A rapid and sensitive enzymatic assay for 2,3-butanediol

Gyu Bi Lee^{1,2} · Yun Jae Kim^{1,2} · Jae Kyu Lim^{1,2} · Tae Wan Kim³ · Sung Gyun Kang^{1,2} · Jung-Hyun Lee^{1,2} · Hyun Sook Lee^{1,2}

Received: 17 January 2019 / Accepted: 3 April 2019 / Published online: 8 April 2019
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Abstract

In this study, we developed a rapid and sensitive enzymatic assay for 2,3-butanediol (2,3-BDO) detection. The concentration of 2,3-BDO was determined by measuring the reduction of NADP⁺ using *Clostridium ljungdahlii* 2,3-butanediol dehydrogenase (CL-Bdh). The enzymatic assay could detect as low as 0.01 mM of 2,3-BDO, while the high-performance liquid chromatography (HPLC) method required a much higher concentration than 0.15 mM. Gratifyingly, the developed method was 15 times more sensitive than the HPLC method. When the enzymatic assay was applied to high-throughput screening, the enzymatic assay detected 14 positive samples out of 23 tested, as compared to 8 by the HPLC method. These results suggest that the enzymatic assay is an effective screening method for the detection of 2,3-BDO-producing microbes in a microtiter plate-based format.

Keywords 2,3-Butanediol · Enzymatic assay · 2,3-Butanediol dehydrogenase · High-throughput screening

Introduction

2,3-Butanediol (2,3-BDO) is a building block for useful chemicals such as methyl ethyl ketone and 1,3-butanediene, and 2,3-BDO can be used as an antifreeze agent and fuel and octane booster and for the production of plasticizers and fumigants. Considerable research on 2,3-BDO production is being carried out using biological methods (Xie et al. 2017; Yang et al. 2017). The quantitative detection of 2,3-BDO in fermentation broth is normally performed using gas chromatography (GC) and high-performance liquid chromatography (HPLC) (Kim et al. 2013; Xiao et al. 2010). These methods

require the pretreatment of samples to remove proteins and other molecules (Dong et al. 2015; Kameya et al. 2014). In addition, 40–60 min is needed to analyze one sample, rendering high-throughput or simultaneous handling of samples extremely difficult. Thus, GC and HPLC are not suitable for screening purposes. Recently, a method to detect 2,3-BDO using thin-layer chromatography (TLC) was suggested (Saran et al. 2014), which is simple and highly sensitive but not suitable for the high-throughput screening system. In this report, we present a new enzymatic assay for high-throughput screening of 2,3-BDO, using 2,3-butanediol dehydrogenase from *Clostridium ljungdahlii* DSM 13528.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-019-1705-9>) contains supplementary material, which is available to authorized users.

✉ Yun Jae Kim
bio1213@kiost.ac.kr

✉ Hyun Sook Lee
leeh522@kiost.ac.kr

¹ Korea Institute of Ocean Science and Technology,
Busan 49111, Republic of Korea

² Department of Marine Biotechnology, University of Science
and Technology, Daejeon 34113, Republic of Korea

³ Department of Biotechnology and Bioengineering, Chonnam
National University, Gwangju 61186, Republic of Korea

Materials and methods

Cloning of the recombinant plasmid

To obtain *C. ljungdahlii* 2,3-butanediol dehydrogenase (CL-Bdh), *C. ljungdahlii* DSM 13528 was cultured anaerobically in a heterotrophic PETC 1754 medium (American Type Culture Collection, Rockville, Maryland, USA) at 37 °C, and its genomic DNA was extracted with a DNA extraction kit (Exgene™ Cell SV, Gene-All, Seoul, South Korea). Next, the gene encoding CL-Bdh was amplified with a primer set (CLJU23220_F,

5'-agaaggagatatacatatgaaagctgtattgtggtatgata-3' and CLJU23220_R, 5'-tggtggtggtggtgctcgcagcaataaggattgtcaggagttac-3') by polymerase chain reaction (PCR). The amplified PCR product was cloned into vector pET-24a(+) via the sequence- and ligation-independent cloning (SLIC) method (Jeong et al. 2012).

Protein expression and purification

To express the CL-Bdh protein, *Escherichia coli* BL21 (DE3) transformed with a plasmid containing the CL-*bdh* gene were grown in Luria–Bertani medium supplemented with 100 µg/ml kanamycin until the OD₆₀₀ reached 0.6. Then, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of CL-*bdh*, and the cultivation temperature was decreased to 16 °C for 15 h. The cells were harvested by centrifugation at 5500×g for 20 min at 4 °C, washed with buffer (50 mM NaH₂PO₄, 300 mM NaCl, 1 mM DTT, pH 8.0), resuspended, and sonicated to break down the cell walls. The crude extracts were centrifuged at 13,000×g for 1 h at 4 °C. The soluble fractions were loaded on a TALON™ metal affinity resin column (BD Biosciences Clontech, Palo Alto, California, USA), and the recombinant proteins were eluted with imidazole. Protein concentrations were determined using the Bradford assay (Bradford 1976), and the purified proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Enzymatic assay

To detect 2,3-BDO in culture broth, enzymatic assays were conducted in a 96-well plate (Bio-Rad, Hercules, California, USA). A buffer mixture was added to each well, consisting of 200 mM Tris–HCl (pH 8.0), 1 mM DTT, and 5 mM NADP⁺, and then 25 µg/ml CL-Bdh and culture broth or serially diluted 2,3-BDO was added immediately before activation at 45 °C. After 20 min of activation, the absorbance of each well was measured with a spectrophotometer (VersaMax microplate reader, Molecular DEVICES, San Jose, California, USA) at 340 nm.

HPLC analysis

All the culture broths tested for enzymatic assay were analyzed with an HPLC equipped with Aminex® HPX-87H (BIO-RAD, Hercules, California, USA) column. All samples were prepared with 1 ml of culture broth and centrifuged to remove cell debris at 4 °C, 13,480×g for 5 min. The supernatants were purified with a syringe filter and analyzed by HPLC under the following operating conditions: 5 mM H₂SO₄, 0.2 ml/min, 60 °C, 80 min.

Results and discussion

SDS-PAGE

Purified proteins were analyzed by SDS-PAGE, and one major band was apparent, which migrated at approximately 40 kDa (Fig. 1). The CL-Bdh used in this study is a Zn²⁺-dependent alcohol dehydrogenase and a strictly NADPH-dependent enzyme catalyzing the conversion of acetoin to 2,3-BDO (Tan et al. 2015). CL-Bdh shows a limited range of substrate specificity for 2,3-BDO and 1,2-propanediol in oxidation reactions, and it is more specific for 2,3-BDO (Tan et al. 2015). CL-Bdh shares 78% amino acid identity with the alcohol dehydrogenase from *C. beijerinckii* NCIMB 8052 (Cbei_1464), which exhibits high activity at pH 8 and 45 °C with (R,R)- and (R,S)-2,3-BDO (Raedts et al. 2014). Thus, the (R,R)-2,3-BDO form was chosen as a substrate for CL-Bdh in determining the limits of detection. In addition, we tested the stability of the CL-Bdh at 4 °C. CL-Bdh was stable enough to maintain 91% of activity when stored at 4 °C for 6 days (Fig. S1). The stability of CL-Bdh does not seem to be a problem for the 2,3-BDO detection assay.

Comparison of sensitivity between the enzymatic assay and HPLC

Using the purified CL-Bdh, the assay was performed at 45 °C for 20 min in a reaction mixture (200 mM Tris–HCl,

Fig. 1 SDS-PAGE analysis of the recombinant protein, CL-Bdh, expressed and purified from *E. coli* BL21 (DE3). Lane M represents the molecular weight marker

pH 8.0, 5 mM NADP⁺, 1 mM DTT, 25 µg/ml CL-Bdh) designed to provide optimal activity conditions in the background of the culture medium. The reduction of NADP⁺ to NADPH was monitored as an increase in the absorbance at 340 nm. The limit of detection of the assay was 0.01 mM (Fig. 2a). However, the activity of the enzyme was inhibited in the presence of acetoin and, consequently, lower concentrations could be detected in the 2,3-BDO determination (data not shown). In contrast, HPLC was applied to determine the limit of detection of (R,R)-2,3-BDO. All samples were analyzed with a YL9100 HPLC system (Younglin, Anyang, Korea) equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, Hercules, California, USA) with a mobile phase of 5 mM H₂SO₄ at a flow rate of 0.2 ml/min (Kim and Hahn 2015; Park et al. 2015). This conventional method could detect 0.15 mM or higher (R,R)-2,3-BDO (Fig. 2b). Consequently, the enzymatic assay was approximately 15 times more sensitive than HPLC.

Screening of 2,3-BDO-producing microbes with culture broths

To examine whether the enzymatic assay can be used as a rapid and reliable method for the screening and selection of 2,3-BDO-producing microbes, culture broths from 23 microbial isolates from various environmental samples were tested in 96-well microplates. Using the enzymatic assay, 14 of 23 samples were identified to be 2,3-BDO producers (Table 1). HPLC detected only eight positive samples. Those samples that were positive by HPLC analysis were also positive in the enzymatic assay. In addition, the concentration of 2,3-BDO detected by the enzymatic assay was 0.1–0.2 mM, which was lower than that determined by HPLC. The differences in the quantity of 2,3-BDO might be caused by the presence

Table 1 High-throughput screening of 2,3-BDO-producing microbes using the enzymatic assay

Isolated strain	2,3-BDO production	
	Enzymatic assay (mM)	HPLC (mM)
D-DJ-16-C2	0.21	0.24
D-DJ-16-C7	0.21	ND
D-DJ-19-C1	0.20	ND
D-SH24-C1	ND	ND
E-DJ17-C1	ND	ND
E-DJ17-C10	ND	ND
E-DJ17-C11	ND	ND
E-DJ17-C16	ND	ND
E-DJ17-C19	ND	ND
E-DJ17-C25	0.21	ND
E-DJ17-C34	0.19	ND
E-HSD45-C29	ND	ND
HSD-45-C1	0.21	ND
HSD-45-C2	0.20	ND
HSD-45-C20	ND	ND
HSD-45-C27	ND	ND
S-NC1-C1	0.20	0.28
S-NC1-C2	0.20	0.28
S-NC1-C3	0.20	0.30
S-NC1-C4	0.21	0.33
S-SH2-C3	0.20	0.33
S-SH2-C4	0.21	0.29
S-SH2-C5	0.20	0.32

The equation used to determine the concentration of 2,3-BDO from the value read during the enzymatic assay was $y = 5.1459x + 0.0014$. The r^2 value was > 0.99

ND not detected

Fig. 2 Comparison of the enzymatic assay (a) and HPLC (b) in the analysis of 2,3-BDO. The CL-Bdh activity oxidizing 2,3-BDO to acetoin was measured spectrophotometrically as the increase in NADPH absorbance at 340 nm. The corresponding peak area, which was auto-

matically measured by an integrator of the HPLC instrument, was plotted against the initial concentration of 2,3-BDO. The calibration curves were all linear with correlation coefficients $r^2 > 0.99$

in culture broths of acetoin, a precursor of 2,3-BDO, which inhibited the oxidation reaction of CL-Bdh. These findings indicate that with a rapid detection time of 20 min, this enzymatic assay could be an effective screening method for simultaneously analyzing hundreds of culture samples.

Acknowledgements This work was supported by the KIOST In-house Program (PE99722) and the understanding the deep-sea biosphere on seafloor hydrothermal vents in the Indian Ridge Program (20170411) of the Ministry of Ocean and Fisheries of the Republic of Korea.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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