（ 続紙 1 ）

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論文題目	Biochemical analysis and genetic engineering of oleaginous fungi for the production of eicosapentaenoic acid and free fatty acid derivatives （エイコサペンタエン酸と遊離脂肪酸誘導体生産のための油糧糸状菌の生化学的解析と遺伝子工学）		

（論文内容の要旨）

The thesis describes biochemical analysis and genetic engineering of oleaginous fungi for the production of functional lipids and oleo-chemicals. ω3-polyunsaturated fatty acids (ω3-PUFAs) and free fatty acid (FFA) derivatives hold great industrial importance as pharmaceutical intermediates and fine oleochemicals, but they are currently obtained from unsustainable sources. For example, eicosapentaenoic acid (EPA) is obtained mainly from depleting marine fisheries, and ricinoleic acid is obtained mainly from castor bean which produces the potentially lethal toxic ricin. Their production from alternative sustainable sources by oleaginous microorganisms is therefore necessary. The results obtained from this thesis can be applied to establishing microbial production of the ω3-PUFA, EPA, and the FFA derivative, 13-hydroxy-*cis*-9-octadecenoic acid (13-OH LA).

Chapter 1 describes characterization of ω3 fatty acid desaturases from oomycetes and their application toward EPA production in *Mortierella alpina*. ω3 desaturases with efficient desaturase activity are vital to produce ω3-PUFAs such as EPA from ω6-PUFAs, but such gene resources are limited. With an aim to isolate efficient ω3 desaturases, oomycetes were selected based on intracellular EPA/arachidonic acid (ARA) ratios. Two oomycetes with the highest EPA/ARA ratios in their respective orders, *Pythium sulcatum* and *Plectospira myriandra*, were selected, and the ω3 desaturase genes, *psulω3*, *pmd17c*, and *pmd17g,* were cloned.

The enzymes were expressed in the oleaginous fungus *M. alpina*, which produces approx. 70 % of total fatty acids (TFAs) as ω6-PUFAs at 28 °C. Two resulting transformants produced EPA comprising 38 % (*psulω3*) and 40 % (*pmd17g*) of TFAs, which are the highest reported EPA/TFA values for *M. alpina* to date. Compared to the strains expressing *pmd17c* and *pmd17g*, the strain expressing *psulω3* showed less accumulation of the C18 ω6 byproducts, linoleic acid (LA) and γ-linolenic acid, as they were presumably converted to the C18 ω3 fatty acids, α-linolenic acid (ALA) and stearidonic acid (SDA). In contrast, *M. alpina* expressing *pmd17c* and *pmd17g* accumulated EPA as a major product with minimal accumulation of C18 ω3 byproducts such as ALA and SDA.

These results show that PSULω3 is a non-specific ω3 desaturase that shows high potential for EPA production through C18 and C20 fatty acids, while PMD17C and PMD17G are of great value for EPA biosynthesis in *M. alpina* via C20 ω6-PUFAs due to their ability to convert endogenous C20 ω6-PUFAs to C20 ω3-PUFAs with minimal byproducts and high conversion efficiency. These ω3 desaturases should thus facilitate the advent of more sustainable EPA sources.

Chapter 2 describes development of a platform host microorganism *Basidiobolus meristosporus* for production of FFA derivative. FFA-derived oleochemicals fermented from biomass are potential alternative sources for various functional lipids and industrial oleochemicals, but no reported organisms possess the metabolic environment to accumulate high levels of FFA without extensive metabolic engineering.

Section 1 of this chapter describes screening and characterization of oleaginous filamentous fungus *B. meristosporu*s accumulating high levels of FFAs.

After characterization and optimization of FFA production, a fed-batch fermentation at 300 mL flask scale was conducted. *B. meristosporus* rapidly grew to a maximum of 60.5 g/L dry cell weight (DCW) after 312 h cultivation at 28 °C. FFAs were produced to high levels from glucose, reaching an FFA titer of 10.0 g/L, a productivity of 61.7 mg/L/h, and a yield of 0.05 g FFA/g glucose (~17 % of the theoretical maximum) after 162 h. These FFA production metrics are the highest reported in any wild type organism to date, with the titer, productivity and yield representing ~500,000-fold, ~100-fold and ~330-fold increases, respectively, compared to the wild types of the current platforms *Escherichia coli*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica*.

Section 2 of this chapter describes a transformation system for *B. meristosporus* utilizing a plasmid vector containing a carboxin resistance gene as a selection marker and a β-glucuronidase (GUS) gene as an expression reporter.

After transformation of the ballistospores by biolistic bombardment, the resulting transformants could be recovered on carboxin selective plates after 72 h and showed stable GUS activity. Transformation efficiency reached approximately 237 transformants/10^6 ballistospores.

Lactobacillus acidophilus FA-HY1 catalyzes the hydration of LA to 13-OH LA, with biochemical characterization and homology models strongly suggesting FFAs to be the sole substrate. To demonstrate that endogenously accumulated FFAs in *B. meristosporus* could be utilized as substrates for FFA derivative production, *de novo* biosynthesis of the hydroxy fatty acid 13-OH LA from glucose was evaluated with *B. meristosporus* transformants. The expression vector pBIG_CarR_FA-HY1 was utilized, which contained FA-HY1 in place of GUS. This vector was introduced into *B. meristosporus* ballistospores. Analysis of the intracellular lipids of a resulting transformant yielded a novel peak with a similar retention time to the 13-OH LA standard. The mass spectrum of the peak produced by the FA-HY1 transformant showed the molecular ions characteristic of 13-OH LA.

13-OH LA production was then evaluated by fed-batch cultivation of the FA-HY1 transformant at 300 mL flask scale. During fermentation, the wild type strain showed no 13-OH LA production and the LA FFA titer reached 1250 mg/L after 162 h. In comparison, the FA-HY1 transformant showed 13-OH LA production throughout fermentation, reaching a maximum of 311 mg/L 13-OH LA after 186 h.

Thus, *B. meristosporus* and the transformation system for the stain facilitate sustainable production of industrially relevant FFA derivatives.

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（続紙 ２ ）

（論文審査の結果の要旨）

ω3-高度不飽和脂肪酸（ω3-PUFA）や遊離脂肪酸（FFA）誘導体といった機能性脂肪酸は、医薬品中間体や精密化学品素材として産業的に非常に重要であるが、現在は持続不可能な供給源から得られている。従って、油糧微生物を活用して、持続可能な供給源から生産する技術の構築に期待が集まっている。本論文は、有用脂肪酸変換酵素の探索、ならびに、FFA生産微生物の探索とその形質転換系の開発をとおして、ω3-PUFAとしてのエイコサペンタエン酸（EPA）、FFA誘導体としての13-ヒドロキシ-*cis*-9-オクタデセン酸（13-OH LA）の生産基盤を構築したものであり、その主な成果として、以下の3点があげられる。

1）3つの新規なω3脂肪酸不飽和化酵素遺伝子（*psulω3*、*pmd17c*、および*pmd17g*）を卵菌類からクローニングし、これらの遺伝子を油糧糸状菌*Mortierella alpina*で発現させた。その結果、*pmd17g*発現株において、全脂肪酸の40%に達するEPAの高生産を実現した。また、形質転換株の脂肪酸組成の解析から、*psulω3*発現産物であるPSULω3が炭素数18及び20の脂肪酸に幅広く作用すること、*pmd17c*、*pmd17g*発現産物であるPMD17C、PMD17Gが炭素数20の脂肪酸に特異的に作用することを明らかにした。

2）FFA高生産微生物の探索を行い、全脂肪酸の87.2%をFFAが占める高FFA生産菌*Basidiobolus meristosporus*を見いだした。本菌は10.0 g/LのFFAを蓄積し（生産効率61.7 mg/L/h）、対糖収率は理論収率の17%に達するなど、野生株としては、従来に報告のないレベルの高いFFA生産性を示した。

3）*B. meristosporus*の形質転換、遺伝子発現制御を可能にする基本的な遺伝子操作技術を確立した。また、内因性FFAからのFFA誘導体生産の実例として、乳酸菌由来の脂肪酸水和酵素を発現する*B. meristosporus*形質転換体を用いて、グルコースからの13-OH LAの効率的発酵生産を実現した。

以上のように、本論文は、新規な脂肪酸不飽和化酵素や遊離脂肪酸生産微生物の生化学的・分子生物学的解析をとおして、高度不飽和脂肪酸や水酸化脂肪酸といった機能性脂肪酸の発酵生産に有用な基盤技術を構築した。従って、応用微生物学、発酵生理学、制御発酵学、分子微生物科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 3年 2月10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。

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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）