Process Biochemistry 52 (2017) 238–242

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Directed bioconversion of Kraft lignin to polyhydroxyalkanoate by *Cupriavidus basilensis* B-8 without any pretreatment

Yan Shi[a,b,c], Xu Yan[b], Qiang Li[a], Xin Wang[a], Mingren liu[b], Shangxian Xie[a], Liyuan Chai[b,*], Joshua Yuan[a,*]

[a] *Synthetic and Systems Biology Innovation Hub, Department of Plant Pathology and Microbiology, Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, 77843, TX, USA*
[b] *School of Metallurgy and Environment, Central South University, 410083, Changsha, PR China*
[c] *School of Environment, Henan Normal University, Xinxiang, 453007, Henan, PR China*

ARTICLE INFO

Article history:
Received 17 August 2016
Received in revised form
30 September 2016
Accepted 2 October 2016
Available online 4 October 2016

Keywords:
Cupriavidus basilensis B-8
Kraft lignin
Lignin depolymerization
PHA production

ABSTRACT

This work presents here a new fundamental strategy for bio-converting Kraft lignin (KL) into useful products. *Cupriavidus basilensis* B-8 (here after B-8) was able to use KL as the sole carbon source. Fully 41.5% of lignin, 37.7% of total carbon (TC) and 43.0% of color were removed after 7 days of incubation. At the same time, lignin was depolymerized into small fragments, which was confirmed by scanning electron microscopy (SEM) and gel permeation chromatography (GPC). Bacterial biomass accumulated to 735.6 mg/L at the initial KL concentration of 5 g L^{-1}, and the corresponding volumetric productivity of polyhydroxyalkanoate (PHA) was 128 mg/L. PHA productivity was significantly improved through fed batch fermentation and reached to 319.4 mg/L. GC–MS analysis showed that PHA polymer was composed of three basic monomers: 98.3 mol% of (*S*)-3-hydroxy-butanoic acid (S3HB), 1.3 mol% of ®-3-hydroxybutyric acid (R3HB) and 0.4 mol% of 3-hydroxy-butanoic acid (3HB).

1. Introduction

Lignin offers potential to be a renewable feedstock for fuels, chemicals and materials. KL that composes the majority of lignin products is produced at a scale of over 6.3×10^8 kg annually [1]. Therefore, development of new strategies for more efficient conversion of KL to useful products is of crucial importance. In addition, as the major by-product washed out in pulp and paper effluent, KL not only causes serious aesthetic problems but also inhibit natural photosynthesis in streams due to the absorbance of sunlight [2]. Hence, the adequate control and utilization of KL prior to Kraft pulping effluent discharge into the environment is warranted.

Biodegradation of lignin and its derivatives by microorganisms has emerged as a potential alternative for pulp and paper effluent treatment. A number of microorganisms including fungi and bacteria were found to be able to break down lignin. The best characterized degraders are white-rot fungi, which have been well studied for treatment of bleached Kraft mill effluent [3]. However, fungi require low pH (pH 4–5) to maintain their growth and enzyme activity. The solubility of high molecular weight lignin and its derivatives is reduced at low pH. Bacteria display versatile pathways that degrade aromatic substances, from simple phenols to highly complex lignin. Given their immense environmental adaptability and biochemical versatility [4], bacteria could play a role in the treatment of pulp and paper effluent. Many strains, such as *Paenibacillus* sp. and *Aneurinibacillus aneurinilyticus*, have been used for the treatment of low strength pulp mill effluent [5,6]. The primary drawback of these strains in this process is the requirement of a co-substrate such as glucose for cell growth and enzyme activity development. The use of a co-substrate may increase COD in Kraft pulping effluent and make direct biotreatment impractical and uneconomical. To our knowledge, little research has been reported on microorganisms that require little or no co-substrate for Kraft pulping waste treatment.

In our previous work, the capability of B-8 to degrade KL without any co-substrate had been confirmed [7]. Genomic sequencing and systematic analysis of the genome identified degradation steps and intermediates from this bacterial-mediated KL degradation [7]. In addition, *phaA*, *phaB*, and *phaC*, the genes responsible for PHA synthesis, were identified based on genomic analysis (unpublished). As a naturally occurring biodegradable polyester produced as energy storage material by many bacteria, PHA is being considered as a

* Corresponding authors.
E-mail addresses: liyuanchaihj@aliyun.com (L. Chai), syuan@tamu.edu (J. Yuan).

http://dx.doi.org/10.1016/j.procbio.2016.10.004
1359-5113/

replacement for conventional plastics [8]. However, the report on PHA produced from KL is limited; thus, a lignin metabolic route connecting the corresponding aromatic catabolism with PHA synthesis represents a new strategy for KL utilization. The objects of this work were to (i) evaluate KL treatment by B-8, (ii) investigate bioconversion of PHA from KL and (iii) characterize PHA produced from KL by B-8.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Strain B-8 isolated from the steeping fluid of eroding bamboo slips has been deposited in the China General Microbiological Culture Collection Center (CGMCC) with the accession number CGMCC 4240. Cells were grown in 10 ml Luria-Bertani broth medium with shaking speed of 150 rpm until the optical density at 600 nm of inoculums reached 1.0. Bacterial cells were collected through centrifugation, washed three times with sterile water and then inoculated into triplicate flasks containing 100 ml sterile KL (CAS no.: 8068-05-1 Sigma) mineral salt medium [7]. Incubation was conducted at 30 ◦ C with shaking at 150 rpm for seven days. To measure lignin degradation, decolorization and TC removal, samples were withdrawn periodically at 24 h interval.

2.2. Measurement of KL degradation, decolorization and TC removal

Bacterial strains in 3 ml samples were removed by centrifugation at 12,000 rpm for 15 min. Supernatant (1 ml) was withdrawn and diluted two-fold with phosphate buffer (pH 7.6). KL degradation and decolorization were determined by monitoring the decrease of absorbance at 280 nm (A_{280}) and 465 nm (A_{465}) with a Hitachi U-4100 spectrophotometer, respectively. For ultraviolet scanning, 20 μl samples withdrawn at four and seven days of incubation were added into 3 ml deionized water, mixed well and then scanned under wavelength ranging from 190 to 400 nm. KL without any treatment was used as a control. To measure TC removal, 1 ml supernatant diluted with 19 ml deionized water was measured with a Shimadzu TOC-V CPH.

2.3. GPC and SEM

Fifty milliliters of control (without inoculation) and samples withdrawn after seven days of incubation were centrifuged at 12,000 rpm for 15 min. The supernatant was dried to constant weight in a vacuum freeze drying apparatus and used for SEM and GPC. For samples containing cells, 50 μl medium after seven days of incubation was placed on glass slides. After 15 min adsorption, glutaraldehyde (2.5%) was added to immobilize the sample for 1 h. Then, the sample was dehydrated in a gradient using 30–70% ethanol and replaced with isoamyl acetate for 30 min. Through critical point drying (HITACHI HCP-2 Critical PointDryer) and ion sputtering (Eiko IB-3 ion plating machine), the sample was observed via SEM in a JEOL JSM-6360LV microscope. GPC was carried out in an AKTA purifier UPC100 using a previously reported method [9].

2.4. Fluorescence microscopy

Samples (2 ml) were withdrawn after two days of incubation. The supernatant was removed, and cells were re-suspended with 150 μl deionized water and 50 μl dimethyl sulfoxide (CAS no.: 67-68-5 VWR). Five microliters of Nile red (0.15 mg/ml) was added to stain for 30 min. Then, the samples were observed under a microscope (Olympus BX60) equipped with a fluorescence source (Olympus U-TV0.5XC-3).

2.5. Extraction and GC–MS analysis of PHA from KL in B-8

Bacterial cells grown two days in N-limited (65 mg/L) KL mineral salt medium were harvested by centrifugation at 12,000 rpm for 15 min, washed twice with deionized water, and then lyophilized for 48 h. PHA was extracted from lyophilized cells using the chloroform-methanol method [10]. Simple fed batch fermentation was performed as follows: bacterial cells were collected and centrifuged at 48 h intervals, and new cells were inoculated as describe above. The water was added in the medium to 100 ml. PHA extracted from bacterial cells from the same bottle were collected together and weighted. The extracted pure PHA (0.5–2 mg) was methanolyzed in 2 ml of methanol containing 15% sulfuric acid and 2 ml of chloroform under 100 ◦C for 4 h. GC–MS was performed in a GC/MS SE plus Gas Chromatograph (Shimadzu, Japan) using the previously described method [10]. All of the experiments were done in triplicate.

3. Results and discussion

3.1. KL degradation and decolorization by B-8

KL degradation by B-8 was evaluated through A_{280} measurement and TC removal. As shown in Fig. 1a, the absorbance at 280 nm was reduced to 1.86 from 3.18 after seven days of incubation; accordingly, 41.5% of lignin in the medium had disappeared. Meanwhile, 37.7% of TC in the culture was removed during this period (Fig. 1b). The loss of lignin content and TC removal demonstrated that lignin was broken down and metabolized by B-8. KL decolorization by B-8 was investigated by A_{465} measurement and ultraviolet scanning. A significant reduction in the absorbance at 465 nm was observed. The initial absorbance of the culture medium was 3.65; it dropped to 2.08, and 43.0% of the color was removed at the seventh day (Fig. 1c). In addition, the UV spectrums of treated samples were obviously lower than that of the control. The peak at 280 nm almost disappeared, while the peak at 200 nm decreased significantly (Fig. 1d), indicating that color was reduced during the process of lignin degradation. Meanwhile, decolorization was assumed to result from lignin depolymerization based on bacterial ligninolytic systems [5]. These data also clearly suggested that B-8 has great potential for Kraft pulping waste treatment.

3.2. Characterization of KL before and after B-8 treatment

To confirm the depolymerization of lignin under B-8 treatment, we first observed lignin morphological changes under a SEM. As a control, lignin without any B-8 addition kept its irregular spherical shapes (Fig. 2a). The diameter of these lignin particles, measured under the SEM, was in a range of 100–150 μm. After seven days of incubation, rod-like bacteria (as indicated by the red row in Fig. 2b) were found to deposit on the surface of broken particles. Accordingly, the lignin/bacteria suspension was centrifuged to remove its bacteria fraction, and then the morphology of the lignin after B-8 treatment was checked. As shown in Fig. 2c, the size of the lignin aggregates after seven days of bacterial treatment had decreased to <10 μm, indicating that lignin was physically or chemically broken down into smaller particles. Therefore, B-8 was confirmed to have the capability of lignin depolymerization. GPC was subsequently utilized to detect the changes in lignin molecular weight (Mw) with B-8 depolymerization treatment. A chromatogram with peak integration is shown in Fig. 2. Untreated KL, as a control, displayed a Mw range from 15.1 KDa to 1.65 KDa, which means this KL is composed of high Mw lignin fractions as well as low Mw fractions. After