Study on The Utilization of Oleaginous Yeast Lipomyces starkeyi as A Microbial Platform for Production of Biochemical Building Blocks

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Dissertation Abstract

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Study on The Utilization of Oleaginous Yeast *Lipomyces starkeyi* as A Microbial Platform for Production of Biochemical Building Blocks

油脂生産における高性能な *Lipomyces starkeyi* の利用に関する研究

Academic Supervisor: Prof. Dr. Chiaki Ogino

Oleaginous microorganisms are able to convert carbon sources into storage lipid as intracellular lipid droplets yielding in more than 20% lipid per dry biomass. These lipids are also known as single cell oils (SCOs)and are produced in the stationary growth phase under nitrogen limitation with simultaneous excess of carbon source. Yeasts are recognized as suitable candidates for biotechnological experiments as they have many advantages over other microbial sources. Compared with filamentous fungi and microalgae, yeasts have a shorter duplication time and higher growth rates. In SCO production, yeasts have a higher lipid content than microalgae and their cultivation is easier to scale up. In the yeast fermentation process, bacterial contamination can be controlled by conditions of low pH growth. Additionally, yeasts have the ability to utilize various types of carbon sources for the production of biomass and lipids. Considering the depletion of crude oil, the controversial use of plant oils for biodiesel production and the overfishing of the oceans, SCO produced from oleaginous yeasts are considered as the suitable oil substitutes for crude, plant and fish oil. However, microbial lipids are not yet economical feasible, apart from small number of production plants for high value fatty acids, e.g. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA). Therefore, strategies are required to reduce the production costs of SCOs and to increase the productivity.

Among oleaginous yeasts, *Lipomyces starkeyi* has considerable potential as a viable SCO producer due to its ability to produce high amounts of oils from hemicellulose-derived sugars including xylose. Since hemicellulose may comprise up to a third of plant-derived biomass, using this oleaginous yeast to produce SCO as a value-added bioconversion platform could provide additional revenue streams for future biomass-based biorefineries. The sugars derived from lignocellulosic biomass are mostly glucose and xylose, accordingly, oleaginous yeast strains that consume both glucose and xylose are preferred, and the ability to consume minor sugars such as arabinose, mannose, or galactose is also desirable. In addition to sugars, unfortunately, inhibitory chemical compounds (ICCs) also are generated as byproducts during biomass pretreatment, and these interfere with microbial growth during the fermentation process. The potent ICCs released during pretreatment are furfural, 5-hydroxymethylfurfurral (HMF), syringaldehyde, and vanillin coupled with some cell-membrane permeative acids such as acetic acid, formic acid, and a small fraction of levulinic acid. These inhibitors are released mainly due to the over-acid hydrolytic degradation of cellulose and hemicellulose. The maximum concentration of each inhibitor that a microorganism can withstand cannot be determined precisely because inhibition greatly depends on factors such as the types of microorganisms, the fermenting process employed, and the synergistic effect of inhibitors present in the medium. The deleterious effect of ICC on cell is considered the most immediate technical barrier for lipid production from lignocellulose. Therefore, oleaginous

yeasts using lignocellulose derived from sugar feedstock should not only be capable of accumulating high levels of intracellular lipid but should also be tolerant to various ICCs. The development of stress tolerance in oleaginous yeasts is a significant challenge for cost-competitive lipid production. Many types of yeast can adapt to growth on medium containing inhibitor. Most studies, however, have focused on strain tolerances in medium containing either a single inhibitor or simple mixtures of furfural, 5-HMF and acetic acid.

Oil palm (*Elaeis guineensis*) is widely planted for its edible oil in tropical countries such as Indonesia and Malaysia. The Indonesian palm oil industry has thrived in recent decades and Indonesia has emerged as one of the world's leading palm oil-producing countries. In general, the palm starts bearing oil-contained fruits in 2.5 years after planted and its productivity becomes lower after 20-25 years. Consequently, it is necessary to cut the old palms and to replant new seedlings in plantations sites. When replanting, old palms are cut and most of them are discarded or burned on site. Therefore, efficient ways for utilizing oil palm trunks is desired for ideal palm plantation and sustainable palm oil industry. Biomass generated as tree trunks from oil palm plantation are often under-utilized and are mainly used by plantations for mulching. Due to the high moisture contents (70-80% on the basis of the total mass), palm trunks are not appropriate as lumber which leads to large warping after drying and only a small percentage of trunks are utilized as plywood and particleboard industries. Oil palm trunks (OPT) sap is rich in sugars, amino acids, organic acids, mineral and vitamins. Sugars form the major constituent of sap and it has been reported to contain 8.3% w/v of total sugar in a freshly logged trunk. Nutrient rich sap from OPT has not yet been commercially exploited and has high potential to be utilized as an oil palm industry waste produce value-added products like biofuels. In addition, as OPT sap contains all the macro and micro nutrients required for yeast growth, it can potentially be exploited as a yeast growth medium. With cheap renewable carbon sources available, complete utilization of these resources are necessary to realize the cost effective value-added bioconversion products. Hereof, employing a microbial strain with efficiently utilizing these resources is urgently needed. *Lipomyces starkeyi* is a well-known strain and promising candidate to produce SCO. This strain has the capability to accumulate over 70% of its cell biomass as lipid under defined culture conditions, and can produce lipid on various carbon sources as well as other wastes. Several studies also have shown that *L. starkeyi* could produce a considerable amount of SCO in hemicellulose hydrolysate under the presence of inhibitory chemical compounds. As concerns of this finding, *L. starkeyi* can be considered a promising oleaginous yeast platform for industrial scale production of lipid. A combined approach has been attempted for the first time to evaluate the potential of OPT sap as a novel inexpensive renewable carbon feedstock for SCO production.

At the first stage of this study, *L. starkeyi* NBRC10381 strain was used as a platform to evaluate cell growth and SCO production in synthetically nitrogen limited mineral media. The effect of inoculum concentration on the cell growth and lipid production were investigated using glucose and/or xylose as carbon source. Fermentation using glucose and xylose as mixed carbon sources generated the highest production of biomass at 40.8 g/L, and achieved a lipid content of 84.9% (w/w). When either glucose or xylose was used separately, the totals for achieved lipid content were 79.6% (w/w) and 85.1% (w/w), respectively. However, biomass production was higher for glucose than for xylose (30.3 vs. 28.7 g/L, respectively). This study describes the first simultaneous achievement of higher levels of cell mass and lipid production using glucose and/or xylose as the carbon sources in different inoculum sizes.

A two-stage selection process was applied to eight oleaginous yeast strains from the *Lipomyces* genera. In the primary selection stage, a nitrogen-limited mineral medium (-NMM) that contained a mixture of glucose and xylose as a carbon source was used to evaluate the lipid-accumulating abilities of the yeast strains. The strains *L. doorenjongii*, *L. orientalis*, and *L. starkeyi* were selected as the potential strains in the primary selection. These three strains exhibited a remarkable ability to simultaneously assimilate glucose and xylose and achieved a cell biomass of more than 30 g/L. The values for lipid content in the selected strains were 57.89±1.92, 56.38±1.93, and 77.14±1.55% for *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*, respectively. In the secondary selection, when the -NMM medium contained an inhibitory chemical compound (ICC), the selected strains showed a different tolerance level against each of the typical inhibitor compounds. However, *L. starkeyi* accumulated the highest lipid content and yield at 68.24±2.48% and 0.19±0.00 (w/w), respectively. *L. starkeyi* accumulated high levels of intracellular lipid and tolerated the ICC. The composition of fatty acid methyl esters (FAMEs) was unaltered by the presence of ICC and the major FAMEs consisted of oleic, palmitic, stearic, palmitoleic and linoleic acids. The results obtained suggest the possibility for the biochemical conversion of lignocellulosic materials as major sources of hexoses and pentoses in biomass hydrolysates that could then be efficiently converted into lipids. Strategies for the selection of the most efficient oleaginous yeast strains should consider not only growth and lipid production but also superior characteristics for the fermentation process that includes sugar utilization and tolerance of lignocellulosic inhibitors.

The ability of oleaginous yeast *Lipomyces starkeyi* to efficiently produce lipids when cultivated on sap extracted from felled oil palm trunk (OPT) as a novel inexpensive renewable carbon source was evaluated. OPT sap was found to contain approximately 85 g/L glucose and 25 g/L fructose. Batch fermentations were performed using three different OPT sap medium conditions:

regular sap, enriched sap, and enriched sap at pH 5.0. Under all sap medium conditions, the cell biomass and lipid production achieved were approximately 30 g/L and 60% (w/w), respectively. *L. starkeyi* tolerated acidified medium (initial pH ≈ 3) and produced considerable amounts of ethanol as well as xylitol as byproducts. The fatty acid profile of *L. starkeyi* was remarkably similar to that of palm oil, one of the most common vegetable oil feedstock used in biodiesel production with oleic acid as the major fatty acid followed by palmitic, stearic and linoleic acids. Results from this study clearly demonstrate the potential of *L. starkeyi* as a microbial lipid producer as well as OPT sap as an important renewable carbon feedstock for SCOs production.

（別紙１）　　　　　　　　　　論文審査の結果の要旨

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論文題目	Study on The Utilization of Oleaginous Yeast *Lipomyces starkeyi* as A Microbial Platform for Production of Biochemical Building Blocks （油脂生産における高性能な *Lipomyces starkeyi* の利用に関する研究）

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要　旨

本学位論文では、微生物から培養することで得られる単一細胞由来油（Single cell oil: SCO）の効率的な生産に関して研究をまとめている。序章では、油性微生物、および単一細胞油（SCO）に関して、これまでの先行研究についてまとめて、自身の研究の振り返りを行っている。以下に一般的な情報を記載する。一般的に油性微生物は、乾燥バイオマスあたり 20%を超える脂質を生じる細胞内脂質液滴として炭素源を貯蔵脂質に変換することができる。SCO は窒素制限下で定常増殖期に生成される。酵母は、他の微生物資源よりも多くの利点を有するため、バイオテクノロジー実験に適した候補として認識されている。酵母は、糸状菌および微細藻類と比較して、より短い複製時間およびより高い増殖速度を有する点で有利であり、SCO 生産においては、その培養はスケールアップが容易である利点も有している。

第１章では、*L. starkeyi* (NBRC10381)株を、合成窒素制限ミネラル培地中の細胞増殖および SCO 産生を評価するためのプラットフォームとして使用した。グルコースおよびキシロースを炭素源として用いて、細胞増殖および脂質生成に対する接種濃度の影響を調べた。結果、混合炭素源としてグルコースおよびキシロースを用いた発酵では、バイオマスの生成量が 40.8g / L と最も高く、脂質含量は 84.9%（w/w）であった。グルコースまたはキシロースを別々に使用した場合、達成された脂質含量の合計はそれぞれ 79.6%（w/w）および 85.1%（w/w）であった。しかしバイオマス生産においては、グルコースやキシロースにおいて高かった（それぞれ 30.3 対 28.7 g/L）。この結果は、炭素源としてグルコースもしくはキシロースを使用し、高い濃度の初期細胞濃度を用いることで、細胞の高濃度化と高い効率で脂質生成の同時を達成することができた、最初の報告例である。またこの結果については、A.B. Juanssilfero, P. Kahar, R.L. Amza, N. Miyamoto, H. Otsuka, H. Matsumoto, C. Kihira, A. Thontowi, Yopi, C. Ogino, B. Prasetya, A. Kondo, Effect of inoculum size on single-cell oil production from glucose and xylose using oleaginous yeast *Lipomyces starkeyi*, J. Biosci. Bioeng. 126(6), 695-702 (2018)として、論文掲載されていることも確認した。

第２章では、*Lipomyces* 属から選抜した８つの油性酵母株に対して、２段階選択プロセスを適用した。第１ステップの選抜では、炭素源としてグルコースとキシロースとの混合物を含む窒素制限ミネラル培地（NMM）を用いて、酵母株の脂質蓄積能力を評価した。その結果、*L. doorenjongii*、*L. orientalis*、および *L. starkeyi* 株を一次選択における候補株として選抜した。これらの３つの菌株は、グルコースとキシロースを同時に資化する顕著な能力を示し、30 g/L を超える細胞濃度（バイオマス濃度）を達成することに成功した。*L. doorenjongii*、*L. orientalis*、および *L. starkeyi* について、各選択された株における脂質含量の値はそれぞれ 57.89 ± 1.92、 56.38± 1.93、および 77.14±1.55 %であった。

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その後、第２ステップ選択において、NMM 培地が阻害性化合物（ICC）を含有する場合、選択された菌株は、それぞれの一般的な阻害剤化合物に対して異なる耐性レベルを示した。しかしながら、その中において、*L. starkeyi* は最高脂質含量を蓄積し、それぞれ 68.24±2.48 %を示した。この結果より、*L. starkeyi* は高レベルの細胞内脂質を蓄積し、ICC に耐性を示した。SCO 中の脂肪酸メチルエステル（FAME）の組成は ICC の存在の有無により変化せず、主な FAME はオレイン酸、パルミチン酸、ステアリン酸、パルミトレイン酸およびリノール酸からなっていた。得られた結果は、バイオマス加水分解物中のヘキソースおよびペントースの主要源としてのリグノセルロース材料への生化学的変換の可能性を示唆し、それはその後、効率的に脂質に変換され得る。本章の研究成果より、最も効率的な油性酵母株の選択戦略は、菌体増殖および脂質生産を指標とするだけでなく、資化可能な糖源の種類、リグノセルロース系由来阻害剤への耐性をも考慮して、発酵プロセスを考慮する必要がある事が示唆された。またこの結果については、Ario Betha Juanssilfero, Prihardi Kahar, Rezky Lastinov Amza, Nao Miyamoto, Hiromi Otsuka, Hana Matsumoto, Chie Kihira, Ahmad Thontowi, Yopi, Chiaki Ogino, Bambang Prasetya, Akihiko Kondo, Selection of oleaginous yeasts capable of high lipid accumulation during challenges from inhibitory chemical compounds, *Biochem. Eng. J.* (2018) として、論文掲載が決定していることを確認した。

第３章では、新規な安価な再生可能炭素源として、油ヤシ幹（OPT）から抽出した樹液で栽培した場合、脂質酵母 *Lipomyces starkeyi* が効率的に脂質を生産する能力に関して評価を行った。OPT 樹液は約 85g / L のグルコースおよび 25g / L のフルクトースを含むことが明らかとなっており、*L. starkeyi* のバッチ発酵において、３つの異なる OPT 樹液条件（pH5.0 のレギュラーサップ、濃縮サップ、および濃縮サップ）を用いて行った。結果、達成された細胞バイオマス量および脂質生成は、それぞれ約 30 g/L および 60 %（w/w）であった。*L. starkeyi* は酸性化された培地（初期 pH 約 3）に耐え、副産物としてかなりの量のエタノールおよびキシリトールを産生し、その脂肪酸プロファイルは、パルミチン酸、ステアリン酸、リノール酸、そしてオレイン酸であり、バイオディーゼル生産に使用される最も一般的な植物油原料の１つであるヤシ油の脂肪酸プロフィールと著しく類似していた。この研究の結果は、微生物の脂質生産菌としての *L. starkeyi* の可能性と、SCO 生産のための重要な再生可能炭素原料としての OPT 樹液の可能性を明確に示している。またこの結果については、Ario Betha Juanssilfero, Prihardi Kahar, Rezky Lastinov Amza, Yopi, Kumar Sudesh, Chiaki Ogino, Bambang Prasetya, Akihiko Kondo, Lipid production by Lipomyces starkeyi using sap squeezed from felled old oil palm trunks として *Biochemical Engineering Journal* への論文投稿が完了しており、現在査読中であることが確認できた。

本研究は油脂生産酵母 *Lipomyces starkeyi* について、その油脂生産能力と培養特性に関して培養工学的な研究したものであり微生物における油脂生産について重要な知見を得たものとして価値ある集積である。提出された論文は工学研究科学位論文評価基準を満たしており、学位申請者の ARIO BETHA JUANSSILFERO は、博士（工学）の学位を得る資格があると認める。