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BRIEF COMMUNICATION

Utilization of Broken Rice for the Production of Poly(3-hydroxybutyrate)

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Abstract The feasibility of utilizing non edible rice (broken rice) for production of fine materials such as poly(3-hydroxybutyrate) (PHB) was considered as one of the alternative ways of keeping the environment clean for sustainable development. Thus, production of PHB from broken rice by simultaneous saccharification and fermentation (SSF) was investigated. During the SSF process, the rice (15% w/v) material was hydrolyzed to glucose, which was utilized by *Cupriavidus necator* for growth and production of PHB. The PHB content reached 38% at 58 h fermentation. The PHB had weight average molar mass (Mw) and polydipersity index of 3.82×10^5 (g/mol) and 4.15, respectively. Differential calorimetric scan of the PHB showed a melting temperature (Tm) of 176 °C. Given that the PHB was a homopolymer (which consisted of (*R*)-3-hydroxybutyric acid monomers), it was thought that broken rice could be a raw material for production of both PHB and (*R*)-3-hydroxybutyric acid. This SSF process would not only help in the utilization of broken rice or non edible rice, but would also serve as a model for utilization of other raw materials that contain starch for production of PHB.

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Introduction

Poly(3-hydroxybutyrate) (PHB) is one of the promising biodegradable plastics, which has very good material properties similar to plastics that are produced from petroleum (e.g., polypropylene). Furthermore, PHB has rapid biodegradation properties under various environmental conditions such as aerobic and anaerobic, which would help in solving the problems of vanishing landfill space [1]. PHB is accumulated by various microorganisms as an intracellular storage material through fermentation processes [2–4]. However, to make PHB economically competitive with petroleum-based plastics, cheap renewable raw materials (e.g., corn, rice, millet and wheat) have to be used in the fermentation with some bacterial strains. Although a lot of work have been done on the production of PHB using various soluble sugars, only very scanty information on the use of starchy materials have been investigated.

Rice is a starchy material, and one of the major staple foods in most parts of the world. During rice processing, a good proportion of it is crushed (i.e., as broken rice) resulting to a considerable loss in the production. High-grade broken rice can find applications in food and alcoholic industries, whereas low-quality ones are hardly consumed. Furthermore, with the increase in industrialization coupled with various human activities, regulations for waste management are being tightened up. There is therefore, an urgent need to develop an alternative and efficient method to utilize these by-products and produce

high value products. One of such high value products that can be produced upon exploitation of contaminated or broken rice is PHB.

This work was therefore, aimed at developing efficient processes for utilization of broken rice for production of PHB. In addition, the possibility of producing (*R*)-3-hydroxybutryic acid using the cell biomass was proposed.

Materials and Methods

Microorganisms and Cuture Media

Cupriavidus necator (JCM 11282) was grown in basal medium, which contained the following components (per L): 5 g yeast extract, 10 g polypeptone, 200 mg $MgSO_4 \cdot 7H_2O$, 100 mg NaCl, 20 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot H_2O$, 0.5 mg $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg $Na_4WO_4 \cdot 2H_2O$, 0.5 mg $MnSO_4$, 1,600 mg K_2HPO_4, 200 mg KH_2PO_4 and 1,000 mg $(NH_4)_2SO_4$ and rice 15% (w/v). The broken rice (imported from Thailand), which contained 75% starch (w/w) and 14% moisture was crushed into powder using a grinding mill. The rice powder was gelatinized at 105 °C for 1 h in an autoclave. Liquefaction was done using α-amylase (0.1% w/w) and 20 mL (0.2% $CaCl_2$; pH 6.0) at 50 °C for 1 h. Seed culture was then prepared in 500 mL flask (which contained 100 mL of the basal medium) on a reciprocal shaker (170 rpm) and cultured at 30 °C for 18 h. The seed culture inoculated in 5 L-fermentor (B.E. Marubishi, Japan) that contained basal medium (working volume = 2.5 L), liquefacted rice (150 g/L) and glucoamylase (0.1% w/v). Temperature and pH were automatically controlled at 30 °C and 7.0 (using 5 M NaOH), respectively. Agitation and aeration were maintained at 300 rpm and 0.5 volume of air per volume of medium per minute (vvm), respectively.

Sample Analysis

Growth of *C. necator* was monitored by plating out the cultures, followed by enumeration of viable cells (i.e., cfu/mL). Cultures were centrifuged (10,000 rpm for 10 min), washed with distilled water and freeze-dried. The concentration of PHB was determined gravimetrically as previously described [5, 6]. Thus, 1 g of lyophilized cells was extracted with 50 mL chloroform in a reflux condenser under heating in an oil bath (75 °C) for 8 h. Cell debris was removed by filtration and the chloroform/PHB solution was then precipitated with ethanol (volume of ethanol was 4 times that of the chloroform). PHB was recovered from the solvents (i.e., chloroform/ethanol mixture) by filtration followed by drying to a constant weight. PHB content was calculated as the quantity of PHB per biomass (g-PHB/g-biomass). Molecular weight distribution of the PHB was measured using gel permeation chromatography (GPC). The GPC (HLC-8020 chromatograph, Tosoh Co., Japan) was equipped with analysis column (TSKgel Multipore H_{XL}-M 7.8 × 300 mm) using chloroform as mobile phase at 0.8 mL/min. The calibration curves for GPC analysis were obtained using polystyrene standards with low Mw (5.87×10^3, 1.71×10^4, 9.89×10^4, 3.54×10^5). Differential scanning calorimeter (DSC) (SSC/5200 SII H, Seiko Instruments, Japan) was used to determine the Tm. (*R*)-3-hydroxybutyric acid was measured using HPLC, which was equipped with UV–Vis, RI detectors, and with Shim-Pack SPR-H column (Shimadzu Corp., Japan). The HPLC was operated at 40 °C and eluted with 4 mM $HClO_4$ at 0.6 mL/min. Glucose concentration was measured using analytical kit (Wako Pure Chemicals, Japan).

Results and Discussions

During the saccharification process, rice was converted to glucose, which was eventually used by the cells for growth and production of PHB. Growth kinetics of *C. necator* and changes in glucose concentration during the SSF are shown in Fig. 1. Rapid growth was observed after 30 h, and cells reached stationary phase at 58 h. The growth of the cells was slow at the beginning of fermentation. However, after 30 h cultivation, cells rapidly increased and then reaching the stationary phase at 58 h. By the end of the fermentation (58 h), viable cells had reached up to 9.7×10^{10} cfu/mL, while the residual glucose was 4.8 g/L.

Production of PHB by microbial fermentation processes has been widely studied with some microorganisms [7–9]. Most of these studies have revealed that production of PHB from carbon substrates (e.g., glucose, sucrose, etc.) is initiated upon condensation of two molecules of acetyl-CoA

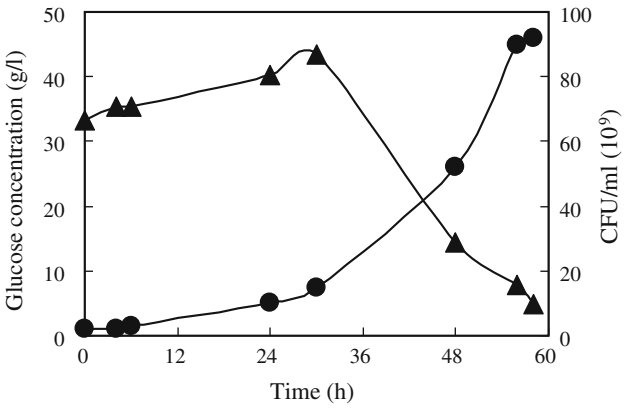

Fig. 1 Time course of the *C. necator* growth (*closed circle*) and changes in glucose concentrations (*closed triangle*) during the SSF process

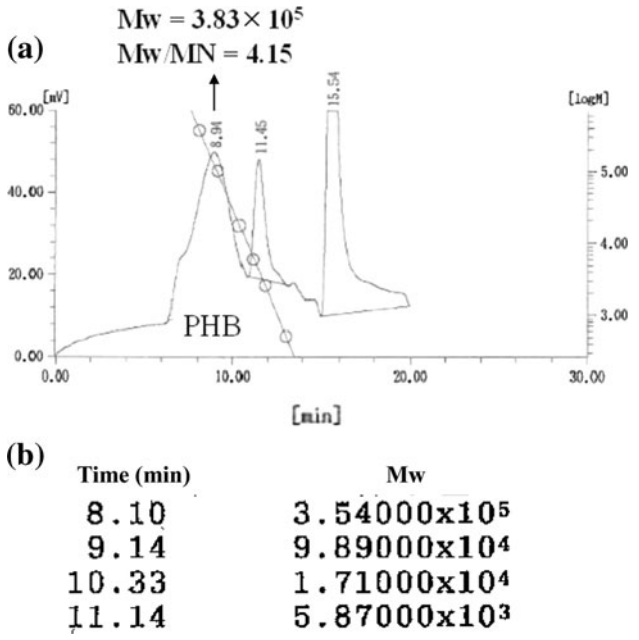

Fig. 2 GPC data showing the molecular weight distribution of PHB produced from rice (**a**) and polystyrene standard (**b**)

to form acetoacetyl-CoA in the presence of *β*-ketothiolase (*phbA*). Acetoacetyl-CoA is then converted to (*R*)-3-hydroxybutyryl-CoA in the presence of acetoacetyl-CoA reductase (*phbB*). Finally, the PHB is produced in the granule in the presence of PHB synthase (*phbC*). Depending on the availability of nutrients (especially under carbon limitation conditions) in the culture, the PHB produced in the granule can be depolymerized to (*R*)-3-hydroxybutyric acid ((*R*)-3-HB). It was necessary to monitor the glucose concentration in the culture to avoid depolymerization. Thus, the cells were harvested just before the glucose became exhausted in the culture broth so as to obtain the maximum yield of PHB.

The PHB yield from biomass at the 48 h of fermentation was 0.22 g/g-biomass (corresponding to PHB content of 22%). The maximum PHB concentration reached 0.38 g/g-biomass (i.e., 38% as the PHB content) at the 58 h. The PHB content obtained in this study is relatively higher than that of Oliviera et al. [10], who reported a PHB content of 33.3% using soycake and 2.5% molasses in solid state fermentation with *C. necator*. Analysis with GPC showed that average molar mass (Mw), and polydipersity index (Mw/Mn) were 3.8×10^5, and 4.15, respectively (Fig. 2). Our previous work [5] indicated that with the same strain (*C. necator*) that utilized sucrose, higher molecular weight PHB ($Mw = 9.3 \times 10^5$; $Mw/Mn = 2.2$) could be obtained. In comparison, molecular weight data reported by Oliviera et al. [10] were 7.2×10^5, and 2.02 for Mw and Mw/Mn, respectively. PHB was confirmed as a homopolymer by New Magnetic Resonance (NMR).

Table 1 Changes in the variables during the fermentation and production of PHB produced from rice

Parameters	Values
Final PHB content (%)	38
Melting point of PHB (°C)	176
Weight average molar mass (g/mol)	3.8×10^5
Polydipersity index	4.15

Furthermore, by differential scanning calorimetery (DSC), the melting temperature (Tm) of the PHB was found to be 176 °C. This value is a little higher than 169.5 °C reported by Oliviera et al. [10]. Material properties of the PHB produced from rice by SSF are summarized in Table 1.

Broken rice was used as model for production of PHB by SSF process. Aside from broken rice, rice paste (by-products) obtained after rice fermentation at the alcoholic production factories contain a lot of starches, and thus can also be used for production of PHB by SSF process. An added advantage of using rice starch is that it contains essential nutrients (e.g., N, P, K, vitamins and minerals) that can supplement the culture media for microbial production of PHB.

Some studies have indicated that PHB can be converted to its monomer by hydrolysis with alkali [11] or enzymatic process [4] or through bioconversion process using suitable substrates [6]. The scheme for production of PHB from rice is shown in Fig 3. Rice is produced (e.g., in paddy fields) when solar light traps down atmospheric carbon dioxide by photosynthetic process. Following the production of rice and its processing, the low quality products and broken rice are subjected to SSF process for production of PHB and (*R*)-3-HB. In other words, lyophilized cells can be used to produce PHB which upon chemical or enzymatic hydrolysis would generate (*R*)-3-HB. On the other hand, cell biomass under resting conditions can be used for production of (*R*)-3-HB. Apparently, (*R*)-3-HB produced through these processes can eventually be used for synthesis of optically pure PHB [4].

Conclusions

Biomass materials such as broken rice can be used for production of PHB by SSF processes with *C. necator*. These processes would not only help in utilizing low quality broken rice, but also for other starchy materials (e.g., cassava, potato, sago, wheat, corn etc.). Proper implementation of this process would help in reducing the cost of producing PHB. The PHB obtained in this work has good characteristics comparable to those obtainable with other carbon substrates as evidenced by its molecular weight distribution and Tm. It would be wise to propose a

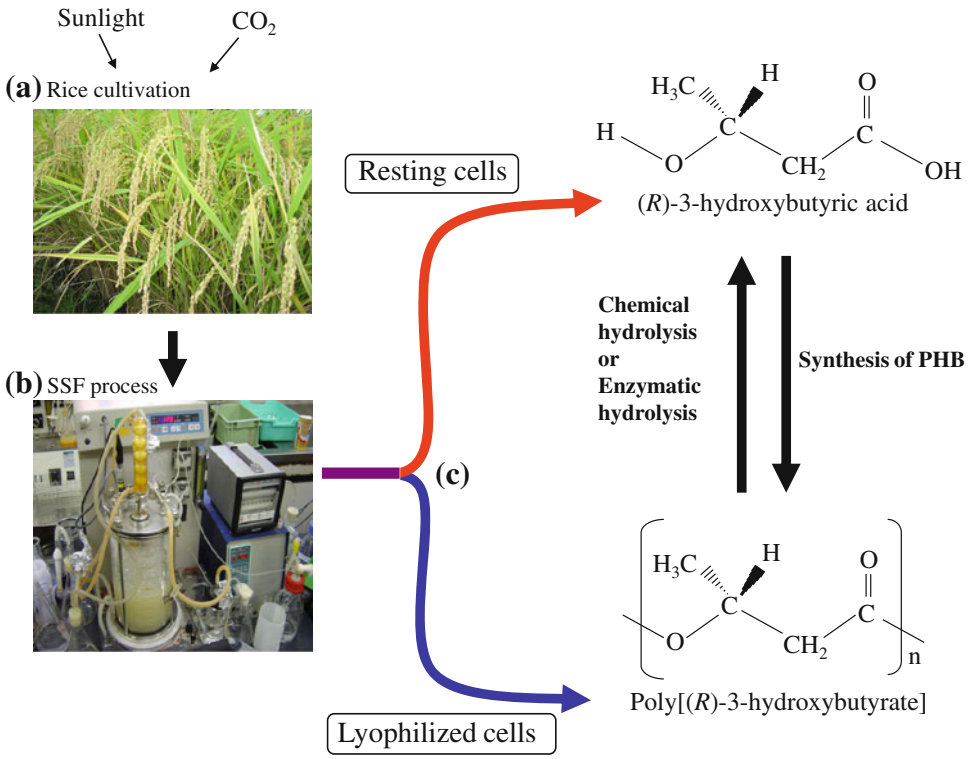

Fig. 3 Mass production of rice for sustainable development (**a**); utilization of low quality or broken rice for production of PHB by SSF process (**b**), and various production methods for (*R*)-3-HB and its utilization for synthesis of optically active PHB (**c**)

strategy that would enable production of PHB by SSF and further transformation of this polymer to (*R*)-3-HB by bioconversion, enzymatic or chemical hydrolysis.

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