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연구논문

Production of Polyhydroxybutyrate from Crude Glycerol and Spent Coffee Grounds Extract by *Bacillus cereus* Isolated from Sewage Treatment Plant

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Abstract: Production of biodegradable polymer polyhydroxyalkanoates (PHAs) from industrial wastes exhibits several advantages such as recycle of waste and the production of high valuable products. To this end, this study aimed at isolating from the sewage treatment plant a PHA producing bacterium capable of utilizing wastes generated from biodiesel and food industries. A *Bacillus cereus* strain capable of producing poly(3-hydroxybutyrate) [P(3HB)] was isolated, which was followed by confirmation of P(3HB) accumulation by gas-chromatographic analyses. Then, the effects of nutrient limitation on P(3HB) production by *B. cereus* was first examined. Cells cultured in a minimal medium under the limitation of nitrogen, potassium and sulfur suggested that nitrogen limitation allows the highest P(3HB) accumulation. Next, production of P(3HB) was examined from both waste of biodiesel production (crude glycerol) and waste from food industry (spent coffee grounds). Cells cultured in nitrogen-limited minimal medium supplemented crude glycerol and waste spent coffee grounds extract accumulated P(3HB) to the contents of 2.4% and 1.0% of DCW. This is the first report demonstrating the capability of *B. cereus* to produce P(3HB) from waste raw materials such as crude glycerol and spent coffee grounds.

Keywords: Polyhydroxyalkanoates, Crude glycerol, Spent coffee ground, Industrial waste, Sewage treatment plant

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1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are microbial polyesters which are intracellularly accumulated as distinct granules as carbon storage material usually when growth is limited in the presence of excess carbon source. PHAs are biodegradable polymers having properties similar to petroleum-based plastics, and thus have potential to replace fossil oil-based plastics [1]. However, its production cost is still relatively high, which can be significantly reduced if inexpensive substrates, and in particular waste raw materials, can be used as substrates [2-4].

Biodiesel production coproduces large amounts of glycerol as a waste. About 1 kg of a crude glycerol is generated in the production of 10 kg of biodiesel [5, 6]. Such waste glycerol has attracted much attention for the production of various bioproducts including acrolein, 1,3-propanediol, butanol, hydrogen and PHAs, both biologically and catalytically [7-11]. Coffee is one of the most popular beverages in the world, thus huge amount of waste are released. Annual global production of coffee is estimated to be about 16 billion pounds [12]. Raw coffee beans contain carbohydrates as well as some oil. The amount of total carbohydrates is about 60% of coffee [13]. Recently, the waste spent coffee grounds, which contain about 15% of oil, have been examined for biodiesel production through catalytic transesterification [12].

Many bacterial species present in sewage sludge have a potential to produce PHAs, because of rich nutrients introduced in the waste stream. Indeed, PHA producing microorganisms have been isolated from the sewage sludge [14]. In addition, the bacterial species found in sewage sludge seem to be able to utilize a variety of carbon substrates due to the varying influx of nutrients in the waste stream. In this study, it was aimed to isolate a PHA producer capable of utilizing wastes including crude gly-

cerol and spent coffee grounds. Additional studies were performed to find the best nutrient limiting condition and to examine wastes that can be used for PHA production by the isolated bacterium.

2. MATERIALS AND METHODS

2.1. Reagents

Nile red (Sigma-Aldrich, MO, USA) was purchased and used for screening bacteria capable of producing PHAs. Glucose (Samyangsa, Seoul, Korea), mannose (Sigma-Aldrich) and glycerol (Avantor Performance Materials, PA, USA) were used as refined carbon sources.

2.2. Industrial waste collection and treatment

During biodiesel production, waste crude glycerol was collected from *Jatropha* oil (see Table 1 for general composition of *Jatropha* [15]) through the alkali-catalytic transesterification. Carbohydrates in the waste spent coffee grounds (Arabica coffee (*Coffea arabica* L.); see Table 1 for general composition of Arabica coffee bean [16]) were extracted with sulfuric acid after decaffeination. Waste spent coffee grounds were dried for 12 h in an oven at 80°C to remove moisture and then refluxed for 2 h with ethyl acetate to remove caffeine from the coffee. Decaffeinated waste coffee grounds were dried. After that, 80 g of decaffeinated waste coffee grounds were mixed gently with 110 mL of 72% (v/v) sulfuric acid for 1 h in a flask at room temperature. After the reaction, 390 mL of distilled water was added into the acidified coffee mixture and autoclaved at 1 atm and 121°C for 1 h to extract carbohydrates form waste coffee grounds. The carbohydrate mixtures were filtered and neutralized with $CaCO_3$, which led to salting out of $CaSO_4$. Calcium sulfate in the extract was removed by filtration to obtain the final carbohydrates solution.

2.3. Waste sludge collection and isolation of bacterium from sewage treatment plant

Waste sludge samples were collected from the waste treatment facility in Daejeon, Korea and used for the isolation of bacteria by serial dilution technique. Waste sludge samples collected were diluted with saline solution for spreading on nutrient agar (Difco, Detroit, MI, USA) plate. Screening of PHA producing bacteria was performed by Nile red staining method [17]. Nile red was added in the agar medium to visualize the bright fluorescent colonies under UV at the wavelength of 312 nm (UV lamp VL-6.M, Vilver Lourmat, Marne-la-Vallée, France).

2.4. Culture conditions for production of PHAs

The stock cultures were initially activated in nutrient broth (Difco) and then inoculated into nutrient broth supplemented with a desired carbon substrate. In order to find the best nutrient limiting condition for P(3HB) production, the pre-cultured cells in nutrient broth supplemented with 10 g/L glucose were transferred to a minimal medium lacking in N, K or S. One milliliter of seed culture was inoculated into a 250 mL flask containing 100 mL nutrient broth and cultured at 37°C and 250 rpm overnight. Then, the cultured cells were centrifuged at 4,000×g and 4°C for 15 min, washed once with distilled water, and transferred into the fresh nitrogen, potassium or sulfur (N-, K- or S-, respectively) limited minimal media for further cultivation at 37°C and 250 rpm for 48 h. The minimal medium contains per liter (pH 6.8): 3.8 g Na_2HPO_4, 2.65 g KH_2PO_4, 0.2 g $MgSO_4$, 2.0 g/L NH_4Cl, 1 mL trace metal solution and a carbon substrate 10 g/L. The trace metal solution was prepared by dissolving 9.7 g $FeCl_3$, 7.8 g $CaCl_2$, 0.218 g $CoCl_2{\cdot}6H_2O$, 0.156 g $CuSO_4{\cdot}5H_2O$, 0.118 g $NiCl_3{\cdot}6H_2O$ and 0.105 g $CrCl_3{\cdot}6H_2O$ in 0.1 N HCl. The N-limited minimal medium was designed by removing NH_4Cl in the minimal medium. The K-limited minimal medium was obtained by adding 2.65 g NaH_2PO_4 instead of KH_2PO_4 in the minimal medium. The S-limited minimal medium was obtained by adding 0.2 g $MgCl_2$ instead of $MgSO_4$ in the minimal medium and by adding 0.1065 g $CuCl_2{\cdot}2H_2O$ instead of $CuSO_4{\cdot}5H_2O$ in the trace metal solution.

2.5. DNA isolation and sequencing

To identify the isolated sewage bacterium, 16S rDNA sequencing was performed. The genomic DNA of the isolate was prepared using a DNeasy blood and tissue kit (Qiagen, German-

Table 1. General compositions of *Jatropha* [15] and Arabica coffee bean [16]

Jatropha		Arabica coffee bean	
Composition	Contents (g/kg)	Composition	Contents (g/kg)
H_2O	66	Trigonelline	8.8-9.7
Protein	182	Caffeine	11.3-12.1
Fat	380	Chlorogenic acid	76.7-86.7
Total carbohydrate	335	Sucrose	64.0-78.3
Fiber	155	Fat	126-155
Ash	45		

town, MD, USA). All DNA fragments were purified using a PCR purification kit (Solgent, Daejeon, Korea). To amplify gene fragments by PCR (T100 thermal cycler, BioRad, Hercules, CA), a proofreading *Pfu* DNA polymerase (Solgent) was used. The 16S rDNA was amplified with primers 27F (5'-AGA GTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTT GTTACGACTT-3') by using the genomic DNA of the isolate as a template. Sequencing of the 16S rDNA was performed with the primers 27F, 1492R, 518F (5'-CCAGCAGCCGCGGTAAT ACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3').

2.6. Extraction of sewage bacterial PHA and structure analysis

Cells were harvested by centrifugation at 4,000×g for 10 min, and washed three times with distilled water. After freeze-drying, PHAs were extracted with chloroform by using the Soxhlet apparatus (Corning, Lowell, MA). Then, cell debris was removed by filtration and chloroform containing PHAs was concentrated by rotary evaporation. The polymer concentrate was precipitated by adding 10 volumes of ice-cold methanol. PHAs obtained as white precipitates were dried on the filter paper. The type of PHA was analyzed by 1H nuclear magnetic resonance (NMR). The 1H NMR spectra of the produced polymer was recorded on a Bruker AVANCE DMX 600 MHz spectrometer (Bruker, Rheinstetten, Germany) using a BBO probe in $CDCl_3$ with tetramethylsilane (TMS) as an internal chemical shift standard at 298K.

2.7. Analytical methods

The PHA concentration was determined by gas chromatography (GC) as reported previously [18-20]. Cells cultured for 48 h in N-, K- and S-limited minimal medium were harvested by centrifugation at 4,000×g for 10 min, and washed three times with distilled water. The final cell pellets were freeze-dried and weighed for measuring the DCW. The freeze-dried cells were suspended in 4 mL of a mixture containing acidified methanol 2 mL, chloroform 2 mL and 0.05 M benzoic acid as an internal standard, and boiled at 100°C for 3.5 h. Then, 1 mL of distilled water was added into the mixture and vortexed. The mixture was left at room temperature to be separated into two phases. The chloroform fraction was used for determining the PHA concentration. GC analysis was performed by using nitrogen as a carrier gas using the Agilent 6890N GC system (Agilent Technologies, Palo Alto, CA) equipped with Agilent 7683 automatic injector, flame ionization detector (FID), and a fused silica capillary column (ATTM-Wax, 30 m, ID 0.53 mm, film thickness 1.20 mm, Alltech, Deerfield, IL). The GC oven temperature was programmed as follows: 80°C for 5 min, ramping to 230°C at 7.5°C/min, increasing to 260°C at 10°C/min and holding for 5 min. The injector and detector temperatures were maintained at 250°C and 300°C, respectively. Cell density was monitored by measuring the OD_{600} using an Ultrospec 3,000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The concentrations of glycerol in crude glycerol and carbohydrates in the spent coffee grounds extract were measured by high performance liquid chromatography (HPLC; Waters 1515/2414/2707, Milford, MA, USA) analyses.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of PHA producing sewage bacterium capable of utilizing glycerol and mannose

Waste crude glycerol and waste spent coffee grounds would be ideal carbon sources for the economical production of PHA. Major carbohydrates solubilized from the acid hydrolysis of the waste spent coffee grounds were mannose and glucose. It was thus aimed to isolate from the waste sludge sample a PHA producing bacterium using glucose (the most abundant carbon source obtainable from lignocellulosics), glycerol (crude glycerol from biodiesel industry) and mannose (a major carbohydrate present in the waste spent coffee grounds extract). Nile red-based screening of PHA producers was performed on the nutrient agar plate supplemented with 10 g/L glucose and 0.5 mg/L Nile red (Fig. 1). *Escherichia coli* strains XL1-Blue (Stratagene, La Jolla, USA) and JLX10 (pPs619C1310-CpPCT540) [18] were used as negative and positive controls, respectively. In the representative plate shown in Fig. 1, three isolates stained by Nile red are shown. In order to isolate a bacterium that can also utilize glycerol and mannose, cells precultured in nutrient broth were washed three times with fresh minimal medium and inoculated into the test tubes containing 10 mL minimal medium supplemented with 10 g/L of glycerol or mannose. The final isolate capable of utilizing glycerol and mannose in addition to glucose was used for further study. A single colony of the final isolate was cultured in nutrient broth overnight followed by transferring to the fresh nutrient broth supplemented with 10 g/L glucose. The broth was incubated 37°C for 96 h in the orbital shaker incubator. GC analysis revealed that this bacterium accumulated P(3HB) to the content of 4.0% of DCW. The polymer accumulated was further confirmed by NMR (see below). To identify the isolate, the 16S rRNA of the microorganism was sequenced and compared with that of other microorganisms. On the basis of the 16S rRNA sequences obtained from the sequencing of the isolate and GenBank database (http://www.ncbi.nlm.nih.gov/genbank/), the phylogenetic tree between the PHA-producing isolate and the genus *Bacillus* was constructed by using MEGA6 [21]. This phylogenetic ana-

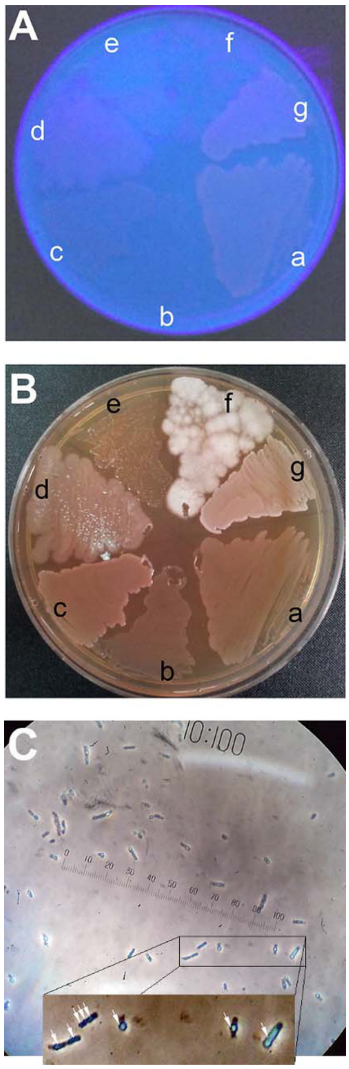

Fig. 1. Screening of PHA producers. Nile red staining of PHAs under (A) fluorescence and (B) light conditions. (C) Accumulation of biopolymer (white arrows) in the bacterial isolate. The letters 'a' and 'b' represent positive control *E. coli* JLX10 (pPs619C1310-CpPCT540) [18] and negative control *E. coli* XL1-Blue (Stratagene), respectively, while 'c-g' represent bacterial isolates from sewage sludge.

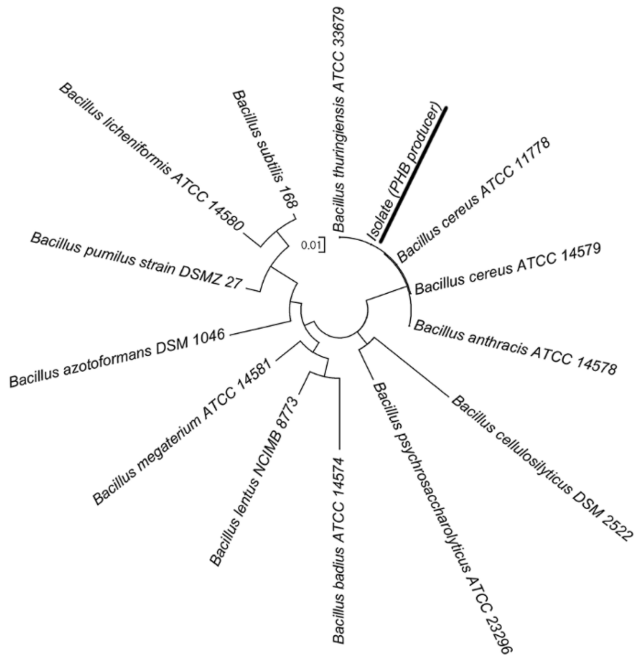

Fig. 2. Phylogenetic tree between the PHA-producing bacterial isolate and the genus *Bacillus*. The phylogenetic tree was constructed by using MEGA6 [21].

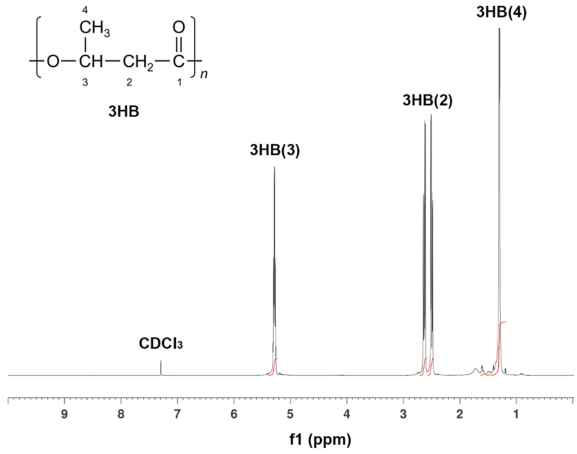

Fig. 3. 1D 1H NMR analysis of PHAs containing 3HB as a monomer synthesized in the *B. cereus* isolate. Polymer was purified from the *B. cereus* isolate that was cultivated in N-limited salt medium supplemented with 10 g/L glucose.

lysis suggested that the isolate belonged to *Bacillus cereus* (Fig. 2).

3.2. Characterization of sewage bacterial polymer

The accumulated sewage bacterial polymers in the *B. cereus* isolate were extracted from dried cell pellets as described in '2. Materials and Methods'. Fig. 3 shows the characteristic P(3HB) peaks at 5.2 (singlet), 2.5 (doublet) and 1.2 (singlet) ppm which correspond to a methine (CH), methylene (CH_2) and methyl ($-CH_3$) groups, respectively, confirming that the accumulated PHA in *B. cereus* is P(3HB).

3.3. P(3HB) production under nutrient limiting conditions

Sewage bacterial polymer, P(3HB) accumulation is often enhanced when one of the growth factors is limiting in the presence of excess carbon source [18,22]. In order to examine the effects of nutrient limitation on P(3HB) production by *B. cereus*, cells were cultured in N-, K- or S-limited minimal medium

Table 2. P(3HB) production of the *B. cereus* isolate in nitrogen, potassium and sulfur limited salt medium supplemented with 10 g/L glucose

Condition	DCW (g/L)	P(3HB) concentration (g/L)	P(3HB) content (wt%)
N-limited	1.25 ± 0.59	0.49 ± 0.30	36.7 ± 9.40
K-limited	0.54 ± 0.14	0.18 ± 0.04	33.0 ± 2.24
S-limited	0.46 ± 0.01	0.15 ± 0.01	32.4 ± 1.11

supplemented with 10 g/L glucose. The *B. cereus* isolate accumulated P(3HB) to the average content of 36.7%, 33.0% and 32.4% of DCW under N-, K- and S-limited conditions, respectively (Table 2). The amount of P(3HB) accumulated was highest in N-limited condition, and the highest P(3HB) content observed for one culture was 44.9%. Cells accumulating P(3HB) under the N-limited condition observed under the phase contrast microscopy are shown in Fig. 1C.

3.4. Production of P(3HB) from waste crude glycerol and waste spent coffee grounds

In order to examine whether the *B. cereus* isolate can produce P(3HB) from waste crude glycerol (obtained as a byproduct of biodiesel industry) and from carbohydrate extract from waste spent coffee grounds, cells were cultured in the N-limited minimal medium containing glycerol or mannose. The P(3HB) contents obtained from glycerol and mannose were 2.6%±0.03 and 1.9%±0.04, respectively.

Having found that the *B. cereus* can produce P(3HB) from glycerol and mannose, P(3HB) production from waste raw materials, such as waste crude glycerol and waste spent coffee ground, was examined in the N-limited minimal medium. Major components of waste crude glycerol and the waste spent coffee grounds extract were analyzed by HPLC. The waste crude glycerol contained mainly glycerol with a purity of 87.5%, while the extract from the waste spent coffee grounds contained 10.5 g/L mannose and 2.7 g/L glucose as major carbohydrates (Fig. 4). The extract from the waste spent coffee grounds also contained 0.4 g/L glycerol, 3.3 g/L formic acid and 1.7 g/L acetic acid (Fig. 4). To examine P(3HB) production from these waste raw materials, the *B. cereus* isolate was cultured in N-limited minimal medium supplemented with 10 g/L waste crude glycerol or the waste spent coffee grounds extract containing 10 g/L carbohydrate equivalents. The *B. cereus* isolate accumulated P(3HB) with the content of 2.4%±0.21 and 1.0%±0.26 from waste crude glycerol and waste spent coffee grounds extract, respectively. Even though the P(3HB) content obtained was rather low, this is the first report on the utilization of the spent coffee grounds as a raw material for the production of P(3HB). Furthermore, flask culture of the *B. cereus* isolate resulted in the accumulation of P(3HB) to the content of 2.4% from crude glycerol, which is not much different from that (2.6%) obtained using pure glycerol. Cell growth of the *B. cereus* isolate also was not retarded in the flask culture in the medium supplemented with crude glycerol compared with the culture containing pure glycerol, which means that the isolate has a tolerance to crude glycerol used in this study. Thus, it is expected that P(3HB) production from these waste raw materials can be improved through strain development by metabolic engineering together with optimization of culture conditions employing a bioreactor.

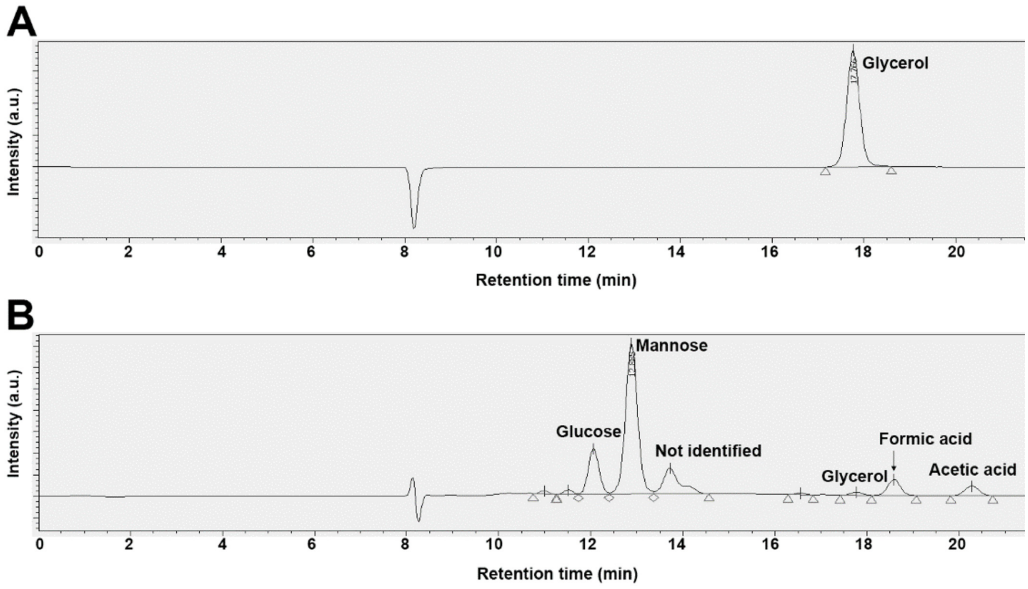

Fig. 4. HPLC analysis of (A) crude glycerol and (B) extracts of spent coffee grounds.

4. CONCLUSIONS

It is important to recycle industrial wastes by using as a substrate for production of value added materials. In particular, it is of great interest to use waste raw materials for the production of PHAs. We were interested in utilizing waste crude glycerol, a major byproduct of biodiesel industry, and the waste spent coffee grounds extract which contains mannose as a major carbohydrate. In this study, a *B. cereus* strain capable of producing P(3HB) from glucose, glycerol and mannose was isolated from the waste treatment facility. First, it was found that the *B. cereus* isolate was capable of producing P(3HB) best under N-limited condition. Next, the possibility of producing P(3HB) by *B. cereus* from waste crude glycerol was examined. Flask culture of the *B. cereus* isolate resulted in the accumulation of P(3HB) to the content of 2.4% from waste crude glycerol. Due to the increased consumption of coffee as a beverage throughout the world, lots of waste spent coffee grounds are generated. Thus, we were interested in the use of waste spent coffee grounds for the production of P(3HB). The major carbohydrate present in the extract of the waste spent coffee grounds is mannose, which is why we isolated a bacterium that is capable of utilizing mannose. The *B. cereus* isolate was able to produce P(3HB) from the waste spent coffee grounds extract. This is the first report on the utilization of the waste spent coffee grounds as a raw material for the production of P(3HB).

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