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

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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

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GRADUATE SCHOOL OF ENGINEERING

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PREFACE

This is a thesis submitted by the author to Kobe University in partial fulfilment of the requirements for the degree of Doctor of Engineering. The studies were carried out between the period of 2015 and 2018 under the supervisory of Professor Chiaki Ogino in the Laboratory of Biochemical Engineering, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University.

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Introduction

Single Cell-Oil

Microbial lipid compounds, known as single-cell oils (SCOs), have piqued industrial interest due to their particular and precise biochemical and physicochemical properties for the biotechnological industry. SCO is commonly defined as neutral storage lipids accumulated by oleaginous eukaryotic microorganisms (e.g. yeast, mold, microalgae) and its often synonymous with lipid, oil, and triacylglycerol (TAG) [1].

Oleaginous yeasts are viable SCO producers that able to accumulate lipid up to 70% (dry mass basis) of their cellular biomass. They are unique compared with other oleaginous microorganisms in their ability to produces SCO heterotrophycally from a variety of low-value input such as agricultural residues, food waste streams and industrial co-products [2]. As suitable candidates for biotechnological experiments, yeasts have many advantages over other microbial sources. Compared with filamentous fungi and microalgae, yeasts have a shorter duplication time and higher growth rates. In lipid production, yeasts have a higher lipid content than microalgae and their cultivation is easier to scale up [3]. In the yeast fermentation process, bacterial contamination can be controlled by conditions of low pH growth. Yeasts have the ability to utilize various types of carbon sources for the production of biomass and lipids [4].

Species of oleaginous yeast include but are not limited to *Lipomyces starkeyi*, *Yarrowiya lypolytica*, *Rhodoturula glutinis*, *Rhodosporidium toruloides*, *Crytococcus curvatus*, and *Trichosporon fermentants* [2,5,6]. *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 [7,8].

The lipid profile of yeast SCO mainly include TAG and lesser amount of phospholipids, sphingolipids, sterols, and free fatty acids (FAs) [9,10]. SCO from oleaginous yeast may serve as a renewable source of edible oil and as an intermediate "building block" for oleochemicals such as fuels, soaps, plastics, paints, detergents, textiles, rubber, surfactants, lubricants, additives for the food and cosmetic industry and many other chemicals [11]. The production of SCO from oleaginous yeast for renewable oleochemicals is still in its early stage. Production of SCO has been successfully commercialized only for specialty oils containing polyunsaturated fatty acids (PUFAs) used in the food and supplement industries, including docosahexanoic acid (DHA), arachidonic acid (ARA), and eicosapentaenoic acid (EPA) [1]. SCO as a commodity-type oil has been hindered by competition from oilseed crops, which cost 10 to 20 times less [12]. Although the use of SCO as a feedstock for biodiesel has received interest in recent years, high manufacturing costs prevent the stand-alone production of biodiesel from SCO [12]. As more emphasis is placed on advancement of integrated biorefineries, the possibility of incorporating a yeast-based SCO biochemical platform for renewable fuels, chemicals, power, and products may become a reality [13].

The Biosynthesis of SCO

The biosynthesis of SCO or TAG accumulation in microorganisms has been reviewed previously [14]. In the oleaginous yeast, the SCO production depends on the composition of the culture medium. Whereas, the initiation of TAG accumulation often occurs under

nitrogen-deficient conditions and the carbon source has to be available in excess. Upon nitrogen limitation, carbon source is diverted from energy production via the tricarboxylic acid cycle (TCA) to TAG synthesis (Figure 1). The carbon source is converted into pyruvate in the cytosol, pyruvate is transported into the mitochondrion, decarboxylized to acetyl-CoA (C_2) which reacts with oxalacetat (C_4) and then further to citrate and subsequently usually to iso-citrate within the citrate cycle. In the case of nitrogen-limitation the enzyme AMP-deaminase is activated by cleaving adenosin-monophosphat (AMP) into inosin-monophospaht (IMP) and $NH4^+$ to provide cell own nitrogen for cell functions. However, AMP is required for the functionality of the enzyme isocitrate-dehydrogenase (ICDH), which converts isocitrate into α-ketoglutarate within the citrate cycle in order to produce $NADH^+ + H^+$ for the production of ATP within the respiratory chain. If no AMP is available, iso-citrate accumulates in the mitochondrion. Because of equilibrium reactions iso-citrate is converted into citrate which accumulates in the mitochondrion as well and is channeled into the cytosol via malat/citrat transporter. At this point citrate is cleaved under the consumption of ATP into oxalacetat (C_4) and one C_2-unit acetyl-CoA which is the chemical precursor for the fatty acid synthesis. This conversion is done by the enzyme ATP-Citrate-Lyase (ACL), which is special in oleaginous microorganisms [10,15,16]. The extent of fatty acid production depends on the malic enzyme (ME) concentration which converts malate to pyruvate via NADPH release. This chemical conversion is the sole source of NADPH for the enzyme fatty-acid-synthase (FAS), which is required in the fatty acid biosynthesis. The fatty acids are constructed by condensations of the C_2-units acetyl-CoA up to the C16 or C18 saturated fatty acids. These saturated fatty acids are extended to longer chain saturated fatty acids (sFA) via the enzyme elongase and can be subsequently converted into unsaturated fatty

acids (PUFAs) via the enzyme complex of desaturases. The enzyme complexes of desaturases and elongases are part of the cell membrane in the periplasmatic reticulum [10,17].

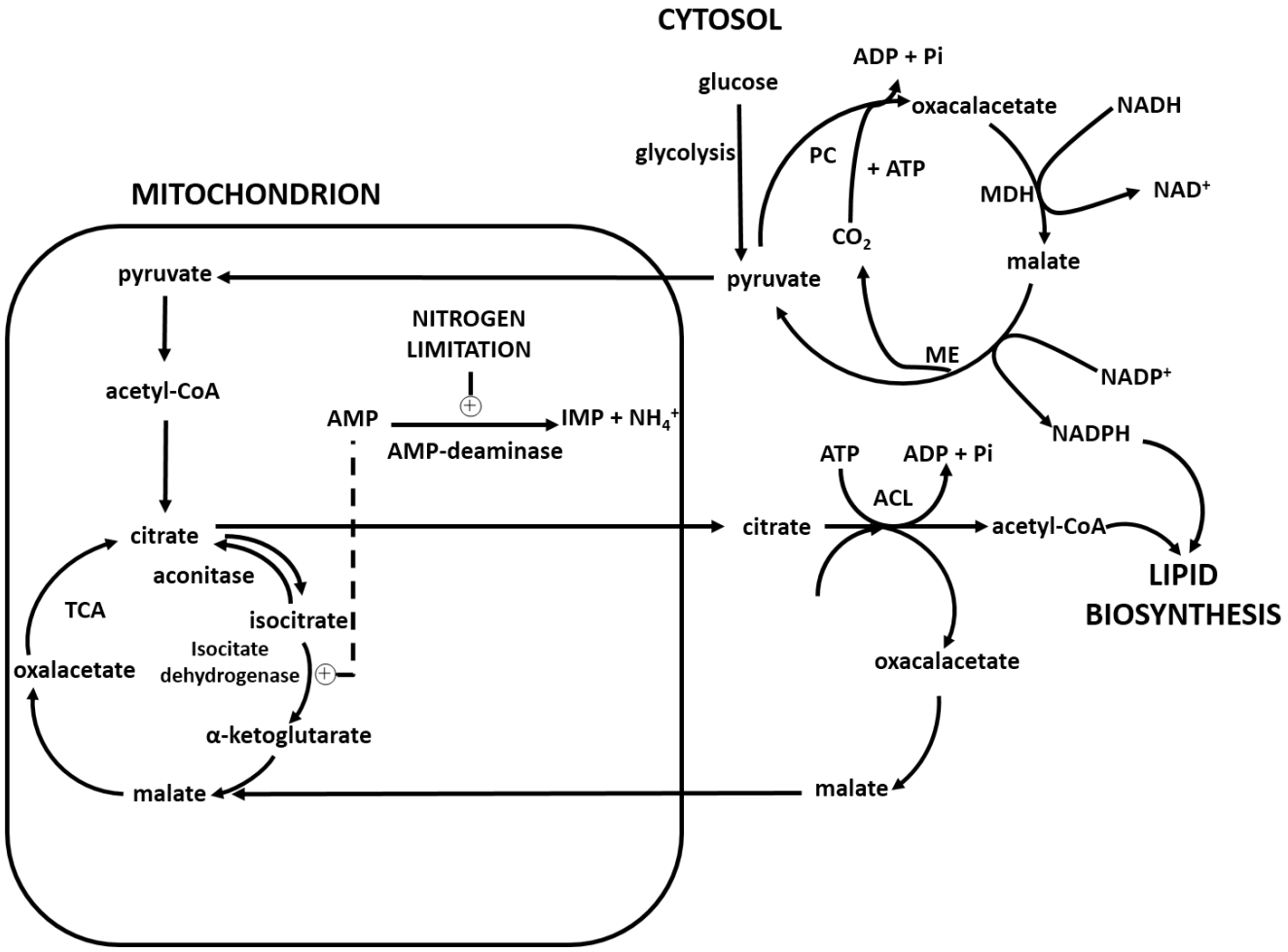

Figure 1. Overview diagram of the biosynthesis single cell-oil in oleaginous microorganisms.

Fermentation Conditions for SCO Production

Fermentation of oleaginous yeasts under limited nutrient condition usually consists of three phases: the exponential phase where cells proliferate rapidly; lipid accumulation phase where cells show minimum growth; and the stationary phase where lipid breakdown may occur [18]. SCO is a secondary fermentation product (i.e. it is produced independent of growth). To induce lipid accumulation, a two-stage fermentation technique is often employed. The first stage aims to promote cellular growth and biomass formation; during the second stage, a stress response is induced which causing metabolism to shift to storage lipid accumulation. As mentioned earlier, the stress-response is accomplished by limiting a specific nutrient, most often nitrogen, but other minerals such as iron and zinc have been reported with similar effects [19,20]. When nitrogen becomes limiting, yeast diverts carbon to FA synthesis and ultimately to TAG accumulation.

Sitepu et al. [4], summarized factors affecting lipid accumulation which include: yeast strains, stage of growth, carbon and nitrogen source, C/N ratio, other micronutrients, temperature, aeration/dissolved oxygen, pH, the presence of alcohol, and acclimatization to a carbon source. The C/N ratio is a critical factor in optimizing SCO production. As the ratio increases, excess carbon becomes available for SCO production. Too high of a ratio (i.e., limited nitrogen) will limit cellular growth, biomass production, and even result in significant secondary metabolite formation; thus, optimal C/N ratios depend on the production method, fermentation conditions and yeast species used [6]. Most researchers have reported C/N ratios that vary from 50-100, but values can be much higher [1,2]. For example, Angerbauer et al. [8] reported that *L. starkeyi* produced a lipid content of 68% (dry mass basis) when grown under a C/N ratio of 150 compared with 40% for a lower

C/N ratio of 60. A critical concentration of nitrogen also may be important in preventing formation of undesirable metabolites such as citric acid [6]. Thus, controlling the C/N ratio during fermentation is essential to direct carbon flux to TAG formation.

The next important aspects of fermentation for SCO production is the pH and aeration of the medium. Average pH used in literature was 5-6.5, however, *L. starkeyi* can grow well in a wide range of acidic pH, but not higher than 6.5 [8]. Angerbauer et al. [8] found that the highest lipid accumulation of *L. starkeyi* grew in sewage sludge was 56% at pH 5, increasing pH to 6.5 led to a lower lipid content of ~ 50% with a corresponding higher biomass. This suggest that lower pH which benefit lipid accumulation may be detrimental to cell growth. Being able to grow in slightly acidic pH is an advantage since it can suppress the growth of microbial contaminant which is important for industrial microbiology [12].

Aeration/ dissolved oxygen is important during fermentation, especially for growth. Higher oxygen transfer rate facilitates cell proliferation, increasing shaking speed leads to increase in dry cell weight (biomass production). On the other hand, SCO production work well in oxygen-limited medium since this condition block glucose oxidation pathway thus induces lipid accumulation [21]. While oleaginous yeast has the advantage to be able to live in a broad range of temperature (10 to ~25 °C) with high total lipid produced, however, if the fermentation is carried out at large scale extra cost will be needed for chilling [4]. Most fermentations were carried out at 30 °C [7,8,21].

Concentration and age of inoculums are also considered as important factors in fermentation. Inoculum concentration employed for fermentation is usually between 5 and 10% (v/v). Higher inoculum concentration will dilute rate-limiting substrate

concentration in the medium, leading to lower specific growth rate, while fewer inoculums would result in lag phase [22]. According to studies carried out in different fermentation mediums: potato starch wastewater [23], MSG wastewater [24] and crude glycerol [25], the optimum inoculums concentration to promote lipid accumulation was 10% (v/v). If higher inoculum was used, more nitrogen was introduced to the medium thus increasing N content which was not favorable for lipid accumulation [25]. In terms of inoculums age, the inoculum was usually taken at its exponential phase, and it may be different in each strain used. Investigation on inoculum age by Liu et al. [25] found that older inoculum seems more mature and more potent in assimilating substrate and lipid accumulation than younger inoculum.

Cultivation mode plays an important role in optimizing lipid productivity during fermentation with a direct economic impact on the process [26]. The commonly used cultivation modes are the batch, continuous, and fed-batch modes. In a batch mode, cells are cultured in a fixed volume of fermentation medium under specific conditions (nutrient type, temperature, agitation, etc.). Many studies in literature performed batch operation since it allows researchers to determine the optimal condition for lipid accumulation [26]. In continuous mode, the fresh nutrient is continuously supplied to a well-stirred medium where products and cells are simultaneously withdrawn [26]. Fermentation was initially run as batch mode (constant C/N ratio at a given dilution rate) for certain time to condition the system before it starts as continuous mode. Under this condition, lipids accumulation highly depends on dilution rate and C/N molar ratio of the culture medium [1]. On the other hand, in fed-batch mode, fermentation is prolonged by intermittent or continuous feeding of nutrients [26], which allows some control over specific growth rate and the flows of nitrogen and carbon utilization [27]. Thus it is possible to maintain

microorganisms in the optimal metabolism state for growth, and lipogenic phase at a later stage for lipid production under limited nitrogen condition with constant C/N ratio to prevent acids production [6]. This mode is usually used to overcome substrate inhibition or catabolite repression by intermittent substrate feeding, thus improves fermentation productivity [26].

After fermentation is terminated, biomass should be harvested (separating spent nutrient from biomass) rapidly to prevent lipid turnover that will reduce lipid production. Common methods to separate wet cells from spent nutrient include centrifugation, ultrafiltration, sedimentation or flocculation [27]. This biomass harvesting process can contribute 2 to 30% of the total biomass production cost [28]. After separation, wet cells are usually dried before extraction (in laboratory scale procedure). Since drying is energy intensive, thus it would be of great interest to develop a less expensive method to dry yeast, or directly use of wet cells in in-situ transesterification to produce FAME.

Challenges for Commercial Production of SCO

The stand-alone production of SCO and other commodities currently is not economically feasible [1]. To enhance the feasibility of commercial SCO production, research and innovation needs to focus on utilizing alternative low-cost nutrient sources and assimilating new technologies to enhance productivity. Area of improvement include developing robust strain that can produce high SCO yields from non-traditional feedstock's, enhancing SCO productivity using biotechnological and molecular techniques, tailoring FA profiles for specific end products, and developing efficient downstream processes for cell lysis and lipid extraction.

One of the major hurdles preventing industrial scale cultivation of oleaginous yeast is the cost of nutrient media (carbon source and other medium components). Synthetic nutrients, such as glucose can serve up to 60% of the fermentation total cost [29-31]. Low-valued carbon sources are often challenging to utilize because of their heterogeneous nature and the presence of inhibitory chemical compounds (ICC). Oleaginous yeasts have been shown to be versatile biocatalysts able to utilize a variety of alternative carbon and nutrient sources to produce SCO, including but not limited to animal fat [32] corn cobs [30,33,34], corn residue [24,35,36], glycerol [32,37,38] molasses [39], monosodium glutamate wastewater [24,40], olive oil wastewater [41], prickly-pear juice [42], pyrolytic waste [43], rapeseed meal [44], rice straw [34], sewage sludge [8], sorghum bagasse [45], sugarcane bagasse [34,46], volatile FAs [29,37], wheat straw [47] and whey permeate [48]. Low-value, carbohydrate-dense lignocellulosic feedstock from plants have the potential to serve as low-cost sources of carbon for SCO production. The heterogeneous composition of cellulose, hemicellulose, and lignin in these feedstock has limited its use in industrial bioprocesses. One way to reduce the total costs is to utilize biomass residues which are available abundantly (low-value-high-volume biomass) as the raw feedstock. If it is plant-based material, ideally it should be available all year, instead of being a seasonal crop [12]. A pretreatment step is required to depolymerize the lignin and hydrolyze the hemicellulose and cellulose fractions into usable sugars. Feedstock high in lignin require harsher pretreatment conditions that can produce inhibitory compounds such as furfural, 5-hydroxymethylfurfural (5-HMF), vanillin, acetic acid, formic acid and levulinic acid [49]. This could be a challenge for SCO production because these inhibitors can affect microbial growth and certain enzymes important in TAG synthesis, such as the malic enzyme [34]. Researchers have shown that

various oleaginous yeasts are nevertheless able to accumulate high amounts of lipid from lignocellulosic hydrolysates, but productivity often suffers Table 1. Robust yeast strains able to handle the presence of inhibitory compounds are necessary to prevent a neutralization step; *C. curvata*, *R. toruloides* and *T. cutaneum* have shown the best resistance to inhibitory compounds [47,49,50].

Monosaccharides produced from the hydrolysis of cellulose and hemicellulose include a mixture of pentose and hexose sugars, mainly glucose and xylose. One of the major challenges in the production of cellulosic ethanol was developing yeast strains capable of utilizing xylose anaerobically [51,52]. Because SCO production is aerobic, xylose metabolism proceeds naturally without the aid of genetic modification, so many oleaginous yeasts are capable of converting xylose to SCO [53]. An early study performed by Evans and Ratledge [19] concluded that the yeast *C. curvata* produced the highest lipid contents when grown using xylose compared with ethanol, lactose, sucrose and glucose. Similar results were obtained for the oleaginous yeast *L. starkeyi*, which is able to utilize xylose to produce SCO [7,54]

Table 1. Single cell-oil production by oleaginous yeast from lignocellulosic biomass.

Yeast strains	Dry Cell Weight (g/L)	Lipid content (%w/w)	Lipid Yield (mg/mg-substrate)	Substrate	Cultivation Mode	Reference
C. curvatus	17.2	33.5	0.20	Non-detoxified wheat straw hydrolysate	Batch-flask	[47]
C. curvatus	6.0	43.3	0.19	Sorghum bagasse hydrolysate	Batch-flask	[45]
C. curvatus	15.5	64.0	0.11	Microwave treated sorghhum bagasse hydrolysate	Batch-flask	[45]
Cryptococcus Sp.	10.8	61.3	0.11	Glucose enriched corn cob hydrolysate	Fed-batch-flask	[33]
L. starkeyi	14.7	31.2	0.16	Non-detoxified wheat straw hydrolysate	Batch-flask	[47]
R. glutinis	13.8	25.0	0.20	Non-detoxified wheat straw hydrolysate	Batch-flask	[47]
R. graminis	48.0	34.0	0.16-0.2	Corn stover hydrolysate	Batch-20L	[35]
R. toruloides	9.9	24.6	0.08	Detoxified wheat straw hydrolysate	Batch-flask	[47]
T. cutaneum	19.3	39.2	0.15	Corn stover hydrolysate	Batch-3L	[36]
T. cutaneum	38.4	32.0	0.13	Corncorb hydrolysate	Batch-3L	[30]
T. dermatis	24.4	40.1	0.17	Corncorb hydrolysate	Batch-flask	[34]
T. fermentans	28.6	40.1	0.10	Detoxified rice straw hydrolysate	Batch-flask	[34]
Y. lipolytica	7.8	4.6	0.01	Non-detoxified wheat straw hydrolysate	Batch-flask	[47]
Y. lipolytica	11.4	58.5	0.31	Detox sugarcane bagasse hydrolysates+peptone	Batch-flask	[46]

Other viable low-value carbohydrate sources may include co-products from the grain-processing industry. High fiber-containing streams from flour milling, such as wheat middling or corn bran, have significant amounts of polysaccharides (≥70%, dry mass basis), including starch, hemicellulose and cellulose fractions that can be selectively targeted. In addition, the low amount of lignin (<5%, dry mass basis) in these feedstock may limit the need for harsh pretreatment conditions and minimize the presence of inhibitory compounds. Additionally, industrial byproducts such as glycerol, molasses or animal fats, can be directly sterilized after characterization (if further pretreatment like removing impurities is not needed). Glycerol is the main byproduct of biodiesel production, which account for 10% (w/w) of biodiesel product [55]. Since a lot of biodiesel is produced nowadays, glycerol could be used as a potential fermentation feedstock.

Single cell oil as a biochemical platform

Integrated biorefineries conceptual processing facilities capable of utilizing a range of feedstock and conversion platforms to produce multiple products (chemicals, fuels, materials, and power) could over an opportunity with contribute to petroleum independence [62-66].

A combined approach of oleaginous yeasts as a biochemical conversion option in integrated biorefineries has many advantages. Yeasts are recognized as suitable candidates for biotechnological experiments as they have many advantages over other microbial sources. Yeasts are diverse and can grow exponentially while utilizing cheap substrates. They are robust (handle high sugar loading, tolerate pretreatment inhibitors, are easily cultivable and are viable for multiple generations), versatile (can utilize a range

of different carbon and nitrogen sources), accumulate high lipid content (up to 70%, dry mass basis) and can be genetically engineered [2]. Furthermore, a study performed by Groenewald et al. [67] deemed the oleaginous yeast *Y. lipolytica* ‘‘safe to use’’ as an industrial microorganism; thus, potential exists for other oleaginous yeast species to be regarded as safe for industrial manufacturing purposes. Due to shorter cultivation times compared with oilseed crops (e.g. days versus months), SCO made for products subject to market instability and price fluctuation could minimize investment risk [66].

Millions tons of oleochemicals are worldwide produced per year, with end-use markets ranging from bulk chemicals to specialty products [68]. The SCO production of oleaginous yeasts are mostly in the form of Triacylglycerols (TAGs) with some in the form of free fatty acids (FAs). The two most prevalent biofuels produced from SCO are biodiesel (via transesterification of TAGs) and renewable diesel (via hydrotreatment) [69]. The major process for transforming SCO into oleochemicals is the hydrolysis of TAGs into glycerol and FAs under the influence of water, temperature, and pressure (Fig. 2A). Furthermore, the SCO can be converted by a variety of chemical, physical and/or biochemical techniques to produce bulk commodity and high value specialty oleochemicals. As shown in Figure 2B, oleochemicals can be made from FAs using different chemical conversion processes. Glycerol, produced as a byproduct during SCO processing, is an important feedstock for various C3 commodity chemicals that are currently produced in petrochemical processes from propylene [70-72] and has many possible applications and transformations (Figure 2C).

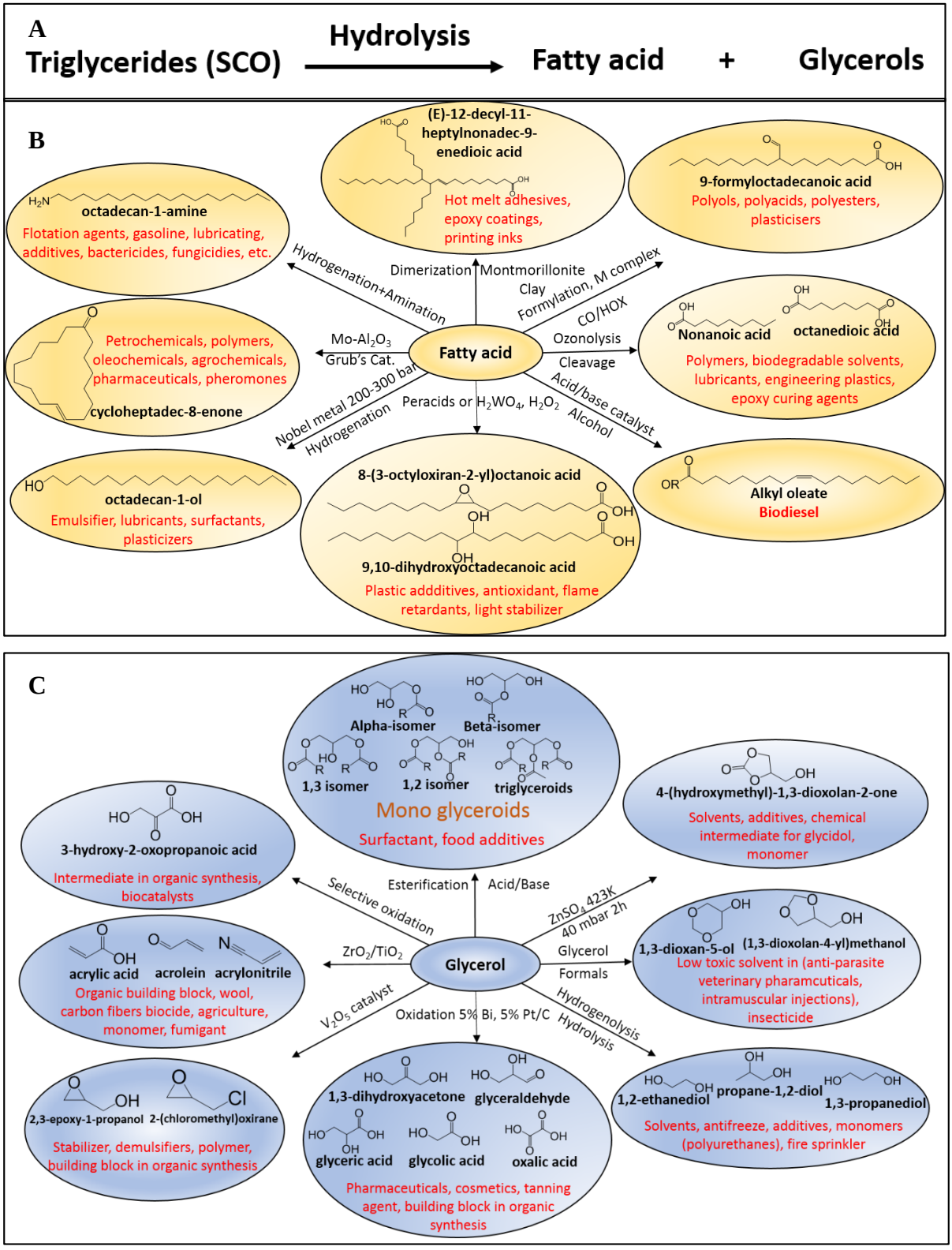

Figure 2. Summary of conversion routes and transformations of fatty acids and glycerol to renewable oleochemicals and their end uses.

SCO could be targeted to specific oleochemicals because some of oleaginous yeast are naturally has the ability to accumulate a higher amount of specific FAs, for instances: *Candida* species produce higher percentages of palmitic acid up to 40% of the total FA profile, *Y. lipolytica* can produce over 50% linoleic acid and *Cryptococcus albidus* and *Lipomyces starkeyi* can produce up to 75% oleic acid [6,14].

Yeast SCO can be hydrotreated to produce drop-in fuels such as diesel and jet fuel (Figure 2). The diverse spectrum of lipids produced by microorganisms also could result in fuels with enhanced performance properties such as lower viscosity and improved oxidative stability [66,69]. Monounsaturated FAs can be transformed via ozonolysis into dicarboxylic acids, which are important intermediates for polyester and polyamide synthesis [73]. Fatty alcohols as the precursors for many industries such as detergents, surfactants, cosmetics, pharmaceuticals, toiletries, and antifoaming agents can be made from long-chain FAs and converted into polyoxyethylene derivatives [72,74] for such applications.

In addition to chemically altering the end group of FAs, a generous work has been to functionalize the double bond of mono- and polyunsaturated oils with an epoxy group. Epoxydized FAs have been used as PVC stabilizers and UV-curable coatings [75]. Monounsaturated oils such as oleic acid have been epoxidized and used to create polymeric formulations such as polyesters and polymers for drug delivery vehicles [76]. Epoxidized FAs can also serve as starting materials for the synthesis of polyurethanes [66]. PUFA such as linoleic acid and α-linolenic acid also can be used to synthesize polyurethanes as renewable replacements for petroleum-based polymers [77].

Glycerol, produced as a by-product of transesterification, can be used to create a variety of highly valued chemicals (Fig. 2C). Opportunities exist to utilize crude glycerol to produce high-value products, including DHA, which is used in many skin-care products; polycarboxylates, which are used in household detergents; glycerol tertiary butyl ether, a replacement fuel oxygenate for the toxic methyl tertiary butyl ether; polyglycerol esters, which are used as wood treatments; acrylic acid esters, which are used as superabsorbent polymers; propylene glycol, which is used as a preservative in food/tobacco products and for the production of polyester resins.; and glycerol carbonate, which can be used to produce renewable polycarbonates and polyurethanes [78-80].

The first chapter of this work focused on the selection of a cultivation strategy for lipid production that could rapidly accumulate lipids with high productivity and high cell biomass simultaneously in typical culture media. The cultivation strategy involves differing the inoculum size in order to increase the microbial growth and initiate the lipid production phase. Although inoculum size is rarely used as a factor in studies of microbial growth, evidence indicates that it may indeed affect microbial growth. Some studies have indicated the importance of inoculum size on the ability of a microbial population to initiate growth [25,56-58]. Therefore, differing the inoculum size promoted appropriate microbial growth and simultaneously initiated lipid production for highly productive yield.

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 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 [59-61]. In the second chapter of this work, a two-tiered selection process to examine eight yeast species from the genus *Lipomyces* to select strains with superior lipid productivity was explored. In the primary selection process, qualities such as the carbon source consumption rate, growth rate, lipid accumulation ability, and fatty acid composition of yeast strains were evaluated in synthetically limited nitrogen mineral medium contained a mixture carbon sources. While in the secondary stage, screening for inhibitory chemical tolerance was initiated for the strains that had demonstrated the highest levels of lipid content, cell mass production, and rates of carbon source consumption in the primary selection.

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. In order to obtain a promising oleaginous yeast platform for industrial scale production of SCO, a combined approach has been attempted to evaluate the potential of selected yeast strain to produce SCO in a novel inexpensive renewable carbon feedstock. This has been explored in chapter 3 of this work.

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Synopsis

Chapter I

Effect of inoculum size on single-cell oil production from glucose and xylose using oleaginous yeast *Lipomyces starkeyi*

Oleaginous microbes can convert substrates such as carbon dioxide, sugars, and organic acids to single-cell oils (SCOs). Among the oleaginous microorganisms, *Lipomyces starkeyi* is a particularly well-suited host given its impressive native abilities, including the capability to utilize a wide variety of carbon sources. In this work, the potential of *L. starkeyi* NBRC10381 to produce SCOs in a synthetically nitrogen-limited mineral medium (-NMM) was investigated by differing the inoculum size using glucose and/or xylose as a 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.

Chapter II

Selection of oleaginous yeasts capable of high lipid accumulation during challenges from inhibitory chemical compounds

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.

Chapter III

Lipid production by *Lipomyces starkeyi* using sap squeezed from felled old oil palm trunks

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.

Chapter I

Effect of inoculum size on single-cell oil production from glucose and xylose using oleaginous yeast *Lipomyces starkeyi*

I.1. Introduction

Yeasts are recognized as suitable candidates for biotechnological experiments as they have many advantages over other microbial sources. Yeasts are diverse and can grow exponentially while utilizing cheap substrates. The ability of certain yeasts to accumulate lipids in high amounts has been known for years, but only in the past decade the real efforts have been invested in unraveling the underlying biochemical pathways [1]. Microbial lipid compounds, known as single-cell oils (SCOs), have piqued industrial interest due to their particular and precise biochemical and physicochemical properties.

Several yeasts have a unique ability to produce and to accumulate lipids from different carbon sources, and even to utilize various forms of fatty acids present in culture media. Yeasts that accumulate lipids at more than 20% (w/w) of their cell mass are classified as oleaginous [2]. Several oleaginous yeasts are known to accumulate more than 60% (w/w) of lipid on their dry cell mass basis, with similar in composition with vegetable oils [3]. Interestingly, the oleaginous yeast is capable of generating SCOs from a variety of low-cost inedible plant-based feedstock [4,5], and the SCOs produced can serve as a non-crop-based renewable replacement for petroleum-based fuels, chemicals, and power [6].

Among oleaginous yeasts, *Lipomyces starkeyi* has considerable potential as a viable SCO producer due to its ability to produce high amounts of oil from hemicellulose-

derived sugars including xylose [7]. Since hemicellulose may comprise up to a third of plant-derived biomass, using oleaginous yeast to produce SCO as a value-added bioconversion platform could provide additional revenue streams for future biomass-based biorefineries.

Lipomyces starkeyi was first reported in the middle of the last century by Starkey [8] during studies on nitrogen-fixing organisms in soils. Recently, in order to utilize this yeast, several physiological studies have been conducted regarding the growth and SCO production by *L. starkeyi*. The batch-flask cultivation mode with glucose as a carbon source was used by Calvey et al. [9] to accumulate lipids from *L. starkeyi*, and generate a lipid content of 55.0% with 18.3 g/L dry cell biomass. Another study was performed by Gong et al. [10] in a batch flask culture using a cellobiose/xylose co-fermentation strategy to improve the oil accumulation in *L. starkeyi*, and they found that the lipid content and cell mass reached 50% and 26.8 g/L, respectively. Fed-batch strategies have also been used to improve cell biomass and oil production beyond that of traditional batch operations. Anschau et al. [7] used a fed-batch operation with mixtures of glucose and xylose as carbon sources in *L. starkeyi*, that resulted in a lipid content of 46.9% production levels of cell biomass as high as 82 g/L. In fact, despite the availability of a fed-batch strategy that has resulted in higher cell biomass production, an improvement in lipid content is yet needed. From an industrial point of view, an oleaginous yeast with the ability to simultaneously produce a high amount of lipid and cell biomass would not only be significant for the ability to improve of the overall economics associated with microbial lipid production, it also suggests an interesting route for effective cell mass conversion [9-13].

Additionally, one of the difficulties in the commercialization of SCOs production by oleaginous yeast may be due to low productivity [13,14]. In general, the growth of oleaginous yeasts is much slower, compared with that of other ascomycetes such as *Saccharomyces cerevisiae*. In addition, SCOs production by oleaginous yeasts requires a prolonged period to produce maximum yields [15,16]. Therefore, strategies that can overcome these challenges are necessary. Given this background, we focused on the selection of a cultivation strategy for lipid production that could rapidly accumulate lipids with high productivity and high cell biomass simultaneously in typical culture media. The cultivation strategy involves differing the inoculum size in order to increase the microbial growth and initiate the lipid production phase. Although inoculum size is rarely used as a factor in studies of microbial growth, evidence indicates that it may indeed affect microbial growth. Some studies have indicated the importance of inoculum size on the ability of a microbial population to initiate growth [17-19]. Therefore, differing the inoculum size promoted appropriate microbial growth and simultaneously initiated lipid production for highly productive yield.

In this study, the *L. starkeyi* NBRC10381 strain was used as a platform to evaluate cell growth and SCO production in synthetically nitrogen-limited mineral media. The effects of inoculum size on the cell growth and lipid production were investigated using glucose and/or xylose as carbon source.

I.2. Materials and Method

I.2.1 Yeast Strain

The yeast strain *Lipomyces starkeyi* NBRC10381 used in this study was selected from the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan. The yeast strain was preserved in 20% (w/w) glycerol at -80 ^{o}C and was revived by streaking onto a potato dextrose agar (PDA) plate. Afterward, the yeast strain was grown on a yeast extract-malt extract-peptone-glucose (YMPG) agar plate (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L and agar 16.0 g/L).

I.2.2. Seed Preparation

One colony from a YMPG agar plate was inoculated into 12 ml of YMPG broth (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L) in a 100 ml Erlenmeyer flask and incubated in an orbital shaker incubator (BioShaker BR-43FH MR, TAITEC Corp., Japan) at 190 rpm for 24 h at 30 ^{o}C. After incubation, the seed was transferred to 12 ml nitrogen limited mineral media (-NMM) for lipid production.

I.2.3. Media Preparation

The nitrogen limited mineral media (-NMM) contained carbon sources (glucose or xylose or a mixture of both) at 50 g/L from a single carbon source and 100 g/L in a mix (unless mentioned otherwise), nitrogen sources (yeast extract 1.5 g/L and $(NH_4)_2SO_4$ 0.25 g/L), $MgSO_4.7H_2O$ 1.5 g/L, phosphate buffer (KH_2PO_4 7 g/L and $Na_2HPO_4.2H_2O$ 5 g/L), and a trace element solution ($FeSO_4.7H_2O$ 0.08 g/L, $ZnSO_4.7H_2O$ 0.01 g/L, $CaCl_2.2H_2O$ 0.1

g/L, $MnSO_4.4H_2O$ 0.1 g/L, $CuSO_4.5H_2O$ 0.002 g/L, $CoCl_2.6H_2O$ 0.002 g/L). The -NMM A, B, and C differed in terms of the carbon source: -NMM A contained glucose as a sole carbon source (50 g/L), and -NMM B contained a mix of glucose and xylose (50 g/L glucose, 50 g/L xylose), whereas -NMM C contained xylose as a sole carbon source (50 g/L). The initial pH of all -NMM was adjusted to 5.5 with 6 N H_2SO_4. All media were sterilized by passing through a 0.2 µm filter. The initial molar carbon to nitrogen (C/N) ratio in the lipid production media was calculated as the ratio of moles of carbon (C) available in usable carbon sugars (glucose and xylose) to the moles of usable nitrogen (N) available (ammonia and yeast extract). It was assumed the yeast extract was 12% (w/w) carbon and 7% (w/w) nitrogen [12].

I.2.4. Fermentation Condition

The seed volume of the pre-culture was adjusted to certain values of initial optical density (OD_{600nm}), i.e., 0.6-0.8 (lower), 4.0-5.0 (middle), and 16.0-18.0 (higher). The highest value of initial OD_{600nm} was achieved by replacing the preculture medium with fresh YMPG broth medium every 24 h and the pre-culture was then continued until the desired value of the initial OD_{600nm} was reached. Afterward, the seed culture from YMPG broth was transferred to -NMM (A, B, C) media for cultivation. These cultures were incubated in an orbital shaker incubator at 190 rpm, 30 ^oC until all of the carbon sources were completely exhausted. Growth was confirmed by measuring the dry cellular weight (DCW, g/L) of the biomass that was analyzed gravimetrically. Samples for the DCW were taken on the initial day of cultivation and every 24 h thereafter until all of the carbon sources were consumed following inoculation. A one ml aliquot of the culture fluid was transferred into a pre-dried and pre-weighed 1.5 ml reaction tube then centrifuged at

14,000×g for 5 min. The cell pellets were washed with deionized water, dried at 60 °C for 24 h and weighed. The cultures were harvested by centrifugation at 14,000×g for 10 min, the supernatants were decanted, and the pellets were washed twice with sterile deionized water. Cell pellets were stored overnight at -80 ^{o}C, and were then lyophilized by freeze-drying (Freez-one® 4.5L Freeze Dry System Model 7750020, Labconco®, Kansas City, MO) for 48 h. Dry cells were weighed and stored in a desiccator until further use. The cultivation flowchart is presented in Figure 1.

I.2.5. Sugar Analysis

The concentrations of glucose and xylose were analyzed using high-performance liquid chromatography (HPLC) (LC-20AB, refractive index detector RID-10A, Shimadzu, Japan) equipped with an ICSep Coregel-87H column (7.8 mm I.D. x 300 mm Transgenomic) operated at a column temperature of 80 ^{o}C. The mobile phase was 5.0 mM H_2SO_4 at a rate of 0.6 ml/min. All samples were centrifuged to remove the cell mass and other water-insoluble substances and then filtered through a 0.22 µm filter before analysis.

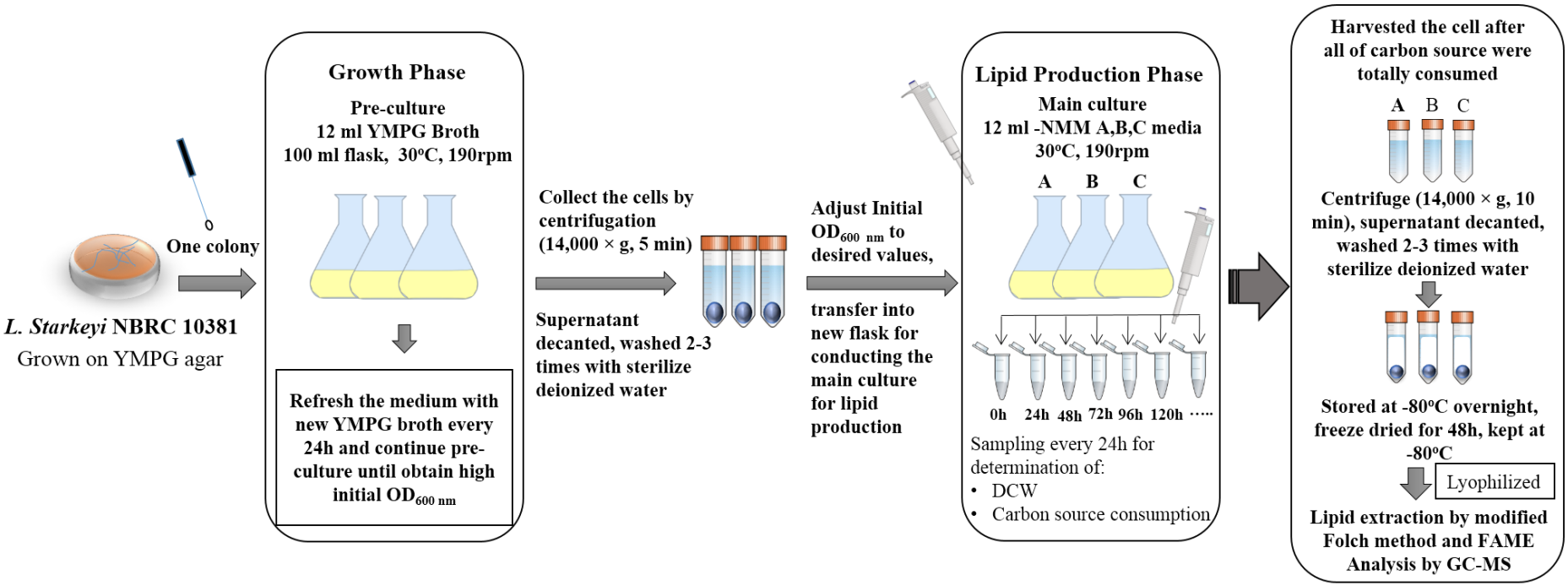

Figure 1. Cultivation flowchart.

I.2.6. Determination of Lipid Content Quantity by Gravimetric Analysis

All chemicals were of analytical grade. Gravimetric analysis was used to measure the SCO production based on the modified Folch method [20], as follows. Triplicate 15 mg samples of freeze-dried cells were transferred to 2.0 ml polypropylene microvial with an O-ring sealed cap containing 0.5 mm zirconia beads and 1.5 ml Folch solvent (2:1 of $CHCl_3$: MeOH, v/v). Cells were pulverized using a Shake Master Neo ver.1.0 (BMS-M10N21, BMS, Tokyo, Japan) at 1,500 rpm for 15 min. Afterward, cells were centrifuged at 14,000×g for 5 min, the supernatant was removed, and the pellets were washed with 1.5 ml deionized water, then pulverized a second time, centrifuged and the remaining water was then removed. The cell pellets were dried at 60 ^{o}C to a constant weight. The lipid content was determined by the weight difference and expressed as a percent of dry cell weight.

I.2.7. BODIPY (493/505) Staining and Visualization

BODIPY or boron dipyrromethene is a class of strong ultraviolet-absorbing molecules that demonstrate a relatively sharp emission peak. This staining method was used to visualize the SCO production. For fluorescence microscopy, a BODIPY stock solution was prepared by adding 0.1 mg/ml with dimethyl sulfoxide (DMSO) [21]. Initially, 10 µl of the culture broth of *L. starkeyi* NBRC10381 grown at different time intervals was pelleted and washed with 0.9% (w/w) phosphate saline buffer (PBS). After washing, the cells were suspended in 10 µl of 0.9% (w/w) PBS to obtain the appropriate cell concentration. Two µl (2.0×10^{-3} µg/µl) of BODIPY stock solution was then added and mixed well. After incubation for 5 min in the dark, the cell suspension was washed with

0.9% (w/w) PBS. Fluorescence images were captured on a digital inverted fluorescence microscope (Biorevo KEYENCE BZ-9000, Tokyo).

I.2.8. Determination and Quantification of Total Fatty Acid Methyl Esters (FAME) by Gas Chromatography

To determine the FAME composition, the oil from lyophilized biomass was derived to methyl esters by direct transesterification following the protocol from a fatty acid methylation kit (Nacalai Tesque, Inc., Japan). The light phase (hexane with methyl esters) was separated, 5-fold diluted, and transferred into GC vials. Chromatographic analysis was performed in a GC-MS QP 2010 Ultra (Shimadzu, Kyoto, Japan) instrument, equipped with a DB-23 capillary column 0.25 mm x 30 m (J&W Scientific). Helium was used as a carrier gas with a flow rate of 0.8 ml/min and with a split ratio of 1:5. The injection temperature was 250 ^{o}C. The primary GC oven temperature was programmed at 50 ^{o}C for 1.0 min, then increased at 25 ^{o}C/min up to 190 ^{o}C, and finally increased at 5 ^{o}C/min up to 235 ^{o}C for 4 min. The temperatures of the ion source and the interface for the MS detector were set 230 and 250 ^{o}C, respectively. SCAN and SIM methods were applied to the MS detector at an injection volume of 1.0 µl. Fatty acids were identified based on the retention times of a SUPELCO™ 37 component FAME mixture (Sigma-Aldrich). Caprylic acid (C8:0) was included in each sample as the internal standard. The percentage of total fatty acid content was calculated as the ratio of the individual FAME peak area to the sum of the all FAME peak areas, excluding the internal standard. The weight of each fatty acid was calculated by multiplying the amount of internal standard (IS) added by the area ratio of the FAME to the internal standard peaks.

I.3. Results and Discussion

I.3.1. Fermentation Profile

Single cell oil (SCO) production by oleaginous yeast could be classified into two steps: the growth phase and the lipid production phase [16]. In this study, after the seed was prepared in YMPG liquid medium, the seed volume was adjusted to the several initial OD_{600nm} to initiate the lipid production phase: 0.6-0.8 (equivalent to 0.5-0.6 DCW g/L), 4.0-5.0 (equivalent to 4.0-7.0 DCW g/L) and 16.0-18.0 (equivalent to 10.0-14.0 DCW g/L). Afterward, the YMPG broth was transferred to the medium -NMM A, -NMM B, and -NMM C, for the lipid production phase.

Figure 2 shows the time courses for the carbon source consumption and the cell growth of the NBRC10381 strain during the cultivation in -NMM A, -NMM B, and -NMM C media at different initial levels of OD_{600nm}. For fermentation with a lower inoculum size (initial OD_{600nm} 0.6-0.8), it was clear that the NBRC10381 strain required more than 5 days to completely consume the glucose when it was used as a sole carbon source (-NMM A). A similar pattern was also observed when xylose was used as a sole carbon source in the medium -NMM C. When the inoculum size increased to the middle (4.0-5.0) or higher (16.0-18.0) levels, the fermentation times were reduced significantly to 3-4 days. This result suggested that the sugar consumption rate over the entire cultivation process was significantly improved by an increase in the initial OD_{600nm}. Kun [22] highlighted the significant effect that inoculum size exerted on the fermentation and found that high inoculum sizes could dilute the rate-limiting substrate concentration in the medium. Otherwise, low inoculum sizes resulted in a prolonged lag phase.

Xylose consumption seemed weak in fermentation medium containing a mixture of glucose and xylose (-NMM B), which led to an incomplete consumption of xylose in the late stages of the cultivation (some residual amounts remained that were not consumed during fermentation) when conducted at a lower inoculum size. Many reports have chronicled the inhibitory effects of high substrate concentrations on the growth of oleaginous microbes due to high osmotic stress [23]. This study returned similar effects in the cultivation of NBRC10381 when the growth was performed at a lower inoculum size in the medium with a mixture of glucose and xylose. When the inoculum size was set to the initial OD_{600nm} 4.0-5.0, however, the xylose consumption was significantly increased while glucose in the medium dropped to a concentration that ranged from 20 to 30 g/L. Microorganisms are known to generally metabolize sugars sequentially when exposed to a mixture of glucose and xylose because glucose can repress the utilization of other sugars via either a catabolic repression mechanism or allosteric competition for sugar transporters [24].

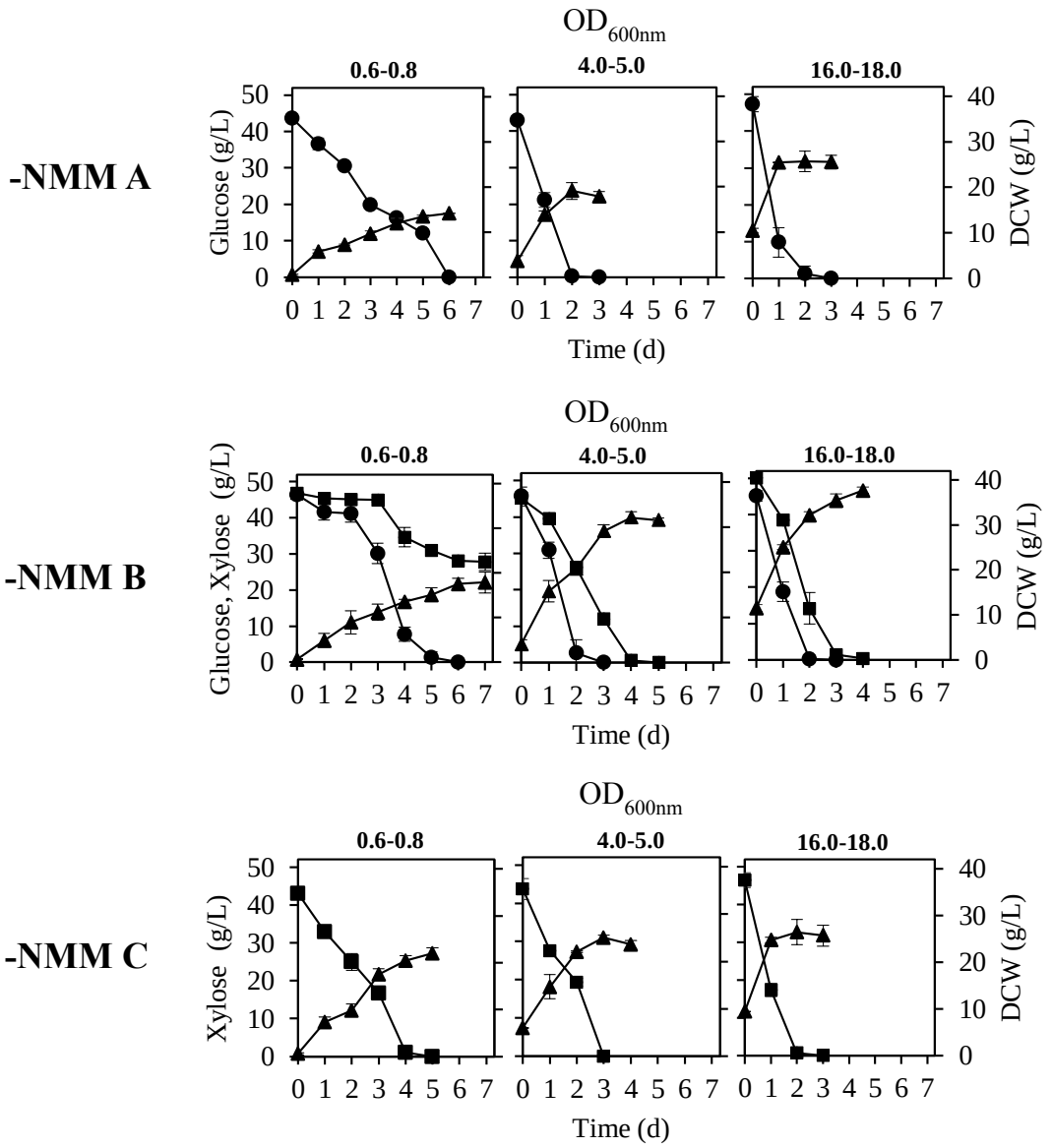

Figure 2. Time course for carbon source consumption and cell growth under different levels of initial OD_{600nm}. The closed circle represents glucose consumption, the closed square refers to xylose consumption, and the closed triangle charts for the cell growth. -NMM A: medium with glucose as sole carbon source; -NMM B: medium with glucose and xylose as mix carbon source; and -NMM C: medium with xylose as the sole carbon source.

Table 1 shows the initial (q_i) and total (q_t) carbon source consumption rate at each level of inoculum concentration for different carbon sources. The initial carbon source consumption rate (q_i) was determined during the first 24 h of the cultivation process, which provided insight into the interaction of assimilation for each carbon source by the NBRC10381 strain before one of them was exhausted. On the other hand, the total carbon source consumption rate (q_t) was determined when the carbon sources were completely consumed. In the single carbon source (-NMM A or -NMM C), the q_i and q_t were achieved almost similar at each level of inoculum size. When the inoculum size increased, an increases in q_i and q_t were also observed. However, the q_t values for glucose and xylose in higher inoculum sizes (16.0-18.0) were similar to those of the middle inoculum sizes (4.0-5.0). This was probably due to the strain under both cultivation conditions required the same amount of total cultivation time to completely consume the carbon sources.

Table 1. Carbon source consumption rate by NBRC10381 cultivated on the -NMM medium at different inoculum size.[a]

Initial OD_{600nm}	Glucose and/or xylose consumption rate (q)								Specific carbon source consumption rate (qs)					
	-NMM A		-NMM B				-NMM C		-NMM A		-NMM B		-NMM C	
	Glucose		Glucose		Xylose		Xylose							
	q_i	q_t	q_i	q_t	q_i	q_t	q_i	q_t	qs_i	qs_t	qs_i	qs_t	qs_i	qs_t
0.6-0.8	0.3±0.1	0.3±0.1	0.2±0.0	0.3±0.1	0.1±0.0	0.1±0.0	0.3±0.1	0.4±0.1	0.051±0.001	0.022±0.001	0.053±0.001	0.021±0.001	0.047±0.001	0.018±0.001
4.0-5.0	0.9±0.1	0.6±0.1	0.6±0.0	0.6±0.0	0.2±0.1	0.5±0.1	0.7±0.1	0.6±0.1	0.069±0.001	0.042±0.001	0.066±0.000	0.034±0.000	0.070±0.001	0.032±0.001
16.0-18.0	1.6±0.1	0.7±0.1	1.1±0.1	0.6±0.0	0.5±0.1	0.5±0.1	1.4±0.1	0.7±0.1	0.098±0.001	0.041±0.001	0.120±0.001	0.042±0.000	0.083±0.001	0.045±0.001

[a]The values are given as mean ± SD of triplicate determinations.

q, carbon source consumption rate (g-substrate/L.h); q_i, carbon source consumption rate during the first 24 h; q_t, carbon source consumption rate over the whole fermentation process; qs, specific sugar consumption rate (g-substrate/g-biomass.h); qs_i, specific sugar consumption rate during the first 24 h; qs_t, specific sugar consumption rate over the whole fermentation process.

In addition, as shown in Table 1, when glucose and xylose were used as a mixed carbon source (-NMM B), the initial carbon source consumption rate (q_i) data indicated that glucose was consumed at a higher rate in the first 24 h. Nevertheless, when the inoculum size was increased, the total carbon source consumption rate (q_t) of a mixture of glucose and xylose was achieved at a similar rate of 0.6 and 0.5 g-substrate/L.h respectively. This condition indicated that both sugars were assimilated simultaneously rather than sequentially. The simultaneous utilization of glucose and xylose is an important process in the use of lignocellulosic biomass to reduce the cost associated with SCO production. Liu et al. [25], studied the inoculum size of *L. starkeyi* and found that the microbial oil production is profoundly related to this parameter.

Accordingly, increasing the initial inoculum size (initial OD_{600nm}) will lead to an increase in the active cell fraction, whereas a higher number of active cell fractions will increase the probability that one or more cells in the population is able to grow [19]. Consequently, this condition leads to a higher cell/carbon source ratio and to an increase in the carbon source consumption rate. Moreover, a higher specific carbon source consumption rate (*qs*) indicates that the NBRC10381 strain is more efficient in converting a carbon source to cell biomass. From an industrial point of view, a higher specific carbon source consumption rate reflects the potential economic benefit of lower capital and operating costs from reduced fermenter capacity [26]. In addition, high inoculum sizes significantly increase the cell mass productivity (Table 2).

Figure 3 shows the profiles of the growth rates throughout the cultivation for different inoculum sizes. The NBRC10381 strain in -NMM A, B, and C, media at inoculum sizes of 0.6-0.8 achieved rates of approximately 0.1-0.2 g/L.h. The rates were

increased by an increase in the initial $OD600_{nm}$ to around 0.2-0.4 g/L.h at inoculum sizes of 4.0-5.0 and to around 0.4-0.6 g/L.h at inoculum sizes of 16.0-18.0. These results confirmed that the growth rate of the strain could be significantly increased by an increase in the initial OD_{600nm}.

To further elucidate the efficiency of carbon source utilization in the media containing different carbon sources, biomass production by the NBRC10381 strain cultured at an initial OD_{600nm} of 16.0-18.0 with a total carbon source at 100 g/L was evaluated, and the results are shown in Table 2. Biomass productions by cultivation in different carbon sources achieved similar results. For instance, the DCW and biomass yields were 40.8 g/L and 43.0 g-cells/g-substrate for cultivation in the medium with a mixture of carbon sources (-NMM B). However, the inhibitory effect of the presence of a highly concentrated carbon source in the medium was not observed (Table 2). These results confirmed that the strain is capable of biomass production from glucose and/or xylose even in high concentrations.

Table 2. Biomass and lipid production of NBRC10381 grown on different inoculum size and carbon source.[*]

Carbon source	Initial lipid content (%)[a]	Initial DCW (g/L)[b] (x_0)	Cult. time (day)[c]	Final DCW (g/L)[d] (x_f)	DCW yield (w/w, %)[e]	Final lipid content (%)[f] (L_c)	Lipid (g/L)[g] (P_L)	Lipid yield (w/w, %)[h] (Y_L)
Initial OD600nm = 0.6-0.8	11.5±1.9							
Glucose, 50 g/L		0.6±0.2	6	21.6±1.5	49.5±1.1	71.5±0.2	14.8±0.1	29.7±0.4
Glucose, 50 g/L; Xylose, 50 g/L		0.7±0.1	7	20.5±0.2	32.1±0.1	73.0±0.2	14.3±0.2	14.3±1.1
Xylose, 50 g/L		0.6±0.2	5	19.1±0.2	44.3±1.2	82.4±1.1	15.1±0.4	30.3±0.8
Initial OD600nm = 4.0-5.0	38.5±0.4							
Glucose, 50 g/L		5.4±1.3	3	25.0±0.6	58.1±0.8	74.8±1.5	13.3±0.6	26.6±1.4
Glucose, 50 g/L; Xylose, 50 g /L		5.1±1.0	5	31.6±0.5	34.8±2.1	84.6±1.2	21.6±0.6	21.6±0.4
Xylose, 50 g/L		5.9±0.1	3	21.9±1.1	50.0±1.6	86.6±1.9	13.1±0.1	26.1±1.5
Initial OD600nm =16.0-18.0	43.4±3.0							
Glucose, 50 g/L		10.5±0.5	3	30.3±1.5	64.1±2.2	79.6±1.2	13.6±0.2	27.2±2.0
Glucose, 50 g/L; Xylose, 50 g/L		11.6±0.8	4	38.2±0.5	41.3±1.8	83.6±1.9	20.3±0.3	20.3±0.9
Xylose, 50 g/L		10.5±0.1	3	28.7±2.3	61.3±1.6	85.1±0.7	13.9±0.6	27.8±0.5
With the same total carbon sources[i]	44.2±1.8							
Glucose, 100g /L		13.4±0.8	3	40.0±0.3	41.6±0.1	83.8±2.0	20.1±0.2	20.1±1.4
Glucose, 50 g/L; Xylose, 50 g/L		13.6±0.7	4	40.8±1.8	43.0±1.4	84.9±3.5	21.0±0.6	21.0±0.6
Xylose, 100 g/L		13.9±1.3	3	40.3±1.6	41.5±0.4	86.9±3.0	21.8±1.1	21.8±0.6

[*]The values are given as mean ± SD of tripicate determination.

[a]Initial lipid content produced in growth phase during preculture

[b]Initial dry cellular biomass DCW (g/L) for cultivated in lipid production phase (x_0)

[c]Cultivation time when the carbon sources were completely exhausted in lipid production phase

[d]Final cellular biomass at the end of cultivation time (x_f).

[e]Gram of dry cell biomass per gram of sugar consumed × 100%.

[f]Final lipid accumulation from the growth phase to the lipid production phase, determinaed by gravimetric analysis (L_c).

[g]Lipid production (P_L) = (($x_f - x_0$) – (x_f × ((100 – L_c) / 100))).

[h]Lipid yield (Y_L) = (P_L / total sugar consumed) × 100%.

[i]Additional experiment with the same total carbon source (100 g/L).

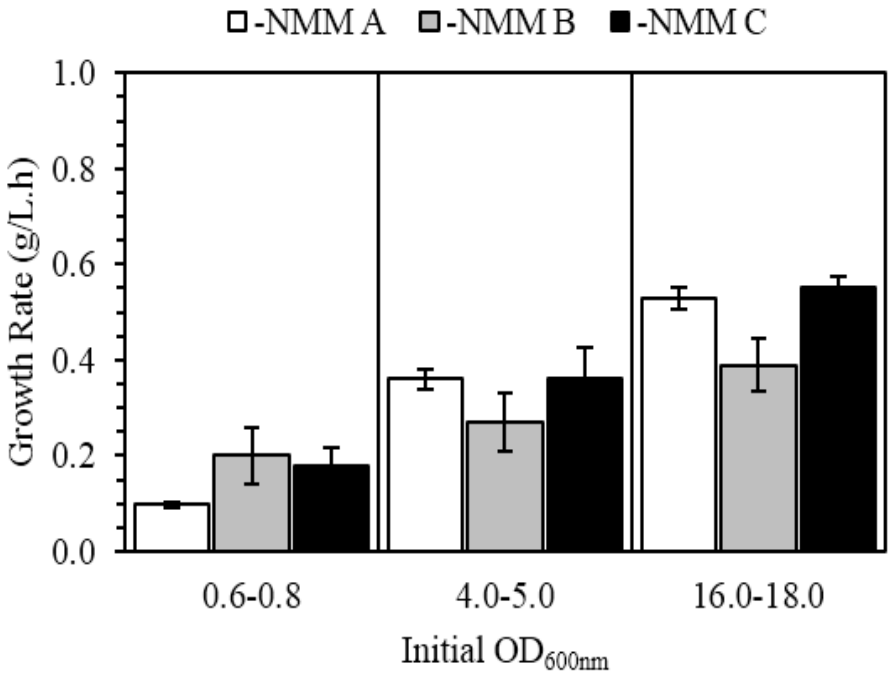

Figure 3. Specific growth rate comparison of different inoculum sizes. The cell growth rate was determined throughout the entire cultivation process. White bars represent -NMM A; medium, gray bars for -NMM B medium, and black bars for -NMM C medium.

I.3.2. SCO Production and Fatty Acids Profile of Intracellular Lipid

The SCO production of a NBRC10381 strain growing on -NMM A and -NMM C generally reached more than 70% (w/w) of dry cell weight, with no significant differences in lipid content observed in the medium with a mixed carbon sources (-NMM B). However, the lipid yield obtained from the cultivation in the media with a single carbon source (-NMM A and -NMM C) was slightly higher when compared with that containing a mixed carbon source (-NMM B), since the total carbon source concentration in the -NMM B medium was twice that of either -NMM A or -NMM C. To confirm the influence that the total concentration of a carbon source exerts the biomass and lipid

production of the strain, an additional experiment was conducted in which the concentrations of either glucose or xylose as the sole carbon source were increased to 100 g/L from an initial OD_{600nm} of 16.0-18.0 in each medium: -NMM A, and -NMM C (Table 2).

Based on the results, when the totals of the carbon sources were increased, increases in the cell biomass and in SCO production were observed. On the contrary, the lipid yield was decreased with increases in the total carbon source, since the lipid yield was determining by dividing the lipid production by the total level of carbon source consumption. Increasing the total carbon source could also increase the molar C/N ratio. For instance, the medium that contained 50 g/L of either glucose or xylose as a sole carbon source showed a C/N ratio of 128. When the total carbon source was raised to 100 g/L (glucose and/or xylose), the rise produced a molar C/N ratio of 254. According to the data shown in Table 2, a higher molar C/N ratio provides excess sugar for a continuation of lipid accumulation instead of the cell growth.

Moreover, the data show that the lipid content from the conversion of xylose as a sole carbon source was significantly higher (at $p<0.05$) than that of glucose at each level of inoculum size. However, the lipid production and the lipid yield data in case when xylose was the sole carbon source, were neither statistically nor significantly higher than that of glucose (Table 2). Evans and Ratledge [27] reported that xylose was a better substrate for lipid production. Additionally, Ratledge [28] has suggested that xylose might be more efficient than glucose in terms of lipid yield, theoretically. This could be because xylose can be exclusively metabolized by the pentose phosphoketolase pathway beside the pentose phosphate pathway [29]. This further reinforces the contention that

xylose utilization during lipid accumulation is a key for generating higher lipid production [6]. Based on Table 2, the biomass and lipid content was increased when the inoculum size was increased to a higher level. These results indicated that the inoculum size had a significant effect on the lipid biosynthesis and cell mass concentration. In terms of further scale-up and industrial production, the results obtained with an initial OD_{600nm} of 16.0-18.0 were markedly more attractive due to high lipid yield (~27%, g-lipid/g-substrate).

Table 3 shows the fatty acid profiles of a NBRC10381 strain cultured on all types of media at different initial levels of OD_{600nm}. The major fatty acids produced in lipids were oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and linoleic acid (C18:2), which is similar to the production of vegetable oils [5]. Oleic acid was accounted for about 50% of the total fatty acids for the NBRC10381 strain. A high amount of oleic acid may provide additional value for the SCOs produced from the NBRC10381 strain. Recently, Tanimura et al., [30] investigated several oleaginous yeasts for practical biodiesel production and found that *L. starkeyi* NBRC10381 could produce an exorbitant amount of oleic acid (around 75%) from a medium with 3% glucose as the sole carbon source. Moreover, there has been considerable interest in high oleic oils for biofuels with improved oxidative stability and cold-flow properties, and as an input for many renewable polymers, lubricants, elastomers, and other oleochemicals [31-33]. In addition, when xylose was used as a sole carbon source at different inoculum sizes, the strain showed a slightly higher composition of palmitic acid. Palmitoleic acid content was also increased when mixed glucose and xylose substrates were used as a carbon source. Hence, the NBRC10381 strain efficiently utilized glucose, xylose and their mixtures.

Table 3. FAMEs profile of NBRC10381 grown in different inoculum size and carbon source.[*]

Carbon source	FAME compositions (%)					
	C16:0	C16:1	C18:0	C18:1(cis)	C18:2 (cis)	Trace element[a]
Initial OD_{600nm} = 0.6-0.8						
Glucose, 50 g/L	26.0±0.1	4.0±0.2	9.0±0.53	27.5±1.4	2.0±0.0	3.4
Glucose, 50 g/L; Xylose, 50 g/L	27.4±0.2	4.0±0.1	9.5±0.58	29.0±1.3	2.1±0.1	3.6
Xylose, 50g/L	30.6±0.5	2.6±0.4	8.3±0.02	37.8±1.2	2.8±0.1	2.8
Initial OD_{600nm} = 4.0-5.0						
Glucose, 50 g/L	27.7±0.1	1.8±0.1	7.9±0.3	30.2±0.1	1.6±0.1	3.2
Glucose, 50 g/L; Xylose, 50 g/L	34.3±0.1	2.9±0.0	8.4±0.3	39.3±0.5	1.4±0.0	3.5
Xylose, 50g/L	35.4±0.9	2.6±0.2	9.0±0.0	29.0±0.8	1.6±0.1	2.5
Initial OD_{600nm} =16.0-18.0						
Glucose, 50 g/L	32.0±0.3	3.3±0.3	9.3±0.3	37.7±0.8	1.9±0.1	3.8
Glucose, 50 g/L; Xylose, 50 g/L	31.1±0.7	4.6±0.1	8.8±0.6	37.3±0.3	1.1±0.4	3.6
Xylose, 50 g/L	35.1±0.3	2.2±0.0	10.5±0.3	30.2±0.9	1.4±0.1	4.1

[*]The values are given as mean ± SD of triplicate determination.

[a]The percentage of individual FAMEs below 1% were categorized as trace elements,

trace element consists of C14:0, C15:0, C15:1, C17:0, C17:1, C18:3 (n-3), C20:0, C21:0, C22:0, C23:0, C24:0.

Fluorescence microscopy and spectroscopy methods have recently been introduced for the quantification and visualization of lipid classes. There are several types of dyes used for the same purposes. Nile Red is a lipophilic stain solution that is used to quantify the neutral lipid accumulation within cells by fluorescence spectroscopy [34,35]. Sudan black B is also used as a staining dye to detect the lipid in the microorganisms, but it cannot distinguish between the lipid classes. To overcome this problem, this study used BODIPY 493/505 for the cell staining to visualize the SCOs that had accumulated in the form of triacylglycerols (TAGs). BODIPY 493/505 has another important characteristic that allows it to easily stain different sizes of lipid droplets in yeast cells, showing bright green culture without pretreatment with an uptake of dye that is affected by neither the cell wall composition nor its structure. This staining method was used to visualize the accumulation of triglycerides occurring in lipid bodies stored inside the cells of the NBRC10381strain, which had been grown in different time intervals. In this study, this staining method was applied to visualize lipid bodies of a moderate inoculum size (initial OD_{600nm} 4.0-5.0) in the manner of mixed glucose and xylose substrates having been used as a carbon source. The lipid droplets shown in Fig. 4 had accumulated in the cells of the NBRC10381 strain following aerobic cultivation. The figure shows a timely increase in the lipid bodies from 0 to 4 d after cultivation in the medium during the lipid production phase. Overall, the stained results confirmed that the SCO production of the investigated strain reached a lipid accumulation of more than 80% (w/w) after 4 d of cultivation.

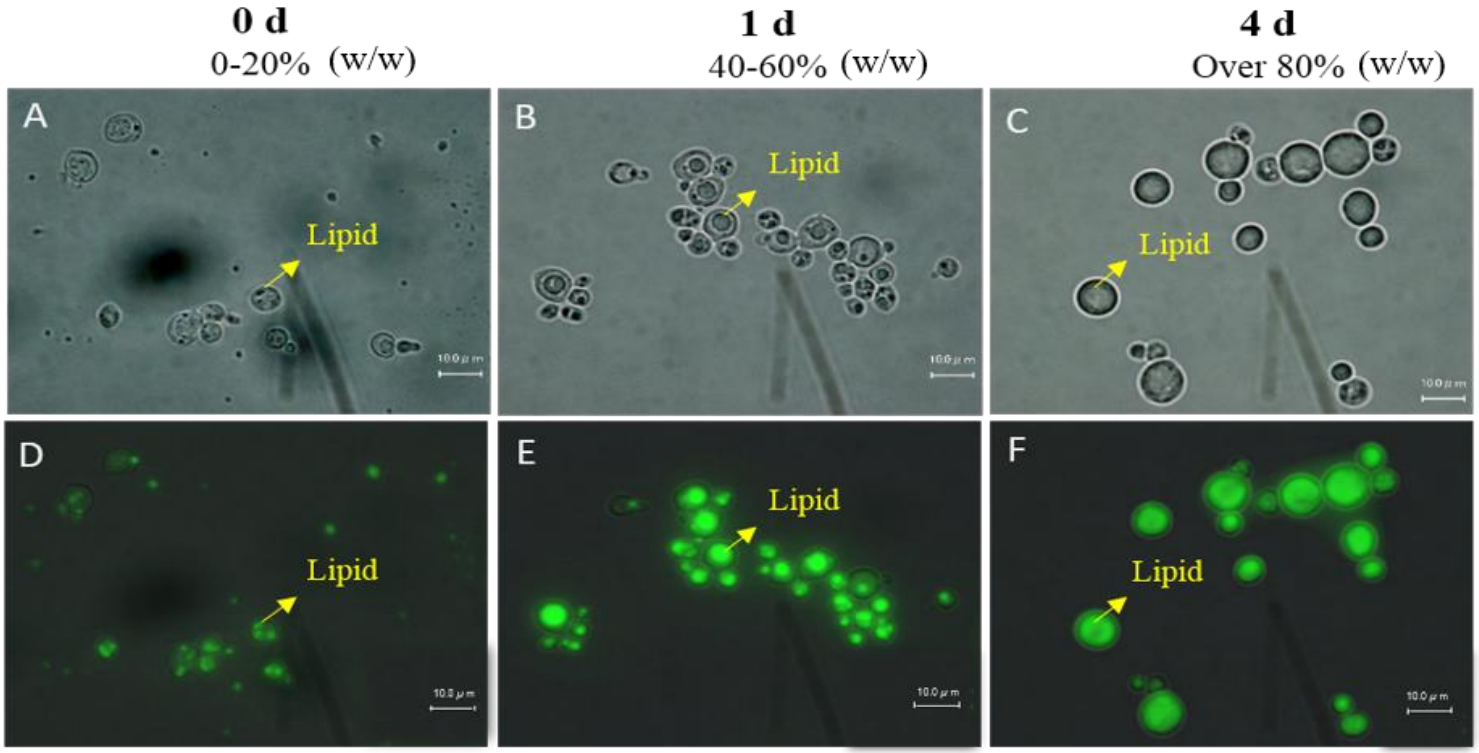

Figure 4. Lipid droplets accumulated in the cells of the NBRC10381 strain following aerobic cultivation. The cells were taken from aerobic cultivation in -NMM B medium at an initial OD of 4.0-5.0 and the lipid droplets were stained with BODIPY (493/505). The alphabetic letters A, B, and C show the microscopic images of *L. starkeyi* NBRC10381 after cultivated in -NMM B medium for 0 d, 1 d, and 4 d, respectively. D, E, and F: microscopic fluorescence image stained by BODIPY (493/505) after cultivated in -NMM B medium for 0 d, lipid content 0-20% (w/w); 1 d, lipid content 40-60% (w/w); and 4 d, lipid content of more than 80% (w/w), respectively. The length of the scale bar for each microscopic image is 10 µm.

Different substrates have been employed to maximize the production of SCOs from oleaginous yeasts. Some representative results are summarized in Table 4. When simple and continuous batch cultures were used to produce SCOs, the biomass yield was usually much lower compared with fed-batch cultivation due to the limited availability of substrates [7,36-38]. The intracellular lipid content is another important aspect in lipid production. Fewer examples have reached lipid content higher than 55% (w/w) [9,10,39]. When xylose was used as a feedstock in continuous batch cultivation, biomass and lipid contents of 22.3 g/L and 57% (w/w), respectively, were achieved [10]. In this study, a higher level of cell biomass and lipid production (as ~ 38 g/L and ~ 85% (w/w), respectively) were obtained using batch flask cultivation. This result was apparently higher than those using the batch and continuous batch cultivations as listed in Table 4. Therefore, this study has provided the first data for the simultaneous achievement of higher cell mass and lipid production using glucose and/or xylose as the carbon sources in different inoculum sizes.

Table 4. Comparison of lipid production from yeasts cultured in the various substrates under batch and continuous flask cultivation.

Yeast strain	Type of carbon source	DCW (g/L)	Lipid content (%)	Y_{lipid} (w/w)	P_{lipid} (g/L)	Ref.
Lipomyces starkeyi	Glucose	18.3	55.0	17.0	10.1	[9]
Lipomyces starkeyi	Glucose and Monosodium glutamate waste water	4.6	24.7	NA	1.1	[25]
Lipomyces starkeyi	Sucrose+Glucose+Fructose	12.3	47.3	NA	5.8	[38]
	Sweet sorghum stalks juice	21.7	29.5	NA	6.4	
Cryptococcus vishniaccii	Paper mill sludge extract	14.6	53.4	NA	7.8	[39]
	Glucose	13.6	40.4	NA	5.5	
Cryptococcus curvatus	Glucose+Xylose	30.6	39.8	17.5	12.2	[37]
	Xylose+Cellobiose	29.8	39.4	16.7	11.7	
	Cellobiose+Glucose	34.3	39.3	18.4	13.5	
Lipomyces starkeyi	Cellobiose	27.9	50.0	20.0	14.0	[10]
	Glucose[a]	23.8	53.0	18.0	12.6	
	Xylose[a]	22.3	57.0	18.0	12.7	
	Cellobiose+Xylose	26.8	50.0	19.0	13.4	
	Glucose+Xylose[a]	23.6	54.0	18.0	12.7	
	Cellobiose+Xylose+Glucose	25.5	52.0	20.0	13.3	
Yarrowia lipolytica	Glycerol or Glucose[a]	8.1	14.5	NA	1.2	[36]
		15.5	10.9	NA	1.7	
Lipomyces starkeyi NBRC10381	Glucose	30.3	79.6	27.2	13.6	This study
	Glucose+Xylose	38.2	83.6	20.3	20.3	
	Xylose	28.7	85.1	27.8	13.9	

DCW, dry cell weight; $Y_{lipid,}$ lipid yield; $P_{lipid,}$ lipid production; NA, not available

[a]Continuous batch cultivation

I.4. Conclusions

This work suggests the plausibility of using the NBRC10381 strain for the production of cell mass that is rich in lipids using glucose and/or xylose as the carbon source in higher inoculum sizes. The identification of microorganisms that can utilize both glucose and xylose simultaneously and efficiently appears to be a key aspect in the utilization of a lignocellulosic feedstock, and elucidation of the mechanism underlying the assimilation of these sugars is crucial for the further development of this industrial process and for the building of a suitable yeast platform for lipid production.

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Chapter II

Selection of oleaginous yeasts capable of high lipid accumulation during challenges from inhibitory chemical compounds

II.1. Introduction

As suitable candidates for biotechnological experiments, yeasts have many advantages over other microbial sources. Compared with filamentous fungi and microalgae, yeasts have a shorter duplication time and higher growth rates. In lipid production, yeasts have a higher lipid content than microalgae and their cultivation is easier to scale up [1]. In the yeast fermentation process, bacterial contamination can be controlled by conditions of low pH growth. Yeasts have the ability to utilize various types of carbon sources for the production of biomass and lipids [2]. Yeasts that accumulate lipids at more than 20% (w/w) of their cell mass are classified as oleaginous [3].

Lipids produced from oleaginous yeasts are considered potential feedstock for biodiesel production since the composition of their fatty acids is similar to that of vegetable oils [4]. Microbial lipids are not yet considered promising alternatives for biodiesel production due to their high processing cost. The search for high lipid-producing microbes using low-cost materials and an improved production process, however, may result in a reduction in overall production cost. The other advantage of oleaginous yeasts is their ability to produce lipids from low-cost biomass, including lignocellulosic biomass [5].

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 [6]. 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 [6,7]. Estimating the varieties of ICCs is difficult, because the species of byproducts released depends not only on the type of biomass processed but also on the type of pretreatment process implemented. 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 [8]. 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 [9,10]. Methods used to solve the inhibitory effects of ICCs have included chemical, biological and physical approaches [11]. However, the above-mentioned approaches to inhibitor removal complicates the production process, which increases the cost. The deleterious effect of ICC on cell is considered the most immediate technical barrier for lipid production from lignocellulose [12]. 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.

Typical oleaginous yeasts are distributed in the genera *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodosporodium*, *Rhodotorula*, *Trichosporon*, and *Yarrowia* [13]. Many studies have utilized mixed carbon sources derived from lignocellulosic biomass for lipid production using the various oleaginous yeasts mentioned above. Oleaginous yeasts from

the genus *Lipomyces* tend to accumulate lipids from various carbon sources [14]. Most microorganisms normally metabolize sugars sequentially. The microorganisms usually do not consume other sugars until the preferred sugar (glucose) is completely consumed [15]. Oleaginous yeasts sequentially metabolize sugar, which increases the fermentation time and reduces the efficiency of lipid production [5]. The simultaneous utilization of glucose and other sugars, however, has been reported in many species [3,5].

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 [16-18]. 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 [6,9,19].

The present study used a two-tiered selection process to examine eight yeast species from the genus *Lipomyces* to select strains with superior lipid productivity. In the primary selection process, qualities such as the carbon source consumption rate, growth rate, lipid accumulation ability, and fatty acid composition of yeast strains were evaluated in synthetically limited nitrogen mineral medium (-NMM) containing a mixture of glucose and xylose as a carbon source. In the secondary stage, screening for inhibitory chemical tolerance was initiated for the strains that had demonstrated the highest levels of lipid content, cell mass production, and rates of carbon source consumption in the primary selection. For the secondary stage, the -NMM medium was supplemented with ICC in a cocktail mixture made up of seven typical major ICCs in lignocellulose hydrolysate. The composition and concentration of the ICC used in this study were similar to those released in real biomass hydrolysate following treatment with hot water. Inhibitor

tolerance was evaluated based on the rates of the complete depletion of ICCs in the cultivation medium.

II.2. Materials and Method

II.2.1. Yeast Strains and Seed Preparation

The eight *Lipomyces* yeast strains (*Lipomyces doorenjongii* NBRC107655, *Lipomyces mesembrius* NBRC107654, *Lipomyces starkeyi* NBRC10381, *Lipomyces kononenkoae* NBRC107661, *Lipomyces orientalis* NBRC107659, *Lipomyces kockii* NBRC107656, *Lipomyces yamadae* NBRC107657, *Lipomyces yarrowii* NBRC107658) used in this study were selected from the NITE Biological Resource Center (NBRC), at the National Institute of Technology and Evaluation, Japan, based on the capability to grow in real biomass hydrolysate. The yeast strains were preserved in 20% (w/w) glycerol at -80 ^{o}C and revived via streaking onto a potato dextrose agar (PDA) plate and subsequently incubated for 3 days. Afterward, the yeast strains were grown on a yeast extract-malt extract-peptone-glucose (YMPG) agar plate (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L, and agar 16.0 g/L) and incubated for 2 days at 30 ^{o}C.

II.2.1.1. Seed Preparation

One colony from a YMPG agar plate was inoculated into 12 ml of YMPG broth (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L) in a 100 ml Erlenmeyer flask and incubated in an orbital shaker incubator (BioShaker BR-43FH MR, TAITEC, Corp., Japan) at 190 rpm for 24 h at 30 ^{o}C for pre-culture (growth phase). After incubation, the seed was transferred to either a 12 ml nitrogen-limited mineral medium (-

NMM) or to a -NMM + ICC medium for lipid production in the primary and secondary selection processes, respectively.

II.2.1.2. Media Preparation

The nitrogen-limited mineral medium (-NMM) contained a mixture of glucose and xylose as a carbon source (50 g/L glucose + 50 g/L xylose), nitrogen sources (yeast extract 1.5 g/L and $(NH_4)_2SO_4$ 0.25 g/L), $MgSO_4.7H_2O$ 1.5 g/L, phosphate buffer (KH_2PO_4 7 g/L and $Na_2HPO_4.2H_2O$ 5 g/L), and a trace element solution ($FeSO_4.7H_2O$ 0.08 g/L, $ZnSO_4.7H_2O$ 0.01 g/L, $CaCl_2.2H_2O$ 0.1 g/L, $MnSO_4.4H_2O$ 0.1 g/L, $CuSO_4.5H_2O$ 0.002 g/L, $CoCl_2.6H_2O$ 0.002 g/L). To investigate lipid production in the presence of a mixture of inhibitory compounds, the -NMM medium was supplemented with ICCs in a cocktail mixture. The ICCs consisted of 5-hydroxymethylfurfural (5-HMF) 2.0 mM, furfural 12.0 mM, acetic acid 12.0 mM, formic acid 6.0 mM, levulinic acid 1.0 mM, vanillin 0.12 mM, and syringaldehyde 0.08 mM. The ICC concentrations were designed to mimic the inhibitory chemical compounds in real biomass hydrolysate released following treatment with hot water. The initial pH of the -NMM medium was adjusted to 5.5 using 6 N H_2SO_4 and sterilized by passage through a 0.2 µm filter.

II.2.1.3. Cultivation Conditions

The seed volume of the pre-culture for each strain in YMPG broth was adjusted to an initial optical density ($OD_{600\ nm}$) of 14.0-16.0 in order to obtain an appropriate active cell fraction that increased the capability of cells to utilize carbon sources and detoxified ICCs. Afterward, the seed culture from the YMPG broth was transferred to -NMM (primary selection) or -NMM+ICC (secondary selection) medium for cultivation with a 12 ml

working volume. These cultures were incubated in an orbital shaker incubator that was operated at 190 rpm and 30 ^{o}C until all of the carbon sources were completely exhausted. Growth was confirmed by measuring the dry cell weight (DCW, g/L) of biomass that was analyzed gravimetrically. Following inoculation, samples for the measuring of DCW were taken on the initial day of cultivation and every 24 h thereafter until all the carbon sources were consumed. For dry cell weight determination, a 1 ml sample of fermentation broth was transferred into a pre-dried and pre-weighed 1.5 ml reaction tube then centrifuged at 14,000 ×g for 5 min. The cell pellets were washed with deionized water, dried at 60 °C for 24 h, and weighed. When they were fermented, the yeast cells were harvested by centrifugation at 14,000 ×g for 10 min, the supernatants were decanted, and each pellet was washed twice with sterile deionized water. Afterwards, Cell pellets were stored overnight at -80 ^{o}C, and were then lyophilized by freeze drying (Freez-one® 4.5L Freeze Dry System Model 7750020, Labconco®, Kansas City, MO) for 48 h. The lyophilized cells were stored in a desiccator until further use for total lipid quantity and fatty acid analysis.

II.2.2. Analysis of Sugars and Inhibitors

The concentrations of glucose and xylose, and those of inhibitors 5-HMF, furfural, acetic acid, formic acid, and levulinic acid were analyzed using high-performance liquid chromatography (HPLC) (LC-20AB, refractive index detector RID-10A, Shimadzu, Japan) equipped with an ICSep Coregel-87Hcolumn (7.8 mm I.D. × 300 mm Transgenomic, USA) operated at a column temperature of 80 ^{o}C. The mobile phase added 5.0 mM H_2SO_4 at a rate of 0.6 ml/min. All samples were centrifuged to remove the cell

mass and other water-insoluble substances and then filtered through a 0.22 µm filter before analysis.

The concentrations of vanillin and syringaldehyde were analyzed using a gas chromatography/mass spectrometry (GC-MS) QP 2010 Ultra (Shimadzu, Kyoto, Japan) instrument equipped with a CP-SIL 8-CB capillary column 0.25 mm x 30 m (Agilent, J&W Scientific). The flow rate of helium gas through the column was 1.69 ml/min. The column temperature was held at 50 ^{o}C for 1 min isothermally, before being increased by 20 ^{o}C/min to 280 ^{o}C where it was maintained for 1 min. The interface- and ion-source temperatures were 250 and 230 ^{o}C, respectively. Ions were generated using a 70 V electron impact and ion fragments were detected in the selected ion-monitoring mode. The GC-analysis standard for vanillin was purchased from Nacalai Tesque (Kyoto, Japan) that for syringaldehyde was purchased from Tokyo Chemical Industry (Tokyo, Japan), and the purity of both phenolic aldehydes standards was 98.0%.

II.2.3. Determination of Total Lipid Quantity by Gravimetric Analysis

All chemicals were of analytical grade. Gravimetric analysis was used to measure the lipid production based on the modified Folch method [20], as follows. Triplicate 15 mg samples of freeze-dried cells were transferred to a 2.0 ml polypropylene microvial with an O-ring sealed cap containing 0.5 mm zirconia beads and 1.5 ml of Folch solvent (2:1 of $CHCl_3$: MeOH, v/v). Cells were pulverized using a Shake Master Neo ver.1.0 (BMS-M10N21, BMS, Tokyo, Japan) at 1,500 rpm for 15 min. Afterward, cells were centrifuged at 14,000×g for 5 min, the supernatant was removed, and the pellets were washed with 1.5 ml of deionized water, then pulverized a second time, centrifuged, and the remaining water was then removed. The cell pellets were dried at 60 ^{o}C to a constant weight. The

lipid content was determined by the weight difference and expressed as a percent of dry cell weight.

II.2.4. Determination and Quantification of Total Fatty Acid Methyl Esters (FAMEs) via Gas Chromatography

To determine the FAME composition, the oil from lyophilized biomass was derived to methyl esters by direct transesterification following the protocol stipulated in the fatty acid methylation kit (Nacalai Tesque, Inc., Japan). The light phase (hexane with methyl esters) was separated, diluted 5-fold, and transferred to GC vials equipped with a micro-tube glass insert. Chromatographic analysis was performed in a GC-MS QP 2010 Ultra (Shimadzu, Kyoto, Japan) instrument, equipped with a 0.25 mm x 30 m DB-23 capillary column (J&W Scientific). Helium was used as the carrier gas at a flow rate of 0.8 ml/min and with a split ratio of 1:5. The injection temperature was 250 ^{o}C. The primary GC oven temperature was programmed at 50 ^{o}C for 1.0 min, then increased at 25 ^{o}C/min up to 190 ^{o}C and finally increased at 5 ^{o}C/min up to 235 ^{o}C for 4 min. The temperatures of the ion source and the interface of the MS detector were set at 230 and 250 ^{o}C, respectively. A full scan (at mass range 46-500 μ) and SIM methods were applied using an MS detector. The volume of injection was 1.0 μL. Fatty acids were identified based on the retention times for a SUPELCO™ 37 component FAME mixture (Sigma-Aldrich). Caprylic acid (C8:0) was included in each sample as the internal standard. The percentage of total fatty acid content was calculated as the ratio of the individual FAME peak area to the sum of all FAME peak areas, excluding the internal standard. The weight of each fatty acid was calculated by noting the amount of internal standard (IS) that had been added, and then multiplying that amount by the ratio of the FAME area to the internal standard peaks.

II.3. Results

II.3.1. Primary Stage Selection of Oleaginous Yeast Strains

During the primary stage selection, the carbon source consumption rates, growth rates, lipid accumulation abilities, and the composition of lipids were evaluated. In this study, a nitrogen limited-mineral medium (-NMM) with a mixture of glucose and xylose as the carbon source was employed because all strains could grow in this medium. The inoculum concentration of all strains was set at $OD_{600\ nm}$ 14.0-16.0 to initiate the fermentation process in -NMM medium. Figure 1 shows the time courses for the carbon source consumption and the cell growth during the primary stage selection for all strains used in this study. Accordingly, the strains *L. doorenjongii*, *L. orientalis*, and *L. starkeyi* showed a remarkable ability to utilize the mixture of glucose and xylose in a total concentration of 100 g/L (50 g/L glucose + 50 g/L xylose) compared with other strains. These three strains could totally consume a mixture of glucose and xylose within only three days, which did not occur with other strains in this selection stage. Additionally, the cell biomass production of *L. doorenjongii*, *L. orientalis*, and *L. starkeyi* were higher than 30 g/L, compared with other strains that produced only 20-28 g/L.

To gain further insight into the ability of each strain to grow and uptake the carbon source, the growth rate and the carbon source consumption rate were evaluated, and the results are shown in Table 1. The growth rates and carbon-source consumption rates were determined when the carbon sources were completely consumed (throughout the entire cultivation process). The highest cell growth rate was reached after 3 days of cultivation at 0.36 g/L.h for *L. starkeyi*, followed by 0.27 and 0.25 g/L.h for *L. doorenjongii* and *L. orientalis*, respectively. The glucose consumption rate indicated that glucose was

consumed at a higher rate compared with that of xylose, except for *L. starkeyi*, *L. orientalis*, and *L. doorenjongii* where the glucose and xylose consumption was achieved at a similar rate of approximately 0.7 g/L.h. The equal consumption rates of glucose and xylose found in *L. starkeyi*, *L. orientalis*, and *L. doorenjongii*, demonstrated that both sugars were assimilated simultaneously rather than sequentially. On the other hand, the xylose consumption rate seemed weak in the fermentations of *L. kockii*, *L. yamadae*, and *L. yarrowii*, which occurred at 0.10, 0.16, and 0.10 g/L.h, respectively. This condition led to an incomplete consumption of xylose in the late stages of cultivation (a residual amount of xylose was not consumed during fermentation)

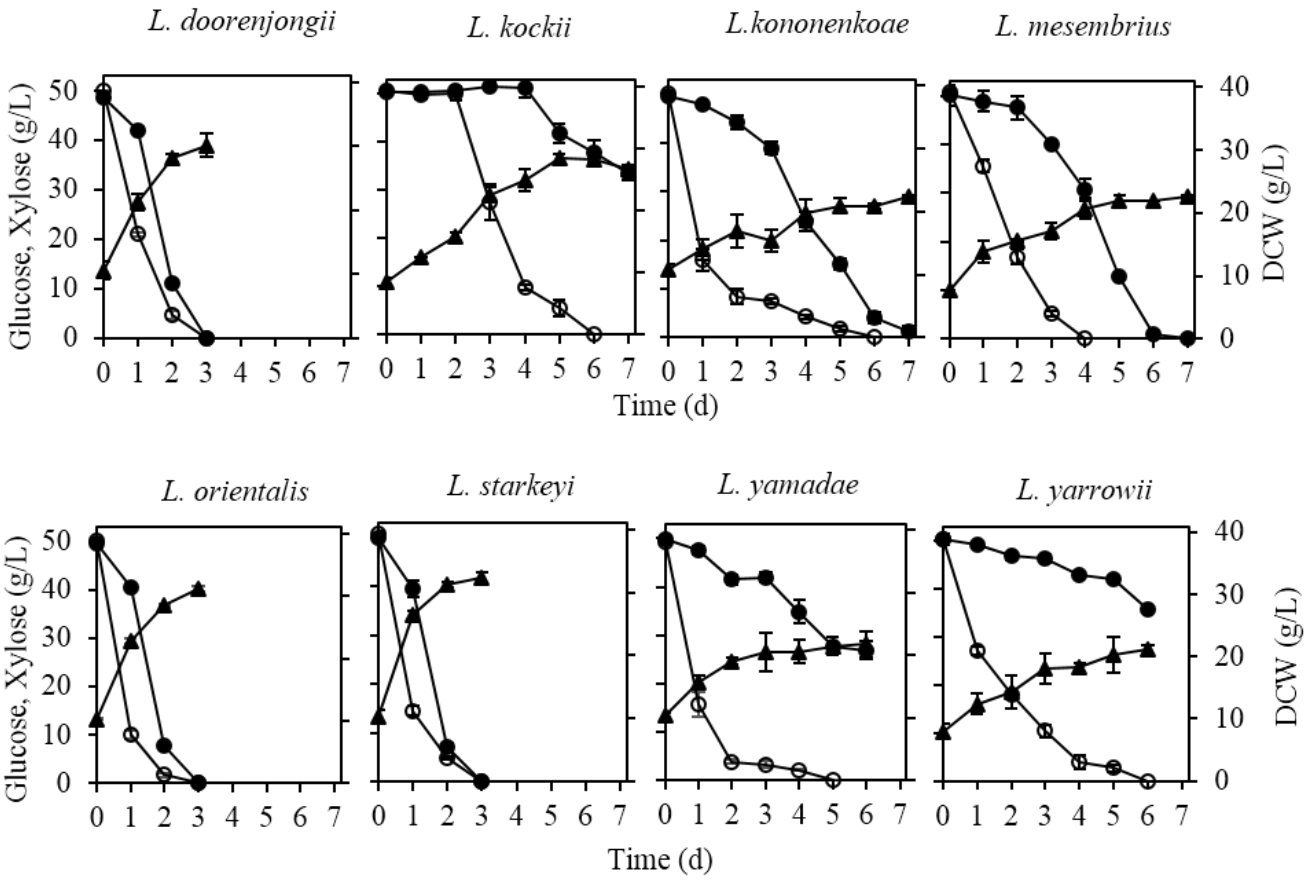

Figure 1. Time course for carbon source consumption and cell growth of eight *Lipomyces* yeast strains in -NMM medium during the primary selection process. The open circle represents glucose consumption, the closed circle refers to xylose consumption, and the closed triangle charts the cell growth.

Table 2 shows the lipid-accumulating abilities and compositions of the fatty acid methyl esters of the strains. The lipid-accumulating ability of the strains was expressed as the lipid content when the carbon sources were completely consumed during each fermentation process. As shown in Table 2, all strains used in this study are classified as oleaginous yeasts since these strains can accumulate lipids at more than 20% (w/w) of their cell mass, and the results were strain specific. Some strains attained a remarkable ability in lipid accumulation and reached levels of more than 50% (w/w). *L. starkeyi* exhibited the highest lipid content at 77.14% (w/w), followed by *L. doorenjongii* and *L orientalis* at 57.89 and 56.38% (w/w), respectively. The fatty acid compositions of the lipids produced by the strains in the -NMM medium containing a mixture of glucose and xylose were examined after the carbon sources were completely consumed (Table 2). No major differences in fatty acid composition were observed from the extracted lipids of the strains. The dominant fatty acids were oleic acid (18:1), the accumulation of which ranged between 35 and 50%, and palmitic acid (16:0), which was produced at 33 to 55%.

Table 1. Carbon source consumption rate and growth rate of eight oleaginous yeast strains during the primary selection process [a].

Strains	Consumption rate (q)		Growth rate (g)	Cultivation time (d)
	Glucose g/L.h	Xylose g/L.h		
Lipomyces doorenjongii	0.69±0.00	0.67±0.01	0.27±0.02	3
Lipomyces kockii	0.35±0.00	0.10±0.01	0.11±0.00	7[b]
Lipomyces kononenkoae	0.35±0.00	0.29±0.00	0.07±0.00	7
Lipomyces mesembrius	0.53±0.01	0.34±0.01	0.10±0.00	7
Lipomyces orientalis	0.70±0.00	0.69±0.00	0.25±0.01	3
Lipomyces starkeyi	0.70±0.00	0.69±0.00	0.36±0.01	3
Lipomyces yamadae	0.42±0.00	0.16±0.02	0.08±0.02	6[b]
Lipomyces yarrowii	0.35±0.00	0.10±0.01	0.09±0.00	6[b]

[a] The values are given as the mean ± SD of triplicate determinations.

[b] Incomplete fermentation process due to a long lag phase in xylose consumption.

Table 2. Lipid content and FAMEs profile of eight oleaginous yeast strains during the primary selection process [a].

Strains	Lipid content (w/w, %)	FAME compositions						
		C16:0	C16:1	C18:0	C18:1 (cis)	C18:2 (cis)	C24:0	Trace[b]
Lipomyces doorenjongii	57.89±1.92	36.83±1.11	1.74±0.63	13.25±1.41	45.74±1.80	1.45±0.61	0.95±0.24	1.40
Lipomyces kockii	44.25±5.94	38.82±0.82	1.35±1.20	10.24±0.67	41.99±1.37	4.70±0.92	1.27±0.83	1.60
Lipomyces kononenkoae	35.92±2.29	33.27±0.37	4.23±0.93	4.84±0.38	49.03±0.94	2.59±0.67	0.70±0.53	2.51
Lipomyces mesembrius	41.89±1.94	35.59±1.15	6.66±1.37	4.37±0.35	43.71±2.10	7.23±1.12	0.43±0.27	1.53
Lipomyces orientalis	56.38±1.93	38.93±0.93	4.86±0.83	9.53±0.87	35.81±1.15	5.46±0.34	0.67±0.01	1.58
Lipomyces starkeyi	77.14±1.55	44.19±0.37	1.85±0.24	14.61±0.71	45.80±1.60	2.16±0.88	0.62±0.35	1.32
Lipomyces yamadae	29.35±1.29	36.70±0.21	4.63±1.18	4.84±0.96	47.22±2.37	3.58±1.12	0.92±0.63	1.84
Lipomyces yarrowii	49.39±2.13	54.48±1.12	1.36±0.66	20.96±0.63	49.58±1.95	2.69±0.23	1.40±0.37	2.50

[a] The values are given as the mean ± SD of triplicate determinations.

[b] The percentage of individual FAMEs below 0.5% were categorized as trace elements, trace elements consist of C14:0, C15:0, C17:0, C17:1, C18:3 (n-3), C20:0, C20:1 (n-9), and C20:5.

Based on these results, *L. doorenjongii*, *L. orientalis*, and *L. starkeyi* strains were selected for further evaluation of cultivation using -NMM+ICC medium as an artificial lignocellulose feedstock.

II.3.2. Secondary Stage Selection in an Artificial Lignocellulose Feedstock

The time courses for carbon-source consumption and cell growth of the selected strains in the -NMM medium supplemented with ICC are shown in Figure 2. The presence of ICC in -NMM medium had an obvious inhibitory effect on the fermentation of the three selected strains (*L. doorenjongii*, *L. orientalis* and *L. starkeyi*), which showed a decrease in cell production and a longer fermentation time. Even so, the yeast strains continued to grow after 4 d of incubation. In this study *L. starkeyi* was the most ICC tolerant strain due to completely consumed carbon sources and produced approximately 30 g/L DCW within 4 days. Of the three selected strains glucose was most quickly consumed followed by xylose.

To attain further insight into the ability of each strain to grow and uptake a carbon source, the growth rates and carbon source consumption rates in the -NMM+ICC medium were evaluated, and the results are shown in Table 3. The glucose consumption rate for the fermentation of *L. doorenjongii* was decreased from 0.69 g/L.h in -NMM medium to 0.53 g/L.h in -NMM+ICC medium, and a similar result was obtained for *L. orientalis*. On the other hand, the glucose consumption rate of *L. starkeyi* in -NMM+ICC medium remained unchanged compared with fermentation in -NMM medium. During fermentation in the -NMM+ICC medium, the xylose consumption rates for the three selected strains were decreased to 0.36, 0.42, and 0.53 g/L.h for *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*, respectively. Unlike fermentation in -NMM medium, the

consumption rate for the three selected strains in the presence of ICC indicated that both sugars were metabolized sequentially. Moreover, the growth rates of the three selected strains were dramatically decreased in the presence of ICC at rates of 0.09, 0.13, and 0.22 g/L.h for *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*, respectively.

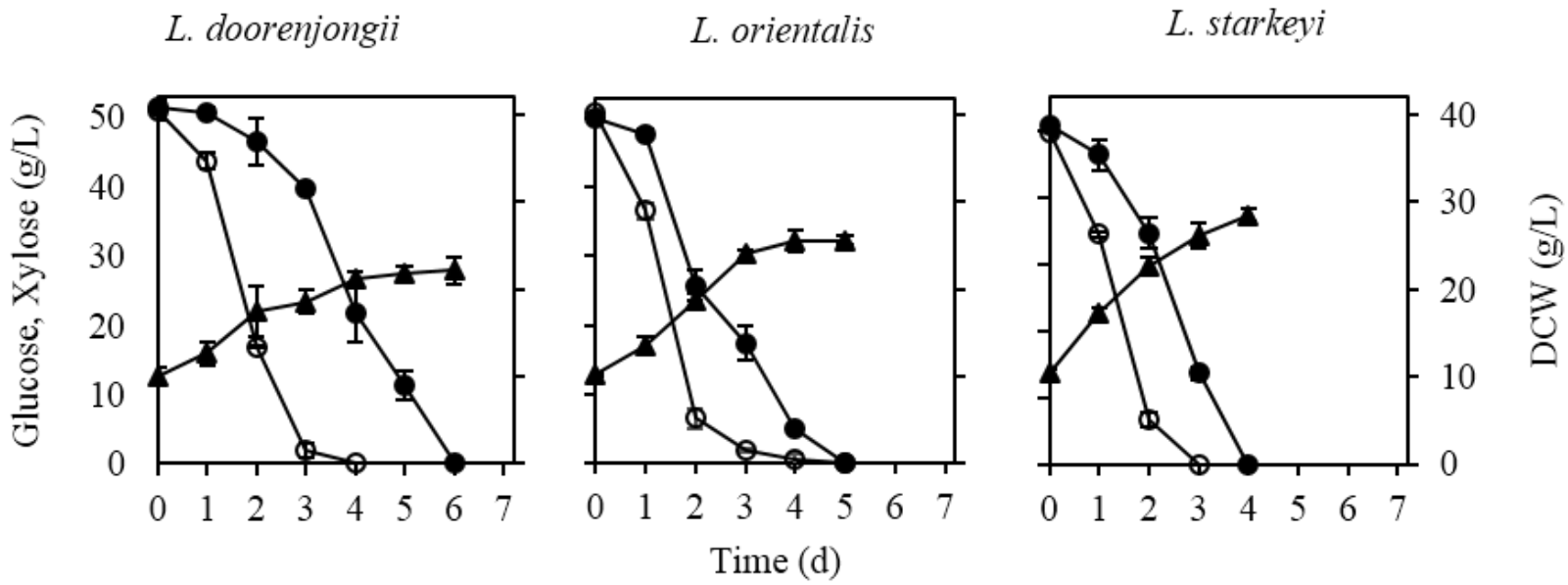

Figure 2. Time courses for carbon source consumption and cell growth of three selected yeast strains in -NMM + ICC medium during the secondary selection process. The open circle represents glucose consumption, the closed circle refers to xylose consumption, and the closed triangle charts the cell growth.

Table 3. Carbon source consumption rate and growth rate of three selected yeast strains during the secondary selection process [a].

Strains	Consumption rate (q)		Growth rate (g)	Cultivation time (d)
	Glucose g/L.h	Xylose g/L.h		
Lipomyces doorenjongii	0.53±0.01	0.36±0.01	0.09±0.00	6
Lipomyces orientalis	0.53±0.01	0.42±0.00	0.13±0.00	5
Lipomyces starkeyi	0.69±0.03	0.53±0.01	0.22±0.01	4

[a] The values are given as the mean ± SD of triplicate determinations.

II.3.3. Inhibitor Tolerance and Degradation Performance of Selected Yeast Strains

The inhibitory chemical compounds (ICCs) were used to investigate the tolerance and degradation performance of selected strains against inhibitors. ICCs containing two furan aldehydes (furfural and 5-HMF) were used to represent the over-degradation of pentose and hexose sugars during the pretreatment of lignocellulosic biomass. Two typical phenolic aldehydes (vanillin and syringaldehyde) were selected to represent the lignin derivatives of guaiacyl and syringyl groups. Furthermore, the most common weak acids released in lignocellulosic hydrolysates included acetic, formic and levulinic acids.

To elucidate the inhibitor tolerance and degradation performance of the selected strains, the time courses and depletion rates for each of the inhibitory chemical compounds were evaluated, and the results are shown in Figs. 3 and 4, respectively. The depletion rates of each typical inhibitory chemical compound were determined during the first 24 h of the fermentation process. The experimental results showed that the three selected strains were able to detract furfural rapidly before the end of the fermentation

process (Fig. 3). For the furfural depletion, *L. starkeyi* had the highest depletion rate at 4.8×10^{-1} mM/h (Fig. 4), and was exhausted after 1 d of cultivation (Fig. 4). *L. doorenjongii* and *L. orientalis* shows the furfural depletion rate at 4.1×10^{-1} and 3.6×10^{-1} mM/h (Fig. 4), respectively, where the furfural was almost exhausted for both strains after cultivation for 3 d. Another furan aldehyde inhibitor compound, 5-HMF, showed that the amount of this compound was nearly constant in the *L. orientalis* fermentation process. However, for the fermentation of *L. doorenjongii* and *L. starkeyi*, 5-HMF was exhausted after 3 d of cultivation. The 5-HMF depletion rates were achieved at 2.2×10^{-2}, 1.0×10^{-2}, and 3.7×10^{-2} mM/h for *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*, respectively.

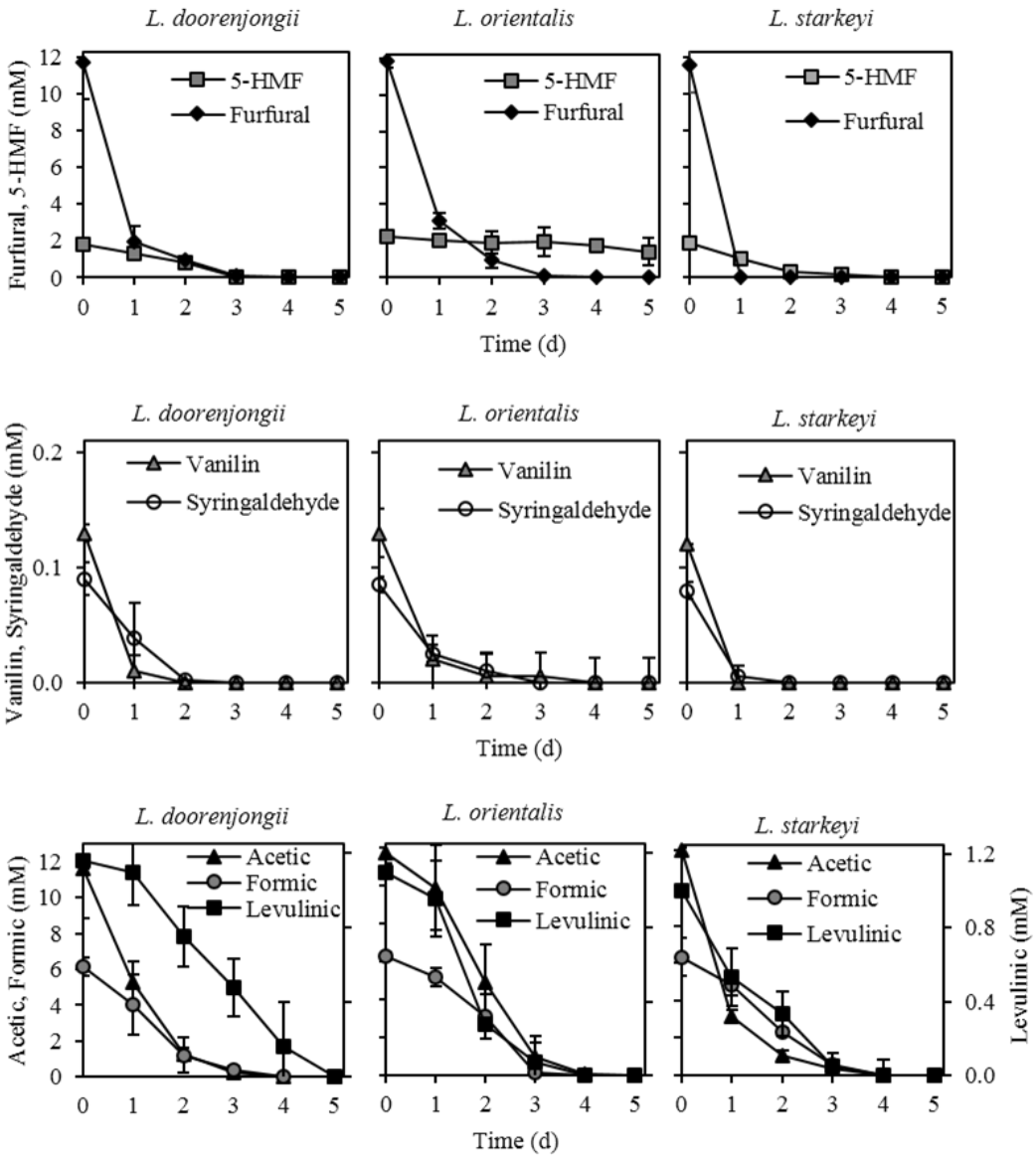

Figure 3. Time course for inhibitor depletion of three selected yeast strains in -NMM + ICC medium during the secondary selection process. Two furan aldehydes (5-HMF, closed grey square and furfural, black diamond), two phenolic aldehydes (vanillin, closed triangle and syringaldehyde, open circle) and the most common weak acids (acetic acid, open triangle; formic acid closed circle; and, levulinic acid, closed black square).

The vanillin depletion rates for the three selected strains were similar at approximately 5.0×10^{-3} mM/h, while those for syringaldehyde were 2.2×10^{-3}, 2.5×10^{-3}, and 3.1×10^{-3} mM/h for *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*, respectively. Consequently, both of these phenolic aldehyde compounds were completely exhausted after 2 d of fermentation with each of the selected strains.

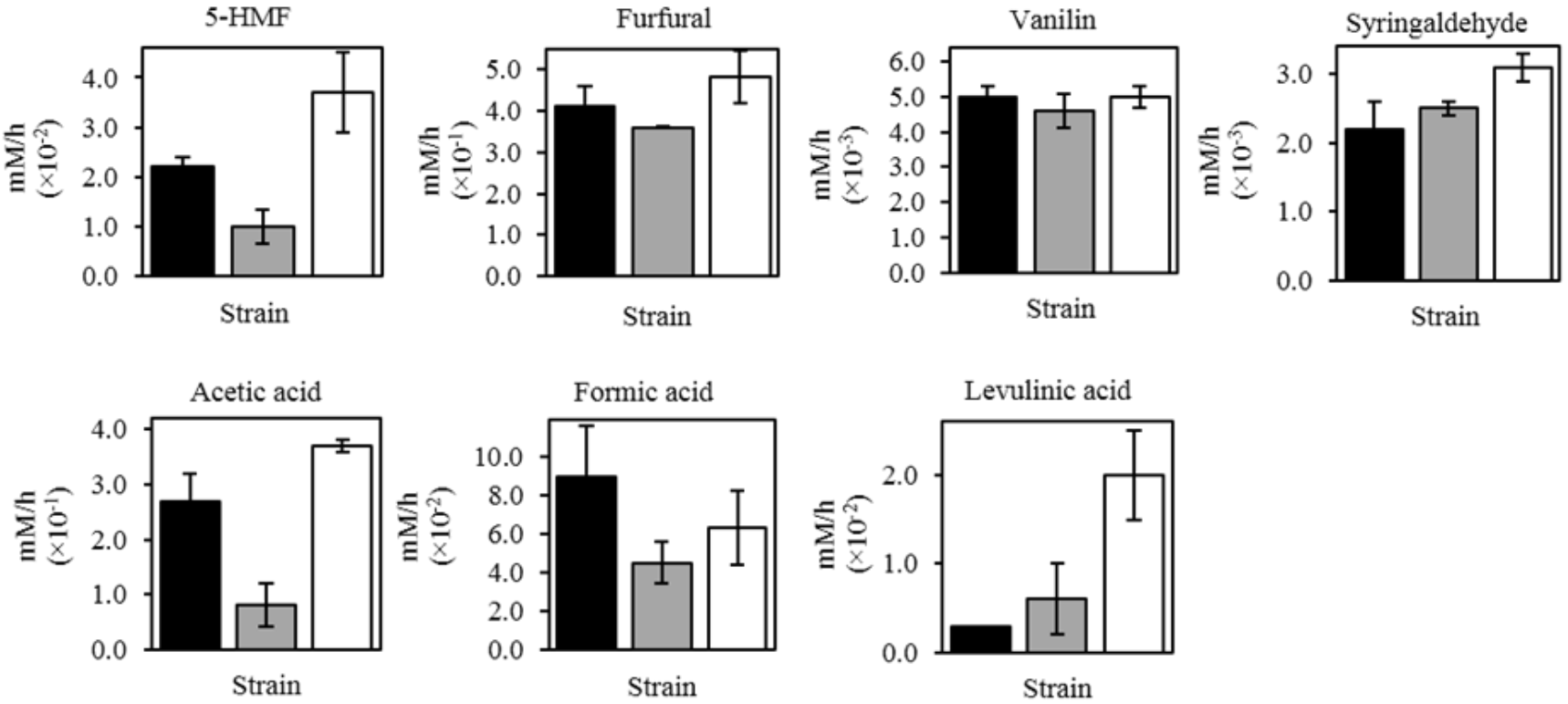

Figure 4. Inhibitory chemical compound reduction rates for three selected yeast strains during the secondary selection process. Black, grey, and white bars represent the strain *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*, respectively.

The sensitivity of the selected strains to several weak organic acids in this study (acetic, formic, and levulinic acids) strongly depended on the microorganism being considered. In particular, *L. doorenjongii* reduced formic acid at the highest rate (8.9×10^{-2} mM/h) compared with other selected strains during the first 24 h of fermentation. In contrast, *L. doorenjongii* showed the lowest depletion rate for levulinic acid (0.3×10^{-2} mM/h) compared with that of both *L. orientalis* and *L. starkeyi* (0.6×10^{-2} and 2.0×10^{-2} mM/h, respectively). Furthermore, *L. starkeyi* showed the highest depletion rate for acetic acid at 3.7×10^{-1} mM/h, followed by *L. doorenjongii* and *L. orientalis* at 2.7×10^{-1} and 0.8×10^{-1} mM/h, respectively. In general, all of the weak acids used in this study were exhausted after 3 to 5 d of the fermentation process for each selected strain.

II.3.4. Effect of ICC on Lipid Accumulation and Fatty Acid Composition

The presence of ICC in the fermentation medium not only inhibited cell growth and reduced the carbon source consumption rate, as mentioned in the above section, it also resulted in a decrease in the content and yield of lipids. Lipid production for selected strains in -NMM and -NMM+ICC media are compared in Table 4. In general, the biomass yield, lipid content, and lipid yield were decreased in the presence of ICC in the fermentation medium: the lipid content of *L. doorenjongii* was decreased from 57.89% (w/w) when cultivated in -NMM to 43.77% (w/w) in -NMM+ICC medium; the content of *L orientalis* was decreased from 56.38% (w/w) to 49.35% (w/w); and, that of *L. starkeyi* was decreased from 77.14% (w/w) to 68.24% (w/w). A similar pattern was also observed in the lipid yields of the three selected yeast strains. In the presence of ICC, the lipid yields of *L. doorenjongii*, *L. orientalis*, and *L. starkeyi* dropped to 0.10, 0.12, and 0.19 (w/w, mg/mg-sugar), respectively, from the previous yield in -NMM medium of 0.17,

0.18, and 0.25 (w/w, mg/mg-sugar). Accordingly, the highest lipid content and yield for fermentation in -NMM+ICC medium was observed for *L. starkeyi* followed by *L. orientalis* and *L. doorenjongii*.

Table 4. Lipid production comparison of three selected strains between fermentation processes in the medium -NMM and medium -NMM + ICC [a].

Strains	-NMM medium				-NMM medium + ICC			
	DCW (g/L)	DCW yield (%, w/w)	Lipid content (%, w/w)	Lipid yield (w/w)	DCW (g/L)	DCW yield (w/w, %)	Lipid content (w/w %)	Lipid yield (w/w)
Lipomyces doorenjongii	30.15±1.46	30.57±0.64	57.89±1.92	0.17±0.00	22.11±1.51	21.58±0.82	43.77±1.02	0.10±0.00
Lipomyces orientalis	31.21±1.29	31.21±0.47	56.38±1.93	0.18±0.00	25.62±0.59	25.42±0.33	49.35±2.71	0.13±0.00
Lipomyces starkeyi	32.34±0.53	32.24±0.27	77.14±1.55	0.25±0.00	28.52±0.68	28.43±0.74	68.24±2.48	0.19±0.00

[a] The values are given as the mean ± SD of triplicate determinations.

Lipid yield: milligram of lipid per milligram of sugar consumed.

DCW yield: gram of dry cell biomass per gram of sugar consumed ×100%.

Table 5. FAMEs profile of three selected yeast strains during the secondary selection process [a].

Strains	FAME compositions						
	C16:0	C16:1	C18:0	C18:1 (cis)	C18:2 (cis)	C24:0	Trace[b]
Lipomyces doorenjongii	42.35±1.13	2.21±0.55	13.06±0.41	42.46±1.23	1.47±0.72	0.36±0.11	1.80
Lipomyces orientalis	42.95±0.87	1.39±0.37	9.99±1.14	48.93±2.49	1.85±0.49	0.55±0.05	1.81
Lipomyces starkeyi	39.27±1.12	5.57±1.21	9.23±0.83	40.56±1.93	2.05±0.13	0.22±0.08	1.77

[a] The values are given as the mean ± SD of triplicate determinations.

[b] The percentage of individual FAMEs below 0.5% were categorized as trace elements, trace elements consist of C14:0, C15:0, C17:0, C17:1, C18:3 (n-6), C20:0, C20:1 (n-9), and C20:5

The fatty acid methyl ester (FAME) compositions of the three selected strains in the medium with ICC are shown in Table 5. In general, there were no major differences in the FAME profiles between the fermentation process in -NMM medium (Table 2) and -NMM+ICC medium (Table 5). For the selected yeast strains, the FAMEs were unaltered by the presence of ICC in the medium, which shows that ICC did not significantly affect the fatty acid biosynthetic pathway. However, the traces of fatty acids found in the fermentation process with -NMM medium were slightly increased in the presence of ICC (data not shown).

II.4. Discussion

To initiate the fermentation process for lipid production in this study, the initial inoculum concentration was set at $OD_{600\ nm}$ 14.0-16.0 for both the primary and secondary stage-selection processes. In our previous study, we found that a higher level of inoculum concentration leads to an increase in the number of active cells to utilize carbon sources, and a higher number of active cells increase the probability that those cells will grow and detoxify the inhibitor chemical compounds [21,22]. Consequently, a higher inoculum concentration leads to a higher cell/carbon source ratio and to an increase in the carbon-source consumption rate. Moreover, the maximum growth rate was also strongly influenced by the inoculum size [21]. For fermentation in ICC medium, a large inoculum size is considered a reliable strategy to decrease the effect of inhibitors. Aldehydes, alcohols, and organic acid compounds have been converted into compounds with low levels of toxicity during the fermentation process by yeast cells, and a large inoculum size could boost the efficiency of this *in vivo* detoxification. Additionally, the effect of inoculum size on the biomass, lipid yield and lipid content is reported to be significant

[6,23-25]. Therefore, based on a literature review, to obtain a potentially effective yeast strain candidate, a large inoculum size was applied in this study.

As shown in Fig. 1, the use of sugars for cell growth was different among the strains tested. Several fermentations in the primary stage selection of oleaginous yeasts, *L. kockii*, *L. kononenkoae*, *L. mesembrius*, *L. yamadae*, and *L. yarrowii*, showed the inhibitory effects of high sugar concentrations on the growth of oleaginous microbes due to high levels of osmotic stress. In the above-mentioned strains both sugars (glucose and xylose) were metabolized sequentially. Glucose was consumed first, and xylose was consumed after glucose depletion (Fig. 1), which resulted in a prolonged lag time and led to an incomplete consumption of xylose in the late stages of the fermentation process. Some microorganisms are known to generally metabolize sugars sequentially when exposed to a mixture of glucose and xylose because glucose can repress the utilization of other sugars via either a catabolic repression mechanism or the presence of allosteric competition sugar transporters [26,27]. By contrast, the inhibitory effects of the presence of a highly concentrated carbon source in the medium were not observed on *L. doorenjongii*, *L.orientalis*, and *L. starkeyi*. These three strains showed higher rates of growth and carbon source consumption compared with other strains in this study (Table 1). In addition, as shown in Table 1, these three strains showed the ability to simultaneously assimilate glucose and xylose in a fermentation medium. The simultaneous utilization of glucose and xylose is an important aspect in the use of lignocellulosic biomass, because it reduces the costs associated with lipid production. For efficient co-cultivation from mixed sugar, it is important to select the appropriate strain of yeast as a platform.

It is well known that several yeasts from *Lipomyces* spp. have a great propensity to accumulate triacylglycerols (TAG). This genus belongs to the *Saccharomycetales* order and represents a unique branch in the evolution of ascomycetes. *Lipomyces* are true soil inhabitants with a worldwide distribution [14]. Many studies have reported that *L. starkeyi* has the capability to accumulate more than 70% of its cell biomass as lipid under defined culture conditions, and can produce lipids on xylose, ethanol, and L-arabinose, or by using a mixture of glucose and xylose [19,28,29]. Furthermore, *L. doorenjongii* was previously identified as a high lipid-producing strain. During an evaluation of 18 potential yeast strains for lipid production in batch-flask fermentation, Dien et al., [30] found that *L. doorenjongii* could produce a considerable amount of lipid content, which amounted to approximately 40% (w/w) of cell biomass at 27 g/L. Another study by Oguri et al., [31] found that *L. doorenjongii* could convert five sugars, glucose, xylose, L-arabinose, galactose, and mannose, to produce lipids in high total yields. They also found that this strain produces lipids by up to 72% (w/w) when glucose is used as the sole carbon source in batch-flask fermentation. A comparable result was observed in the present study during the primary stage of the selection process as high lipid contents were obtained for *L. starkeyi* and *L. doorenjongi* when cultivated in -NMM medium using a mixture of glucose and xylose as the carbon source in a batch-flask fermentation process (Table 2). On the other hand, we could find no previous reference to lipid production from *L. oreintalis*. This strain was isolated for the first time by Thanh [32] during a survey of the diversity of soil yeast in Vietnam. This strain is worthy of further investigation because *L. orientalis* in this study could produce a considerable amount of lipid content (Table 2) with a high rate of sugar consumption (Table 1) during the primary selection process. In addition, *L. orientalis* also showed the potential to detoxify ICCs and could produce a

considerable amount of lipids during the secondary selection process. To the best of our knowledge, no study has examined lipid production from *L. orientalis*, particularly in the presence of inhibitory chemical compounds in the fermentation medium.

Lignocellulosic hydrolysates generally contain more than one inhibitor, and the synergistic effect of different inhibitors is complex [33]. Therefore, a cocktail mixture of seven typical inhibitory compounds was used to evaluate the effects of ICCs on lipid production as well as testing the tolerance and degradation performance of selected strains against inhibitory compounds. Furan derivatives (furfural and 5-HMF) and phenolic compounds (vanillin and syringaldehyde) are the two main types of aldehydes in lignocellulosic hydrolysates [34]. Among the aldehydes examined, furfural was less toxic to the three selected yeast strains in this study (Figs. 3 and 4), followed by aromatic aldehydes (vanillin and syringaldehyde) and 5-HMF. There is no known correlation between the hydrophobicity and toxicity of aldehydes. The hydrophobicity of an aldehyde, however, is not the only determinant of its toxicity [6,35]. Additionally, many yeast species could convert aldehydes into less-toxic compounds [18,23]. For instance, Wang et al., [7] found that furfural and 5-HMF were converted to the corresponding alcohols (furfuryl alcohol and HMF alcohol) then into the corresponding acids (furoic acid and HMF acid) during the fermentation process when cultivating *Trichosporon cutaneum* in a synthetic medium containing inhibitor mimic pretreated corn stover hydrolysate. They also found that vanillin and syringaldehyde could converted to less toxic derivatives. In this study, we detected no furfural after fermentation for 4 days in the medium (Fig. 3), suggesting that the furfural was metabolized by *L. doorenjongii*, *L. orientalis*, and *L. starkeyi*. Intrigue, neither furfuryl alcohol nor HMF alcohol were detected in the medium during the fermentation process (data not shown). This result suggests that the strains may

use these derivatives as a carbon source for growth and/or lipid production. Even though many previous studies have shown that yeast could metabolize furans [36], 5-HMF [37], and vanillin [38], the precise mechanism involved remains unknown in most cases and should be clarified in future study.

In the case of weak acids, acetic acid can also contribute to cell growth since the selected yeast strains consumed this organic acid during fermentation (Fig. 3). This finding is very encouraging since the inhibition posed by acetic acid was rapidly neutralized during early yeast growth on the three selected yeast strains. Acetic acid could be involved in lipid production because it is one of the sources of carbon for Acetyl-CoA fatty acid synthesis [39]. Yeast sensitivity to acetic acid also depends on the species [17,35,40]. On the other hand, the highest depletion rate of acetic and levulinic acids throughout the fermentation processes of the three selected yeast strains resulted in a rapid depletion of both weak acids. Hence, this phenomenon indicated that *L. doorenjongii*, *L. orientalis* and *L. starkeyi* strains could use these compounds as a carbon source for producing lipids. Furthermore, James and Russel [41] found that un-dissociated acetic acid in the cultivation media could enhance lipid solubility, which would allow increased fatty acid accumulation on the cell membrane or in other cell wall structures. Based on these results (Figs. 3 and 4), formic acid showed a stronger inhibitory effect compared with acetic and levulinic acids due to its higher acidity. The acidity (pKa) for formic, levulinic, and acetic acids are 3.75, 4.5, and 4.75, respectively, where smaller pKa means higher acidity. The higher acidity and smaller molecular size of formic acid could facilitate diffusion across the plasma membrane to exert an inhibitory effect [17].

Lipid yields and content vary depending on the oleaginous yeast species, feedstock type, and cultivation conditions, and the conversion of sugars in the hydrolysate

of lignocellulosic biomass to lipids using oleaginous yeasts is feasible. Lipid yield is an essential parameter that affects the cost of lignocellulosic lipid production. The theoretical yield of lipid production is 0.32 mg/mg-sugar from glucose and 0.34 mg/mg-sugar from xylose [42,43]. In this study, during the primary selection process, the lipid yield of the theoretical maximum for *L. doorenjongii* and *L. orientalis* was 50% compared with 73% for *L. starkeyi*. However, for the secondary selection process in the presence of ICC, the lipid yield was drastically decreased for both *L. doorenjongii* and *L. orientalis* to levels of 29 and 38% of the theoretical maximum, respectively. On the other hand, for *L. starkeyi*, the lipid yield was slightly decreased to 57% of the theoretical maximum. Lipid yields were often below 0.25 mg/mg-sugar (<74% of the theoretical maximum), with one exception, which reached as high as 0.33 mg/mg-sugar (almost the theoretical maximum) in a detoxified hydrochloric acid hydrolysate of sugarcane bagasse using *Yarrowiya lipolytica* Po1 g. The same strain, however, reached a lipid yield of only 0.10 mg/mg-sugar in a detoxified sulfuric acid hydrolysate of rice bran [12,44].

L. starkeyi proved to be a promising candidate that could be important for industrial lipid production using lignocellulosic biomass due to demonstrating the highest rate of carbon source consumption as well as the highest lipid content and lipid yield during the primary and secondary selection processes in this study. The other two strains, *L. doorenjongii* and *L. orientalis*, also showed potential ability for such application since these strains also demonstrated a capability for inhibitor tolerance. Interestingly, based on a phylogenetic study of these strains (Fig. 5), we found that *L. starkeyi*, *L. doorenjongii*, and *L. orientalis* showed a closely related taxon for clades with a good consumption of glucose and xylose for lipid production under the presence of ICC. In this study, only three selected strains (*L. starkeyi*, *L. doorenjongii*, and *L. orientalis*) were systematically

evaluated due to their fast consumption of carbon sources. On the other hand, we could not reject the potential of *L. mesembrius* and *L. kononenkoae* strains for lipid production under the presence of ICC, because they also showed a closely related taxon to *L. starkeyi* and the other two selected strains. However, further study is required to enable high lipid production from real biomass hydrolysates using lipid yeasts based on a comprehensive understanding of the metabolism of these stains to withstand the stress from ICCs.

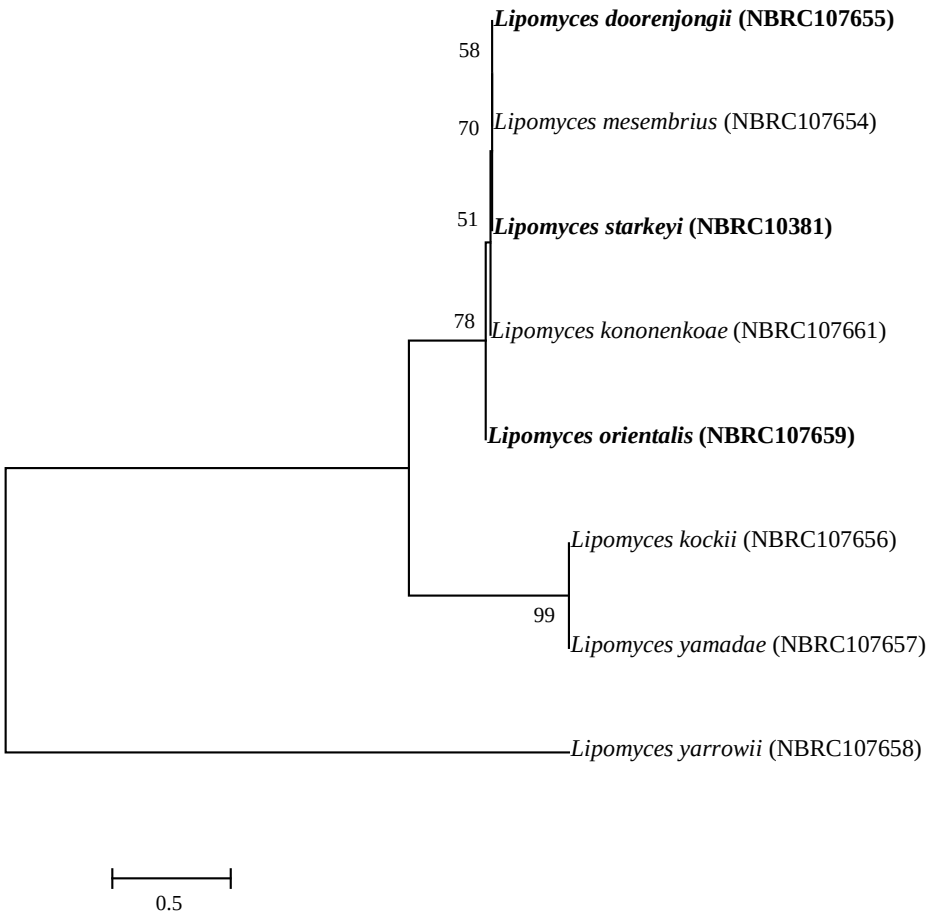

Figure 5. Neighbor-joining analysis showing the extent of divergence among closely related species in *Lipomyces starkeyi* NBRC10381clade determined from the variable D1/D2 domain of an LSU rRNA gene. The species *L. yarrowii* NBRC107658 was designated as the outgroup for analysis. Bar represents the 0.5 substitutions per nucleotide position. Bold lines means the strains under consideration for high lipid production in the medium containing ICCs.

II.5. Conclusions

Lipomyces yeasts, as the genus name implies, are oleaginous, but they have yet to be systematically screened for lipid production. Eight oleaginous yeast strains from the *Lipomyces* genera were applied to a two-tier selection process. Three yeast strains (*L. doorenjongii*, *L. orientalis*, and *L. starkeyi*) were obtained from the primary selection process based on their ability to assimilate a mixture of glucose and xylose at a high rate and to accumulate a high level of lipid content. During the secondary selection process *L. starkeyi* exhibited the most promising strain and accumulated a high lipid content and yield at 68.24% and 0.19 (w/w, mg /mg-sugar), respectively. 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.

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Chapter III

Lipid production by *Lipomyces starkeyi* using sap squeezed from felled old oil palm trunks

III.1. Introduction

Under the condition of nutrient stress (particularly nitrogen-limited) and in the presence of excess carbon source, oleaginous yeasts accumulate single cells-oils (SCOs) as intracellular carbon and energy reserves. The SCOs have attracted much attention due to their similar fatty acid profile to that of oils from agricultural oil crops, which make them generally suitable for processing into several other products in addition to biodiesel, such as fungicides, bactericides, emulsifier, plasticizers, surfactant and lubricants [1-3]. However, the costs of SCOs or microbial oil production are currently higher than those vegetable oils due to the carbon feedstock which is estimated to be about 80% of the total medium cost, but there are many methods to drastically improve the techno-economics of SCO production processes [4-6]. Various research groups around the world have been working to develop efficient strategies for reducing the production cost, based on cost effective fermentation, a robust strain and with an efficient recovery process [7-9]. It has been shown that the cost of carbon source is the major factor that contributes to the production cost of SCOs. Hence, utilization of cheap renewable carbon source for SCOs production would have a positive influence on its economics.

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. Together with neighboring Malaysia, it accounts for approximately 88% of the world palm oil production [10]. 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 [11]. According to Kosugi et al. [12], replanting of palm oil trees would generate on an average of 64-80 million palm trees every year as lignocellulosic residues in Indonesia and Malaysia for the next 25 years. 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.

Previous study by Kosugi et al. [12], was able to obtain 0.65 g of sap from 1 g of the trunk (inner part) using laboratory-scale press. Oil palm trunks (OPT) sap is rich in sugars, amino acids, organic acids, mineral and vitamins [12]. 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 [11]. 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 [12,13]. 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. Fewer than 30 of the about 600 yeast species are known to be oleaginous [14,15]. Oleaginous yeasts are typically found, but not exclusively, in genera such as *Candida*, *Crytococcus*, *Rhodotorula*, *Rhizopus*, *Trichosporon*, *Lipomyces*, and *Yarrowiya* [16]. *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 [17,18]. In our previous study, *Lipomyces starkeyi* was used as a platform to evaluate the SCO production in synthetically nitrogen-limited mineral media and lipid production was achieved at higher than 80% (w/w) in a mixture of glucose and xylose as carbon sources [19]. 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 [20-23]. In regards 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.

III.2. Materials and Methods

III.2.1. Yeast Strain

The yeast strain *Lipomyces starkeyi* NBRC10381 used in this study was selected from the NITE Biological Resource Center (NBRC), National Institute of Technology and

Evaluation, Japan. The yeast strain was preserved in 20% (w/w) glycerol at -80 ^{o}C and was revived by streaking onto a potato dextrose agar (PDA) plate. Later, the yeast strain was grown on a yeast extract-malt extract-peptone-glucose (YMPG) agar plate (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L and agar 16.0 g/L).

III.2.2. Seed Preparation

A single colony from a YMPG agar plate was used to start the pre-culture which contained 12 ml of YMPG broth (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L) in a 100 ml Erlenmeyer flask and incubated in an orbital shaker incubator (BioShaker BR-43FH MR, TAITEC Corp., Japan) at 190 rpm for 24 h at 30 ^{o}C. Then the pre-culture was transferred to 12 ml OPT sap medium for lipid production.

III.2.3. Extraction of OPT sap

Oil palm trees (*Elaeis guineensis* var. Tenera) aged around 25 years, from the oil palm plantations of Pahang located in the central region of Peninsular Malaysia were felled for this experiment. Trunks from the felled trees were cut transversely into discs at the thickness of approximately 10 cm and transported immediately to the laboratory and stored in -20 ^{o}C freezer until further use. The discs were then cut into smaller sections using a band saw and the central moist region was retained while discarding the harder peripheral bark region. The central sections were then squeezed with the help of a laboratory-scale press machine (Moulding Test Press, GT-7014-A30, FABRICATE) at 80 MPa to extract the sap. The extracted sap was collected in Schott bottles and stored immediately in a -20 ^{o}C freezer for further use.

III.2.4. Preparation of Extracted OPT Sap as Fermentation Medium

Extracted OPT sap stored in a -20 ^{o}C freezer was thawed to room temperature. To evaluate the capability of OPT sap as the fermentation medium, the OPT sap was divided into three categories i.e. regular sap, enriched sap, and enriched sap with adjusted pH. Enriched sap was supplemented with mineral medium (MM), consisting of the following compounds dissolved in deionized water: $(NH_4)_2SO_4$ (0.25 g/L); $MgSO_4.7H_2O$ (1.0 g/L); KH_2PO_4 (1.5 g/L); $Na_2HPO_4.2H_2O$ (5 g/L). The trace element solution consisting of $FeSO_4.7H_2O$ 0.08 g/L, $ZnSO_4.7H_2O$ 0.01 g/L, $CaCl_2.2H_2O$ 0.1 g/L, $MnSO_4.4H_2O$ 0.1 g/L, $CuSO_4.5H_2O$ 0.002 g/L, $CoCl_2.6H_2O$ 0.002 g/L was also included in MM. The enriched sap with adjusted pH was prepared in the same way with enriched sap and the initial pH was adjusted to 5.0 with 2 N NaOH. Whereas, the regular sap was prepared with neither of additional mineral medium nor adjusted pH, where the initial pH of regular sap was 3.2. Hereinafter, the three types of OPT sap used in this study are: regular sap (Reg-Sap), enriched sap (Sap+MM) and enriched sap with adjusted pH (Sap+MM pH 5.0). The OPT sap medium was sterilized by the addition of both ampicillin and kanamycin to prevent contamination.

III.2.5. Cultivation Conditions

The seed volume of the pre-culture in YMPG broth was adjusted to an initial optical density ($OD_{600\ nm}$) of 14.0-16.0 in order to obtain an appropriate active cell fraction which increase the capability of cells to utilize carbon sources and detoxified any inhibitor chemical compounds. Afterward, the seed culture from the YMPG broth was transferred to regular-sap, enriched-sap, and enriched-sap with adjusted pH media for cultivation

with 12 ml working volume. These cultures were incubated in an orbital shaker incubator that was operated at 190 rpm and 30 ^{o}C until all of the carbon sources were completely exhausted. Growth was confirmed by measuring the dry cell weight (DCW, g/L) of biomass that was analyzed gravimetrically. Following inoculation, samples for the measuring of DCW were taken on the initial day of cultivation and every 24 h thereafter until all the carbon sources were consumed. For dry cell weight determination, a 1 ml sample of fermentation broth was transferred into a pre-dried and pre-weighed 1.5 ml reaction tube then centrifuged at 14,000 ×g for 5 min. The cell pellets were washed with deionized water, dried at 60 °C for 24 h, and weighed. At the end of fermentation time the yeast cells were harvested by centrifugation at 14,000 ×g for 10 min, the supernatants were decanted, and each pellet was washed twice with sterile deionized water. Afterwards, Cell pellets were stored overnight at -80 ^{o}C, and were then lyophilized by freeze drying (Freez-one® 4.5L Freeze Dry System Model 7750020, Labconco®, Kansas City, MO) for 48 h. The lyophilized cells were stored in a desiccator until further use for total lipid quantity and fatty acid analysis.

III.2.6. Sugar Analysis

At the preliminary stage, the thin layer chromatography (TLC) was used for quantitative analysis of sugars component in the OPT sap. Diluted sample (10 µl) was loaded on TLC Silica gel plate 60F $_{254,\ 20\text{-}20\ cm}$ (Merck, Germany) and left at room temperature for drying. Plate was run in a pre-equilibrated TLC container with a mixture of n-butanol/acetic acid/water (2:1:1, v/v/v) as the mobile phase. After 10 cm of front migration the plate was removed from the TLC container and air dried. To visualize the spots, the dried plate was sprayed using DAP that contained diphenylamine, aniline, acetone and phosphoric acid

(Merck, Germany), then subsequently placed in an oven at 120 ^{o}C for approximately 20 min. The pure chemicals such as glucose, sucrose, fructose, xylose, galactose were used as standards.

The concentrations of sugars during fermentation were analyzed using high-performance liquid chromatography (HPLC) (LC-20AB, refractive index detector RID-10A, Shimadzu, Japan) equipped with an ICSep Coregel-87H column (7.8 mm I.D. x 300 mm Transgenomic) operated at a column temperature of 80 ^{o}C. The mobile phase was 5.0 mM H_2SO_4 at a rate of 0.6 ml/min. All samples were centrifuged to remove the cell mass and other water-insoluble substances and then filtered through a 0.22 µm filter before analysis.

III.2.7. Determination of Total Lipid Quantity by Gravimetric Analysis

All chemicals were of analytical grade. Gravimetric analysis was used to measure the lipids production based on the modified Folch method [24], as follows. Triplicate 15 mg samples of freeze-dried cells were transferred to a 2.0 ml polypropylene micro-vial with an O-ring sealed cap containing 0.5 mm zirconia beads and 1.5 ml of Folch solvent (2:1 of $CHCl_3$: MeOH, v/v). Cells were pulverized using a Shake Master Neo ver.1.0 (BMS-M10N21, BMS, Tokyo, Japan) at 1,500 rpm for 15 min. Afterward, cells were centrifuged at 14,000×g for 5 min, the supernatant was removed, and the pellets were washed with 1.5 ml of deionized water, then pulverized a second time, centrifuged, and the remaining water was then removed. The cell pellets were dried at 60 ^{o}C to a constant weight. The lipid content was determined by the weight difference and expressed as a percent of dry cell weight.

III.2.8. Determination and Quantification of Total FAMEs via Gas Chromatography

To determine the FAME composition, the oil from lyophilized biomass was derived to methyl esters by direct transesterification following the protocol stipulated in the fatty acid methylation kit (Nacalai Tesque, Inc., Japan). The light phase (hexane with methyl esters) was separated, diluted 5-fold, and transferred to GC vials equipped with a micro-tube glass insert. Chromatographic analysis was performed in a GC-MS QP 2010 Ultra (Shimadzu, Kyoto, Japan) instrument, equipped with a DB-23 capillary column 0.25 mm x 30 m (Agilent J&W Scientific, Santa Clara, CA). Helium was used as the carrier gas at a flow rate of 0.8 ml/min and with a split ratio of 1:5. The injection temperature was 250 ºC. The primary GC oven temperature was programmed at 50 ºC for 1.0 min, then increased at 25 ºC/min up to 190 ºC and finally increased at 5 ºC/min up to 235 ºC for 4 min. The temperatures of the ion source and the interface of the MS detector were set at 230 and 250 ºC, respectively. A full scan (at mass range 46-500 μ) and SIM methods were applied using an MS detector. The volume of injection was 1.0 μL. Fatty acids were identified based on the retention times for a SUPELCO™ 37 component FAME mixture (Sigma- Aldrich). Caprylic acid (C8:0) was included in each sample as the internal standard. The percentage of total fatty acid content was calculated as the ratio of the individual FAME peak area to the sum of all FAME peak areas, excluding the internal standard. The weight of each fatty acid was calculated by noting the amount of internal standard (IS) that had been added, and then multiplying that amount by the ratio of the FAME area to the IS peaks.

III.3. Results and Discussion

III.3.1. Fermentation Profiles

The result for qualitative analysis of sugar components in OPT sap by TLC is shown in Figure 1. Accordingly, TLC analysis indicated that glucose and fructose were found as sugar components in OPT sap. Further analysis by HPLC prior to its usage for fermentation process, the glucose and fructose concentration approximately were found 85% and 25%, respectively.

The time courses for the carbon source consumption and the cell growth of *L. starkeyi* in different OPT sap medium conditions are shown in Fig. 2. In general, the yeast rapidly utilized glucose and fructose due to its complete carbon source consumption and produced approximately 30 g/L DWC within 4 d under different sap medium conditions. Under all condition tested, glucose was most quickly consumed followed by fructose. To gain further insight into the ability of *L. starkeyi* to utilize carbon source and grow, the carbon source consumption rates (Fig. 3A) and growth rates (Fig. 3B) were evaluated. The carbon source consumption rates and growth rates were either determined in the first 24 h (q_i) of fermentation process or when the carbon sources were completely consumed (q_t).

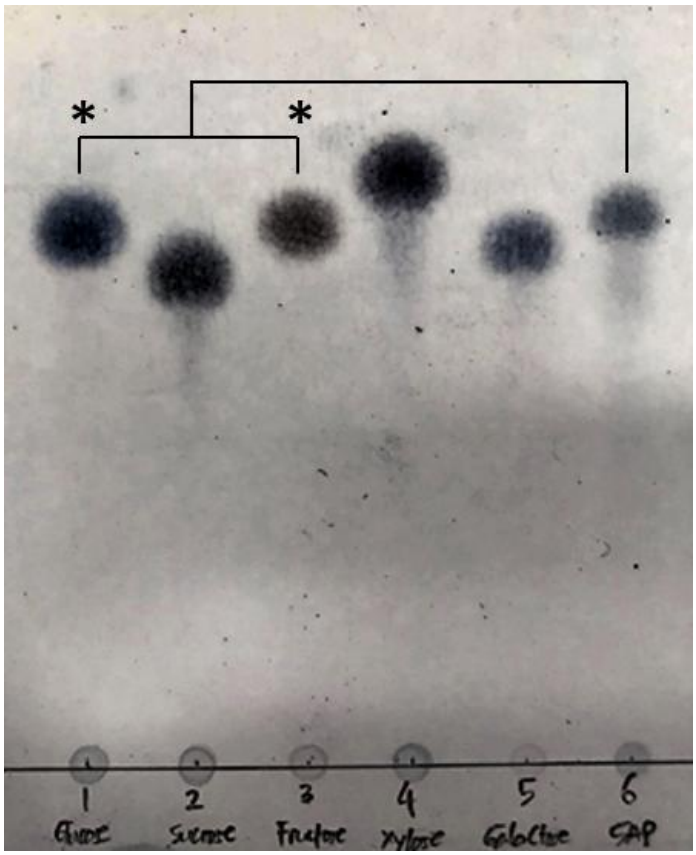

Figure 1. Sugar composition of OPT sap analyzed by thin layer chromatography. The sample (sap, No. 6) was diluted at 10 ppm. Several sugar standards: Glucose (No. 1); Sucrose (No. 2); Fructose (No. 3); Xylose (No. 4); Galactose (No. 5), were used. The concentration of each standard was set at 10 mg/L. 10 μl of each sample and standard were loaded on the plate. Symbol (*) indicates the major sugars contained in the SAP.

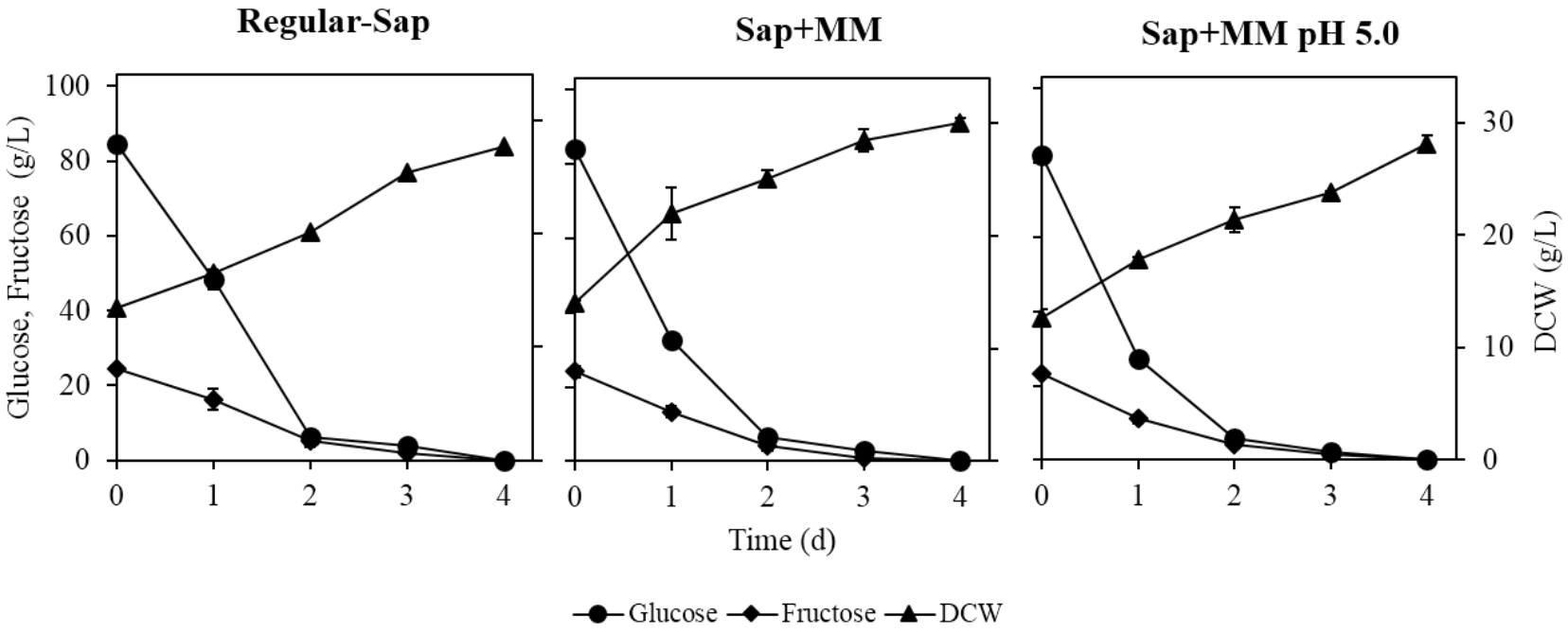

Figure 2. Time courses for carbon source consumption and cell growth of *Lipomyces starkeyi* in different sap medium. The closed circle represents glucose consumption, the closed diamond refers to fructose consumption, and the closed triangle charts for the cell growth.

L. starkeyi achieved the highest q_i for glucose at 2.3 g/L.h in the Sap+MM (pH 5.0) medium, following 2.2 and 1.5 g/L.h in Sap+MM and Reg-Sap mediums, respectively (Fig. 3A). A similar pattern was also observed for fructose, where high q_i was achieved at 0.5 g/L.h in Sap+MM (pH 5.0) and Sap+MM mediums, following 0.3 g/L.h in Reg-Sap medium. Enrichment of the OPT sap with mineral medium and adjustment of the initial pH into suitable fermentation condition seemed to induce the ability of yeast cells to consume carbon sources with higher rates at the first 24 h. Nevertheless, the consumption rates in the entire cultivation process (q_t) were indistinguishable for all medium conditions and achieved approximately at 0.9 g/L.h for glucose and 0.3 g/L.h for fructose. This condition indicated that although in the absence of mineral medium and without adjusting the initial pH, the fermentation process in Reg-Sap medium could achieve the carbon source consumption at the similar rate to Sap+MM and Sap+MM (pH 5.0) mediums. This could be attributed to the fact that the OPT sap was a complex mixture with various sugars, organic acids, vitamins, minerals and amino acids [12,13] which indicating that OPT sap has sufficient nutrients to support fermentation by *L. starkeyi*. Additionally, high level of initial inoculum concentration was applied in this study (at $OD_{600\ nm}$ 14.0-16.0) to initiate the fermentation process. A higher level of inoculum concentration leads to an increase in the active cell fraction, consequently, will leads to a higher cell/carbon source ratio and to an increase in the carbon-source consumption rate [19].

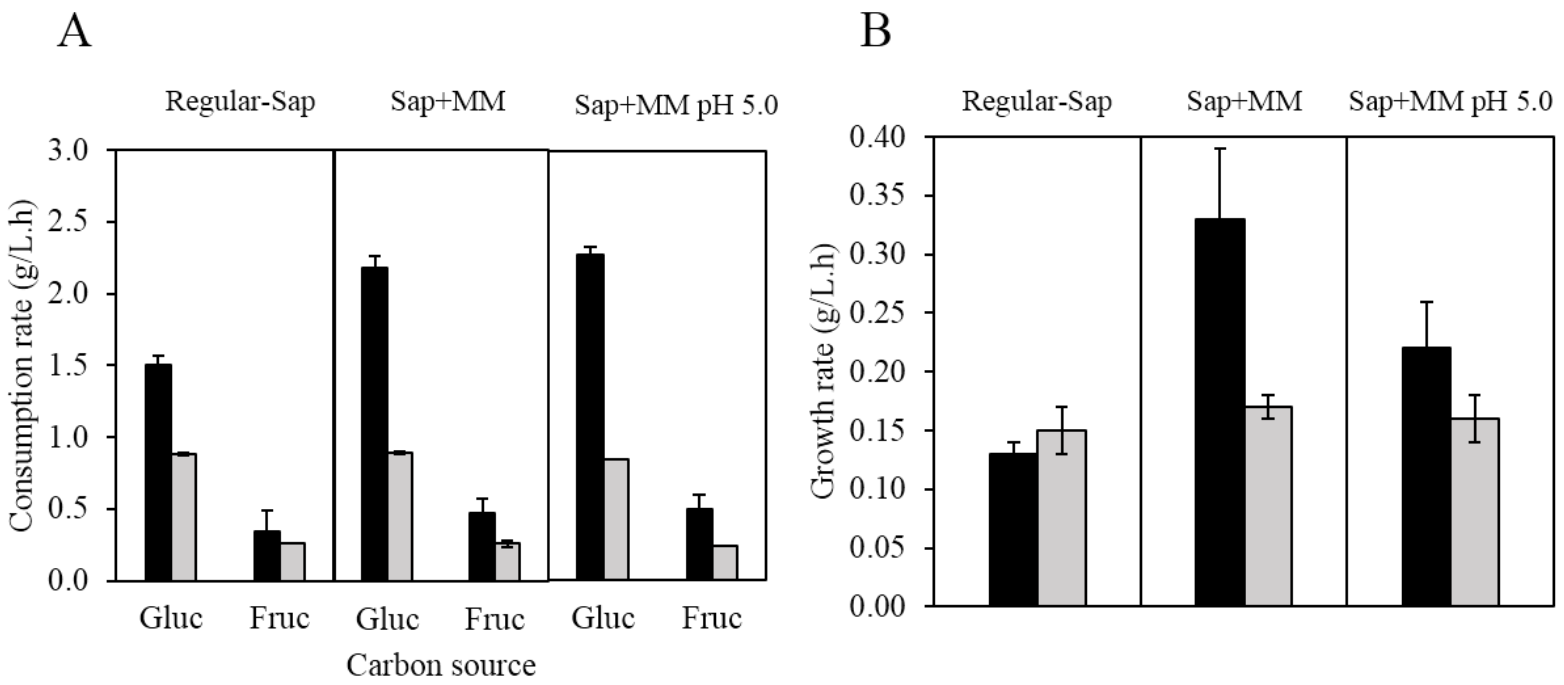

Figure 3. **A**: Carbon source consumption rate of *Lipomyces starkeyi* at the first 24 h (q_i: black bar) and over the whole fermentation process (q_t: grey bar) in the different sap mediums. **B**: Growth rate of *Lipomyces starkeyi* at the first 24 h (g_i: black bar) and over the whole fermentation process (g_t: grey bar)) in the different sap mediums.

The growth rate of *L. starkeyi* at the first 24 h (g_i) and over the whole fermentation process (g_t) in different condition of sap mediums are shown in Fig. 3B. Following the similar patterns of consumption rates, the growth rates of *L. starkeyi* in Reg-Sap medium at the first 24 h was lower (0.1 g/L.h) compared with two others medium condition (0.3 and 0.2 g/L.h, for Sap+MM and Sap+MM (pH 5.0) mediums, respectively). However, the growth rates of *L. starkeyi* over the whole fermentation process (g_t) were found at similar level, approximately at 0.2 g/L.h for all conditions tested. Hence, the OPT sap containing almost all the macro and micronutrients, therefore its suitable for enhance the cell growth of *L. starkeyi* even without mineral medium supplementation. Moreover, the maximum growth rate was also strongly influenced by the inoculum size.

III.3.2. Ethanol and Xylitol as Byproducts

Pentose fermenting yeasts have been reported to accumulate ethanol and a variety of polyols, including xylitol, ribitol, arabitol, glycerol, acetoin and 2,3-butanediol [25]. Among the oleaginous yeasts, only *Yarrowiya lipolytica* is known to be a significant producer of ethanol and other polyols [26]. However, recent studies by Calvey et al. [21], found that *L. starkeyi* produces significant amount of ethanol and other polyols compound under lipogenic conditions. In this study, a considerable amount of ethanol and xylitol were also produced as byproducts during *L. starkeyi* fermentation under all different sap medium conditions. The time course for ethanol and xylitol production under different sap medium conditions are shown in Fig. 4.

Under all condition tested, concentration of ethanol and xylitol increased overtime, and catabolism of accumulated alcohol only began after the exhaustion of carbon sources.

Due to the faster glucose and fructose utilizations in the medium Sap+MM (pH 5.0) (higher consumption rates in the first 24 h, Fig. 3A) led to complete assimilation of previously produced alcohol (ethanol and xylitol as shown in Fig. 4). Fermentation with Regular sap and Sap+MM as the medium were unable to metabolize the remaining ethanol in the time allotted (Fig.4). Lower initial pH might be responsible for this condition which reduce the number of active cells fraction to metabolize the remaining ethanol and xylitol. As a result, the pH was decreased over the course of fermentation process which could retarded cell growth and led premature death. The optimal initial pH for lipid production in *L. starkeyi* depends upon the carbon and nitrogen sources present in the media [18]. In our preliminary experiments, it was observed an optimum SCO production could be achieved by adjusting initial pH media approximately at pH 5.0 (data not shown). However, *L. starkeyi* has been characterized as a yeast tolerant to low pH and grows at pH 3.5 or even lower [27,28].

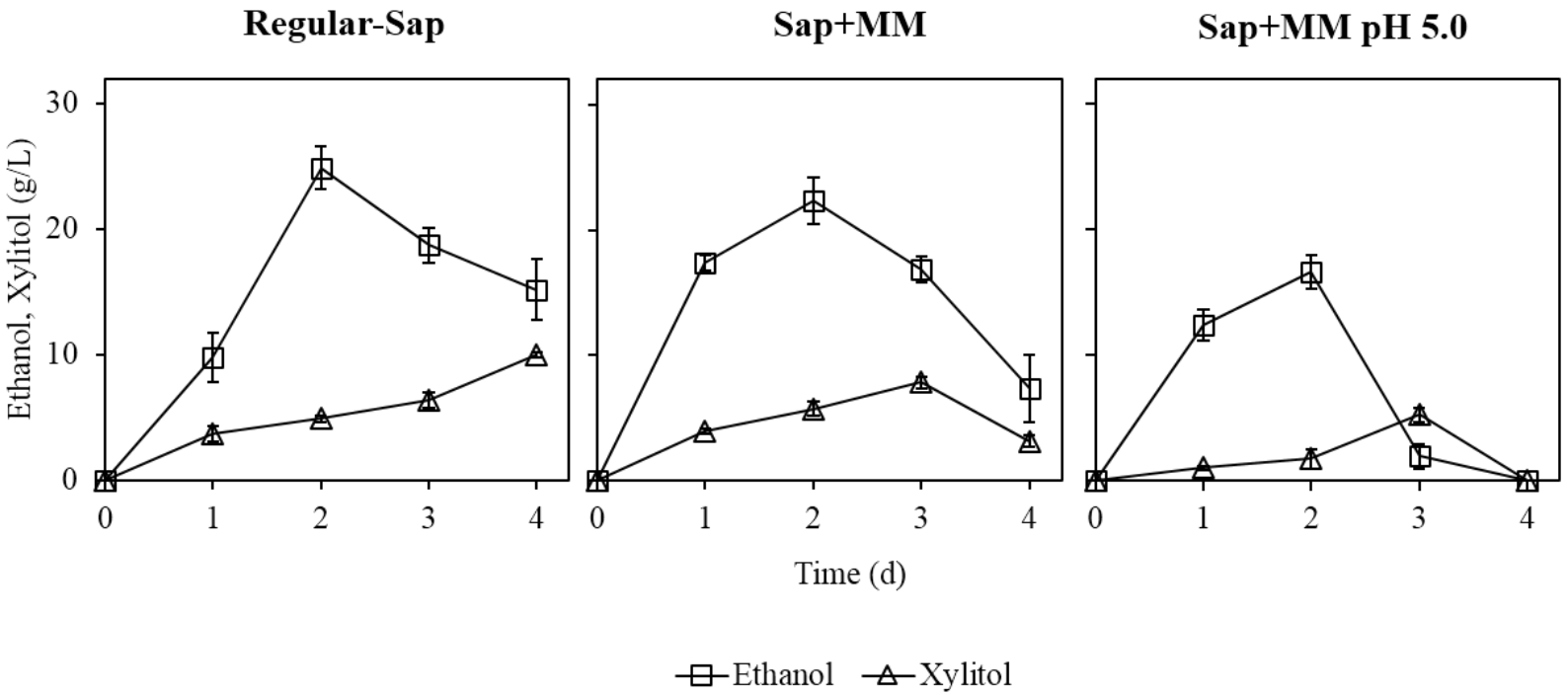

Figure 4. Time course for ethanol and xylitol as byproducts from fermentation of *L. starkeyi* under different sap medium conditions. The open square represents ethanol production; the open triangle refers to xylitol production.

Alcohols production was observed under all condition tested (Fig. 4). Accumulation of ethanol and xylitol began at the first 24 h. The higher ethanol accumulation was observed during 2 d fermentation in Reg-Sap medium with concentration achieved approximately 25 g/L. Higher xylitol accumulation was also observed in the fermentation with Reg-Sap medium with concentration approximately 10 g/L during 4 d. In general, the concentrations of each alcohol increased progressively. After the exhaustion of carbon sources, some alcohols were consumed by cells and probably used for production of cell mass and more lipids. Catabolism of alcohols followed a similar pattern in all conditions tested, except for xylitol in Reg-Sap medium. Supplementation of OPT sap with mineral medium and adjusted initial pH to the optimum fermentation condition for *L. starkeyi* led to more rapid catabolism of ethanol and xylitol after carbon sources completely consumed. As noted in *Y. lipolytica* by Workman et al. [26], the production of alcohols as by product in oleaginous yeast is occur due to cofactor imbalances, particularly an excess of NADPH, which are restored by the reduction of sugars into alcohols or by the reduction of pyruvate into ethanol or butanediol. Additionally, in the SCO production process, high sugar concentration in the medium may also result in the secondary metabolite formation [8], since the total sugar concentration in OPT sap was higher than 100 g/L.

III.3.3. SCO Production and Fatty Acid Profiles of Intracellular Lipid

Table 1 shows the SCO production of *L. starkeyi* grown on different sap medium conditions. The SCO production was expressed as the lipid content when the carbon sources were completely consumed during fermentation process. According to Table 1, the SCO productions from approximately 110 g/L total carbon sources (glucose+fructose)

generally reached more than 50% (w/w) of DCW. The highest lipid content of 64.4±1.9% (w/w) was achieved from the fermentation of *L. starkeyi* in Sap+MM medium with a lipid yield of 0.19±0.02 (w/w), followed by fermentation in Sap+MM (pH 5.0) medium which produced 63.1±1.6% (w/w) lipid content with lipid yield of 0.17±0.00 (w/w). For the fermentation in Reg-Sap medium, the lipid content achieved was 55.2±1.9% (w/w) with lipid yield of 0.15±0.01 (w/w). The high lipid accumulation in fermentation process with Sap+MM and Sap+MM (pH 5.0) media was probably due to complete assimilation of alcohols (ethanol and xylitol) as byproducts, which led to high biomass (DCW) and lipid production. Additionally, the supplementation of OPT sap with mineral medium and adjusting initial pH could provide beneficial nutrition and suitable medium environment for continuous cell growth. However, the use of Reg-Sap as the fermentation medium also produced good lipid content of higher than 50% (w/w) with no requirement for any additional mineral medium or adjusting initial pH, which is beneficial for the process economics.

Table 1. SCO production comparison by *L. starkeyi* in the different sap medium conditions [a]

Medium types	DCW (g/L)	DCW yield (w/w, %)	Lipid content (w/w %)	Lipid yield (w/w)	Cultivation time (d)
Regular-Sap	27.7±0.7	25.4±0.6	55.2±1.9	0.15±0.01	4
Sap+MM	30.1±0.4	27.9±0.5	64.4±1.9	0.19±0.02	4
Sap+MM pH 5.0	28.1±0.8	26.7±0.3	63.1±1.6	0.17±0.00	4

[a] The values are given as the mean ± SD of triplicate determinations.

DCW yield: gram of dry cell biomass per gram of sugar consumed ×100%.

Lipid yield: milligram of lipid per milligram of sugar consumed.

The pH of oleaginous yeast cultures is an important consideration, as lipid production is significantly reduced at low pH values in some species [7]. The SCO production results in the media Reg-Sap and Sap+MM indicated that *L. starkeyi* is capable to thriving and accumulating lipids under acidic conditions (pH at the initial fermentation process was 3.2). This also may be beneficial in consideration of biodiesel economics, as pH control adds to the production cost of any fermentation process [29]. Furthermore, acidic pH can help to suppress the growth of bacterial contaminants, which is the major concern in industrial microbiology [30].

A summary of the fatty acid methyl ester profiles obtained from these experiments is shown in Table 2. On average, the profile of total accumulated lipids was comprised of approximately 33% palmitic acid (C16:0), 2% palmitoleic acid (C16:1), 6% stearic acid (C18:0), 42% oleic acid (C18:1), and 4% linoleic acid (C18:2). These results are similar to lipid profiles obtained by others for this oleaginous yeast strain [21,30-32], although slight differences were observed which can be explained by variability between *L. starkeyi* strains and culture conditions [29]. The fatty acid profile of *L. starkeyi* was remarkably similar to that of palm oil, which is one of the most common vegetable oil feedstock used in biodiesel production (Table 2). This observation suggest that biodiesel produced from lipids extracted from this species would have desirable fuel properties and would be compatible with existing diesel engine technology. This is not the case in most oleaginous fungi and algae, which have elevated levels of polyunsaturated fatty acids which are undesirable in biodiesel fuels [21,33,34]. Moreover, there has been considerable interest in high oleic oils for biofuels with improved oxidative stability and cold-flow properties, and as input for many renewable polymers, lubricants, elastomers, and other oleochemicals [35-37].

For the first time, we have successfully demonstrated the potential of OPT sap as a novel inexpensive renewable carbon feedstock for SCO production by *L. starkeyi* NBRC10381. High lipid content at around 55% (w/w) could be achieved in 4 d, even when the raw material (Reg-Sap) was used directly as the medium. Addition of some minerals (MM) could enhance the lipid content up to around 66% (w/w). Moreover, the profile of fatty acids indicated that the quality of lipid produced by the strain was considerable as a material not only for biodiesel production but also for high-valued fatty acid production.

Table 2. Comparison of fatty acid profiles of *L. starkeyi* at different sap mediums [a]

Medium types	FAME compositions										
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	C24:0
Regular-Sap	0.0±0.0	0.4±0.1	30.3±1.1	1.6±0.6	6.3±1.4	43.7±1.8	5.7±0.6	0.3±0.0	0.1±0.0	0.0±0.0	0.5±0.1
Sap+MM	0.0±0.0	0.1±0.0	34.6±0.8	1.22±1.0	8.2±0.7	39.3±2.4	3.0±0.9	0.7±0.1	0.1±0.0	0.0±0.0	0.2±0.0
Sap+MM pH 5.0	0.0±0.0	0.2±0.0	33.3±0.4	2.8±0.9	4.8±1.4	42.0±1.9	3.6±0.7	0.4±0.1	0.1±0.0	0.0±0.0	0.3±0.1
Average	0.0	0.2	32.7	1.9	6.4	41.7	4.1	0.5	0.1	0.0	0.3
Palm oil[b]	0.1	0.7	36.7	0.1	6.6	46.1	8.6	0.3	0.4	0.1	0.0

[a] The values are given as the mean ± SD of triplicate determinations.

[b] Palm oil fatty acid profile as reported by Ramos et al. [38].

III.4. Conclusions

This work has demonstrated that *L. starkeyi* could grow well in each condition of OPT sap medium and efficiently accumulate lipids. The OPT sap represents almost all the macro and micronutrients required for good cell growth. Moreover, the supplementation of OPT sap with MM and adjusting initial pH prior to the fermentation process was not necessary, which is beneficial for the process economics. Results from this study clearly demonstrate the potential *L. starkeyi* as a microbial lipid producer as well as OPT sap as an important renewable carbon feedstock for SCOs production.

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General Conclusion and Future Research

Single cell-oil (SCO) from oleaginous yeast is a renewable noncrop-based oil source that can be used for the production of bio-based fuels, chemicals and other petroleum counterparts. Development of an integrated approach will be necessary for economical production including: improved fermentation and utilization of low-valued feedstock. The purpose of this doctoral research was to evaluate the important step for an integrated approach for efficient SCO production from low-cost renewable resources. Key findings from our studies include:

1. The development of appropriate cultivation strategy for SCO production that could rapidly accumulate lipids with high productivity and high cell biomass simultaneously in typical culture media.
2. SCO from *Lipomyces starkeyi* is naturally high in oleic acid (C18:1), which is a key feature for improved biofuel performance and as an input for promising oleochemicals.
3. The development of stress tolerance in oleaginous yeasts is a significant challenge for cost-competitive lipid production. 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 by *Lipomyces starkeyi*.
4. 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. A combined approach has been attempted to

evaluate the potential of a novel inexpensive renewable carbon feedstock for SCO production.

One of the promising aspects of oleaginous yeast is their ability to utilize hemicellulose. As mentioned earlier, this has been a major challenge for the cellulosic biofuels industry. With the advent of biorefineries capable of generating multiple products including fuels, chemicals, and power from plant-derived biomass feedstock, oleaginous yeast could be adopted as a value-added platform for hemicellulose bioconversion.

Further work is required to commercialize SCO biochemical platform for renewable fuels, chemicals, and polymer products. Currently, lower cost alternatives from petroleum and oilseed crops have a limitation in the production of SCO as a commodity-type oil. Developing of processing technology that can improve the productivity and cost competitiveness of SCO will be important for industrial scale implementation. To address this, innovative approaches will be needed across the major processing steps including effective pretreatment techniques, improved SCO fermentation and efficient downstream processing. Additionally, performing economic and life cycle analysis would be helpful to determine the profitability and sustainability of SCO platforms. Key areas of future research include:

1. Fermentation strategy: to improve the cell biomass and SCO productivities by developing novel fermentation technique to meet the different demands of growth and oil accumulation.
2. Metabolic engineering: to increase oil yield and titer as well as tailored lipid profiles for specialty oils via biochemical pathway engineering.

3. Identifying and characterizing robust and efficient wild type yeast species for industrial scale of SCO production
4. Downstream processing: to improve oil extraction and purification by employing efficient, cost effective and sustainable extraction systems
5. Sustainable process development: to determine the economic and environmental impact of a renewable SCO biochemical platform by performing economic and life cycle analyses.

Publication List

Chapter I

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. 125 (2018) 695-702.

Chapter II

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, Selection of oleaginous yeast capable of high lipid accumulation during challenges from inhibitory chemical compounds. Biochem. Eng. J. 137 (2018) 182-191.

Chapter III

A.B. Juanssilfero, P. Kahar, R.L. Amza, K. Sudesh, Yopi, C. Ogino, B. Prasetya, A. Kondo, Lipid production by *Lipomyces starkeyi* using sap squeezed from felled old oil palm trunks. (Submitted to Biochemical Engineering Journal)

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