHB production and cell growth of *R. eutropha* H-16 were significantly higher in the fed-batch cultivation than those in the batch fermentation. The above results are expected because controlling the C/N ratio to 20:1 by the addition of glucose solution, not only influenced cell growth, but also PHB production. ML contains various ingredients, such as organic acids, esters, glucose, starch, and proteins [21]. Therefore, it could be inferred that the presence of various elements in MLEH facilitated the growth of the microorganisms. However, the amount of carbon source was approximately 2-fold higher in the fed-batch condition relative to that in the batch fermentation condition, and cell growth was approximately 1.5-fold higher in the fed-batch condition. In addition, PHB production was 30-fold higher in the fed-batch condition than that observed in the batch fermentation condition. The above results suggested that adjusting the C/N ratio resulted in optimal cell growth of *R. eutropha* H-16 and led to optimal PHB production.

Table 3 shows properties of PHB produced using glucose or MLEH with glucose by *R. eutorpha* H-16. The PHB produced using glucose as a sole carbon source was performed in batch fermentation (Fig. 1a). On the other hand, PHB obtained by using MLEH with glucose was performed in fed-batch fermentation (Fig. 2). Because lower cell growth and PHB yield were obtained when glucose was used as a sole carbon source in the same fed-batch culture condition (data not shown). As shown in Table 3, there were no significant difference according to the carbon source type. The two

Table 3 Properties of PHBs produced by using glucose and MLEH with glucose as carbon source

Carbon source	T_d (°C)	T_g (°C)	ΔH_m (J/g)	T_m (°C)	M_n (Da)	M_w (Da)	PDI
Glucose	270.4	–	102.6	177.0	211957	405348	1.91
MLEH with glucose	269.7	–	108.5	172.9	186999	356146	1.90

Table 4 Comparison of PHB accumulation using various waste hydrolysates as carbon source

Substrate	DCW (g/L)	PHB yield (g/L)	PHB content (%)	Microorganism	Reference
Bagasse	8.0	4.3	54.0	*Cupriavidus necator*	[6]
Cassava	6.0	2.4	61.6	*C. necator* KKU38	[7]
Office paper	7.7	4.5	57.5	*C. necator*	[8]
Wheat straw	3.0	0.9	30.9	*C. necator* DSM 545	[9]
Wheat straw	3.7	1.8	48.4	*C. necator* NCIMB 11599	[9]
Wheat straw	2.1	1.1	52.6	*Bacillus licheniformis* IMW KHU 3	[9]
Wheat straw	2.2	1.2	55.2	*B. megaterium* IMW KNaC 2	[9]
Brown seaweed	5.4	2.9	54.7	*C. necator* PTCC 1615	[10]
Rice straw	45.4	10.6	23.3	*B. cereus* PS 10	[11]
MLEH with glucose	36.9	24.1	79.3	*R. eutropha* H-16	Present study

differently produced PHB showed similar thermal properties and molecular weight.

Table 4 shows the DCW and PHB content of various strains utilizing different waste hydrolysates as carbon source [6–11]. The above findings indicated that cell biomass and PHB production by *R. eutropha* obtained in the present study were higher than those reported in other studies. As shown in Table 4, the total carbon content obtained in the present study was different from those reported in previous studies. These results indicated that cell growth and PHB content were significantly higher when MLEH was used as the carbon source relative to other systems using other waste mixtures as carbon sources. In particular, when rice straw was used as a carbon source, the DCW and PHB production were 45.4 g/L and 10.6 g/L, respectively [11]. DCW was 1.2-fold higher, whereas PHB production was 2.4-fold lower than those obtained in the present study. Therefore, the above findings demonstrated that MLEH is a suitable carbon source for use in PHB production compared to other hydrolysates.

Conclusion

This study suggested that enzymatic pretreatment of ML could produce MLEH with a high glucose yield. MLEH was found to be a suitable carbon source for supporting cell growth because of the presence of complex components; however, MLEH have abundant total nitrogen leading to unbalanced C/N ratio. To optimize the C/N ratio (20:1), fed-batch fermentation was conducted using MLEH supplemented with sterile glucose solution, which achieved the biomass and PHB yield of 36.9 g/L and 24.1 g/L after 72 h, respectively. Therefore, these results indicated that MLEH is a suitable carbon source for PHB production by *R. eutropha* H-16. Furthermore, the results of the study demonstrated the potential use of waste ML as a carbon feedstock for industrial PHB production and have the advantage of reduced cost.

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