Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–5, 2017

www.elsevier.com/locate/jbiosc

A search for microorganisms producing medium-chain alkanes from aldehydes

Masakazu Ito,[1] Hiromi Kambe,[2] Shigenobu Kishino,[3] Masayoshi Muramatsu,[1] and Jun Ogawa[3,*]

Future Project Division, Frontier Research Center, Toyota Motor Corporation, 1 Toyota-cho, Toyota, Aichi 471-8572, Japan,[1] Toyota Biotechnology and Afforestation Laboratory, Toyota Motor Corporation, 1099 Marune, Kurozasa-cho, Miyoshi, Aichi 470-0201 Japan,[2] and Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan[3]

Received 17 April 2017; accepted 4 August 2017
Available online xxx

Microorganisms with medium-chain alkane-producing activity are promising for the bio-production of drop-in fuel. In this study, we screened for microorganisms producing tridecane from tetradecanal. The activity of aldehyde decarbonylation was found in a wide range of microbes. In particular, the genus *Klebsiella* in the *Enterobacteriaceae* family was found to have a high ability to produce alkanes from aldehydes via enzyme catalyzed reaction.

[**Key words:** Hydrocarbon; Biofuel; Medium-chain alkanes; *Klebsiella* sp.; Aldehyde decarbonylase]

Biofuels are expected to be an attractive alternate energy source to fossil fuels. The total carbon dioxide emissions of biofuels are far lower than those formed by the combustion of fossil fuels, as the former includes carbon dioxide fixation from the air via plant photosynthesis (1). Therefore, the widespread use of biofuels is being promoted around the world to reduce greenhouse gas emissions (2,3).

The biofuels currently used, including ethanol and fatty acid methyl ester, cause problems such as metallic corrosion and resin degradation for automobile engine systems (4,5). In addition, they have low energy density and poor cold-start performance (6). Thus, engine and infrastructure repair will be needed, which might impose a large burden on drivers and car manufacturers. This situation makes biofuels being increasingly mixed with existing petroleum-based fuels (7).

To avoid these concerns, attention is being paid to "drop-in fuel," which is a hydrocarbon biofuel that would not damage infrastructure and could be mixed at an arbitrary rate with pre-existing petroleum-based fuels. The manufacturing methods for drop-in fuels include bio hydro-fined diesel produced by hydrotreatment of vegetable fat and oil (8), biomass-to-liquid that integrates biomass gasification with Fischer–Tropsch synthesis technology (9), and production of hydrocarbons using algae (10), or genetically modified microorganisms such as bacteria, yeasts, and fungi (11). However, these manufacturing processes are all still in the early stages of development, and many challenges remain to be overcome in terms of catalyst cost reduction, energy balance, and productivity. Thus, it is not yet economically efficient to produce biofuel using these current methods.

In this study, we searched for microorganisms with the ability to produce alkanes among various types of bacteria and yeasts, and we discovered that many microbial species are capable of producing medium-chain alkanes from corresponding medium-chain aldehydes. In particular, the genus *Klebsiella* in the *Enterobacteriaceae* family of bacteria was found to have higher ability to produce alkanes among all strains tested. It is assumed that the microorganisms with alkane-producing activity, especially for medium chain length alkane showing excellent drop-in fuel properties, will provide potential genes for alkane production and might lead to an efficient and economically feasible method for bio drop-in fuel production.

MATERIALS AND METHODS

Reagents, microbial strains, and media Tridecane and tetradecanal standards were purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Beta-nicotinamide adenine dinucleotide 2′-phosphate reduced (NADPH) tetrasodium salt hydrate, proteinase K, and trypticase soy broth (TSB; 17.0 g/L casein peptone, 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L glucose, 5.0 g/L sodium chloride, 3.0 g/L soya peptone, pH 7.2) were purchased from Sigma–Aldrich Japan (Tokyo, Japan). The other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Bacteria and yeasts were obtained from the culture collections of microorganisms as listed in Table S1. Bacteria were cultivated in basal succinate medium (BSM, 2.5 g/L succinate, 1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L $(NH_4)_2SO_4$, 2.0 g/L KNO_3, 0.1 g/L NaCl, 0.017 g/L $FeCl_3 \cdot 4H_2O$, 1.0 g/L yeast extract, pH 7.2) or TSB. Yeasts were cultivated in yeast growth medium (5 g/L malt extract, 5 g/L yeast extract, pH 5.5).

Cultivation of microorganisms A single bacterial colony on TSB agar medium was cultured in 1 mL of TSB medium at 30°C for 24 h with shaking (130 strokes per minute). For the first screening, an aliquot (50 μL) of this broth was inoculated into 5 mL of TSB (containing 1 mM tetradecanal) in a glass test tube (16 × 100 mm) with a stainless steel molten cap (16 mm), and cultured at temperatures of 25–37°C for 120 h with shaking (130 strokes per minute). For the second screening, an aliquot (50 μL) of the pre-cultured broth in TSB was inoculated into 5 mL of BSM (containing 1 mM tetradecanal) in a glass test tube (16 × 100 mm) with a stainless steel molten cap (16 mm), and cultured at 30°C for 72 h with shaking (100 strokes per minute). The AnaeroPack-Anaero system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) was used for anaerobic cultivation of microorganisms. A single yeast colony on yeast growth agar medium was cultured in 1 mL of yeast growth medium at 30°C and at 130 strokes per minute for 24 h. An aliquot (50 μL) of this broth was inoculated into 5 mL of yeast growth medium (containing 1 mM tetradecanal) in a glass test tube (16 × 100 mm) with a stainless steel molten cap (16 mm), and cultured at 30°C for 72 h with shaking (100 strokes per minute). One milliliter of

* Corresponding author. Tel.: +81 75 753 6115; fax: +81 75 753 6113.
E-mail address: ogawa@kais.kyoto-u.ac.jp (J. Ogawa).

1389-1723/$ – see front matter © 2017, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2017.08.002

post-culture was centrifuged at room temperature at 3000 ×*g* for 10 min, and the supernatant was then used for gas chromatography–mass spectrometry (GC–MS) analysis.

GC–MS analysis Alkane production was measured using GC–MS (GC–MS system HP6890/5973; Agilent, Tokyo, Japan) equipped with a headspace sampler (HP7694; Agilent) with an analytical column, HP-INNOWAX (30 m × 0.32 mm × 0.5 μm; Agilent). One milliliter of post-culture supernatant was transferred to a 20-mL headspace vial and tightly sealed with a 20-mm silver aluminum crimp cap-PTFE/silicone septa. The sealed vial was incubated at 80°C for 15 min and pressurized to 15 psi, then immediately subjected to GC–MS (injection time 1 min, fill flow 50 mL/min, loop temperature 150°C, transfer line temperature 200°C). Helium at a flow rate of 1.0 mL/min was used as the carrier gas. The heating program of the column consisted of the following steps: the temperature was held at 60°C for 10 min, increased to 260°C at a rate of 25°C/min, and was then held again at 260°C for 1 min. MS was used in the electron impact mode at 70 eV, with a source temperature of 230°C.

TABLE 1. Results of alkane synthesis.

Strain	TSB (tetradecanal −)	TSB (tetradecanal +)
Bacillus cereus NCIB10404	N.D.	N.D.
Clostridium acidiurici ATCC7906	N.D.	N.D.
Clostridium tetanomorphum H-1 ATCC15920	N.D.	N.D.
Desulfovibrio desulfuricans NCIB8307	N.D.	N.D.
Escherichia coli W3110	N.D.	N.D.
Micrococcus luteus JCM1464	N.D.	N.D.
Micrococcus luteus JCM20344	N.D.	N.D.
Micrococcus roseus JCM20437	N.D.	N.D.
Pseudomonas fluorescens JCM5963	N.D.	N.D.
Pseudomonas putida NCIB10408	N.D.	1.0 μg/L of tridecane
Vibrio furnissii NCTC11218	N.D.	N.D.

ND, not detected.

Optimization of alkane production by *Pseudomonas putida* NCIMB10408 A single colony of *P. putida* NCIMB10408 on TSB agar medium was cultured in 1 mL of TSB medium at 30°C for 24 h with shaking (130 strokes per minute). A 50 μL aliquot of this broth was inoculated into 5 mL of TSB or BSM (containing 1 mM tetradecanal) in a glass test tube (16 × 100 mm) with a stainless steel molten cap (16 mm), and cultured at temperatures of 25–37°C for 24–120 h with shaking (0–130 strokes per minute). The AnaeroPack-Anaero system was used for anaerobic cultivation. One milliliter of post-culture was centrifuged at room temperature at 3000 ×*g* for 10 min, and the supernatant was then used for GC–MS analysis.

Enzyme assay using *Klebsiella* sp. NBRC100048 crude extract *Klebsiella* sp. NBRC100048 was cultured in 1 mL of TSB at 30°C for 24 h with sahking (130 strokes per minute). A 50-μL aliquot of seed culture was used to inoculate 5 mL of TSB and cultured at 30°C with shaking (130 strokes per minute) before reaching an OD_{600} of 1.0. Three milliliters of *Klebsiella* sp. NBRC100048 broth was pelleted by centrifugation at 3000 ×*g* at 4°C, washed three times with 1 mL of 0.1 mM phosphate buffer (pH 7.2), and suspended in 1 mL of 0.1 mM phosphate buffer (pH 7.2). Suspended cells were transferred to a 10-mL spitz tube and disrupted at 4°C for 5 min (30 s ON/30 s OFF) using a Bioruptor equipped with a cooling pump (Cosmo Bio), set to "High" (220 W). The lysate was centrifuged at 12,000 ×*g* for 30 min, and the supernatant was used as the crude cell extract.

The reaction mixture for measuring enzymatic activity contained 200 μM tetradecanal, 1 mM NADPH, and 10 mg/mL crude cell extract in 1 mL of phosphate buffer (pH 7.2). The mixture was incubated in a sealed 20-mL headspace vial at 30°C for 16 h and then used for GC–MS analysis as mentioned above.

To examine the effect of proteinase K on tridecane production, 1 unit of proteinase K was added to 10 mg crude cell extract in 1 mL of phosphate buffer (100 mM, pH 7.2), incubated at 37°C for 1 h, and 200 μM tetradecanal and 1 mM NADPH were then added and vigorously shaken with a vortex. The mixture was then incubated in a sealed 20-mL headspace vial at 30°C for 16 h and used for GC–MS analysis.

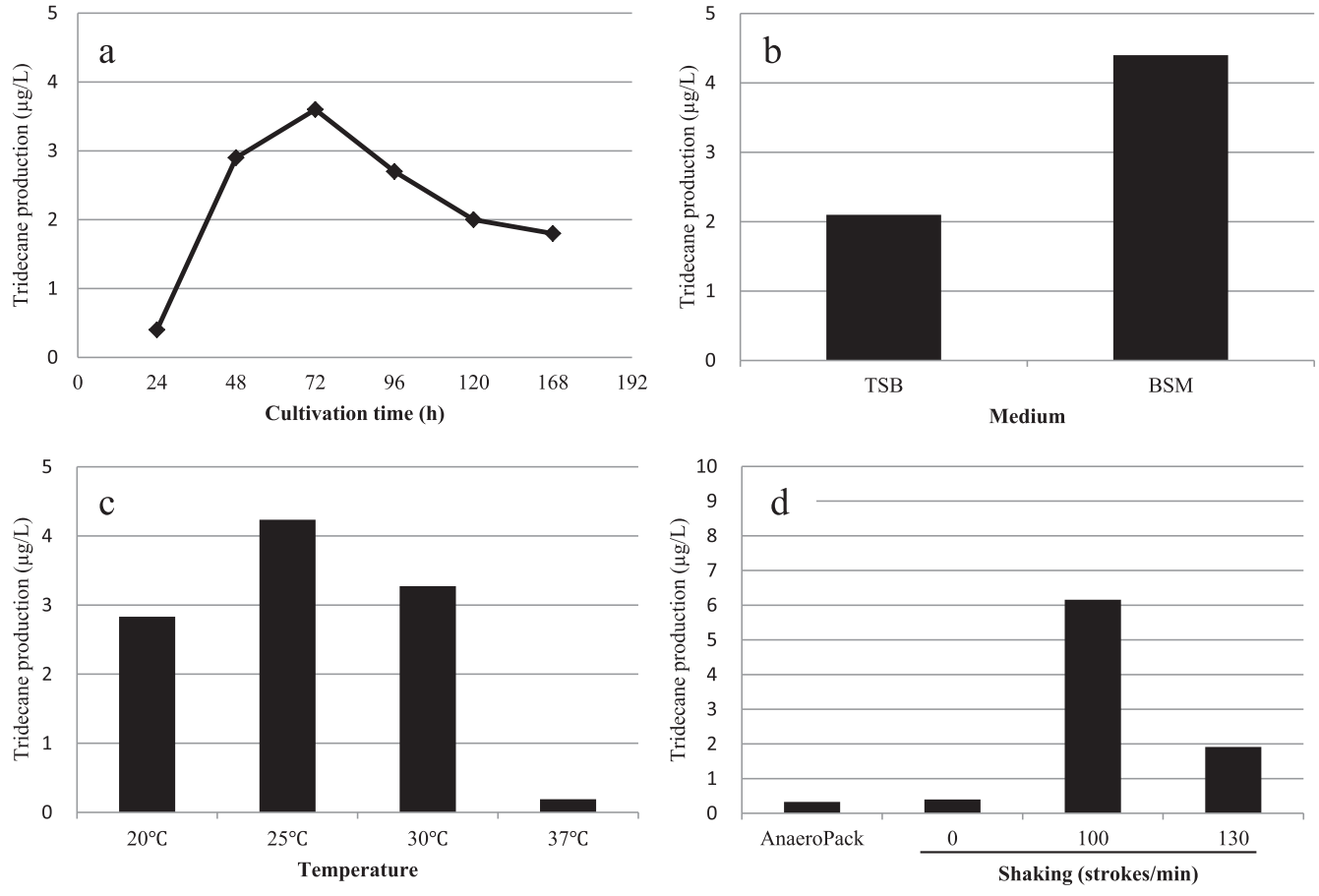

FIG. 1. The effects of cultivation time (a), medium (b), temperature (c), and shaking rate (d) on tridecane production from tetradecanal by *P. putida* NCIMB10408.