Biotransformation of oleic acid into 10-ketostearic acid by recombinant Corynebacterium glutamicum*-based biocatalyst*

Byeonghun Lee, Saebom Lee, Hyeonsoo Kim, Kijun Jeong, Jinbyung Park, Eunyeol Lee & Jinwon Lee

Biotechnology Letters

ISSN 0141-5492

Biotechnol Lett
DOI 10.1007/s10529-015-1775-7

Springer

Biotechnol Lett
DOI 10.1007/s10529-015-1775-7

ORIGINAL RESEARCH PAPER

Biotransformation of oleic acid into 10-ketostearic acid by recombinant *Corynebacterium glutamicum*-based biocatalyst

Byeonghun Lee · Saebom Lee · Hyeonsoo Kim · Kijun Jeong · Jinbyung Park · Eunyeol Lee · Jinwon Lee

Received: 17 December 2014 / Accepted: 20 January 2015

Abstract
Objective To produce 10-ketostearic acid from oleic acid.
Results Oleic acid was converted to 10-ketostearic acid by a recombinant *Corynebacterium glutamicum* ATCC 13032 expressing oleate hydratase from *Stenotrophomonas maltophilia* and a secondary alcohol dehydrogenase from *Micrococcus luteus* under the control of a synthetic constitutive promoter. Optimal conditions for 10-ketostearic acid production were pH 7.5 and 30 °C with 5 g cells l^{-1} and 2.5 g oleic acid l^{-1}. Under these conditions, the cells produced 1.96 g 10-ketostearic acid l^{-1} from oleic acid in 6 h, with a conversion yield of 78 % (w) and a maximum volumetric productivity of 1.67 g l^{-1} h^{-1}.
Conclusion This is the first report of 10-ketostearic acid production using a recombinant *C. glutamicum*.

Keywords Biocatalysts · Biotransformation · *Corynebacterium glutamicum* · 10-Ketostearic acid · Oleate hydratase · Oleic acid · Secondary alcohol dehydrogenase

Introduction

Corynebacterium glutamicum has traditionally been used in the industrial production of various amino acids (e.g., lysine and glutamic acid) (Hermann 2003) and, furthermore, has been engineered to produce a variety of bio-based chemicals, materials, and fuels such as 2-ketoisovalerate, succinate, cadaverine, putrescine, 1,2-propanediol, ethanol, 1-butanol, and polygalacturonic acid (Becker and Wittmann 2012).

Many microorganisms, including *Staphylococcus* spp. (Lanser 1993), *Flavobacterium* sp. strain DS5

Byeonghun Lee and Saebom Lee have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-015-1775-7) contains supplementary material, which is available to authorized users.

B. Lee · S. Lee · H. Kim · J. Lee (✉)
Department of Chemical and Biomolecular Engineering, Sogang University, Seoul 121-742, Republic of Korea
e-mail: jinwonlee@sogang.ac.kr

K. Jeong
Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon 305-701, Republic of Korea

J. Park
Department of Food Science and Engineering, Ewha Womans University, Seoul 120-750, Republic of Korea

E. Lee
Department of Chemical Engineering, Kyung Hee University, Yongin-si, Gyeonggi-do 446-701, Republic of Korea

Published online: 21 February 2015

(Hou 1994), *Sphingobacterium* sp. strain 022 (Kuo et al. 1999), *Staphylococcus warneri* (Lanser and Nakamura 1996), *Mycobacterium, Nocardia, Aspergillus terreus, Sphingobacterium thalpophilum,* and *Bacillus sphaericus* (Hou 2009), have been used for the production of 10-ketostearic acid from oleic acid.

In this study, the genes encoding oleate hydratase (*OhyA*) in *Stenotrophomonas maltophilia* (Joo et al. 2012) and secondary alcohol dehydrogenase (ADH) in *Micrococcus luteus* (Huang et al. 2010) were cloned into the pCES208-L10 plasmid and then introduced into *C. glutamicum*. Part of a previously-designed synthetic biotransformation pathway (Song et al. 2013) in *C. glutamicum* was constructed using a constitutive expression vector for the production of 10-ketostearic acid from olive oil and oleic acid. To increase the conversion yield using whole cells, reaction conditions (pH, temperature, substrate concentration, and cell concentration) were optimized, after which a time-course assessment of the conversion of oleic acid and olive oil to 10-ketostearic acid was carried out under optimal conditions. This is the first report of microbial 10-ketostrearic acid production using recombinant *C. glutamicum* cells.

Materials and methods

Bacterial strains, cloning vectors, and culture media

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *Escherichia coli* DH5α was used as a host for gene cloning and plasmid maintenance, and *C. glutamicum* ATCC 13032 was used as the main host for the production of 10-ketostearic acid. The *OhyA* and *ADH* genes were cloned into *E. coli*/*C. glutamicum* shuttle vectors (pCES208, KAIST, Daejeon, Korea) (Park et al. 2008) containing one of three synthetic promoters: H36, I16, or L10 (Yim et al. 2013). *E. coli* DH5α was cultivated in lysogeny broth (10 g tryptone l^{-1}, 5 g yeast extract l^{-1}, and 5 g NaCl l^{-1}) at 37 °C and for protein production *C. glutamicum*-pCES208-L10-OhyA-ADH was cultivated in a 500 ml baffled flask containing 100 ml brain heart infusion (BHI) broth (Becton–Dickinson, Sparks, MD) and 50 μg kanamycin ml^{-1} at 30 °C for 12 h with shaking at 200 rpm.

Plasmid construction for expression of *OhyA* and *ADH* in *C. glutamicum*

The primers used for PCR amplification of genes of interest are shown in Supplementary Table 1. Restriction enzymes were purchased from Takara (Kyoto, Japan) and oligonucleotides were synthesized by Macrogen Inc. (Seoul, Korea). The *OhyA* gene of *S. maltophilia* KCTC 1773 was ligated into pCES208 vectors containing synthetic promoters (H36, I16, or L10) using the 'sequence and ligation independent cloning (SLIC)' method (Li and Elledge 2007). The *ADH* gene of *M. luteus* NCTC 2665 was ligated into the vectors containing the *OhyA* gene using an In-fusion cloning kit (Clontech, USA) (Clontechniques 2002). A ribosomal-binding site (RBS) was encoded upstream of the *ADH* gene. All PCR constructs were confirmed by Macrogen, Inc. (Seoul, Korea) and then transformed into *C. glutamicum* using electroporation with a Gene Pulser Xcell electroporator (Bio-Rad, USA).

Optimization of reaction conditions

The OD600 values of the cultures were measured and converted to dry cell weights (DCW). Unless otherwise stated, reactions were performed at 30 °C in 50 mM Tris/HCl (pH 7.5) containing 2.5 g oleic acid l^{-1}, 5 g cells l^{-1}, and 0.05 % Tween 80 (w/v) for 1 h under aerobic conditions. To examine the effects of pH and temperature on 10-ketostearic acid production using whole recombinant cells, pH was varied from 6.5–8.5 using 50 mM phosphate/citrate buffer (pH 6.5–7.5) or 50 mM Tris/HCl buffer (pH 7.5–8.5) at 30 °C and temperature was varied from 20 to 40 °C at pH 7.5.

Cell concentration was assessed at 0.5–10 g l^{-1} with 5 g oleic acid l^{-1} to determine the optimal cell concentration for 10-ketostearic acid production and substrate concentration was assessed at 0.5–6.25 g l^{-1} with a constant cell concentration of 5 g l^{-1} to determine the substrate concentration required for maximum production of 10-ketostearic acid. A fixed reaction time (4 h) was used for the cell and substrate concentration experiments.

Biotransformation

Recombinant *C. glutamicum* expressing the *OhyA* and *ADH* genes was cultivated in BHI medium at 200 rpm

and 30 °C until the stationary phase (for 12 h). Cells were harvested by centrifugation at 4 °C, washed, and resuspended in 50 mM Tris/HCl buffer (pH 7.5) to a concentration of 5 g cells l^{-1}. Reactions were initiated by the addition of 2.5 g oleic acid l^{-1} and 0.05 % Tween 80 (w/v) to the buffer and were performed in a shaking incubator at 200 rpm and 30 °C for 6 h.

For the biotransformation of olive oil to 10-ketostearic acid, olive oil was hydrolyzed by the lipase from *Candida rugosa* (Sigma-Aldrich, USA) (Song et al. 2013) and the resulting hydrolysate was subjected to biotransformation by recombinant *OhyA*- and *ADH*-expressing *C. glutamicum*. The biotransformation reaction was performed under optimal conditions.

Analysis of products

For product analysis, reactions were terminated by acidification to pH 2 with HCl and reaction products were then extracted with ethyl acetate containing methyl palmitate as an internal standard. The solvent was removed from the extracts using a rotary evaporator and fatty acids present in the extract were silylated with a 3:1 mixture of pyridine:*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (TMS). TMS derivatives were analyzed by GC/MS analysis which was performed on an Agilent 5975 series mass spectrometer and a 7890A GC system equipped with non-polar capillary column (30 m length, 0.25 m film thickness, HP-5MS, Agilent Technologies, Palo Alto, CA, USA). Helium was used as the carrier gas. The column temperature gradient was programmed as follows: initially 80 °C, increased by 5 °C min^{-1} to 125 °C, then by 15 °C min^{-1} to 200 °C, and finally by 5 °C min^{-1} to 255 °C. The injection port temperature was 230 °C and mass spectra were obtained using an electron impact ionization source at 70 eV.

Results and discussion

Selection of recombinant *Corynebacterium* strains and product identification

In the expression of genes for protein production in microorganisms, it is important to choose an appropriate promoter. Constitutive promoters are considered more cost-effective than inducible promoters (Yim et al. 2013; Lee 2014) and thus for cloning purposes, three pCES208 vectors each containing a synthetic promoter (H36, I16, or L10) allowing for the constitutive expression of heterologous genes in *C. glutamicum* were obtained. Of the three pCES208 transformants, *C. glutamicum*-pCES208-L10-*OhyA*-*ADH* (*C. glutamicum* expressing *OhyA* and *ADH* under control of the constitutive L10 promoter) exhibited the highest 10-ketostearic acid yield (data not shown) and was thus selected for 10-ketostearic acid production by biotransformation.

The biotransformation products of oleic acid and olive oil (10-hydroxystearic acid and 10-ketostearic acid) were analyzed by GC/MS and the chemical structures of these products were identified by comparison with the authentic reference compound reported in a previous study (Song et al. 2013).

Optimization of reaction conditions for the biotransformation of oleic acid to 10-ketostearic acid by whole recombinant *C. glutamicum* cells

The biotransformation of oleic acid to 10-ketostearic acid by whole recombinant *C. glutamicum* cells expressing the *OhyA* and *ADH* genes was optimized by assessing the biotransformation efficiency at various pH (6.5–8.5) (Fig. 1a) and temperatures (20–40 °C) (Fig. 1b) using a constant cell concentration of 5 g l^{-1}. The optimal reaction conditions were selected for maximal conversion of oleic acid to 10-ketostearic acid in terms of conversion yield (1 h reaction time). The maximal conversion yield (%, w/w) of oleic acid to 10-ketostearic acid was achieved at pH 7.5 and 30 °C.

The optimal cell concentration for 10-ketostearic acid production was determined by carrying out biotransformation for 4 h (Fig. 2a). The optimal cell concentration was therefore determined to be 5 g l^{-1}. Moreover, to determine the optimal substrate concentration for maximum conversion of oleic acid to 10-ketostearic acid, oleic acid concentrations in reactions were varied (0.5–6.25 g l^{-1}) while the cell concentration was fixed (5 g l^{-1}) (Fig. 2b). With oleic acid concentrations of $\leq$2.5 g l^{-1}, increasing substrate concentrations resulted in proportional increases in conversion, with a conversion yield of $\sim$78 % (w/w) being measured for 2.5 g oleic acid/l; however, conversion yields decreased with increasing concentrations of oleic acid >2.5 g l^{-1}, and a conversion yield of 15 % (w/w) was measured for 6.25 g

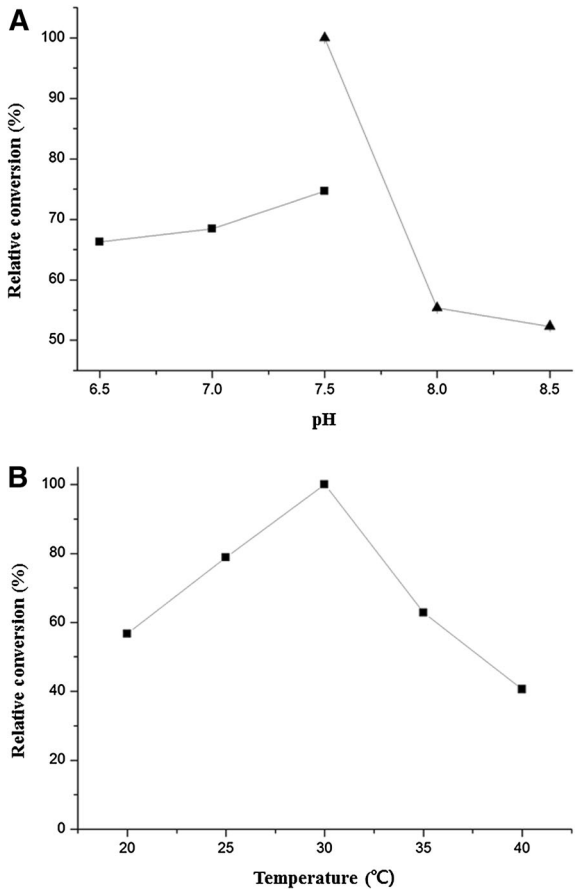

Fig. 1 Effects of reaction conditions on biotransformation. **a** Effect of pH: reactions were performed in 50 mM phosphate/citrate buffer (pH 6.5–7.5, filled square) or 50 mM Tris/HCl buffer (pH 7.5–8.5, filled triangle) with 2.5 g oleic acid l^{-1} and 5 g cells l^{-1} for 1 h. **b** Effect of temperature: reactions were performed in 50 mM Tris/HCl buffer (pH 7.5) containing 2.5 g oleic acid l^{-1} and 5 g cells l^{-1} for 1 h. Data represent means of triplicate experiments and error bars represent standard deviation. At the relative 100 % value, conversion of oleic acid to 10-ketostearic acid was the highest in terms of conversion yield

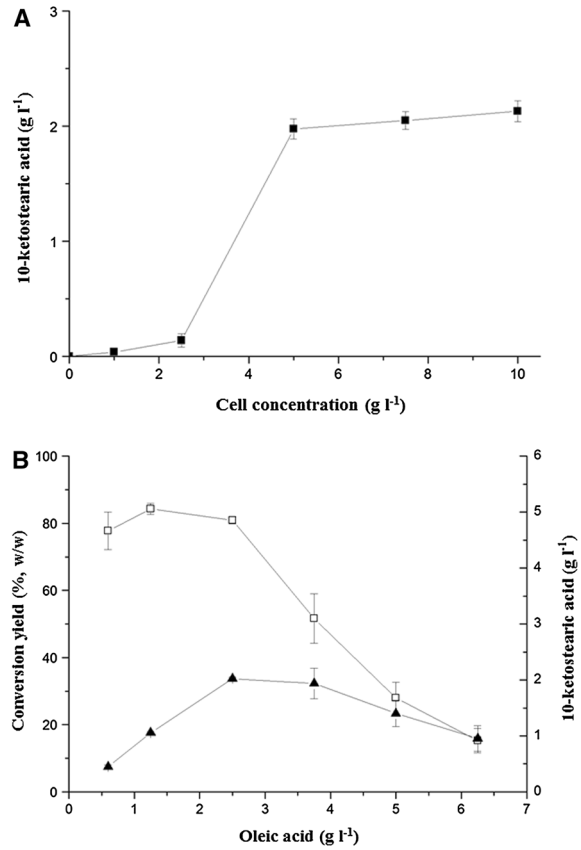

Fig. 2 Effects of cell concentration and substrate concentration on 10-ketostearic acid production from oleic acid by recombinant *C. glutamicum*. **a** Effect of cell concentration. **b** Effect of substrate concentration. Conversion yield (*open square*) and 10-ketostearic acid concentration (*filled triangle*). Data represent the means of triplicate experiments and *error bars* represent standard deviation

oleic acid l^{-1}. With substrate concentrations of >2.5 g l^{-1}, the viscosity of the reaction solutions became so high that resistance to mass transfer in the aqueous phase became dominant and the conversion rate decreased (Yu et al. 2008).

Production of 10-ketostearic acid by whole recombinant *C. glutamicum* cells under optimized conditions

The optimal reaction conditions for the conversion of oleic acid to 10-hydroxystearic acid by whole recombinant *C. glutamicum* cells were found to be pH 7.5, 30 °C, 5 g cells l^{-1}, and 2.5 g oleic acid l^{-1}. Under these optimal conditions, a time-course assessment was carried out and the whole recombinant *C. glutamicum* cells were found to produce 1.96 g 10-ketostearic acid l^{-1} after 6 h, with a conversion yield of 78 % (w/w) and a maximum volumetric production rate of 1.67 g l^{-1} h^{-1} (Fig. 3). For biotransformation, a multistep approach by one biocatalyst unit enables simplification and intensification of the conversion process and eliminates the need for expensive isolation of intermediates (Ladkau et al. 2014).

To extend the applicability of the biotransformation described in this report, a plant oil (olive oil) with oleic acid as its major fatty acid component was used as a substrate instead of oleic acid. Olive oil was

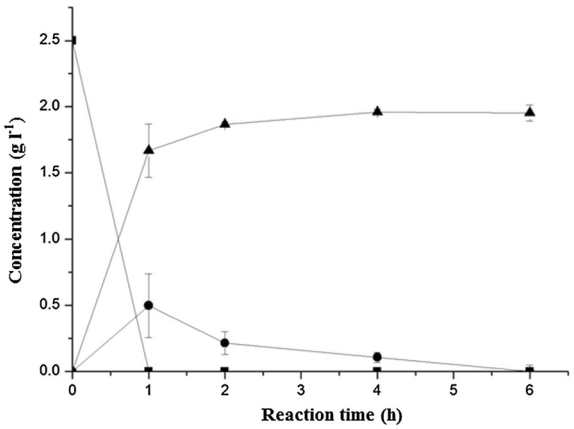

Fig. 3 Biotransformation of oleic acid into 10-ketostearic acid by whole recombinant *C. glutamicum* cells. Time-course assessment. The bioconversion of oleic acid (*filled square*) to 10-hydroxystearic acid (*filled circle*) and 10-ketostearic acid (*filled triangle*) are shown. Reactions were performed in 50 mM Tris/HCl buffer (pH 7.5) containing 2.5 g oleic acid l^{-1}, 5 g cells l^{-1}, and 0.05 % Tween 80 for 6 h. Data represent the means of triplicate experiments and *error bars* represent standard deviation

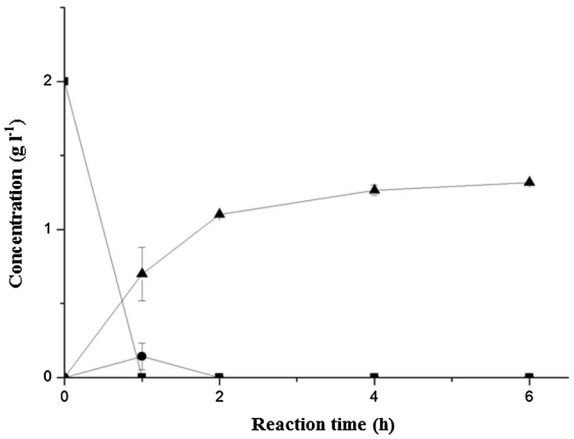

Fig. 4 Biotransformation of olive oil with lipase and recombinant *C. glutamicum* expressing oleate hydratase from *S. maltophilia* and ADH from *M. luteus.* Olive oil was hydrolyzed with lipase in 50 mM Tris/HCl buffer (pH 7.5; data not shown). The bioconversion of oleic acid (*filled square*) to 10-hydroxystearic acid (*filled circle*) and 10-ketostearic acid (*filled triangle*) are shown. Reactions were performed in 50 mM Tris/HCl buffer (pH 7.5) containing 5 g cells l^{-1}, 0.05 % Tween 80 for 6 h. Data represent the means of triplicate experiments and *error bars* represent standard deviation

hydrolyzed with lipase and lipase treatment of 2.5 g olive oil l^{-1} yielded 2 g oleic acid l^{-1} (data not shown). The subsequent biotransformation was carried out using recombinant *C. glutamicum* expressing the *OhyA* and *ADH* genes, and 10-ketostearic acid was produced at 1.3 g l^{-1} from oleic acid (Fig. 4).

Conclusion

The recombinant *C. glutamicum* expressing the *OhyA* and *ADH* genes was first applied to the conversion of oleic acid to 10-ketostearic acid. The conversion reaction conditions (pH, temperature, cell concentration, and substrate concentration) for this system were optimized and extended to produce 10-ketostearic acid from olive oil, renewable oil. These findings thus contribute to achieving the initial stage of the biological process required for the industrial production of 10-ketostearic acid.

Acknowledgments This work was supported by the MOTIE/KEIT R&D Program (10044604, Bioproduction of long-chain dicarboxylic acids from fatty acids and lipids).

Supporting information Supplementary Table 1—Bacterial strains, plasmids and oligonucleotides used in this study.

References

Becker J, Wittmann C (2012) Bio-based production of chemicals, materials and fuels *Corynebacterium glutamicum* as versatile cell factory. Curr Opin Biotechnol 23:631–640

Clontechniques (2002) BD In-fusion cloning kit. Precise, directional cloning of PCR products without restriction enzymes. Clontechniques XVII:10–11

Hermann T (2003) Industrial production of amino acids by coryneform bacteria. J Biotechnol 104:155–172

Hou CT (1994) Production of 10-ketostearic acid from oleic acid by *Flavobacterium* sp. strain DS5 (NRRL B-14859). Appl Environ Microbiol 60:3760–3763

Hou CT (2009) Biotechnology for fats and oils: new oxygenated fatty acids. Nat Biotechnol 26:2–10

Huang JK, Park JK, Dhungana BR et al (2010) A novel secondary alcohol dehydrogenase from *Micrococcus luteus* WIUJH20: purification, cloning, and properties. FASEB J(Meeting Abstract Supplement) 24:835.5

Joo YC, Seo ES, Kim YS et al (2012) Production of 10-hydroxystearic acid from oleic acid by whole cells of recombinant *Escherichia coli* containing oleate hydratase from *Stenotrophomonas maltophilia*. J Biotechnol 158:17–23

Kuo TM, Lanser AC, Kaneshiro T et al (1999) Conversion of oleic acid to 10-ketostearic acid by *Sphingobacterium* sp. strain 022. J Am Oil Chem Soc 76:709–712

Ladkau N, Schmid A, Bühler B (2014) The microbial cell - functional unit for energy, dependent multistep biocatalysis. Curr Opin Biotechnol 30:178–189

Lanser AC (1993) Conversion of oleic acid to 10-ketostearic acid by a *Staphylococcus* species. J Am Oil Chem Soc 70:543–545

Lanser AC, Nakamura LK (1996) Identification of a *Staphylococcus warneri* species that converts oleic acid to 10-ketostearic acid. Curr Microbiol 32:260–263

Lee JH (2014) Development and characterization of expression vectors for *Corynebacterium glutamicum*. J Microbiol Biotechnol 24:70–79

Li MZ, Elledge SJ (2007) Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods 4:251–256

Park JU, Jo JH, Kim YJ et al (2008) Construction of heat-inducible expression vector of *Corynebacterium glutamicum* and *C. ammoniagenes*: fusion of lambda operator with promoters isolated from *C. ammoniagenes*. J Microbiol Biotechnol 18:639–647

Song JW, Jeon EY, Song DH et al (2013) Multistep enzymatic synthesis of long-chain α, ω-dicarboxylic and ω-hydroxycarboxylic acids from renewable fatty acids and plant oils. Angew Chem Int Ed 52:2534–2537

Yim SS, An SJ, Kang MS et al (2013) Isolation of fully synthetic promoters for high-level gene expression in *Corynebacterium glutamicum*. Biotechnol Bioeng 110:2959–2969

Yu IS, Kim HJ, Oh DK (2008) Conversion of linoleic acid into 10-hydroxy-12(Z)-octadecenoic acid by whole cells of *Stenotrophomonas* nitritireducens. Biotechnol Prog 24:182