Bioresource Technology 176 (2015) 156–162

Contents lists available at ScienceDirect

Bioresource Technology

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

Improved ethanol tolerance and ethanol production from glycerol in a streptomycin-resistant *Klebsiella variicola* mutant obtained by ribosome engineering

Toshihiro Suzuki [a,1], Kohei Seta [a,1], Chiaki Nishikawa [a], Eri Hara [a], Toshiya Shigeno [b], Toshiaki Nakajima-Kambe [a,*]

[a] Faculty of Life and Environmental Sciences (Bioindustrial Sciences), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[b] Tsukuba Institute of Environmental Microbiology, 8-1 Sakuragaoka, Tsukuba, Ibaraki 300-1271, Japan

HIGHLIGHTS

- Streptomycin-resistant *K. variicola* TB-83D was obtained by ribosome engineering.
- Ethanol tolerance and ethanol production were improved by *rpsL* mutation.
- Ethanol production was increased significantly by addition of YE.
- Highest ethanol concentration of 34 g/L was obtained by using YE and CSL.
- CSL was suitable for reducing by-product production and production costs.

ARTICLE INFO

Article history:
Received 22 July 2014
Received in revised form 27 October 2014
Accepted 29 October 2014
Available online 13 November 2014

Keywords:
Glycerol
Ethanol tolerance
Ribosome engineering
Klebsiella variicola
Ethanol production

ABSTRACT

To improve the ethanol tolerance of the *Klebsiella variicola* strain TB-83, we obtained the streptomycin-resistant, ethanol-tolerant mutant strain TB-83D by a ribosome engineering approach. Strain TB-83D was able to grow in the presence of 7% (v/v) ethanol and it showed higher ethanol production than strain TB-83. Examination of various culture conditions revealed that yeast extract was essential for ethanol production and bacterial growth. In addition, ethanol production was elevated to 32 g/L by the addition of yeast extract; however, ethanol production was inhibited by formate accumulation. With regard to cost reduction, the use of corn steep liquor (CSL) markedly decreased the formate concentration, and 34 g/L ethanol was produced by combining yeast extract with CSL. Our study is the first to improve ethanol tolerance and productivity by a ribosome engineering approach, and we found that strain TB-83D is effective for ethanol production from glycerol.

1. Introduction

Recently, increased biodiesel fuel (BDF) production has led to increased production of glycerol because BDF waste contains a large amount of glycerol (Yazdani and Gonzalez, 2007). Glycerol is used as a carbon source (Chi et al., 2007) for microbial fermentative production such as that of ethanol (Ito et al., 2005; Nwachukwu et al., 2012) and 1,3-propanediol (Mu et al., 2006), which is a valuable chemical product (Horng et al., 2010). Therefore, many researchers have attempted to produce useful compounds by microbial fermentation for resource recovery of BDF-waste-derived glycerol (Choi et al., 2011; Nwachukwu et al., 2012). Among fermentation products, ethanol is a promising alternative energy source (Sun and Cheng, 2002) and has potential as a biofuel substitute for fossil fuels.

Klebsiella variicola strain TB-83, a glycerol-assimilating bacterium, was isolated by our laboratory as an ethanol producer, and it showed maximum ethanol productivity under alkaline conditions. The addition of yeast extract (YE) was effective for producing ethanol, and the ethanol concentration was increased by

Abbreviations: YE, yeast extract; CA, casamino acid; PEP, peptone; CSL, corn steep liquor; GB medium, glycerol basal medium; GB medium w/o YE, glycerol basal medium without yeast extract; GB medium w/o CA, glycerol basal medium without casamino acid; GB medium w/o YCN, glycerol basal medium without yeast extract, casamino acid, and NH_4Cl.

* Corresponding author. Tel./fax: +81 29 853 4619.
E-mail address: nakajima.toshiaki.ga@u.tsukuba.ac.jp (T. Nakajima-Kambe).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biortech.2014.10.153
0960-8524/

approximately 1.7 times by addition of YE. In addition, when ethanol was produced using two types of BDF wastes, namely, new frying oil-derived BDF (wBDF) and waste cooking oil-derived BDF (bBDF), 1.2 times more ethanol was produced by using bBDF. Although strain TB-83 is a discriminatory bacterium with regard to ethanol production from glycerol, ethanol production and bacterial growth are not affected by salt concentrations but are inhibited significantly by the addition of at least 2% ethanol (Suzuki et al., 2014), indicating that ethanol tolerance is an important limiting factor for ethanol production of this strain.

To date, the improvement of ethanol production from glycerol has been attempted by two approaches: a genetically modified approach (gene disruption or gene over-expression) (Yazdani and Gonzalez, 2007; Oh et al., 2012; Wong et al., 2014) and a non-genetically modified approach (natural mutation) (Oh et al., 2011; Nwachukwu et al., 2012). The genetically modified approach is difficult to perform because of the target specification required for modification of genes, and its use is limited to strengthening of specified functions. In addition, it is difficult to adapt to practical application from the standpoint of production costs and containment level. In contrast, in the non-genetically modified approach, it is necessary to screen the desired mutant from various mutant strains. Thus, a highly efficient approach is needed for mutant screening.

Recently, bacteria have been engineered for antibiotic resistance, and various secondary metabolites such as antibiotics have been obtained (ribosome engineering). The ribosome engineering technique is a powerful method to activate the potential ability of bacteria to produce various secondary metabolites involved in the effects of antibiotics (Shima et al., 1996; Hosoya et al., 1998; Kurosawa et al., 2006). Antibiotic-resistant bacteria show improvement of not only enzymatic activity but also organic chemical tolerance (Hosokawa et al., 2002; Liu et al., 2013). We consider that an adaptation of this technique is useful for the construction of marker strains and may be adapted not only for the molecular breeding of an ethanol-tolerant strain but also for overproduction of the fermentation product. For instance, Kurosawa et al. (2006) reported that the streptomycin-resistant *Bacillus subtilis* showed improved α-amylase production. However, applications to fermentative production have not been reported.

In this study, we aimed at high ethanol production from glycerol through two approaches: acquisition of a non-genetically modified high-ethanol-tolerant mutant and improvement of ethanol production of the mutant through optimization of culture conditions. To improve the ethanol production of strain TB-83, we obtained an ethanol-tolerant strain by the ribosome engineering approach and investigated its microbiological characteristics. In addition, the ethanol production of this mutant strain was evaluated under various culture conditions, including cost reduction of culture ingredients to improve ethanol production.

2. Methods

2.1. Bacterial strain and culture media

K. variicola strain TB-83 was used as the parent strain; it has been described previously (Suzuki et al., 2014). GB medium (10.0 g of KH_2PO_4, 1.0 g of NH4Cl, 0.50 g of YE (Becton Dickinson, Franklin Lakes, NJ, USA) 1.0 g of CA (Becton Dickinson), and 20.0 g of glycerol in 1 L of distilled water) was used for bacterial culture and for ethanol production. The culture pH was adjusted to 8.0 with 6 N NaOH before inoculation.

In the case of the use of BDF waste, glycerol was replaced by BDF waste that was provided by the Nagasaki Prefectural Institute for Environmental Research and Public Health. The CSL used for examination of the nutrient source was corn steep liquor powder (Solulys AST; Oriental Yeast Co., Tokyo, Japan). The total organic carbon (TOC) and total nitrogen (TN) contents of YE and CSL were analyzed using a Shimadzu TOC-V device, which is a total organic carbon analyzer (Shimadzu, Kyoto, Japan).

2.2. UV irradiation mutagenesis

TB-83 cells (1.0×10^8 cells/ml) that grew in nutrient broth (NB) (Becton Dickinson) were poured onto a Petri dish and UV-irradiated by a GL-15 low-pressure germicidal lamp (Toshiba, Tokyo, Japan, 254 nm; 15 W) at a distance of 30 cm from the Petri dish. Samples (100 μl) were collected every 20 s for a duration of 1 min and immediately inoculated into 2 ml of NB liquid medium and cultured at 30 °C overnight under dark conditions. The cells were collected by centrifugation at 3000*g* for 5 min, and then the pellet was washed twice with 0.85% NaCl and re-suspended in the same solution. Moreover, 100 μl of the cell suspension was spread onto an NB agar medium containing 50 mg/L streptomycin (Wako, Tokyo, Japan) and cultured at 30 °C to obtain the streptomycin-resistant strain TB-83D.

2.3. Genetic manipulations

Total DNA isolation from the *K. variicola* strains TB-83 and TB-83D was described previously (Suzuki et al., 2014). DNA manipulations of *Escherichia coli* were performed according to the procedure reported by Sambrook and Russell (2001), which is a standard protocol. The 30S ribosome protein S12 coding sequences were amplified using the total DNA of strains TB-83 and TB-83D as a template and the 5′-end phosphorylated primers S12-SeqF (5′-AAGCAAA AGCTAAAACCAGGAGCTATTT-3′) and S12-SeqR (5′-AATCAGTTTGG CCTTACTTAACGGAGAA-3′), which were designed from the *K. variicola* At-22 chromosome complete genome sequence (gene accession number: NC_013850). PCR amplification was performed with a BIO-RAD iCycler™ (Bio-Rad, Hercules, CA, USA) using KOD-Plus- ver.2 DNA polymerase (Toyobo, Osaka, Japan). PCR was performed with one step at 94 °C for 2 min followed by 25 cycles of 94 °C for 15 s, 58 °C for 30 s, and 68 °C for 1 min. The amplified fragments were cloned into the pUC118 *Hinc*II/BAP cloning vector (Takara Bio, Otsu, Japan) and then transformed into *E. coli* DH5 alpha (Takara) as a host strain. DNA sequencing was performed by cycle sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISMTM 3130^{NT} genetic analyzer (Applied Biosystems). The accumulated sequencing data and amino acid alignment were analyzed using GENETYX-MAC software (Genetyx Corporation, Tokyo, Japan).

2.4. Culture conditions for ethanol production

All culture processes for ethanol production were performed at 25 °C and pH 8.0, and are described in detail as follows. On the test-tube scale, culture was performed under static culture conditions (Suzuki et al., 2014). When a 1-L culture vessel was used, the culture was performed by agitation (80 rpm) without aeration. In the case of constant pH conditions, the culture pH was maintained at approximately pH 8.0 with 6 N NaOH using a pH electrode (CE-108C, Nissin, Tokyo, Japan), a pH controller (NPH-660, Nissin), and a peristaltic pump (Model SJ1211H, ATTO Corporation, Tokyo, Japan) during cultivation.

2.4.1. Pre-cultivation of strain TB-83D

One loopful of strain TB-83D that grew on a 2% (w/v) GB agar plate (pH 8.0) containing 25 mg/L streptomycin was suspended with 0.85% NaCl. The cell suspension (0.5 ml) was inoculated into