（ 続紙 1 ）

京都大学	博士（ 農 学 ）	氏名	SUI YU-AN
論文題目	Screening of fatty alcohol dehydrogenase and its application on alkane production （脂肪族アルコール脱水素酵素の探索とそのアルカン生産への応用）		

（論文内容の要旨）

Bio-alkanes, which are produced from biomass and designed to share the same chemical composition as gasoline, are expected to serve as the next-generation biofuel. Alkanes are produced by many organisms in nature, including plants, insects, and microorganisms, where in most cases, fatty aldehydes derived from fatty acid metabolism are converted to alkanes through a decarbonylation reaction mechanism. It has been reported that heterologous expression of cyanobacterial fatty acyl-ACP reductase (AR) and aldehyde deformylating oxygenase (ADO) resulted in alkane production in *Escherichia coli*. However, the alkane titer was limited by the endogenous aldehyde reductases (ALR) which are active in converting fatty aldehydes, the precursor of alkanes, into fatty alcohols. This thesis describes discovery of a fatty alcohol dehydrogenase, PsADH, from a soil isolate of *Pantoea* sp., its characterization, and introduction of the enzyme into engineered *E. coli,* resulting in improved alkane production.

Chapter 1 describes the selection of alcohol-oxidizing bacteria from the natural environment and characterization of an alcohol dehydrogenase, PsADH, from a selected bacterium, *Pantoea* sp. 7-4. It has been reported that ADO converts medium-chain fatty aldehydes into medium-chain alkanes, which can be used as jet fuels. To utilize alcohol for alkane production, an enzyme capable of oxidizing medium-chain fatty alcohols to fatty aldehydes remains in need. This chapter describes screening of 1-tetradecanol-assimilating microorganisms. The screening was started with the isolation of microbial strains from soil samples and was followed by optimization of the resting cell reaction using 1-tetradecanol as the substrate. Among the 355 strains screened, 19 strains showed the activity to produce tetradecanal, while 41 strains showed the activity to produce tetradecanoic acid, including 14 strains that produced both. Regarding that this study aims to select an aldehyde-producing enzyme, the strain *Pantoea* sp. 7-4 was chosen since it showed the highest tetradecanal production.

To identify the enzyme involved in the alcohol-oxidizing reaction, the cofactors used by *Pantoea* sp. 7-4 was investigated, and then, *Pantoea* sp. 7-4 was found to possess a NAD^+-dependent dehydrogenase. Next, the target enzyme was purified from *Pantoea* sp. 7-4 via a series of column chromatography, and its single protein band on SDS-PAGE was subjected to N-terminal amino acid sequencing. By applying the obtained sequence to BLAST analysis, whole sequence of the dehydrogenase gene which showed 100 % homology with a putative alcohol dehydrogenase from *Pantoea* sp. MSR2 strain was revealed. The target protein from *Pantoea* sp. 7-4 was named as PsADH. Furthermore, PsADH was heterologously expressed in *E. coli* Rosetta 2(DE3), and the recombinant PsADH was purified and used for the characterization of its biochemical properties. The results suggested that PsADH is a NAD^+-dependent dehydrogenase. The optimal reaction pH of PsADH was pH 9.0 when catalyzing oxidation reaction of alcohols, while the optimal reaction pH was pH 7.0 when catalyzing reduction reaction of aldehydes. Besides, the

optimal reaction temperature was found to be 40 °C, which is also the highest temperature where PsADH showed sufficient thermal stability. In PsADH catalyzing oxidation reaction of 1-tetradecanol, K_m value of 0.17 mM and k_{cat} value of 29 s^{-1} were revealed. PsADH showed activity to oxidize alcohols including C6 – C18 primary alcohols as well as secondary alcohols and unsaturated alcohols.

Chapter 2 describes the application of PsADH to alkane production from fatty alcohol. To evaluate the potential of PsADH on alkane production using *E. coli* as host, PsADH was co-expressed with a cyanobacterial aldehyde deformylating oxygenase together with a reducing system, including ferredoxin, Fd, and ferredoxin reductase, FNR, from *Nostoc punctiforme* PCC73102. After inducing the gene expression in the transformed *E. coli* with IPTG, the production of each target protein was confirmed by SDS-PAGE. The activity of induced *E. coli* cells was checked by resting cell reaction, and the results showed that the *E. coli* harboring *PsADH-NpAD-Fd-FNR* genes successfully converted 1-tetradecanol to tetradecanal and tridecane, while the *E. coli* without *PsADH* gene could not convert the alcohol substrate to alkane. The products produced during the resting cell reaction were confirmed by GC-MS. Optimum reaction conditions for obtaining efficient conversion rate of 1-tetradecanol to tridecane with *E. coli* cells harboring *PsADH-NpAD-Fd-FNR* were as follows: pH 7.5 and 30 °C with 80 mg of washed cells. Under the conditions, 2.0 mM 1-tetradecanol was converted to 1.0 mM tridecane in 24 hours. The conversion rate of alcohol to alkane was increased to 52 % after optimization of the reaction conditions. Furthermore, the substrate specificity was revealed with resting cells of the *E. coli* transformant harboring *PsADH-NpAD-Fd-FNR* using fatty alcohols ranging from C6 – C18 as substrates. The results demonstrated the broad product spectrum of this biosynthetic pathway, in which alkanes ranging from C5 – C15 could be produced from their corresponding alcohol substrates. The production of C17 alkane and alkene was detected when using cell-free extracts as the enzyme source, indicating that substrate permeation limited the specificity of the resting cell reaction.

Chapter 3 describes application of PsADH to the alkane fermentation process. The bottle neck of fermentative alkane production using *E. coli* as host is due to the endogenous ARs which are active in converting fatty aldehydes into fatty alcohols. To utilize these alcohol byproducts, the alcohol dehydrogenase, PsADH, was further introduced to the alkane fermentation process. This was achieved by co-expression of a fatty acyl-ACP reductase, SeAR, originated from cyanobacteria *Synechococcus elongatus* PCC7942, with NpAD and PsADH using *E. coli* as host. The alkane production by fermentation was performed in 2.5 L of 2×YT medium in a jar fermenter for 6 days, and the time course of the production were analyzed. As a result, with the strain expressing *PsADH-NpAD-SeAR* genes, the concentration of alkanes, including tridecane, pentadecane, and heptadecene, in the culture medium reached 1.3 mM, and the concentration of alcohols, including 1-tetradecanol, 1-hexadecanol, and oleyl alcohol reached 2.1 mM. On the other hand, with the control strain without *PsADH* genes, the concentration of alkane and alcohol reached 0.36 mM and 4.4 mM, respectively. The results indicated that PsADH was effective in reducing alcohol byproducts and facilitating the fermentative alkane production.

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（続紙 2 ）

（論文審査の結果の要旨）

近年、持続可能かつカーボンニュートラルな石油代替エネルギーの開発が進められている。その一つとして、微生物機能を利用してバイオマスからバイオアルカンを生産する研究が行われている。微生物によるアルカン生産の課題として、副産物であるアルコールが大量に生成し、原料のほとんどがアルカン生産に結びついていないことが挙げられる。本論文は、新規アルコール酸化酵素を見いだし、本酵素によるアルコール再酸化反応の導入によりアルカン合成中間体であるアルデヒドに誘導することで、蓄積したアルコールをアルカン合成の基質として供給することを試みた。その主な成果として、以下の3点が挙げられる。

1. 脂肪族アルコールを酸化する活性が高い菌株のスクリーニングを、土壌からの分離菌355株を対象にテトラデカノールを基質として用いて行った結果、目的化合物であるテトラデカナールの生成量が最も高い株*Pantoea* sp. 7-4を見いだした。本菌から新規アルコール脱水素酵素PsADHを精製し、N末端アミノ酸配列情報をもとにPsADHをコードする遺伝子*PsADH*を特定した。さらに、*PsADH*を導入した大腸菌形質転換株より精製酵素を調製し、速度論的パラメータ、pH依存性、基質特異性など、PsADHの生化学的性質を明らかにした。
2. PsADHと*Nostoc punctiforme*由来アルデヒドデカルボニラーゼNpADを大腸菌で共発現することにより、アルコールからのアルカン生産が可能となることを示した。本形質転換株を用いて反応条件を最適化した結果、アルコールからアルカンへの変換率52 %の高効率で、炭素数C5 – C17のアルカン生産が可能となることを示した。
3. 前述したPsADHとNpADに加え、*Synechococcus elongatus*由来アシルACPレダクターゼを共発現した大腸菌を作製し、グルコースからのアルカンの発酵生産におけるPsADHの効果を検証した。その結果、蓄積したアルコールをPsADHによりアルデヒドに再変換する反応の導入が、アルカンの効率的発酵生産に寄与することを明らかにした。

以上のように、本論文は、新規脂肪族アルコール脱水素酵素の探索及び生化学的解析を通じて、副生するアルコールのアルデヒドへの再酸化系の導入が、バイオアルカンの発酵生産に有用な基盤技術となることを示した。従って、応用微生物学、発酵生理学、制御発酵学の発展に寄与するところが大きい。

よって、本論文は博士（農学）の学位論文として価値あるものと認める。

なお、令和 5年 2月 9日、論文並びにそれに関連した分野にわたり試問した結果、博士（農学）の学位を授与される学力が十分あるものと認めた。

注）論文内容の要旨、審査の結果の要旨及び学位論文は、本学学術情報リポジトリに掲載し、公表とする。

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要旨公開可能日：　　年　　月　　日以降（学位授与日から３ヶ月以内）