Bioresource Technology 177 (2015) 381–386

Contents lists available at ScienceDirect

Bioresource Technology

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

Short Communication

Bacterial production of short-chain organic acids and trehalose from levulinic acid: A potential cellulose-derived building block as a feedstock for microbial production

Hiroshi Habe [a,*], Shun Sato [a], Tomotake Morita [a], Tokuma Fukuoka [a], Kohtaro Kirimura [b], Dai Kitamoto [a]

[a] Research Institute for Innovation in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan
[b] Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, Tokyo 169-8555, Japan

HIGHLIGHTS

- Six bacterial strains capable of using levulinic acid were isolated and identified.
- The strains produced short-chain organic acids or trehalose from levulinic acid.
- Two strains degraded up to 70 g/L levulinic acid in a high-cell-density system.
- *Burkholderia stabilis* produced trehalose extracellularly from levulinic acid.

ARTICLE INFO

Article history:
Received 20 September 2014
Received in revised form 10 November 2014
Accepted 11 November 2014
Available online 18 November 2014

Keywords:
Levulinic acid
Cellulosic biomass utilization
Microbial production
Short-chain organic acid
Trehalose

ABSTRACT

Levulinic acid (LA) is a platform chemical derived from cellulosic biomass, and the expansion of LA utilization as a feedstock is important for production of a wide variety of chemicals. To investigate the potential of LA as a substrate for microbial conversion to chemicals, we isolated and identified LA-utilizing bacteria. Among the six isolated strains, *Pseudomonas* sp. LA18T and *Rhodococcus hoagie* LA6W degraded up to 70 g/L LA in a high-cell-density system. The maximal accumulation of acetic acid by strain LA18T and propionic acid by strain LA6W was 13.6 g/L and 9.1 g/L, respectively, after a 4-day incubation. Another isolate, *Burkholderia stabilis* LA20W, produced trehalose extracellularly in the presence of 40 g/L LA to approximately 2 g/L. These abilities to produce useful compounds supported the potential of microbial LA conversion for future development and cellulosic biomass utilization.

1. Introduction

A shift from petroleum to bio-based feedstocks is important to address greenhouse gas emissions and the exhaustion of fossil fuel resources. The use of cellulose, the most abundant and non-edible biomass resource, is important for synthesis of useful compounds and energy as it represents an inexpensive and renewable source of sugars. Cellulose in plants forms highly crystalline microfibrils embedded in a hemicellulose, pectin and lignin matrix; this recalcitrant structure restricts access to and decomposition of cellulose into simple sugars by hydrolytic enzymes. To overcome this problem, many studies on the chemical and physicochemical pretreatment of lignocellulose for subsequent enzymatic saccharification have been performed; however, the enzymatic hydrolysis of cellulose remains a major factor limiting efficient utilization (Olson et al., 2011; Hasunuma et al., 2013).

Of the diverse chemicals other than sugars that can be synthesized from cellulose, levulinic acid (LA) is a promising building block for chemical production; indeed, it is considered one of the top 12 building blocks by the US Department of Energy (Werpy and Petersen, 2004). It has for more than 130 years been known that LA is formed from sugars in the presence of large quantities of mineral acids. Thus, many reports on LA production from biomass have been published. In the 1990s, Biofine Inc. (Framingham, MA) developed the first practical production process of LA from cellulose-containing biomass with more than 300 mol% of H_2SO_4. According to Biofine's estimation of operating costs and byproduct revenues from a plant processing 1000 dry tonnes of feedstock/day, the production cost of ethyl levulinate is $291/ton (Hayes

* Corresponding author. Tel.: +81 29 861 6247; fax: +81 29 861 4457.
E-mail address: hiroshi.habe@aist.go.jp (H. Habe).

http://dx.doi.org/10.1016/j.biortech.2014.11.048
0960-8524/

et al., 2006). Tominaga et al. (2011) reported an efficient and recyclable catalyst system for synthesizing methyl levulinate from cellulose by combining two acids—a Lewis acid and a Brønsted acid—in methanol. This reaction proceeds in two steps, in which cellulose is first hydrolyzed into sugars by sulfonic acids, followed by conversion to the LA derivative by metal triflates (Tominaga et al., 2011). This green and sustainable chemical process uses less acid, and can be used for the production of LA itself. More recently, Ren et al. (2013) reported selective conversion of cellulose to LA using microwave-assisted synthesis in ionic liquids (Ren et al., 2013).

LA can be converted into various useful chemicals, such as fuel additives, polyacrylates, polycarbonates, biodegradable herbicides, and photosynthesis promoters using chemical processes (Bozell et al., 2000). However, few reports focus on microbial conversion of LA to compounds such as alcohols, organic acids and amino acids in fermentation, because the short-chain organic acids (e.g., acetic acid and LA) and furfural-like compounds produced by acid-catalyzed thermal hydrolysis of saccharides inhibit microbial growth at moderate concentrations (Park et al., 2013). Isolation and identification of LA-utilizing microorganisms using media containing 10 g/L LA as a sole carbon source have been reported; however, little is known regarding the microbial LA metabolic pathway. Recently, a kinetic analysis of 1 g/L LA utilization by *Cupriavidus necator* and *in vitro* bioconversion of LA with its crude enzymes provided the first direct evidence of both acetyl-CoA and propionyl-CoA formation from LA, suggesting that the inducible membrane-bound enzyme, LA acyl-CoA synthetase, is involved in the initial step of LA metabolism (Jaremko and Yu, 2011). However, detailed information on LA metabolic genes and enzymes, as well as LA metabolites excreted extracellularly, remain unknown. In addition, a higher concentration of LA as a feedstock for microorganisms is necessary for chemical synthesis.

Investigation of microbial metabolites that can be produced from LA may facilitate the synthesis of a range of non-sugar-based compounds by fermentation, which will broaden the use of lignocelluloses. In this study, to explore the potential of LA as a platform chemical for bioprocesses, we isolated several LA-utilizing bacteria and characterized their products, such as propionic acid and trehalose. In addition, we showed that some strains tolerated up to 70 g/L LA in the culture medium.

2. Methods

2.1. Bacterial strains, media, and chemicals

LA-utilizing bacteria isolated from environmental samples were routinely cultivated on nutrient-rich LA medium (LA medium A; composition: 20–50 g/L LA, 5 g/L polypeptone, 5 g/L yeast extract, 1 g/L $MgSO_4{\cdot}7H_2O$; pH 7.0). Strain LA20W isolated in this study was deposited as NITE P-01893 on the NITE Patent Microorganisms Depositary (NPMD, Chiba, Japan). All chemicals were of the highest purity available commercially (98–100%; Sigma–Aldrich, Tokyo Chemical Industry, Wako Pure Chemical, Nacalai Tesque).

2.2. Screening of LA-utilizing strains

LA-utilizing bacteria were isolated from environmental samples taken from Tsukuba, Japan. Each environmental sample—such as soil, water of pounds, or duff in forests—was added to 5 mL of nutrient-poor LA medium in a test tube (LA medium B; composition: 20 or 50 g/L LA, 2 g/L K_2HPO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$; pH 7.0), and incubated aerobically at 30 °C on a reciprocal shaker (200 rpm). When bacterial growth was observed, 10 μL of each culture were transferred into new tubes containing freshly prepared medium. After three subcultures, the appropriate dilution of the resultant culture was spread onto a YPD agar plate (5 g/L glucose, 5 g/L polypeptone, 5 g/L yeast extract, 1 g/L $MgSO_4{\cdot}7H_2O$). Colonies were isolated and their ability to grow on LA media re-checked.

2.3. 16S rRNA gene analysis of LA-utilizing bacteria

Total DNA was isolated from bacteria (strains LA1S, LA6W, LA18T, LA20W, LA22B, and LA24Y) grown on YPD medium, according to standard protocols. Using total DNA from each strain as the template, the 16S rRNA genes were amplified by PCR using the forward primer 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and reverse primer 1406R (5′-ACGGGCGGTGTGTAC-3′). PCR reactions were performed using *Premix Taq*™ (Takara Shuzo, Kyoto, Japan). Sequencing reactions were performed using the ABI PRISM™ BigDye™ Terminator, v3.1 Kit (Applied Biosystems, CA, USA) with the 9F and 1406R primers for the total 16S rRNA gene region and the 9F and 536R (5′-GTATTACCGCGGCTGCTG-3′) primers for a partial 16S rRNA gene region. The PCR products were sequenced using the ABI PRISM™ 3100 genetic analyzer system (Applied Biosystems). The 16S rRNA gene sequences were compared with those of closely related species obtained from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp) using the BLAST search software and alignment using the Clustal W software. The sequences of the 16S rRNA genes of strains LA6W, LA18T, and LA20W were registered in the DDBJ, EMBL, and GenBank databases under accession numbers AB973362, AB973361, and AB938315, respectively.

2.4. Effect of initial LA concentration on strain growth

Following pre-cultivation of the LA1S, LA6W, LA18T, LA20W, LA22B, and LA24Y strains for 1 day in 5 mL of YPD medium in a test tube, seed cultures (0.5 mL each) were transferred to 30 mL of LA medium A (LA concentration: 20, 30, 40, or 50 g/L) and cultured for 4 days at 30 °C on a rotary shaker (200 rpm). Cell growth was evaluated based on optical density (OD) measurement at 600 nm.

2.5. Quantification of LA metabolites (short-chain organic acids) using HPLC

After cultivation of the strains, cells were then pelleted by centrifugation, and the respective supernatants analyzed by high-performance liquid chromatography (HPLC) with an LC-20AD HPLC pump (1.0 mL/min flow rate) and an RID-10A detector (Shimadzu) equipped with an Aminex® HPX-87H column (300 × 7.8 mm, BIO-RAD) to determine the concentrations of LA, α-ketoglutaric acid, acetic acid, and propionic acid. A mobile phase of 5 mM H_2SO_4 was chosen for the column. During the analysis, the column temperature was maintained at 60 °C. The concentration of each product was determined from a calibration curve generated using the corresponding authentic reagents. We also confirmed these compounds using the same HPLC system (1.0 mL/min flow rate) with another UV detector and column [a SPD-20AV detector (Shimadzu) and a TSK-GEL ODS-100V column (150 × 4.6 mm, TOSOH)]. A mobile phase of 74 mM H_3PO_4 and column temperature of 30 °C were used. Co-chromatography experiments involving a mixture of culture samples and standard samples were performed using both columns.

2.6. Identification of trehalose

HPLC analysis of LA20 W culture using the RID-10A detector (Shimadzu) and an Aminex® HPX-87H column (BIO-RAD) revealed a peak of an unknown metabolite with a retention time of 4.7 min. Levulinate (sodium salt) was removed by passage through