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ISOLATION OF OLEAGINOUS YEASTS AND OPTIMIZATION OF SINGLE CELL OIL FOR BIODIESEL PRODUCTION

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Approved by Examining Board

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Abstract

Oleaginous yeasts are known to produce oil with high potential as source of biodiesel. In this study, 340 yeast colonies were isolated from 200 samples that were collected from natural sources in Ethiopia. All the yeast isolates were screened using Sudan III staining for oil production. Among these, 18 were selected as possible oleaginous yeasts. Identification of the 18 isolates was done using morphological and physiological methods as well as sequencing of the internal transcribed spacer regions (ITS; ITS 1, ITS 2 and the intervening 5.8S rRNA gene), and the D1/D2 domain of the 26S rRNA gene. Molecular phylogenetic analyses indicate that isolates PY39, SY89 and SY94 are species of *Cryptococcus curvatus*, *Rhodosporidium kratochvilovae* and *Rhodotorula dairenensis*, respectively, while the rest (SY09, SY18, SY20, PY21, PY23, PY25, SY30, PY32, SY43, PY44, SY52, PY55, PY61, SY75, and PY86) were identified as *Rhodotorula mucilaginosa*. From these *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were selected for further activities based on their substantial lipid producing capacities. To determine the optimal cultivation conditions for oleaginous yeasts, different carbon and nitrogen sources, carbon to nitrogen ratio, pH and inoculum size were investigated. Moreover, incubation temperature, shaking speed, culture volume (aeration rate) and duration of cultivation were investigated. Wide variations were recorded in the cultivation conditions that lead to maximum lipid production by the yeasts under test. The maximum lipid production was attained within 120-144 h, using 50-70 g/L glucose as a carbon source, 0.50 g/L yeast extract and 0.31-0.85 g/L, $(NH_4)_2SO_4$ as nitrogen sources, at C/N ratio of 100-140, pH range 5-6, 10% inoculum size as seed culture, 30^oC incubation temperature, shaking speed of 200- 225 rpm and 50 mL culture medium. Lipid content was determined by solvent mixture of chloroform and methanol (2:1). Under the optimized cultivation conditions,

Cryptococcus curvatus PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 accumulated lipids up to 7.22±0.26, 5.73±0.62, and 6.47±0.05 and 7.65±0.77 g/L, respectively on dry weight basis. Such values correspond to lipid content of 48.66±0.60, 38.38±3.90, 40.74±0.54 and 51.17±0.72%, respectively. These strains were further grown on media containing peel mixtures of papaya and mango. Under the optimized conditions, *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 gave lipid yields and lipid contents of 3.95±0.67 g/L and 35.02±1.63%, 2.66±0.49 g/L and 28.15±1.63%, 3.84±0.19 g/L and 36.76± 0.61%, and 4.31± 0.30 g/L and 35.18±1.40%, respectively. The fatty acids profiles were analyzed using gas chromatography. Data revealed the presence of high amount of oleic acid (47.44±2.14–54.40±1.15%), palmitic acid (10.69±0.66–24.04±0.39%), linoleic acid (6.34±0.64–21.24± 0.36%) and low amount of other fatty acids in the extracted yeast oils which indicate that the fatty acid profiles fit well with that of conventional vegetable oil. Furthermore, lipid production capacity of *Rhodosporidium kratochvilovae* SY89 was evaluated using molasses as a substrate in a bioreactor and gave a maximum lipid concentration of 4.82±0.27 g/L which corresponds to 38.25±1.10% of lipid content. The extracted lipid was transesterified into biodiesel and gave a yield of 85.30%. The properties of this biodiesel were determined and found to be comparable to the specifications established by ASTM D6751 and EN14214 related to biodiesel quality. In conclusion, this study revealed the possibility of using the promising yeast isolates in biodiesel production.

Keywords/phrases: Biodiesel, biomass, cultivation conditions, fatty acid, lipid content, lipid concentration, oleaginous yeast, single cell oil

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I praise Almighty God!!!

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List of Abbreviations and Acronyms

AA	Arachidonic Acid
Acetyl- COA	Acetyl coenzyme A
ACC.NO.	Accession Number
AFLP	Amplified Fragment Length Polymorphism
ALA	Alpha-Linolenic Acid
AMP	Adenosine Mono Phosphate
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine Tri Phosphate
DBB	Diazonium Blue B
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic Acid
DNS	Di Nitrosalicilic Acid
EPA	Eicosapentaenoic Acid
FAME	Fatty Acid Methyl Esters
FAS	Fatty Acid Synthase
GC	Gas Chromatography
g/L	Gram per Liter
ITS	Internal Transcribed Spacer
LA	Linoleic Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acids
MLST	Multilocus Sequence Typing

NADPH	Nicotine Amine Dinucleotide Phosphate Hydrogen
NCBI	National Center for Biotechnology Information
NJ	Neighboring Joint
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
PUFA	Polyunsaturated Fatty Acids
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RPM	Revolution per Minute
SCO	Single Cell Oil
SD	Standard Deviation
SFAs	Saturated Fatty Acids
SPSS	Statistical Packages for Social Sciences
STEs	Steryl Esters
TAGs	Triacylglycerols

Published and Submitted Papers from the Dissertation

This dissertation is based on the following papers which are included in different chapters.

Tamene Milkessa Jiru and Dawit Abate (2014). Oleaginous microorganisms, diversity, lipid biosynthesis pathway and strain improvement. *WebPub Journal of Scientific Research*. **2**: 55–65.

Tamene Milkessa Jiru, Dawit Abate, Nicholas Kiggundu, Carolina Pohl, Marizeth Groenewald. (2016). Oleaginous yeasts from Ethiopia. *AMB Express*.**6**:78.

Tamene Milkessa Jiru, Dawit Abate, Nicholas Kiggundu, Laurina Styen, Carolina Pohl, Marizeth Groenewald. (2017). Optimization of cultivation conditions for production of lipid by *Rhodotorula kratochvilovae* (syn, *Rhodosporidium kratochvilovae*) SY89 for biodiesel preparation. *3 Biotech*.**7**:145. DOI: 10.1007/s13205-017-0769-7.

Tamene Milkessa Jiru, Laurina Styen, Carolina Pohl and Dawit Abate. (2017). Production of Single Cell Oil from Cane Molasses by *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) SY89 as a Biodiesel Feedstock (Submitted to BMC Biotechnology Journal).

Chapter 1: Introduction

1.1 General background

Microorganisms capable of accumulating lipids more than 20 to 70% of their dry weight are termed oleaginous (Ratledge and Wynn, 2002; Wynn and Ratledge, 2006). They can be used to extract microbial lipids commonly, triacylgycerols (TAGs), investigated and exploited as alternative sources of oils and fats for human consumption (Ratledge, 2005) and, increasingly, for renewable energy (biodiesel) production (Li *et al*., 2008). Oleaginous microorganisms include some selected species of yeasts, filamentous fungi, microalgae and bacteria (Sijtsma *et al*., 1998; Subramaniam *et al*., 2010). The well-known oleaginous yeasts belong to the genera *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodotorula*, *Rhodosporidium*, *Yarrowia* (Li *et al*., 2008; Rossi *et al*., 2009) and *Trichosporon* (Gujjari *et al*., 2011; Hu *et al*., 2011). The most investigated oleaginous filamentous fungi include *Mortierella alpina* (Sakuradani *et al*., 2009), *Mucor circinelloides* (Wynn *et al*., 2001) and *Umbelopsis isabellina* (Meeuwse *et al*., 2011) and *Mortierella isabelina*, *Cunninghamella echinulata* and *Thamnidium elegan* (Fakas *et al*., 2008). Microalgae such as *Chlorella vulgaris* and *Spirulina platensis* can also accumulate oils (Chisti, 2007; Shalaby, 2011). Bacterial species belonging to *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Streptomyces* can accumulate oils under suitable cultivation conditions (Alvarez and Steinbuchel, 2002).

Oil accumulation by oleaginous microorganisms depends on the culturing conditions such availability of carbon and nitrogen sources, and supply of mineral salts, carbon to nitrogen (C/N) ratio, cultivation period and incubation temperature, pH of the medium, agitation and aeration rates (Li *et al*., 2008; Ageitos *et al*., 2011; Zhao *et al*.,2015).

Optimum C/N ratio of greater than 20 is needed for maximum accumulation of lipids by oleaginous microorganisms (Papanikolaou and Aggelis, 2011). The specific need for C/N ratio depends on the nature of the microorganism, the medium composition as well as the carbon and nitrogen sources (Ykema *et al*., 1986). Lipid accumulation in oleaginous yeasts occurs when the growth medium is replete (excess) in carbon source with nitrogen limitation (Wu *et al*., 2011).

The initial pH of the culture medium also plays a critical role on lipid synthesis by oleaginous microorganisms. Different optimum pH values for maximum production of lipids by different oleaginous yeasts were reported in previous studies (Tao *et al*., 2008; Zhu *et al*., 2008; El-Fadaly *et al*., 2009; Kraisintu *et al*., 2010). The optimum pH for specific oleaginous yeast for biomass production and single cell oil (SCO) is also influenced by the different carbon sources in the growth medium (Angerbauer *et al.*, 2008).

Cellular lipid accumulation is critically affected by culture temperature. Too high or too low temperature affects the cell growth and lipid accumulation (Sha, 2013). The composition of the lipid also varies at different temperatures (Saxena *et al*., 2009).

Incubation period has an effect on lipid production (Leesing *et al*., 2011). It is recommended that cells should be harvested at early stationary phase to prevent lipid degradation (Beopoulos *et al*., 2008).

Other factors such as aeration rate and agitation speed (Higashiyama *et al*., 2002; Liang *et al*., 2006; El-Fadaly *et al*., 2009; Liu *et al*., 2010) have pronounced effect on lipid accumulation by oleaginous microorganisms. Availability of metal ions such as Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Ca^{2+} can also affect oil accumulation to a varied extent (Li *et al*., 2008).

Fatty acid synthesis in general involves the conversion of acetyl-COA into the long chain fatty acid palmitic acid (C16:0). Acetyl-COA carboxylase catalyzes the formation of malonyl-CoA from acetyl-COA. This is the first step in the formation of fatty acid. Subsequently 7 malonyl-COA molecules come together with acetyl-COA converted to palmitic acid (Sijtsma *et al*., 1998). Fatty acid synthase enzyme complexes are involved in chain elongation processes (Sijtsma *et al*., 1998). Desaturases are other groups of enzymes involved in the formation of polyunsaturated fatty acids (PUFAs) (Wynn and Ratledge, 2006).

Petroleum-based fuel is a limited and non-renewable energy resource. It is not environmentally friendly since it has high CO_2 and sulphur emissions to the atmosphere and hence contributes to the increase in emissions to global warming (Ahmad *et al*., 2013).To substitute such petroleum based-diesel, the use of biodiesel which is a useful alternative biodegradable, nontoxic renewable energy resource has been recommended (Li *et al*., 2008; Kraistinu *et al*., 2010). Currently, biodiesel is produced from plant oils and /or animal fats by transesterification with short chain alcohols like methanol (Vicente *et al.,* 2004; Pan *et al*., 2009; Liu *et al*., 2010). Manufacturing biodiesel from vegetable oil or animal fat is undesirable because this competes with the use of these oils/fats for the food market and the high cost of such oils as feedstocks (Vicente *et al.,* 2009; Kraistinu *et al*., 2010). Microbial oils are potential sources for biodiesel production and are hoped to substitute the above feedsocks (Li *et al*., 2008).

The desirable biodiesel properties include cetane number, cloud point, cold filter plugging point, kinematic viscosity, flash point, iodine value, oxidation stability, pour point, acid value, carbon residue, lubricity, fuel stability, heating value, solidifying point, free glycerin, total glycerin, sulphated ash and ash content (Knothe, 2006; Minnesota Statutes, 2008;Dunn, 2011). They are the most important parameters in determining the quality of diesel fuel.

1.2 Statement of the problem

Ethiopia is a developing country with no crude (petroleum) oil but with increasing energy demand. As a result, the use of petroleum oil is increasing steadly. Even if the use of petroleum oil has its own negative effect, it is still useful for countries like Ethiopia. To purchase and transport petroleum oil from aboard, a considerable amount of foreign currency is spent to meet the annual demand of the country. Therefore, efforts in the direction of biodiesel research and development are crucial.

Biodiesel is produced from vegetable oil or animal fat feedstocks. Manufacturing biodiesel from vegetable or animal fat competes with the use of these oils for human consumption. Especially, the biodiesel produced from edible plant feedstocks competes with the food supply as demand for more biodiesel is increased. Furthermore, manufacturing biodiesel from plant feedstocks such as *Jatropha*, Castor (*Ricinus*) etc. takes up farm land useful for food crop production. Still with these limitations of producing biofuel using *Jatropha*, *Ricinus* bean, Palm oil and sugar cane, the government of Ethiopia has drafted a strategy, Ethiopian Biofuel Development and Utilization Strategy. This strategy is targeted to supply fuels from locally produced biofuel (*Jatropha*, *Ricinus* bean, Palm oil and sugar cane) and the objective of the strategy is to ensure the production of biofuel without significantly affecting food self-sufficiency, to improve balance of payment and to reduce environmental impacts.

This necessitates the need for alternative substitution of petroleum oil for the national demand for development. Thus biodiesel which is an environmentally friendly energy source has to be produced from oleaginous microorganisms. This is the first systematic study on evaluation of microorganisms for biodiesel in the country.

In the current study, yeasts were selected for the production of single cell oil and biodiesel over algae, bacteria and filamentous fungi. Unlike algae, yeasts do not require sunlight for their growth and biomass accumulation. Moreover, yeasts can be cultivated in a confined environment in conventional fermenters, and are also able to metabolize a number of carbon sources. While bacterial species accumulate triacylglycerols, others accumulate poly-β-hydroxybutyrte and alkanaoates. Some others accumulate diacylglycerols and wax esters. On the other hand, yeasts accumulate purely triacylglycerols which is the desirable lipid for biodiesel production. Filamentous fungi accumulate mostly polyunsaturated fatty acid which is not desirable for biodiesel production, while yeasts accumulate saturated and monounsaturated fatty acids which are desirable for biodiesel production.

1.3 Research Objectives

1.3.1 General objectives

- To isolate, screen, identify and characterize high oil producing yeasts from natural sources and optimize conditions for high single cell oil accumulation and production of biodiesel from yeast oils.

1.3.2 Specific objectives

- To isolate, screen, and identify potential oleaginous yeasts from natural sources.
- To characterize and optimize cultivation conditions of selected yeasts for high lipid production.
- To transesterify yeast oil to biodiesel.

Chapter 2: Literature Review

2.1 Diversity and ecology of yeasts

Microorganisms are diverse and are found in almost every natural habitat. Yeasts, like other microorganisms are ubiquitous in nature. Yeasts represent a part of the microbiota in all natural ecosystems, such as soils, fresh and marine waters from the ocean surface to the deep sea (Kutty and Philip, 2008) and are found distributed in the different natural ecosystems and environments. Some group of yeasts also colonize more extreme environments, such as low temperatures, low oxygen availabilities, and oceanic waters (Butinar *et al*., 2007). Yeasts inhabit moist environments where there is an abundant supply of sugar-rich sources. Carbon and energy sources as well as the mineral content of a specific habitat influence the type of yeasts present in that habitat (Phaff, 1986; Lachance, 1996). They are known to be common inhabitants of leaf, fruit surfaces and roots as well as various types of food. Only few types of yeasts have the capacity to degrade polymers like starch and cellulose. Both agricultural and forest soil (Sláviková and Vadkertiová, 2003) are habitats of certain groups of yeasts. Yeasts also occur in the intestinal flora of mammals (intestinal commensals) (Lachance, 1996) and some insects (Martini, 1992).Yeasts play a pivotal role in the food chain, and in the carbon, nitrogen and sulphur cycles. Yeasts act as agents of pollution, bioremediation or biological pest control as well as central components of some industrial processes (Pöggeler, 2001).

In a similar fashion, oleaginous yeasts have been isolated from diverse natural habitats such as from various soils, water bodies and flower surfaces (Breuer and Harms, 2006; Dai *et al*., 2007; Pan *et al*., 2009; Amaretti *et al*., 2010). The common habitats of an oleaginous yeast, *Yarrowia lipolytica* are oil-polluted environments and foods such as cheese, yoghurt, kefir, meat, and poultry

products (Rossi *et al*., 2011). *Debaromyces hansenii* is an osmo-, halo- and xerotolerant lipid-accumulating oleaginous yeast (Breuer and Harms, 2006). It is known to be a contaminant of various foods with low water activity. *Debaromyces hansenii* is found in hypersaline waters, such as the salterns on the Atlantic coast in Namibia and the Great Salt Lake (Breuer and Harms, 2006). Pan *et al.* (2009) isolated 20 potential oleaginous yeasts from soil. Dai *et al*. (2007) isolated the known oleaginous yeast *Rhodotorula glutinis* from flower samples. An oleaginous psychrophilic yeast, *Rhodotorula glacialis* AS4.7 was isolated from glacial environment and the strain abundantly grew and accumulated lipids from a temperature of -3 up to 20ºC (Amaretti *et al*., 2010). About 65% of *Candida* species are unable to grow at 37°C (non -pathogens) and are potential oil producing yeasts (Calderone, 2002). Among these, *Candida curvata* D (Holdsworth and Ratledge, 1991) and *Candida freyschussii* (Amaretti *et al*., 2011) synthesize and store significant amount of lipids. Yeasts of the genus *Cryptococcus* are widely distributed in nature and may be isolated from various substrates such as air, soil, bird excreta, water, animal surfaces and mucosa, leaves, flowers and decomposing wood. Most species of *Cryptococcus* are considered as free-living (non-symbiotic) and only a few have medical importance being responsible for disease in man and animals (*Cryptococcus neoformans* and *Cryptococcus gattii*). An oleaginous yeast, *Cryptococcus curvatus* (Meesters *et al*., 1996) is also recognized as an opportunistic pathogen of animals, including humans (Findley *et al*., 2009). *Trichosporon* are basidiomycetous yeasts widely distributed in nature, consisting of soil and water-associated species, predominantly found in environmental substrates, such as decomposing wood. They are occasionally found in the gastrointestinal microbiota of humans as well as transiently colonize the skin and respiratory tract (Rossi *et al*., 2011). *Trichosporon cacaoliposimilis* and *Trichosporon oleaginosus* (Gujjari *et al*.,

2011) and *Trichosporon cutaneum* (Hu *et al*., 2011) are the best studied oleaginous yeasts belonging to this genus.

2.2 Isolation and cultivation of yeasts

As heterotrophic organisms, yeasts require carbon, nitrogen, phosphorus, trace elements and other growth factors for metabolism and growth. Yeasts mostly occur together with bacteria and/or molds. Thus, selective techniques should be used for recovery of yeasts, employing media that permit the yeasts to grow, while suppressing molds and bacteria (Kurtzman *et al*., 2011). Isolation procedures include direct plating, dilution, and enrichment (Kurtzman and Fell, 2004).

In direct plating, samples are placed directly on an agar medium and allowed to grow for at least 24 h (Kurtzman and Fell, 2004). Selected yeast colonies are transferred into another agar medium as streak and grown as pure cultures. Dilution procedures involve thoroughly mixing a known quantity of sample with a given volume of sterile water in a test tube and then making a dilution series. At each successive step of the series, the sample is maintained in an increasingly dilute aqueous suspension. The suspensions are spread onto isolation media, and colonies are recorded and enumerated (Kurtzman and Fell, 2004). Enrichment procedures consist of placing a given sample in conical flask with a nutrient medium tailored to the needs or preferences of the target yeast strain being sought. The composition of the growth medium is, therefore, customized by adding specific carbon and nitrogen compounds, vitamins, or other growth factors (Kurtzman and Fell, 2004).

Yeast isolation medium include peptone yeast extract glucose medium agar (PYG) or peptone, yeast extract, malt extract, glucose medium agar (YM) (Yarrow, 1998). Various carbon sources can be substituted for glucose. Such growing media are acidified using hydrochloric acid or

phosphoric acid as most yeast species prefer acidic pH. The growing media may include antibiotics such as chloramphenicol, tetracycline, and combination of penicillin G and streptomycin sulfate for suppression of bacteria or fungistatic agents such as propionic acids for suppression of molds. Acidification of the media is preferred over the incorporation of antibiotics and fungistatic agents (Lachance and Starmer, 1998). Fungistatic agents are to be used with caution as some of these compounds may also inhibit certain yeasts (Yarrow, 1998).

Mesophilic yeasts are incubated at 20 to 30°C. Optimum temperatures for growth are higher for some yeasts and lower for others. Psychrophilic yeasts grow at temperatures between 4°C and 15°C. Yeasts that are associated with warm-blooded animals grow in the range of 30–37°C (Yarrow, 1998).

2.3 Yeast taxonomy and identification

2.3.1 Oleaginous yeast taxonomy

Before the molecular era of yeast taxonomy, species and genera including oleaginous ones were defined on the basis of phenotypic characters such as cell morphology, mode of sexual reproduction, physiological and biochemical activities. In recent years, however, research with genetic crosses, as well as molecular comparisons, have shown that many of the phenotypic characters considered to be taxonomically definitive vary among strains of the same species (Kurtzman and Fell, 2004). As a consequence, divergent strains of known species may be mistaken for novel taxa, whereas new species may go unrecognized. Currently, DNA nucleotide sequences of unknown strains of yeasts are being compared with those of authentic reference strains for species identification (Kurtzman and Fell, 2004). The molecular era started with the determination of the mol% G+C relations of nuclear DNA, followed by the DNA re-association technique and sequencing.

Yeasts are a polyphyletic group of fungi with the ability to grow unicellularly. Currently, there are more than 2000 recognized yeast species (www.mycobank.org). They are estimated to constitute 1% of all described fungal species (Kurtzman and Piškur, 2006).Yeasts are grouped into three major subdivisions: Ascomycotina, Basidiomycotina and Deuteromycotina (Phaff, 2001). Ascomycetous yeasts produce variously shaped ascospores following meiosis (reduction division) of a diploid nucleus inside an ascus (a sac-like structure) that is not enclosed in a complex fruiting body or ascocarp (Phaff, 2001). Basidiomycetous yeasts produce a basidium (in some genera form thick-walled teliospore) on which external basidiospores are formed following meiosis. Deuteromycotina are asexual, imperfect or anamorphic yeasts (Phaff, 2001) and lack sexual state (Deak, 2008).

2.3.2 Yeast identification

2.3.2.1 Conventional methods

Cultural characteristics

Cultural characteristics are conventional yeast identification and classification methods which involve observation of colony color, shape and texture on agar media. For example, the presences of red, orange or yellow carotenoid pigments are characteristic feature of genera *Phaffia*, *Rhodosporidium* and *Sporidiobolus* (Kurtzman *et al*., 2011). Majority of yeasts produce color ranging from whitish or cream to buff (Yarrow, 1998; Kurtzman *et al*., 2011).The texture can vary from mucoid, viscous, butyrous, friable to membranous. Muciod growth is often associated with the production of extracellular polysaccharides (Kurtzman *et al*., 2011).

Morphology of asexual structures

Morphological characteristics like cultural and reproductive features usually help to delimit genera (Kurtzman *et al*., 2003). Important morphological features include shape and size of vegetative

cells, budding pattern (unipolar, bipolar or multipolar), fission, mode of conidia formation (absence or presence of arthroconidia, blastoconidia, ballistoconidia, clamp connections, endoconidia), germ tubes, hyphae, pseudohyphae, sporangia and sporgangiospores (McGinnis, 1980). Shape of cells indicate the mode of vegetative reproduction. Budding is characteristic of the *Saccharomycetales* (Yarrow, 1998; Kurtzman *et al*., 2011) as asexual reproduction. Some yeasts reproduce asexually by fission, e.g., *Schizosaccharamyces pombe* (Yarrow, 1998; Barnett *et al*., 2000). Others, such as species in the genus *Trichosporon* reproduce by both budding and fission (Barnett *et al*., 2000).

Shapes of vegetative cells vary from globos to ellipsoidal, ovoidal, cylindrical, elongate, apiculate, lunate or triangular are useful characteristics for identification of yeasts (Yarrow, 1998).

Characteristics of sexual reproduction

Both ascomycetous and basidiomycetous yeasts reproduce sexually. The generation of ascospores is a defining feature of the ascogenous yeasts. Ascospores are generally found in clusters of four to eight haploid spores within a single mother cell, the ascus (Neiman, 2005). On the other hand, basidiomycetous yeasts reproduce sexually by basidiospores (Yarrow, 1998; Kurtzman *et al*., 2011). A basidiospore contains one haploid nucleus that is produced in the basidia. Mature state of basidia has the base usually topped with four basidiospores which contain one from the two haploid nuclei obtained from the process of meiosis. The arrangement, cell wall ornamentations, number, shape and size of such sexual structures (ascospores or basidiospores) are useful for identification of yeasts (McGinnis, 1980).

Physiological tests for yeast identification

Physiological tests are necessary for the description and identification of species. Some of the most common routinely used physiological tests are discussed below.

Fermentation of carbohydrates: It has been proved that yeasts differ in their ability to ferment sugars and this is measured by the production of carbon dioxide using Durham tubes (Yarrow, 1998; Kurtzman *et al*., 2011). Species of yeasts in the genera *Saccharomyces*, *Kluyveromyces* and *Zygosaccharomyces* ferment at least glucose vigorously, while some others are capable of only weak fermentation or no fermentation at all, e.g., *Rhodotorula* (Yarrow, 1998). The sugars used for fermentation test include glucose, galactose, sucrose, maltose, lactose, raffinose, trehalose, inulin, starch, melibiose, cellobiose, xylose, etc. These sugars are utilized for routine fermentation tests (Yarrow, 1998; Kurtzman *et al*., 2011). If a yeast fails to ferment glucose by no means it is able to ferment other carbon sources (Kurtzman *et al*., 2003).

Assimilation of carbon compounds: This is a test for the ability of a yeast to grow aerobically on a particular carbon compound that could be monosaccharide (hexose, pentose), disaccharide, trisaccharide, polysaccharide sugar, alcohol, organic acid or a glycoside which is utilized as the sole source of carbon and energy (Yarrow, 1998; Kurtzman *et al*., 2011).The tests can be done either on solid media (auxanographic method) or in liquid broth.

Assimilation of nitrogen compounds: Yeasts are capable of utilizing a wide range of nitrogen compounds including nitrate, nitrite, ethylamine hydrochloride, cadaverine dihydrochloride, L-lysine, imidazole, glucosamine, creatine and creatinine (Yarrow, 1998; Barnett *et al*., 2000). One fourth of all yeast species investigated utilize nitrate as nitrogen source (Barnett *et al*., 2000). The methods for testing growth are similar to those described for growth on sources of carbon, using liquid or solid media (auxanographic method), with carbon base instead of nitrogen base.

Hydrolysis of urea (urease test): The ability to hydrolyze urea is generally absent in ascogenous species (except *Schizosaccharomyces pombe*, *Yarrowia lipolytica* and *Lipomyces* species), whereas basidiomycetous yeasts such as *Cryptococcus* and *Rhodotorula* can hydrolyze urea

(Yarrow, 1998). Hydrolysis of urea is tested using Christensen's urea agar. Urease is an enzyme responsible for the utilization of urea by yeasts (Yarrow, 1998). Change of color of agar media to deep red after 4-20 h indicates urease activity (Kurzman *et al*., 2003).

Fat splitting: Lipase activity or fat splitting ability has been detected in few yeasts such as *Yarrowia lipolytica*, *Candida rugosa*, *Zygoascus hellenicus*, and *Trichosporon pullulans*. This method is often employed as a confirmatory test. For the detection of lipolytic activity in microorganisms oily/fatty substances like tributyrin, olive oil, tallow, Tween 40, 60, and 80 can be used (Yarrow, 1998).

Diazonium Blue B (DBB) color reaction: This test differentiates between ascomycetous and basidiomycetous yeasts (Hagler and Ahearn, 1981). The cell wall of basidiomycetous yeasts reacts with the dye, resulting in dark red colonies, in contrast to ascomycetous yeasts, which fail to react with a buffered solution of the DBB dye (Yarrow, 1998).

Other useful physiological tests for identification are splitting of arbutin, cyclohexamide resistance, growth at different temperatures, gelatin liquefaction, tolerance to acetic acid, starch formation, growth in high osmotic concentration of sugar or salt, acid production from glucose, and growth in vitamin free medium and vitamin requirements (Kurtzman *et al*., 2011).

2.3.2.2 Kits for yeast identification

Identification kits are commercially available tools which could simplify the identification of some yeast species. They are rapid, precise and permit rapid processing of multiple isolates at the same time. Commonly used commercial miniaturized systems (kits) include Vitek, API ID 32C, API 20C AUX (bioMèrieux), Yeast Star (Clarc Laboratories, Heerlen), Auxacolor, RapID Yeast Plus system (Remel), API Candida (Verweij *et al*.,1999), Auxacolor (Sanofi,Paris,France) (Willemsen *et al*.,1997) and CHROMagar (Ainscough and Kibbler, 1998). These are miniaturized version of

existing conventional methods. They are manufactured by combining some conventional tests. These kits are designed to shorten the identification time of yeasts.

2.3.2.3 Molecular methods of yeast identification

Morphological, physiological and biochemical tests have commonly been used for phenotypic identification and classification of yeast species for the last hundred years (Yarrow, 1998; Barnett *et al.*, 2000). However, this is complex, laborious, easily influenced by operation approach, expensive to perform and time-consuming, sometimes ambiguous and can lead to incorrect identification and classification (Deak and Beuchat, 1996; Kurtzman and Robnett, 1998). To overcome these problems, yeast identification techniques have been shifted to molecular methods. These include RFLPs of chromosomal DNA (Versavaud and Hallet, 1995), mitochondrial DNA analysis (Guillamo´n *et al.*,1997) and ribosomal DNA sequencing (Kurtzman and Robnett, 1998), karyotyping electrophoresis (Hierro *et al*., 2004; Flórez *et al*., 2007), Random Amplified Polymorphic DNA (RAPD), microsatellites analysis, Amplified Fragment Length Polymorphism (AFLP) analysis, Multilocus Sequence Typing (MLST), real time PCR (Flórez *et al*., 2007) and DNA-DNA hybridization (Hierro *et al*., 2004). RFLP analysis of the entire ITS fragment is also one of the most popular tools for yeast identification (Guillamòn *et al*., 1998; Esteve-Zarzoso *et al*., 1999).The majority of yeast species can be identified using D1/D2 analyses although the internal transcribed spacer region (ITS1 and ITS2) is required to distinguish closely related species. The intergenic spacer region is recommended for additional differentiation of species and strains (Fell *et al*., 2000).

Polymerase Chain Reaction for yeast identification

In the year 1983, Kary Mullis invented the Polymerase Chain Reaction (PCR) (Bartlett and Stirling, 2003). PCR is an indispensable scientific technique used in medical and biological

research works (Saiki *et al*., 1988). The technique allows the amplification of specific DNA sequences with adequate primers in a total genomic DNA. A PCR- based method has become powerful tool in intraspecies differentiation and species identification of yeast isolates (Lopes *et al*., 1998). By using this method, Ness *et al*. (1993) identified different strains of *Saccharomyces cerevisiae.*

In PCR procedure, thermal cycling is crucial. Thermal cycling consists of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using a PCR machine (thermocycler). Primers that bind to single stranded DNA templates along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

PCR applications make use of a thermostable DNA polymerase, commonly Taq polymerase. This DNA polymerase enzymatically assembles a new DNA strand from the nucleotides, by using single- stranded DNA as a template and oligonucleotide primer, which is required for initiation of DNA synthesis. The essential components for PCR amplification include template DNA, two synthetic oligonucleotide primers, thermostable DNA Taq polymerase, the four deoxyribonucleotides and reaction buffer containing magnesium ions and other components (Glick *et al*., 2010). After isolation of DNA, three steps are required in PCR (Glick *et al*., 2010):

- Denaturation by heating at 95ºC for 20−30 sec. This step separates the double stranded DNA into single stranded components.
- Primer annealing/renaturation at 55°C for 20−40 sec. This step helps to cool the single DNA, so that one primer can form hydrogen bond to each existing single strand DNA template.

- Primer extension/elongation or synthesis at 75^{o}C for 30 sec. Average time for each cycle is approximately 3−5 min. DNA polymerase extends the primers by adding nucleotides complementary to the template strand.

Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is among one of the earliest method of characterizing yeast strains in a DNA-based procedure based on variations in the DNA structure by detecting RFLPs. This involves amplification of a specific DNA region followed by cleavage into fragments by DNA restriction enzymes called restriction endonucleases (Cutler *et al.*, 1988; Merz, 1990). These restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences (recognition sites) depending on the particular enzyme used. In most cases, enzyme recognition sites are usually 4−6 bp (Merz, 1990). Sample DNA is digested with one or more restriction endonucleases and the resulting fragments are separated according to molecular size using gel electrophoresis (Magee *et al.*, 1992). RFLPs can be better observed after transferring the DNA fragments from the gel onto membrane filters (Southern blotting) and hybridizing them with labeled DNA probes (Deak, 2008). Molecular size standards are used to estimate fragment size. Ethidium bromide, an intercalating agent, is stained to reveal the fragments under UV (260nm) light. The observed differences result from base substitutions, additions, deletions or sequence rearrangement within restriction enzyme recognition sequences or sites (Magee *et al.*, 1992). RFLP is most suited to studies at the intraspecific or among closely related taxa (Dlauchy *et al.*, 1999). Presence or absence of fragments resulting from changes in recognition sites are used to identify species or even populations of organisms from one another (Merz, 1990). Esteve-Zarzoso *et al. (*2003) generated the restriction patterns from the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8s rRNA genes to identify a total of 132 yeast

species belonging to 25 different genera. This method has helped the rapid identification of yeasts involved in the spoilage of yoghurt (Caggia *et al.*, 2001) and medically important yeasts (Trost *et al.*, 2004).

Internal Transcribed Spacer (ITS)

The genes coded by the subunits 18S, 5.8S, and 26S of rDNA are highly repeated and separated by two different regions: ITS1 and ITS2 (Molina *et al*., 1992). The ITS1 region lies between the genes of the subunits 18S and 5.8S rRNA genes, whereas the ITS2 region lies between the genes of the subunits 5.8S and 26S/28S. The ITS 1 and ITS 2 have been shown to play a role in primary rRNA processing (Musters *et al.*, 1990). Similar species highly preserve sequence of these regions and this concept explains the low degree of intraspecific polymorphism and high interspecific variability (Kurtzman and Robnett, 1998). Currently, RFLP analysis of the entire ITS fragment is one of the most popular tools for yeast identification (Guillamòn *et al*.,1998; Esteve-Zarzoso *et al*.,1999) due to the high copy number of rRNA genes easy to amplify even from small quantities of DNA, and has a high degree of variation even between closely related species. ITS is useful for elucidating relationships among congeneric species and closely related genera in clinically important yeast species (Chen *et al*., 2001). Other yeast researchers also reported that ITS1 and ITS2 regions are useful for differentiation of species within *Saccharomyces* (Oda *et al.*, 1997; Fernandez-Espinar *et al.*, 2000), rapid identification of yeasts (Guillamón *et al.*, 1998), a rapid method for species identification and differentiation of flour yeasts (Fernandez-Espinar *et al.*, 2000) and rapid identification of yeasts involved in spoilage of yoghurt (Caggia *et al.*, 2001), yeast associated with table olives (Arroyo-López *et al*., 2006) and identification of yeast species belonging to the genus *Yamadazyma* (Groenewald *et al*., 2011).

Sequencing of D1/D2 domain

The variable domain of the large subunit, 26S ribosomal DNA, is approximately 600 bases in size. It represents a partial sequence of the 26S rDNA. The D1/D2 domain has been broadly used for taxonomic purposes. As reported by Kurtzman and Robnett (1998), conspecific strains are separated by less than 1% nucleotide substitution, whereas biological species are separated by a greater than 1% nucleotide substitution. According to Kurtzman and Robnett (1998) most ascomycetous yeast species can be identified from sequence divergence in this region. They were able to identify 500 species of ascomycetous on the basis of the sequence of this domain. The D1/D2 domain sequences for most basidiomycetous yeast species have recently become available as well (Fell *et al*., 2000). They were able to determine sequence analysis of the D1/D2 region of the large subunit renal of 337 strains of basidiomycetous yeasts and yeastlike fungi, which represent 230 species in 18 anamorphic and 24 teleomorphic genera.

For taxonomic purposes and reliable identification yeasts sequencing of the D1/D2 domain, which is universally accepted, is extensively used (Hong *et al.*, 2001; Scorzetti *et al.*, 2002). Fortunately, databases of the D1/D2 sequences have been generated for all currently accepted yeasts as ascomycetous or basidiomycetous (Kurtzman and Robnett, 1998; Guffogg *et al.*, 2004). This extensive available database makes the task of species identification much easier and fast (Kurtzman, 2001; Starmer *et al.*, 2001; Wesselink *et al.*, 2002).

Sequence-based analysis of the D1/D2 region of the large ribosomal subunit was also used for molecular identification of new yeasts in the *Pichia anomala* clade (Kurtzman, 2000), veterinary yeast isolates (Garner *et al*., 2010) and yeast species belonging to the genus *Yamadazyma* (Groenewald *et al*., 2011).

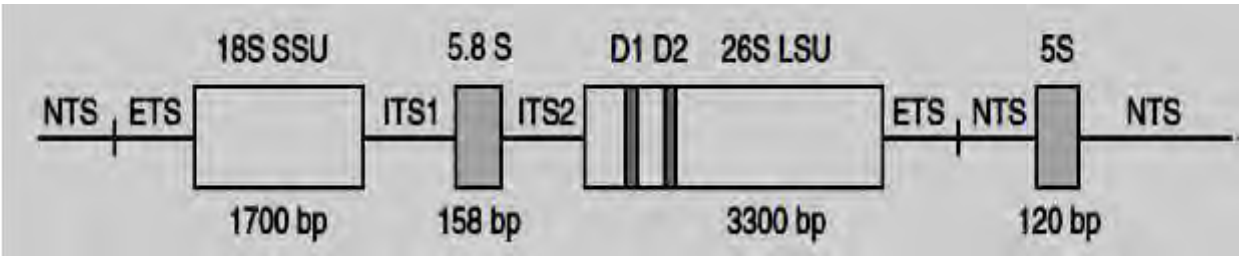

Figure 2.1: Structure of nuclear ribosomal DNAs (Source: Deak, 2008).

2.4 Oleaginous microorganisms

Oleaginous microorganisms, including some selected species of yeasts, bacteria, filamentous fungi and microalgae contain more than 20% of their dry weight (Ratledge and Wynn, 2002; Wynn and Ratledge, 2006), can be used to obtain microbial lipids (TAGs), studied and used as alternative sources of oils and fats for human consumption (Ratlege, 2005). Furthermore, such microbial lipids are recommended for biodiesel production (Li *et al*., 2008).

There are several advantages of developing oleaginous microorganisms for oil production over plants or animal sources (Certik and Shimizu, 1999; Mamatha, 2009; Papanikolaou and Aggelis, 2011): High (fast) growth rates on wide variety of substrates (cheap agro-industrial wastes), microorganisms can be cultured in various carbon sources including waste materials presenting simultaneously rapid and significant biomass and oil production, oil production can be carried out throughout the year (climatic independent) and there is no need for light energy to produce biomass in heterotrophic microorganisms, microorganisms are appropriate vehicles for cloning foreign genes (plant or animals) for production of the oils of interest, for example, production of specific PUFAs, metabolic regulations are simple compared to plants and animals.

Microbes can grow under controlled conditions with nutritional regimes that may stimulate or repress the key enzymes and allow for manipulation of lipid concentration and fatty acid profile.

In addition, microorganisms provide useful models for studying lipid biochemistry, metabolic control and function of complex multicellular systems of other organisms and production of microbial oil can be scaled up relatively easily.

2.4.1 Oleaginous molds

Production of biodiesel from oleaginous filamentous fungi has been given less attention; rather such fungi were studied for production of PUFA, such as arachidonic acid, γ-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid (Wynn and Ratledge, 2006; Sakuradani *et al*., 2009). Like other oleaginous microorganisms,the culture medium for molds must be low in nitrogen and high in carbon availability (Moreten, 1998).The most deeply studied and exploited molds to produce microbial oils containing PUFAs are *Mortierella alpina* (Sakuradani *et al*., 2009) and *Mucor circinelloides* (Li *et al*., 2011). Also the phylogenetically related *Umbelopsis isabellina* has emerged as a potential species to produce oil TAG as starting material for production of biodiesel (Meeuwse *et al*., 2011). *Aspergillus oryzae* is another mold that produces lipid, however, limited attention was given to this mold with respect to oil production (Adachi *et al*., 2011).

Yet other lipid producing molds reported recently are *Mortierella isabelina*,*Cunninghamella echinulata* and *Thamnidium elegans* (Papanikolaou *et al*., 2004; Fakas *et al*., 2008) and *Tilletiopsis albescens*, *Backusella ctenidia*, *Plectosphaerella* sp. and *Gibberella fujikuroi* (Li *et al*., 2011). A filamentous fungus, *Ashby gossypii* mostly accumulates unsaturated fatty acids, with more than 50% of the total fatty acid content corresponding to oleic acid (Ledesma-Amaro *et al*., 2014).

2.4.2 Oleaginous algae

The average lipid contents of algal cells vary between 1 and 70% but can reach 90% of dry weight under certain conditions (Thevenieau and Nicaud, 2013). Authotrophic microalgae are capable of producing large amounts of oils (Liang *et al*., 2006; Chisti, 2007) and hydrocarbons (Subramaniam

et al., 2010) in the presence of sunlight and carbon dioxide. It has been found that many autotrophic algae such as *Chlorella vulgaris, Botryococcus braunii, Navicula pelliculosa, Scendesmus actus, Dunaliella primolecta, Monallanthus salina, Neochloris oleoabundans, Phaeodactylum tricorntum,Tetraselmis sueica, Spirulina Maxima, Scendesmus obliquus* and *Scendesmus dimorphus* can accumulate oils (Liang *et al*., 2006; Chisti, 2007; Shalaby, 2011). Algae can be sources of several different types of renewable biofuels, including biodiesel, biohydrogen, hydrocarbons, ethanol and methane (Subramaniam *et al*., 2010). In addition to autotrophic microalgae, some heterotrophic microalgae have been found producing oils. Docosahexaenoic acid (DHA) is recently extracted from two marine heterotrophic microalgae, that is, from *Cypthecodinium cohnii* and *Schizochytrium* (Wynn and Ratledge, 2006).

2.4.3 Oleaginous bacteria

Some species of bacteria belonging to the actinomycetes group in the genera *Mycobacterium*, *Streptomyces*, *Rhodococcus*, *Nocardia* and *Gordonia* can accumulate TAGs at high concentrations under nitrogen-limited conditions (Alvarez and Steinbuchel, 2002).The Actinomycete groups of bacteria are capable of accumulating significantly high amounts of intracellular fatty acids from simple carbon sources like glucose (Kalscheuer *et al*., 2006). Not all bacterial species accumulate purely TAGs, some accumulate poly-β-hydroxybutyrte and alkanaoates (Ratledge, 2004) or some others accumulate diacylglycerols (DAGs) and wax esters (Alvarez *et al*., 1997).

2.4.4 Oleaginous yeasts

Yeasts were the first group of microorganisms recognized over a century ago to accumulate lipids (Leman, 1997). The lipids accumulated by yeasts are TAGs (Papanikolaou *et al*., 2002). Among oleaginous yeasts less than 5% of them can accumulate more than 25% of lipid under nitrogen-limited conditions (Ageitos *et al.*, 2011). Most of the identified lipids producing strains are

basidiomycetous yeast species; nevertheless, some useful oleaginous species such as *Yarrowia lipolytica* and *Debaryomyces hansenii* which are ascomycetes have also been identified. The most deeply studied oleaginous yeasts belong to the genera *Candida, Cryptococcus, Lipomyces, Rhodotorula, Rhodosporidium, Yarrowia* (Li *et al*., 2008; Rossi *et al*., 2009) and *Trichosporon* (Gujjari *et al*., 2011; Hu *et al*., 2011). Two *candida* species, namely *Candida curvata* D (Holdsworth and Ratledge, 1991) and *Candida freyschussii* (Amaretti *et al*., 2011) synthesize and store significant amount of lipids. *Cryptococcus curvatus* is a yeast with industrial potential as SCO because it can grow and accumulate lipid on a variety of substrates. It requires minimal nutrients for growth, accumulating up to 60% of its cellular dry biomass (Meesters *etal*., 1996). *Cryptococcus albidus* is the other potential oleaginous yeast (Li *et al*., 2008). Species in the *Lipomyces* genus present a great potential to accumulate TAGs. *Lipomyces starkeyi* has the capability to accumulate over 70% of its biomass as lipid under nitrogen - limited culture conditions, and can accumulate lipid when grown on substrates such as xylose, ethanol and L-arabinose or using a mixture of glucose and xylose (Zhao *et al*., 2008). Another species of *Lipomyces, Lipomyces lipofer* has high capacity to produce SCO too (Li *et al*., 2010).

The mesophilic red yeast, *Rhodotorula glutinis* is known to synthesize and store lipids, whereas the psychrophilic species *Rhodotorula glacialis* accumulates lipids in a range of temperature between -3 and 20°C (Amaretti *et al*., 2010a). Another strain of oil producing red yeast in the genus *Rhodotorula*, namely *Rhdotorula mucilaginosa* was able to synthesize lipid under favorable conditions. Different researchers investigated different strains of this yeast for the production of SCO on different substrates such as, on hydrolysate of cassava starch (Li *et al*., 2010), *Rhodotorula mucilaginosa* TJY15a on inulin and extract of tubers of Jerusalem artichoke (Zhao *et al*., 2011) and on other lignocellulosic wastes (Enshaeieh *et al*., 2015a). Another species of mesophilic

oleaginous red yeast, *Rhodosporidium toruloides* was found to convert glycerol and lignocellulosic biowastes into lipids (Hu *et al*., 2009; Yu *et al*., 2011). Researchers recently identified some oil producing yeast species in the genus *Trichosporon.* These include *Trichosporon cacaoliposimilis, Trichosporon oleaginosus* (Gujjari *et al*., 2011) and *Trichosporon cutaneum* (Hu *et al*., 2011). A model yeast, *Yarrowia lipolytica* was discovered as an excellent candidate for microbial lipid production by Beopoulos *et al*. (2008). This oleaginous yeast has the ability to degrade hydrophobic substrates such as n-paraffins and oils efficiently (Bankar *et al*., 2009). Various *Yarrowia lipolytica* strains (*Yarrowia lipolytica* strain DSM 70561 and JDC 335) also showed significant growth on glycerol used as sole carbon source and grew best in the presence of 1.0 g/L urea as nitrogen source (Sriwongchai *et al*., 2013). *Yarrowia lipolytica* DSM 70561 and JDC 335 accumulated 37.1 and 54.4% lipid in their cells, respectively. The accumulated lipids were mainly saturated and monounsaturated with C16 and C18 fatty acids. Li *et al*. (2010) also reported *Hansenula saturnus* as oleaginous yeast species. Recently, high level of oil produced from *Metschnikowia pulcherrima* cultures was reported (Santamauro *et al*., 2014).

2.5 Cultivation of oleaginous yeasts on agro-industrial wastes

Oleaginous microorganisms under suitable cultivation conditions convert the carbon sources contained in various substrates into storage lipid (Economou *et al*., 2011). In order to reduce the cost of microbial oil production, low-cost raw materials like lignocellulosic hydrolysates such rice straw (Huang *et al*., 2009) and rice hull (Economou *et al*., 2011), glycerol (Sriwongchai *et al*., 2013; Moustogianni *et al*., 2014), beer factory wastewater (Lutzu *et al*., 2016) and products of fruit processing industry (Kulkarni *et al*., 2013; Park *et al*., 2014) are used.

Various juice producing industries and local juice sellers produce a vast amount of different fruit wastes including fruit peels. The peels from these fruits are disposed as wastes. Utilization of such

wastes needed to be emphasized to save the resources and protect the environment from pollution (Kulkarni *et al*., 2013). Fruit waste has high level of sugars, including sucrose, glucose and fructose (Choi *et al*., 2015). These fruit wastes are suitable not only for the production of SCO (Kulkarni *et al*., 2013; Park *et al*., 2014) but also, they are useful for the production of several bioproducts including ethanol, biogas, lactic acid, enzymes and single cell protein (Puligundla *et al*., 2014). Banana peel can also be used for the production of wine (Faturoti *et al*., 2006). To improve the economic competitiveness of microbial oil (TAGs), the use of agricultural products or by- products that have low or even negative value can be used as natural substrates (Hassan *et al*., 1994; Pan *et al*., 2009).These microbial oils in turn can be used as feedstocks for the production of biodiesel (Li *et al*., 2008).

Molasses, a by-product of sugar processing industry, is produced in large amount in Ethiopia. It is a residue after the crystallization of the main fraction. Based on its origin, it can be called cane molasses or beet molasses (Crueger and Crueger, 1990) and is the cheapest source of carbohydrate (Goksungur and Zorlu, 2001). It contains 45−55% fermentable sugars including sucrose, glucose, fructose, raffinose, melibiose and galactose (Bekatorou *et al.*, 2006). Molasses is available in a wide range of geographical locations. This is because it is extracted from at least two sources of plant adapted to tropical and temperate climate. The use of molasses for yeast biomass production has simplified the manufacturing process in many ways. Its cost is reduced as compared with the use of grains (Crueger and Crueger, 1990).

Molasses is used as a source of carbon, energy and other essential nutrients. Molasses could not supply all the essential nutrients for yeast growth. Therefore, the addition of supplements such as $(NH_4)_2SO_4$, yeast extract or peptone as nitrogen source, KH_2PO_4, H_3PO_4 as phosphorus source, other macroelements such as calcium in form of calcium salts, magnesium in the form of

magnesium salts, microelements such as iron, zinc, copper, manganese are necessary for maximizing biomass yield of any type of yeast. Vitamins such as biotin, inositol, panthotenic acid and thiamine are also required for yeast growth (Cook, 1958).

The composition of molasses varies depending on the location, soil type, climatic conditions and the production process of sugar factories (Curtin, 1983; Crueger and Crueger, 1990). Due to its availability and low price, molasses has long been used as cattle feed (Cleasby, 1963). Molasses has also been used in the production of bioethanol **(**Periyasamy and Venkatachalam, 2009; Fadel *et al*., 2013; Arshad *et al*., 2014). Recently, molasses has been used as a primary feedstock for the cultivation of lipid producing microorganisms (Sabry *et al*., 1990). For example, Johnson *et al*. (1995) cultivated an oleaginous yeast *Rhodotorula glutinis* IP-30 for the production of fat using a fed-batch fermentation method and got a good result. PUFAs were also produced from *Mucor recurvus* sp. which was grown on sugar cane molasses (Li *et al.*, 2008). Still other researchers were able to produce SCO for the production of biodiesel using molasses as a substrate (Almazan *et al*., 1981; Karatay and Donmez, 2010; Gajdoš *et al*., 2015).

2.6 Cultivation conditions for lipid production in oleaginous yeasts

Oil accumulation by oleaginous microorganisms depends highly on the culturing conditions such as the available carbon and nitrogen sources in the growing medium, C/N ratio, the available inorganic salts, pH, incubation temperature, cultivation period and agitation and aeration rates (Li *et al*., 2008; Zhao *et al*., 2015). Each will be discussed in detail below.

2.6.1 Nutrient composition and lipid production

C/N ratio of the culture medium is one of the critical factors that affect oleaginous potential of microorganisms (Li *et al*., 2008). Optimum C/N ratio is needed for maximum production of lipid. Excess sugar (commonly glucose, glycerol and/or polysaccharides) and limitation of other

nutrients (usually nitrogen) in a medium are prerequisites for good lipid production (Anderson and Wynn, 2001). In most cases, C/N ratio must be greater than 20 (Papanikolaou and Aggelis, 2011). C/N ratio depends on the type and nature of microorganism, the medium composition in which the cultivation is undertaken, types of carbon and nitrogen sources (Ykema *et al*., 1986). Optimum C/N ratio for maximum lipid production from glucose or fructose with a nitrogen source from either $(NH4)_2SO_4$, NH_4NO_3 or urea by *Rhodosporidium toruloides* ATCC 10788 was 77 (Turcotte and Kosaric, 1989). *Trichosporon fermentans* exhibited maximum biomass (28.1 g/L) and lipid content (62.4%) at the C/N ratio of 163(Zhu *et al*., 2008). Kraisintu *et al*. (2010) also studied the influence of C/N ratio on biomass and lipid production by varying C/N ratios (65, 90, 115 and 140) in a nitrogen-limited media and reported that the C/N ratio as an important factor for lipid accumulation. The highest cellular lipid content (62.30% of dry biomass) and 4.23 g/ L of lipid concentration were attained at the C/N ratio of 140 but with the lowest biomass (6.79 g/L). However, the lowest C/N ratio (65) produced the highest biomass (10.52 g/L) and the lowest lipid concentration (2.7 g/L).

On the other hand, limitation of carbon sources resulted in a decrease in the growth rate (Gill *et al*., 1977). The decrease in the growth rate found to cause a decrease in the lipid content. The decrease both in growth rate and lipid content could be due to diversion of a greater percentage of the glucose to energy production and maintenance of the organism at slower growth rates.

The medium of composition containing the different nitrogen sources is another factor to be considered for biomass production and lipid accumulation in oleaginous microorganisms. Kraisintu *et al*.(2010) grew *Rhodosporidium toruloides* DMKU3-TK16 and achieved highest biomass (8.71 g/L) when yeast extract with $(NH_4)_2SO_4$ were used in combinations, while higher cellular lipid contents of 53.71 and 53.10% of dry biomass were produced, when peptone was used

with NH_4Cl and with $(NH_4)_2SO_4$, respectively. *Trichosporon fermentans* yielded a maximum biomass (23.1 g/L) when grown in a medium containing urea, while maximum lipid content (54.9%) and lipid concentration (10.8 g/L) were exhibited when the yeast was grown in a medium containing peptone (Zhu *et al*., 2008).

The effects different carbon sources (glucose, sucrose, xylose, lactose and fructose) for maximum biomass and lipid production by *Trichosporon fermentans* was evaluated by Zhu *et al*. (2008). The maximum biomass (24.1 g/L) and lipid content (56.6%) were obtained when glucose was used as the carbon source, followed by fructose, sucrose, xylose and lactose. All the carbon sources tested gave relatively high lipid content. It can be observed that *Trichosporon fermentans* has a broad spectrum of carbon sources (Zhu *et al*., 2008).

Addition of KH_2PO_4 and $MgSO_4.7H_2O$ also resulted in higher biomass and lipid contents in *Rhodosporidium toruloides* DMKU3-TK16 (Kraisintu *et al*., 2010).

2.6.2 pH and lipid production

pH of the medium also plays a critical role on lipid production by oleaginous microorganisms. Different investigators found different optimum pH values for maximum production of lipids for different oleaginous microorganisms. In general, increasing the pH of cultivation medium, the amount of saturated and monounsaturated fatty acids tend to decrease, whereas PUFAs content tend to increase (Mamatha, 2009). Highest cellular lipid content (71.30% of dry biomass) and lipid production (9.26 g/L) were exhibited by *Rhodospooridium toruloides* DMKU3-TK16 when the yeast was cultured in a medium with adjusted pH of 5.5 (Kraisintu *et al*., 2010). Best growth pH of *Rhodotorula glutinis* EF081370 was 5.0 (Tao *et al*., 2008). A maximum lipid production of 66% w/w was obtained by growing *Rhodotorula glutinis* IIP-30 in a fed-batch fermentation under nitrogen-limiting conditions at 30^{o}C using glucose as a substrate. The optimum pH obtained for

this maximum lipid production was pH 4.0 (Johnson *et al*., 1992). *Cryptococcus curvatus* NRRLY−1511 was cultivated for biomass production and lipid accumulation with various pH values. Optimum pH for both biomass production and lipid accumulation obtained was at pH 5.5 with dry weight produced, 2.4 g/L and microbial lipid produced, 1.7 g/L, respectively (El-Fadaly *et al*., 2009). *Trichosporon fermentans* yielded a maximum biomass (28.1 g/L) and lipid content (62.4%) at pH value of 6.5 (Zhu *et al*., 2008). For the same oleaginous yeast, Wang *et al*. (2005) reported maximum biomass (19.4 g/L) and lipid content (50.8%) at pH 6.0. Angerbauer *et al*. (2008) reported that pH of the medium had an effect on lipid production and seemed to depend on carbon sources. An optimal pH of 4.0 for lipid production by *Lipomyces starkeyi* was found when ethanol was used as a carbon source (Yamauchi *et al*., 1983). *Lipomyces starkeyi* had optimum pH at 5.5 with glucose (Holdsworth and Ratledge, 1988), while Angerbauer *et al*. (2008) got highest lipid content when *Lipomyces starkeyi* was cultivated at pH 5.0.

2.6.3 Temperature and lipid accumulation

Cellular lipid accumulation is critically affected by incubation temperature of the culture medium. Temperature affects all living organisms and controls the growth rate, lipid synthesis and alters the composition of fatty acid in cellular level (Mamatha, 2009). Temperature has a fundamental effect both on biosynthesis of saturated and unsaturated fatty acids. Generally, degree of unsaturation increases with decreasing temperature (Mamatha, 2009). Temperature ranges between 20 −30^{o}C are most favorable for the growth of laboratory and industrial yeasts.

The proportions of unsaturated fatty acids present in membrane lipids regulate membrane fluidity and are critical for proper membrane function. The proportion of these lipids in turn varies with temperature and it might have a possibility to contribute to temperature adaptation in poikilothermic organisms (Saito *et al*., 2005).

Every oleaginous microorganism requires specific optimum temperature for maximum lipid production. For example, El-Fadaly *et al*. (2009) found that 28ºC was the optimum temperature needed for biomass and lipid production by *Cryptococcus curvatus* NRRLY−1511. With this optimum temperature, the oleaginous yeast produced 5.83 g/L biomass and 1.21 g/L lipid. *Rhodotorula glutinis* EF081370 also accumulated the maximum lipid when the growth condition was adjusted at 30ºC (Tao *et al*., 2008). The psychrophilic oleaginous yeast, *Rhodotorula glacialis* DBVPG 4785, was cultivated for biomass production and lipid accumulation by varying different temperatures, i.e., from the range of -3 to 20ºC, the optimum temperature for maximal biomass productivity(0.101 g/L h^{-1}) and rate of lipid accumulation (0.034 g/L h^{-1}) was 15ºC(Amaretti *et al*., 2010a). The effect of temperature on lipid accumulation by *Rhodosporidium toruloides* TISTR 5123 was also investigated (Ruangudom and Punpeng, 2011). The experiments were performed at 10, 15, 20, 25, 30 and 35ºC.The results revealed that lipid production decreased at lower temperature. The optimal temperature was 15 to 30ºC and the lowest lipid content of 22.58% was obtained when the yeast was grown at 10°C. At 20°C the yeast showed its maximum as 55.50% lipid content, 6.85 g/L dry biomass and 3.80 g/L total lipid. Both biomass and lipid content reached the maximum of 27.5 g/L and 60.6%, respectively at 25ºC by *Trichosporon fermentans* (Zhu *et al*., 2008). It was also noted that in the range from 20ºC to 35ºC, the total amount of unsaturated fatty acids of the lipid decreased from 71.8% to 52.0%, indicating that a low temperature was favorable for the formation of unsaturated fatty acids in *Trichosporon fermentans* (Zhu *et al*., 2008).

2.6.4 Aeration and agitation rates on lipid production

The effect of both agitation and aeration rate on the production of SCO by oleaginous microorganisms has been investigated by various researchers. For example, Dai *et al*. (2007)

obtained a maximum lipid content of 49.25% at agitation rate (shaking speed) of 180 rpm in *Rhodotorula glutinis* after 96 h of cultivation. El-Fadaly *et al*. (2009) investigated the effect of agitation speed on the SCO production by *Cryptococcus curvatus* NRRLY−1511. Highest dry cell biomass (SCP) and lipid concentration was recorded when *Cryptococcus curvatus* NRRLY−1511 was grown in medium that was kept at the agitation speed of 200 rpm. Sugar consumption in *Cryptococcus curvatus* NRRLY−1511 also increased with the increase in agitation speed used.

Dissolved oxygen concentration in the culture medium has a positive correlation with the accumulation of oils (Liang *et al*., 2006; Yi and Zheng, 2006). Oxygen has a pronounced effect on the growth and general metabolism of any organism. Aeration in shake flasks with 80−100 mL medium in a 500 mL flask was found to be nearly equivalent to an air sparging ratio of 0.33. Spotholz *et al*. (1956) studied that supplying 3.0 g/L h^{-1} dissolved oxygen to *Rhodotorula gracilis* satisfied the requirements for the yeast to grow exponentially until the substrate become limiting. Gill *et al*. (1977) showed that oleaginous microorganisms can be grown successfully in a continuous culture, and Yamuchi *et al*. (1983) demonstrated that very high cell densities could be achieved in fed-batch conditions. However, lipid yields were very low. The oxygen requirement under nitrogen-limitation was 50−60% of that required under carbon limiting conditions, i.e., 3 mM g/h for *Candida* 107 and 2.25 mM g/h for *Rhodotorula gracilis* NCYC1549 in continuous culture (Hall and Ratledge, 1977), suggesting maximum productivity of lipid achieved could be 1−2 g/L h^{-1}. *Rhodotorula gracilis* produced more lipids when grown in phosphate molasses medium under high aeration (Allen *et al*., 1964). Both biomass and its lipid content increased with increase in dissolved oxygen concentration in *Rhodotorula gracilis* (Choi *et al*., 1982). It is argued that *Rhodotorula gracilis* depends on oxygen for its energy metabolism and synthesis of cellular components and hence lipid production (Choi *et al*., 1982).

2.6.5 Incubation period and lipid production

Culturing time (incubation period) also has an effect on lipid production. Different oleaginous microorganisms require different incubation period for maximum biomass production and lipid yield. Usually lipid content reaches its highest value at stationary phase of microbial growth (Beopoulos *et al*., 2008). It is recommended that cells should be harvested at early stationary phase to prevent lipid degradation (Beopoulos *et al*., 2008).

According to earlier studies 72 h, 168 h,7 days and 8 days were found to be optimum culture times for maximum lipid production by *Rhodotorula glutinis* (Dai *et al*., 2007), *Rhodosporidium toruloides* DMKU3-TK16 (Kraisintu *et al*., 2010), *Trichosporon fermentans* (Zhu *et al*., 2008) and *Torulaspora globosa* YU5/2 (Leesing and Baojungharn, 2011), respectively.

2.6.6 Mineral salts for lipid accumulation

Biomass and oil content could be improved significantly by the addition of ions of different mineral salts including Ca2+, Zn^{2+}, Mn^{2+}, Cu^{2+},Fe^{+3} etc.(Li *et al*., 2007; Li *et al*., 2008).

2.7 Microbial lipids and their application

Lipids are naturally occurring organic compounds that include fats, waxes, sterols, and fat- soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, steroids (like cholesterol), TAGs, phospholipids and others. The main biological functions of lipids include energy storage, as structural components of cell membranes, and as important signaling molecules (Fahy *et al*., 2009; Subramaniam *et al*., 2011). Fats are composed of fatty acids and glycerol. They store energy, aid in insulating the body, and cushion and protect internal organs.

A TAG is an ester derived from glycerol and three fatty acids. The hydroxyl groups of the glycerol join the carboxyl groups of the fatty acid to form ester bonds. TAGs are the main components of

vegetable oils (Santala *et al*., 2011) and animal fats (Alvarez and Steinbuchel, 2002). In addition to their occurrence in vegetable, oleaginous microorganisms, such as yeasts, fungi and microalgae, can accumulate high amounts of such lipids under appropriate cultivation conditions (Ratledge and Wynn, 2002; Wynn and Ratledge, 2006; Santala *et al*., 2011). TAGs also occur in bacteria (Alvarez and Steinbuchel, 2002). Other components present in oleaginous microorganisms in non-negligible quantities are free fatty acids, neutral lipids (smonoacylglycerols, diacylglycerols, and steryl-esters), sterols, phospholipids, sphingolipids and glycolipids (Fakas *et al*., 2006; Papanikolaou and Aggelis, 2011).

Yeast strains produce predominantly SFAs or MUFAs with carbon lengths of C16 and C18 (Papanikolaou and Aggelis, 2011) that are used in biodiesel preparation ((Ramos *et al*., 2009; Christophe *et al*., 2012).Oleaginous fungi and microalgae accumulate PUFA (Wynn and Ratledge, 2006) and have been principally used to produce lipids rich in PUFAs of medical and dietetical interest like γ-linolenic acid, arachidonic acid,docosahexaenoic acid, eicosapentanoic acid, etc. (Ratledge and Wynn, 2002;Ward and Singh, 2005; Sakuradani *et al*., 2009).

2.8 Biosynthesis of microbial fatty acids

The ability to accumulate high amount of lipid depends on the regulation of the biosynthetic pathway and the supply of precursors, namely acetyl-CoA, malonyl-CoA and glycerol-3-phosphate and the cofactor NADPH. Fatty acid synthesis involves the conversion of acetyl-CoA into palmitic acid (16:0). For the formation of acetyl-CoA, for example, in yeasts glucose is used as a primary substrate. The carbon source (glucose) is converted into pyruvate in the cytosol, pyruvate is transported into the mitochondrion, decarboxylized to acetyl-CoA which reacts with oxalacetate 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

adenosine-monophosphate (AMP) into inosin-monophospahte (IMP) and NH_4^+ to provide cell own nitrogen for cell functions. However, AMP is required for the functionality of the enzyme isocitrate-dehydrogenase (ICDH), which converts iso-citrate 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. At this point citrate is cleaved into oxalacetate and acetyl-CoA by an enzyme essential for lipid accumulation, ATP: citrate lyase (Ratledge, 2002; Ratledge, 2004). It does not occur in non-oleaginous species into oxalacetate. The acetyl-CoA acts as the precursor for the fatty acid synthesis. The oxaloacetate is converted via malate dehydrogenase to malate, which is then used as the counterion in the citrate efflux system. This process is illustrated in Figure 2.2.Then acetyl-CoA carboxylase catalyzes the formation of malonyl-COA from acetyl-CoA. Subsequently, 7 malonyl-CoA molecules are together with acetyl-CoA converted to palmitic acid (Sijtsma *et al*., 1998).

Fatty acids are highly reduced chemical compounds and to achieve their synthesis as a ready supply of reductant as NADPH is essential and required for further chain lengthening. 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, which is involved in chain elongation processes (Ratledge, 2004).

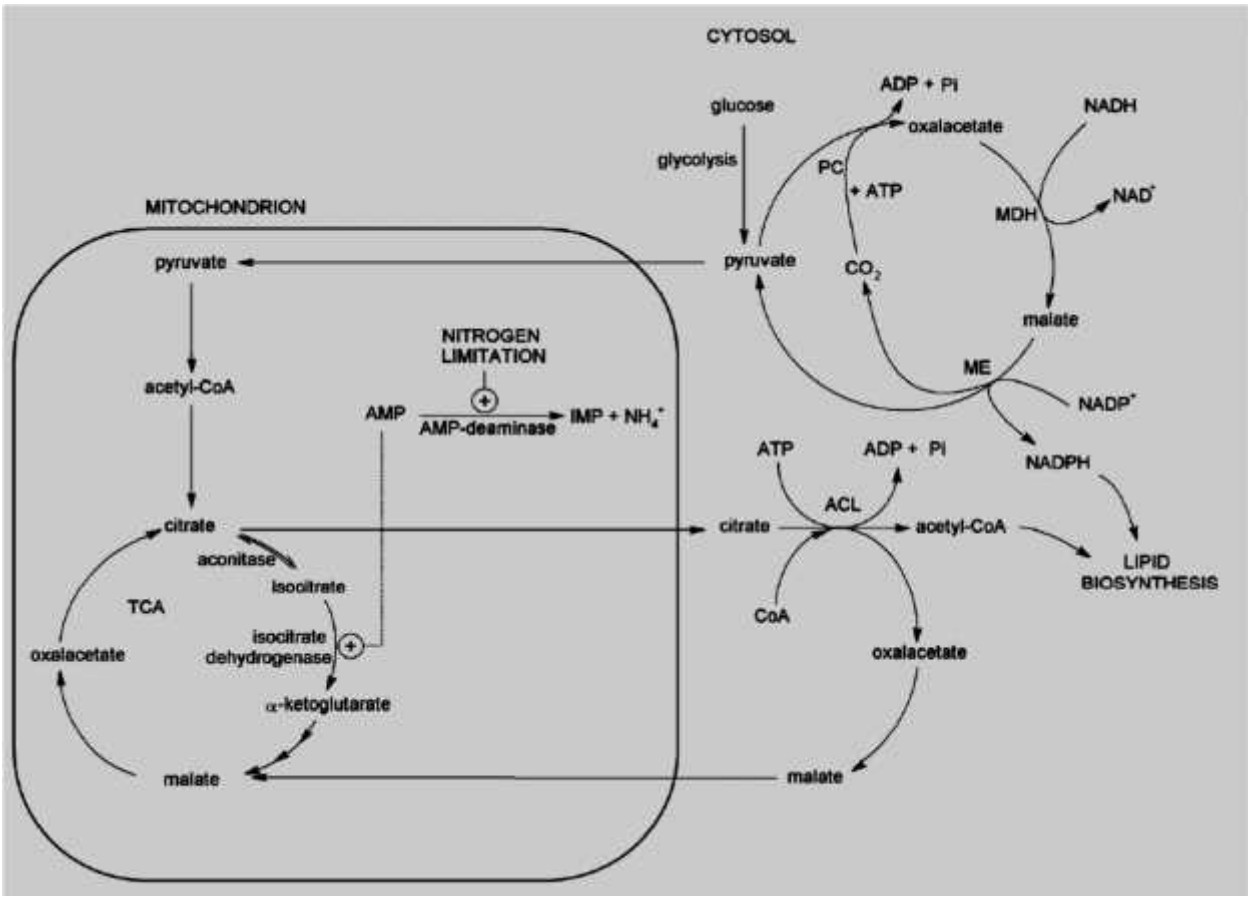

Figure 2.2: Diagram of the biosynthesis of single cell oils in oleaginous microorganisms (Source: Rossi *et al*., 2011).

Desaturases are other groups of enzymes involved in the formation of PUFAs. The biosynthesis of the two major groups of PUFAs, the ω -6 and ω -3 series are shown in Figure 2.3. These two groups are synthesized, respectively, from linoleic acid (C18:2) and α-linolenic acid (C18:3). Both linoleic acid and α-linolenic acid are essential fatty acids, as they cannot be synthesized in animals which lack the Δ-12 desaturase required for linoleic acid biosynthesis from oleic acid and the Δ-12 and Δ-15 desaturases required for α-linolenic acid biosynthesis from oleic acid. As a result of this metabolic deficiency, animals require sources of both linoleic acid and linolenic acid in their diet. Luckily, plants possess a Δ-12 desaturase and Δ-15 desaturase and are able to synthesize these essential fatty acids. Human beings and herbivores obtain these essential fatty acids from the plants

they eat. Once taken up by animal cells, both linoleic acid and linolenic acid can be further elongated and desaturated to the full complement of long chain PUFAs (LC-PUFA), i.e., fatty acids containing 20 or more carbon atoms and three or more double bonds, (Figure 2.3) (Wynn and Ratledge, 2006).

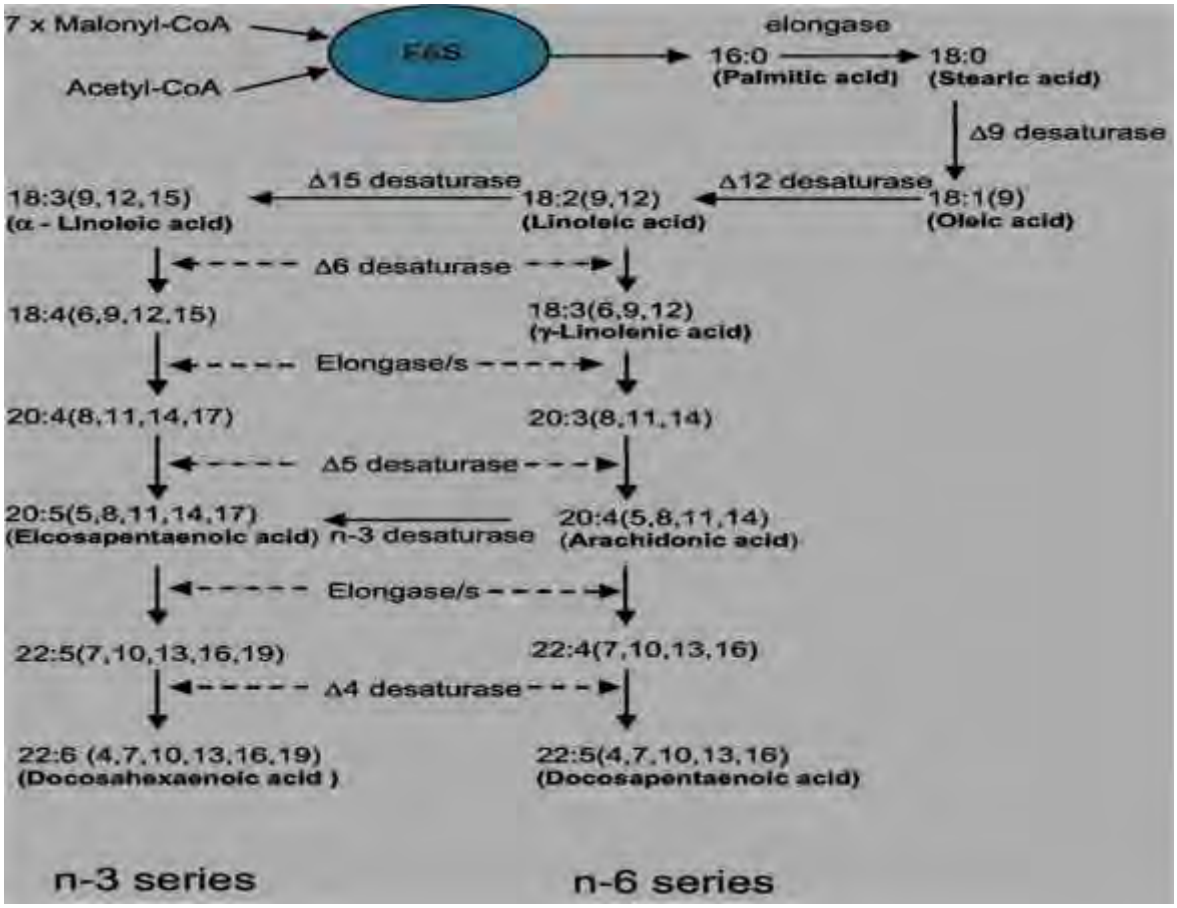

Figure 2.3: The routes of biosynthesis of PUFAs of the ω -3(n-3) and ω-6 (n-6) series (Source: Wynn and Ratlegde, 2006).

2.9 Methods of oil/lipid extraction

There are three components for extracting lipids from tissues and microbial biomass (Xiao, 2010): Firstly, exhaustive extraction and solubility of the lipids in organic solvent. Secondly, the removal of non-lipid contaminants from the extracts. Thirdly, the potential toxicity of solvents to the analysts.

Prior to extraction of lipids from source material using selective solvent extraction, the starting material may be subjected to drying, particle size reduction and acid hydrolysis.

Drying: It is often necessary to dry samples before proceeding to solvent extraction, because many organic solvents cannot easily penetrate into foods or any sample containing water, and therefore extraction would be inefficient. Drying makes the sample easier to grind for better extraction. In the case of extracting microbial oil, the microbial cell is first harvested. The cells can be kept moist or they can be completely stabilized by flash or drying, spray drying or by lyophilization. The moist cells can be directly extracted using hexane. The spray dried cells are also extracted in a similar fashion (Wynn and Ratledge, 2006).

Particle size reduction: Dried samples are finely ground prior to extraction to produce a homogeneous sample and to increase the surface area of lipid exposed to the solvent. Grinding is often carried out at low temperatures to reduce the tendency for lipid oxidation to occur.

Acid hydrolysis: Some lipids occur complexed with lipoproteins or glycolipids. To determine the concentration of these components it is necessary to break the bonds which hold the lipid and non-lipid components together prior to solvent extraction. Acid hydrolysis is commonly used to release bound lipids into easily extractable forms, e.g., a sample is digested by heating it for some time in the presence of 3N HCl acid prior to extraction. The most common lipid extraction methods will be discussed below.

Soxhlet extraction method

Soxhlet method is among the most commonly used extraction method of lipids from foods. According to AOAC (1995), the procedures of Soxhlet extraction are the following. Lipid sample from solid material is extracted by repeated washing (percolation) with an organic solvent. The most common used solvents are hexane, petroleum ether or ethyl acetate. The lipid sample is dried,

ground into small particles and placed in a porous cellulose thimble. The thimble is placed in an extraction chamber, which is suspended above a flask containing the solvent and below a condenser. The flask is heated and the solvent evaporates and moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample. The extraction chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. At the end of the extraction process, the flask containing the solvent and lipid is removed. The solvent in the flask is then evaporated and the mass of the remaining lipid is measured. The percentage of lipid in the initial sample can then be calculated (Shahidi, 2001).

Mass of lipid = (weight of the flask + boiling chips + extracted oil) − (weight of the flask + boiling chips)

Lipid content (%) = mass of lipid extracted (g)/sample weight (g) × 100

Goldfisch method of lipid extraction

Goldfisch extraction procedure is another lipid extraction method from biological material. This procedure allows the solvent to continuously flow over the sample held in a ceramic thimble. The solvents used in this extraction method are hexane, petroleum ether, or diethyl ether. This extraction method has advantages over Soxhlet method in that it is helpful for determination of non-polar lipids and is faster and more efficient.

Goldfisch lipid extraction unit is operated inside an explosion proof fume hood to exhaust the vapors from flammable solvents used during normal operation. The fume hood should be equipped with a fire suppression device. In Goldfisch extraction method, porous extraction thimbles and extraction containers are prepared, pre-dried and weighed. A known amount of pre-dried sample is added to the thimble and weighed again. The ceramic extraction thimble is placed into a glass

holding tube. Known amount of solvent is placed in the extraction container. The apparatus is set to extract for 4 to 7 h. Then the sample is left to cool. The extraction container is removed and then the solvent is evaporated in the air overnight with subsequent heating at 95^{o}C to 100^{o}C for 30 min using a forced air oven. The beaker is cooled in a desiccator. Then the beaker and its contents are weighed and the weight of the lipid can be calculated:

Weight of lipid = (weight of container + extracted lipid) − (weight of container)

Calculate the percentage of lipid in the sample as given below (Shahidi, 2001).

Lipid content (%) = mass of lipid extracted (g)/sample weight (g) × 100

Folch method

The Folch method is one of the most popular lipid extraction methods (Folch *et al*., 1957). In this extraction method, the sample tissue may be dried, ground and homogenized with chloroform/methanol (2:1). Then, the whole mixture is agitated in an orbital shaker at room temperature. The homogenate is either filtrated or centrifuged to recover the liquid phase. Solvent is washed with specified volume of water. After vortexing, the mixture is centrifuged at low speed to separate the two phases. The upper phase is removed by siphoning. After siphoning of the upper phase, the lower chloroform phase containing lipids is evaporated under vacuum in a rota vapor or under a nitrogen stream.

Bligh and Dyer method

The Bligh and Dyer method (1959) is among the best methods for extraction of polar and non-polar substances. The Bligh and Dyer method is a simple adaptation of the Folch (Folch *et al*., 1957) procedure and was developed for cost reduction (in terms of solvent volumes) of extracting lipids. The lipid in the sample is extracted by solvent mixture consisting of chloroform, methanol and water, which gives a one-phase system. After extraction, the one-phase system is separated

into the lower chloroform layer and the top methanol-water layer by addition of more chloroform and water and bi-phasic formed. The lipids will follow the chloroform phase. The lipid in the chloroform layer is isolated using a separatory funnel. The fat content is determined in an aliquot of the chloroform phase by weighing the lipids after evaporation of the solvent.

Weight of lipid = (weight of container + extracted lipid) − (weight of container)

The content of lipids can also be in the sample by weight difference (Shahidi, 2001).

Lipid content (%) = amount of lipid extracted (g)/weight of original sample (g) × 100

In addition to the above mentioned popular lipid extraction methods, there are other extraction methods such as supercritical fluid extraction (King, 2000; Johnson and Barnett, 2003), accelerated solvent extraction (Schafer, 1998; King, 2000) microwave assisted extraction (Virot *et al*., 2007; Jyothi *et al*., 2010), ultra-sound assisted extraction and pressurized fluid extraction (Zhu *et al*., 2000). These methods are modifications of the traditional methods with the demand for higher recovery, faster analysis, and increased possibilities for automation and reduced solvent usage (beneficial from a cost and environmental standpoint).

2.10 Biodiesel from single cell oil

Due to environmental problems (especially concerns about greenhouse gas emissions), the impact of drastic increase in energy consumption, the rising price of fossil fuels and limited petroleum resources on the earth, it is becoming challenging to exploit fossil resources for sustainable use (Pahl, 2005; Hill *et al.*, 2006; Pan *et al*., 2009).This situation has initiated investigators to search for the alternative renewable resources such as biodiesel, biohydrogen and/or bioethanol (Alper and Stephanopoulos, 2009). Biodiesel, long chain alkyl esters (fatty acid methyl, ethyl or propyl ester) is a useful ecofriendly alternative energy resource. It has attracted much attention recently because it is made from renewable resources (Li *et al*., 2008) and may be used as a substitute for

petroleum based-diesel (Pan *et al*., 2009). It is biodegradable and nontoxic (Kraistinu *et al*., 2010). It has been reported that biodiesel reduces net carbon dioxide and total COx gas emissions by 78% on a lifecycle basis when compared to conventional diesel fuel (Tyson, 2001).When such biodiesel is used as fuel in diesel engines and heating systems, biodiesel has many merits, it is compatible with current automotive engines, it has high energy density, it has more favorable combustion emission profile, it has improved lubricating properties etc. (Tyson, 2001).

Currently, biodiesel is produced from plant oils (canola and soya bean etc.) and /or animal fats by transesterification with short chain or low molecular weight alcohols like methanol (Vicente *et al.,* 2004; Pan *et al*., 2009; Liu *et al*., 2010). Transesterification (also called alcoholysis) is the reaction of vegetable oil and or animal fats with an alcohol to form esters and glycerol in the presence of a suitable catalyst (Ma and Hanna, 1999). Transesterification is the displacement of an alcohol from an ester by another alcohol in a process similar to hydrolysis except that an alcohol is employed instead of water. During transesterification process an animal fat or plant oil reacts with an alcohol to form esters and glycerol. The alcohols used in the transesterification process are generally short chain alcohols such as methanol, ethanol, propanol and buthanol. Methanol is utilized most frequently because of its low-cost and its physical and chemical advantages (Fukuda *et al*., 2001).

Transesterification process is a sequential reversible reaction (Figure 2.4). TAGs are first reduced to diacylglycerols. Diacylglycerols then reduced to monoacylglycerols. Lastly, monoacylglycerols are reduced to fattyacyl esters. Glycerol is released as a by-product (Fukuda *et al*., 2001).

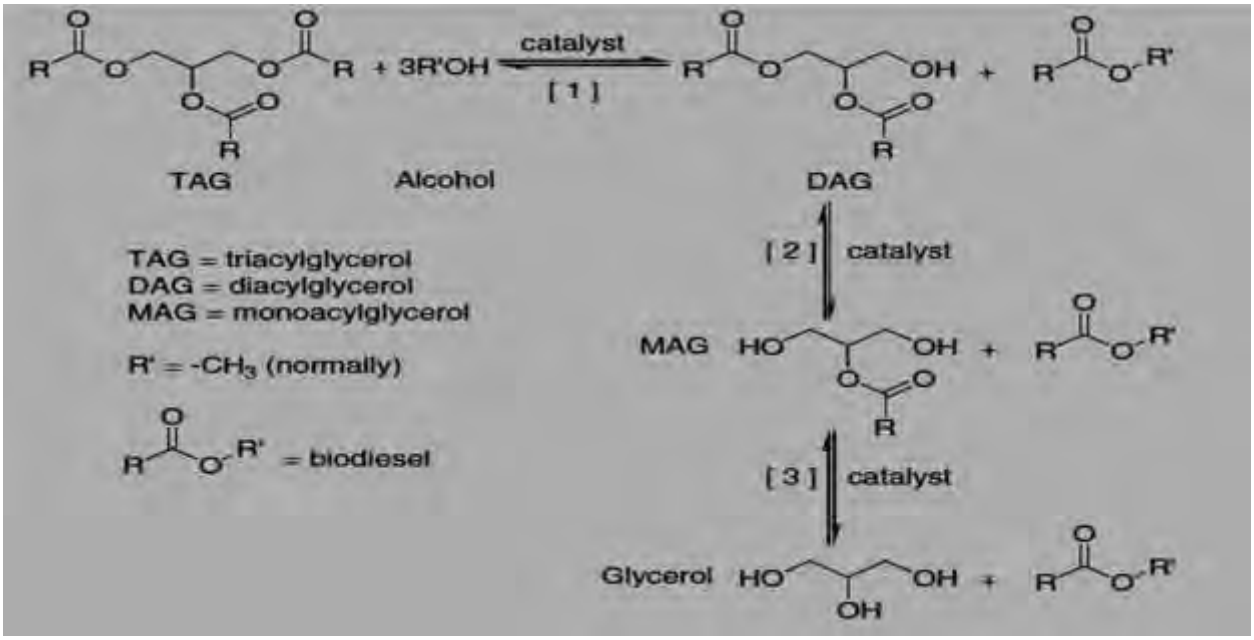

Figure 2.4: Schematic representation of biodiesel production from triacylglycerols (Source: Moser, 2011).

Making biodiesel from oils or fats is undesirable because this competes with the food market since these oils and fats are used for human consumption and the high cost of such oils as raw materials, especially vegetable oils (70−90%) and more time and man power is needed for their production (Vicente *et al.*, 2009; Kraistinu *et al*., 2010).

All the above limitations have initiated scientists to search other alternative feasible renewable resources, such as production of biodiesel from lipid feedstocks of algae, bacteria, molds and yeasts. Such cheap lipid feedstocks from oleaginous microorganisms is getting more attention because they like conventional vegetable oils have similar compositions, i.e., microbial oils are basically TAGs that require transesterification process to convert them into biodiesel. From this view point, microbial oils are potential sources for biodiesel production in the future (Li *et al*., 2008). The advantages biodiesel include (Tyson, 2001; Dai *et al*., 2007): It is renewable energy

resource; it is easily available probability in the future; it is easily biodegradable; it is free from sulphur and aromatic hydrocarbons, the combustion of which makes an important contribution to the greenhouse effect; it has low emission profiles; it is environmentally friendly; it is essentially carbon dioxide neutral; it is nontoxic and it is safe to store, handle and use.

2.10.1 Major properties of biodiesel

The major desirable properties of biodiesel are discussed below.

Cetane Number: Cetane number (CN) is dimensionless descriptor and indicator of the combustion speed of diesel fuel and is required for good engine performance (Knothe, 2006). CN is one of the most important parameters in determining the quality of diesel fuel. Conventional diesel must have a CN of at least 40 in the United States (Minnesota Statutes, 2008). Higher CN helps to ensure good cold start properties and minimize the formation of white smoke.

Cloud Point: The temperature at which the first crystals form and are visible to the naked eye (Minnesota Statutes, 2008). This is the most commonly used measure of low-temperature operability; fuels are generally expected to operate at temperatures as low or lower than their cloud point (Minnesota Statutes, 2008). Prolonged exposure of the biodiesel to temperatures at or below cloud point causes crystals to grow and cling together forming agglomerates that restrict flow (Dunn, 2011). Biodiesel has a higher cloud point than petroleum diesel.

Cold Filter Plugging Point (CFPP): This is the temperature under a standard set of test conditions (ASTM D6371) at which the filter plugs (Minnesota Statutes, 2008). It is the lowest operating temperature in which a vehicle will operate. The sample is cooled and tested at intervals of 1^{o}C until the wax crystals precipitate out of solution and are sufficient to slow or stop the flow of fuel through the filter.

Kinematic Viscosity: A minimum viscosity level is required for some engines because of the potential for power loss caused by injection pump and injector leakage. Actual viscosity of biodiesel depends on the fatty acid composition of the oil or fat from which it is made, and also on the extent of oxidation and polymerization of the biodiesel (Tat and Van Gerpen, 1999). The maximum viscosity is limited by the design of the engine fuel injections systems (Minnesota Statutes, 2008). At low temperatures, viscosity of biodiesel increases. Higher viscosity fuels can cause poor fuel combustion that leads to deposit formation as well as higher in-cylinder penetration of the fuel spray, which can result in elevated engine oil dilution with fuel.

Flash Point: The flash point (FP) is the lowest temperature at which a combustible mixture can be formed above the liquid fuel (Van Gerpen *et al*., 2004). The FP as specified is not directly related to engine performance. It is dependent on both the lean flammability limit of the fuel as well as the vapor pressure of the fuel constituents. It is, however, of importance in connection with legal requirements and safety precautions involved in fuel handling and storage, and it is normally specified to meet insurance and fire regulations (Minnesota Statutes, 2008). Naturally, biodiesel has higher FP than petroleum diesel.

Iodine Value: Iodine value (IV) is a measure of the degree of unsaturation of a lipidic material (Knothe, 2006). Highly unsaturated compounds have been linked with decreased oxidation stability, causing the formation of various degradation products, which can negatively affect engine operability (Schober and Mittelbach, 2007). The IV has been found to correlate with viscosity and CN, which both decrease with increasing degree of unsaturation (Schober and

Mittelbach, 2007). Upon oxidation, biodiesel properties like the acid value, peroxide value and viscosity increase, while the IV and the content of methyl esters decrease (Zuleta *et al*., 2012).

Oxidation Stability: Biodiesel can oxidize during storage and handling, leading to the formation of peroxides, acids, gums and deposits (Minnesota Statutes, 2008). It is the most discussed parameter in connection with the iodine value. The IV can be classified as a parameter giving an idea of stability of biodiesel but cannot be taken as a defined indicator for how long a sample is stable (Schober and Mittelbach, 2007). Oxidation stability describes the degradation tendency and consequences of the stability of biodiesel. The minimum oxidation stability requirement is intended to ensure the storage stability of B100 and biodiesel blends (Minnesota Statutes, 2008).

Pour Point: Pour Point the lowest temperature where the fuel flows or can be pumped (Dunn, 2011). It is a rough indication of the lowest temperature at which the liquid is readily pumpable (Dunn, 2011).That temperature is a good bit colder than the cold filter plugging point.

Other important biodiesel properties include acid value, carbon residue, lubricity, fuel stability, heating value, solidifying point, free glycerin, total glycerin, sulphated ash and ash content.

2. 10.2 Current status of biodiesel production in Ethiopia

The development of the country`s infrastructure, transport, industry, agriculture, households and societal service has increased the demand for fuel. To fulfill this demand Ethiopia imports three million metric tons of fuel annually (EPSE, 2016). Such imported fuel absorbs worth of 2.8 billion dollars (EPSE, 2016). The demand for fuel will increase when the economic growth of the nation increases. To fulfill this demand and to save foreign currency that is expended for purchasing and transport, the Ethiopian government has drafted a strategy (Ethiopian Biofuel Development and Utilization Strategy). This strategy is targeted to supply fuels from locally produced biofuel (*Jatropha*, Castor or *Ricinus*) bean, palm oil and sugar cane) and the objective of the strategy is to

ensure the production of biofuel without affecting food self- sufficiency, to improve balance of payment and to reduce environmental impacts (Abreham Berta and Belay Zerga, 2015).

Biodiesel production from *Jatropha curcas* has been started in Ethiopia. The most potential sites for *Jatropha* production in Ethiopia are Harar and Welo (Abreham Berta and Belay Zerga, 2015). There is also an interest to produce biodiesel from Castor (*Ricinus*) and palm oils. However, production of biodiesel from these plants is not effective since a minimum of seven years to harvest seeds from these plants and a large acre of land is needed to grow these plants which compete with the production of food plants. Furthermore, a lot of man power is needed to harvest the seeds. So that production of biodiesel from microbial oil feedstock is recommended.

Chapter 3: Isolation and Characterization of Oleaginous Yeasts from Different Geographical Locations in Ethiopia

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Abstract

Oleaginous microorganisms can produce relatively high amounts of oil under suitable cultivation conditions. In the current study, from 200 environmental samples collected, three hundred and forty yeast strains were isolated. All yeast isolates were screened using Sudan III staining for oil production. Among these, 18 were selected as possible oleaginous yeasts. Identification of these 18 isolates were done using morphological and physiological methods as well as sequencing the 3'end of the small-subunit rRNA gene, the internal transcribed spacer regions (ITS; ITS 1, ITS 2 and the intervening 5.8S rRNA gene), and the D1/D2 domain of the 26S rRNA gene. Molecular phylogenetic analyses indicate that isolates PY39, SY89 and SY94 are species of *Cryptococcus curvatus*, *Rhodosporidium kratochvilovae* and *Rhodotorula dairenensis*, respectively, while the

rest (SY09, SY18, SY20, PY21, PY23, PY25, SY30, PY32, SY43, PY44, SY52, PY55, PY61, SY75, and PY86) were identified as *Rhodotorula mucilaginosa*. Under nitrogen-limited cultivation conditions, *Rhodotorula mucilaginosa* PY44 produced the highest biomass (15.10± 0.54 g/L), while *Rhodotorula mucilaginosa* PY32 produced the lowest biomass (10.32 ±0.18 g/L). The highest lipid concentration of 6.87±0.62 g/L and lipid content of 46.51±0.70% were attained by *Cryptococcus curvatus* PY39. On the other hand, *Rhodotorula mucilaginosa* PY61 gave the lowest lipid concentration (2.06±0.52 g/L) and *Rhodotorula mucilaginosa* SY52 gave the lowest lipid content of 16.99±0.85%. On the basis of their substantial lipid production abilities, *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were selected as best candidates and recommended for oil production. Furthermore, *Cryptococcus curvatus* PY39 is recommended for large scale and commercial production of yeast oil for biodiesel production.

Keywords: Oleaginous yeast, single cell oil, yeast identification

3.1 Introduction

Oleaginous microorganisms can produce relatively high amounts of oil under suitable cultivation conditions (Wynn and Ratledge, 2006) and are of great interest due to their ability to accumulate high amounts of lipids in separate lipid bodies (Drucken, 2008; Li *et al.*, 2008). Moreover, their relatively high growth rates and the resemblance of their TAGs to plant oils (Pan *et al*., 2009; Kosa and Ragauskas, 2010), makes them promising organisms for biofuel production. Their production is not influenced by external factors such as their origin, and season or climatic changes (Thiru *et al*., 2011). The accumulated oil (stored TAGs) in the cells of oleaginous microorganisms acts as a carbon store to maintain essential metabolic processes in the event of subsequent carbon starvation (Anderson and Wynn, 2001). It is also suggested that, in the case of marine microorganisms, the

accumulation of large lipid droplets in the cytosol acts as an aid to buoyancy (Anderson and Wynn, 2001). The oil produced can be investigated and exploited as alternative sources of oils and fats for human consumption (Ratledge, 2005) and can also be used as feedstocks for biodiesel production (Li *et al*., 2008).

Oleaginous yeasts can be identified through the use of both conventional and molecular techniques. The conventional methods of oleaginous yeast identification include morphological and physiological tests (Yarrow, 1998; Kurtzman *et al*., 2011).These traditional or conventional methods are laborious, lack discriminatory power, time consuming, expensive and most of the times lead to misidentification (Kurtzman and Robnett, 1998). To avoid such problems, progress in molecular biology in the last decade has opened up possibilities of characterizing and identifying yeasts at the genomic level. The sequencing of the genes coding for 18S and 26S ribosomal RNA (rRNA), as well as ITS regions, has brought about many changes in the identification and classification of yeasts (Kurtzman and Robnett, 1998; Fell *et al*., 2000).

The objective of this study was to isolate, characterize and identify oleaginous yeasts from natural sources, i.e, from soil, plant surfaces (leaves, flowers and fruits), traditional oil mill wastes and dairy products in Ethiopia and produce SCO as a feedstock for biodiesel production.

3.2 Materials and methods

3.2.1 Sample collection

Two hundred samples of soil, dairy products (cheese, yogurt, and whey), flower, leaves, roots, fruits and wastes from traditional oil mill houses were collected. This was done to increase the chance of obtaining a high variety of oleaginous yeasts. Samples were collected from various parts of the country: Addis Ababa and surroundings (Menagesha Suba, Yeka Terara, Intoto, Gurara,

Atikilt Tera, Kebena, Megenagna and Arat Kilo),Gerbe Guracha, Ambo, Butajira, Buee, Gondar Zurea, Amba Giorgis, Debark, Gorgora, Nekemte, Bichena, Lumame, Dejen, Mota, Bure, Finote Selam, Dembecha, Adet, Debre Birhan, Jimma, Adama, Wolayita and Adigrat. All samples collected, were properly labeled and coded for further use. Figure 3.1 shows sample collection sites.

An amount of 1g of each sample was aseptically milled, ground or homogenized and added into 50 mL sterile Yeast Malt extract (YM) broth (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract and 5 g/L peptone) in a 250 mL Erlenmeyer flask, and incubated at 30^{o}C for 24 h with shaking speed at 200 rpm according to Yarrow (1998).

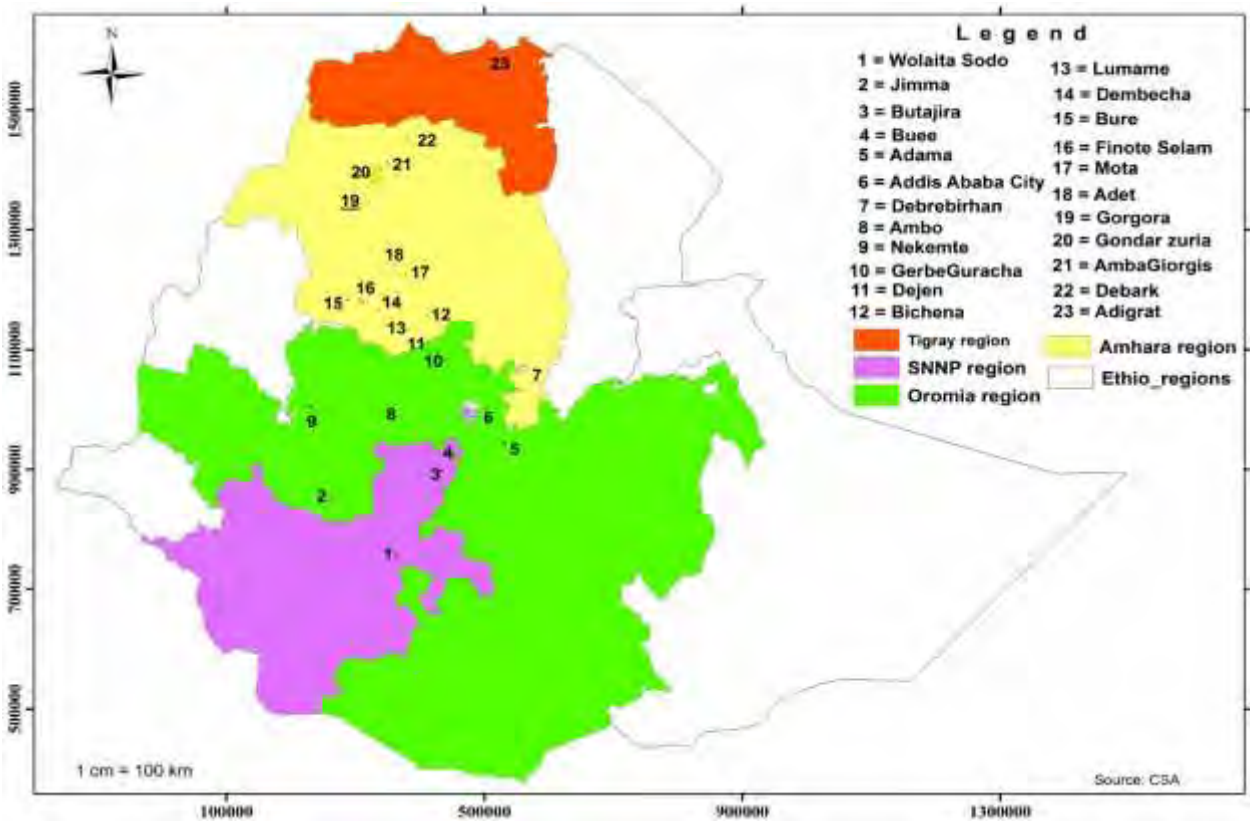

Figure 3.1: Map showing sample collection sites in Ethiopia.

3.2.2 Isolation of yeasts from different natural samples

Volumes of 1 mL of the prepared cultures were added to 9 mL of distilled water and 10-fold serial dilutions were made. A volume of 0.1 mL from each dilution ranging from 10^{-1} to 10^{-5} was spread onto plates made with YM agar plates (10 g/L glucose, 3 g/L yeast extract,3 g/L malt extract,5 g/L peptone and 20 g/L agar). Chloramphenicol (1 g/L) was added to the media to inhibit bacterial growth and sodium propionate (2.5 g/L) was added to inhibit the growth of filamentous fungi. The plates were incubated at 30^{o}C for two to four days and those containing isolated colonies with the morphology typical of yeasts were picked and transferred as pure cultures to YM agar slants and maintained at 4^{o}C until used.

3.2.3 Screening for oleaginous yeasts

The isolated yeast cultures were screened for their lipid producing abilities by qualitative analysis with the Sudan III staining technique (Thakur *et al*., 1988; Xing *et al*., 2012) and all yeasts positive for lipid production were further characterized and identified.

Sudan III staining procedure

In the preliminary study, 340 yeast strains were isolated from 200 samples, each strain was cultivated under nitrogen-limited media containing (g/L): glucose (70), $(NH_4)_2SO_4$(0.30), yeast extract (0.30), KH_2PO_4 (2.0), $MgSO_4.7H_2O$ (1.8), $CaCl_2.2H_2O$ (0.1), $FeSO_4.7H_2O$ (0.035), $ZnSO_4.7H_2O$ (0.011), $MnSO_4.H_2O$ (0.007), $CoCl.6H_2O$ (0.002), $Na_2MoO_4.2H_2O$ (0.0013), and $CuSO_4.5H_2O$ (0.001). The pH of the medium was adjusted to 5.5 using 1 NaOH and 1 N HCl. Cultures were kept at temperature of 30^{o}C for 4 days with shaking speed at 200 rpm. They were harvested by centrifugation (3000x*g* for 15 min). A smear of yeast was prepared on a slide, and then it is let dry thoroughly in the air, and was heat fixed. The entire slide was flooded with Sudan III solution (0.3 g of the powdered stain in 100 mL of 70% ethanol), and the slide was allowed to

remain undisturbed at room temperature for 15min. The excess stain was drain off, counterstained with safranin red for 30 sec, washed with water and air dry. It was observed under a microscope (Olympus BX51) using oil immersion objective. Sudan III stains red. Photomicrographs of each stained strain were taken from a computer attached to the microscope. The potential oleaginous yeast colonies then were maintained on YM slant agar at 4^{o}C and were transferred once every 2 months. Stock cultures were incubated for 2 days, and then stored in a refrigerator before use.

3.2.4 Identification of oleaginous yeasts

An integrated approach including morphological, biochemical and physiological characterization following the method of Yarrow (1998) and Barnett *et al*. (2000) and genotypic (sequencing of both ITS domain and D1/D2 domain of the 26S rRNA gene) was used for the identification of the oleaginous yeasts.

3.2.5 Characteristics of vegetative reproduction

3.2.5.1 Investigation of non-filamentous vegetative cells

Examination of vegetative cells was made following the method described by Yarrow (1998) using sterile YM broth. Cells from a young actively growing culture were then inoculated into 30 mL of medium in a 100 mL, cotton-plugged Erlenmeyer flask and incubated for 2−3 days. Then shape of the cells, their mode of asexual reproduction (budding or fission) was examined.

3.2.5.2 Investigation of filamentous growth (pseudohyphae)

The formation of pseudohyphae was observed through the inoculation of yeasts cells in Petri dishes containing Potato Dextrose Agar (PDA) with ingredients potato extract 4 g/L, glucose 20 g/L and agar 15 g/L), the cultures were covered with a glass slide. The Petri dishes were incubated at 30^{o}C and examined microscopically for the formation of filaments from 5 to 21 days of incubation (Yarrow, 1998; Maldonade *et al*., 2007).

3.2.5.3 Formation of ballistoconidia

The formation of ballistospores or ballistoconidia was verified using an inoculated dish having PDA inverted over another petri dish containing malt extract agar (malt extract 50 g/L and 20 g/L). The two dishes were taped together. The dishes were incubated at 20°C for one month. The presence of discharged spores (if any) that germinate to form colonies on the bottom dish which were collected on the glass slide were tested. This was done by removing the slide from the bottom petri dish and examining under the microscope (Yarrow, 1998; Maldonade *et al*., 2007).

3.2.6 Ascospore formation and induction

Yeast isolates examined for ascospore formation and induction was brought to active growth by cultivating them on a pre-sporulation media 30^{o}C for 48 h. This medium was prepared by mixing 10 g/L yeast extract, 100 g/L glucose, 20 g/L potassium acetate and 2 g/L of agar. The medium was then sterilized by autoclaving at 121^{o}C for 15 min. In addition, Gorodkowa Agar Sporulation Medium (1 g/L of glucose, 5 g/L of sodium chloride, 10 g/L of peptone, and 20 g/L of agar) was prepared. Then, the ingredients were sterilized by autoclaving at 121^{o}C for 15 min. The media was then poured into plates of sporulation media. The plates of sporulation media was inoculated from the culture in pre-sporulation media and incubated at 30^{o}C. The cultures were examined under the microscope by staining them at intervals for 6 weeks.

Staining procedures: - Heat-fixed preparations were flooded with a solution of 0.5% malachite green and 0.05% basic fuchsine and heated to steaming for 1 min, washed thoroughly in flowing water and blotted dry. The preparations were then flooded with a 5% solution of malachite green, heated to 80^{o}C for 5 min, washed for 30 sec and counter stained with a 0.5% solution of safranin for 10 sec.

3.2.7 Physiological tests

3.2.7.1 Fermentation tests

Fermentation test showed that none of the yeasts fermented glucose and there was no need undertake further fermentation tests on other carbon sources. Before inoculating yeasts, fermentation basal medium was prepared as follows. Powdered yeast extract with 4.5 g and peptone 7.5 g were dissolved in 1 L of distilled water. A basal medium of 2 mL was then put into tubes. Then the ingredients were sterilized at 121^{o}C for 15 min. A 1 mL concentrated, filter-sterilized sugar solution was added into the tubes to give a final sugar concentration of 2% (w/v). The tubes were inoculated with cells from a 24−48 h old culture and incubated at 30^{o}C for 28 days. The tubes were shaken and inspected for accumulation of gas in the insert. The results were recorded and scored.

3.2.7.2 Assimilation tests of carbon sources

An assimilation test of yeasts was done using auxonographic method following the methods of Yarrow (1998) and Barnett *et al*. (2000). For this purpose, 0.5% w/v $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05 % $MgSO_4.4H_2O$, 2% noble agar and 0.1% w/v vitamin solution (biotin,0.2 mg/L; calcium pantothenate,40 mg/L; folic acid,0.2 mg/L,inositol,200 mg/L; niacin,40mg/L, para-aminobenzoic acid, 20 mg/L; pyridoxine hydrochloride,40 mg/L; riboflavin,20 mg/L and thiamine hydrochloride, 100 mg/L) were dissolved in 1 L of distilled water and sterilized at 121^{o}C for 15min. The basal agar medium was melted and cooled to 45^{o}C. A suspension of young cells was added to the medium, mixed and then poured into a plate, the plate was gently swirled to mix the contents. The plates then were left on a level surface for the agar to set and then allowed to stand for 2 h to allow the surface of the agar to dry. The plates were then seeded with carbon sources at various points around the periphery. The results were later read by inspecting the plates for an

opaque zone of growth around the point where a carbon source was applied. The bottom of the plate was marked around the periphery to locate and identify the carbon sources. The plates were examined for a week.

3.2.7.3 Test for assimilation of nitrogen compounds

Assimilation of nitrogen compounds (potassium nitrate, L-lysine, creatine and peptone as a control) was tested in petri dishes with the following basal medium (Yarrow, 1998). This basal medium contained 2% w/v of glucose, 0.1% w/v of KH_2PO_4, 0.05% w/v of $MgSO_4.7H_2O$, and 2% w/v of high quality agar and 0.1% w/v vitamin solution(biotin,0.2 mg/L; calcium pantothenate,40 mg/L; folic acid,0.2 mg/L,inositol,200 mg/L; niacin,40mg/L, para-aminobenzoic acid,20 mg/L; pyridoxine hydrochloride,40 mg/L; riboflavin,20 mg/L and thiamine hydrochloride, 100 mg/L) and autoclaved at 121^oC for 15 min. The basal agar medium was melted and cooled to 45^oC. A suspension of young cells was added to the medium, mixed and then poured into a plate, the plate was gently swirled to mix the contents. The plates then were left on a level surface for the agar to set and then allowed to stand for 2 h to allow the surface of the agar to dry. Aseptically the nitrogen sources were placed on the surface of medium. Then the plates were incubated at 30^oC temperature and inspected after 2 and 4 days for zones of growth around the sites of the nitrogen sources.

3.2.7.4 Hydrolysis of urea

Christensen's urea agar (containing 1 g/L of peptone, 5 g/L of NaCl, 2 g/L of KH_2PO_4, and 12 µg/L phenol red and 20 g/L of agar with pH to 6.8) was used for hydrolysis of urea test. About 4.5 mL medium was dispensed into 10 mm diameter plugged glass tubes and autoclaved at 121°C for 15min. About 0.5 mL of a filter-sterilized 20% solution of urea was added at last. After mixing, the tubes were slanted and allowed for the agar to set. Cells from a 24 h old culture were inoculated

onto a slant of Christensen's urea agar and a control tube of the basal medium without urea and grown at 30^{o}C. The cultures were inspected daily for up to 4 days and the result was recorded.

3.2.7.5 Diazonium Blue B color reaction

The yeast strains tested were inoculated into the sterilized YM agar media and incubated for 3 weeks. Two drops of prepared chilled DBB reagent was applied well to the surface of each colony. The DBB reagent was prepared as follows. It was prepared by dissolving 15 mg of DBB salt in 15 mL of chilled 0.25 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.0. The freshly prepared DBB reagent was kept in an ice bath (4^{o}C). Those which showed dark-red to violet-red color within 2 min at room temperature were recorded as positive.

3.2.7.6 Growth in media of high osmotic pressure

The ability of a yeast to grow with high concentrations of sugar was tested using agar media containing 60% w/w of glucose. For this 60% glucose, 1% yeast extract and 2% agar were dissolved in distilled 100 mL water. The contents were dispensed into tubes and were autoclaved at 110°C for 10 min before slanting. The tubes were inoculated with the target yeast isolate and incubated at 30^{o}C and checked every week up to 4 weeks for the development of colonies.

3.2.7.7 Splitting of fat/Lipolytic activity

Suet from beef was melted, filtered, and autoclaved at 121^{o}C for 15min. About 0.5 mL amount was poured into a slightly warmed sterile plate and the plate was tilted from side to side so the fat evenly spread over the bottom. The plate was then put in a refrigerator for two h to allow the fat to harden. About 20 mL YM agar was melted, cooled to 45^{o}C, poured over the fat, and allowed to set. The plate was inoculated with the test strains, cutting slightly into the agar medium with the needle and incubated at 30^{o}C. The plate was inspected daily for the formation of an opaque zone which is usually apparent along the streak within a week.

3.2.7.8 Formation of extracellular amyloid compounds

To test for the formation of starch-like material by a yeast, 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, and 20 g glucose were dissolved in 1 L of distilled water in an Erlenmeyer flask. The pH was adjusted to 4.5. In addition, 40 g of agar was dissolved in 1 L of distilled water separately. The two portions of the media were autoclaved at 121^oC for 15 min. They were put together aseptically and 20 mL of 20% solution of yeast extract was added. The two solutions were mixed gently, while the agar was still molten and dispensed aseptically into petri dishes. The test strain was inoculated onto agar medium in petri dishes. Cultures were incubated for 2 weeks, then flooded with dilute Lugol's iodine, and inspected for formation of a dark blue to green color. The test was conducted following the procedure of Yarrow (1998).

3.2.8 Molecular characterization of oleaginous yeasts

Genomic DNA was extracted from cultures grown on 40 g/L glucose, 5 g/L peptone, 5 g/L yeast autolysate, and 20 g/L agar (GPYA) medium for three days using the FastDNA kit (BIO101, Carlsbad, CA, USA) with the "FastPrep" Instrument (Q-Biogene). Primers V9G (-TTACGT CCCTGCCCTTTGTA-) (de Hoog and Gerrits van den Ende, 1998) and LR5 (-ATCCTGAGGGAAACTTC-) (Vilgalys and Hester, 1990) were used to amplify the region of the rRNA gene operon that includes the 3'end of the small-subunit rRNA gene, the ITS regions (ITS 1, ITS 2 and the intervening 5.8S rRNA gene), and the D1/D2 domains of the 26S rRNA gene of the large subunit, as described by Knutsen *et al*. (2007). The PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8% (w/v) agarose gel containing 0.1 µg/mL ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and examined under UV-light. The amplicons were sequenced in both directions using the primers LR0R (-ACCCGCTGAACTTAAGC-) (Vilgalys and Hester, 1990) and LR5 for the D1/D2 domain, while

the primers V9G and ITS4 (White *et al*., 1990) were used for the ITS domain (ITS 1, ITS 2 and the intervening 5.8S rRNA gene). The BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) was used according to the manufacturer's recommendations and the products were analyzed on an ABI Prism 3730XL DNA Sequencer (Perkin-Elmer). A consensus sequence was computed from the forward and reverse sequences with SeqMan version 8 from the Lasergene package (DNASTAR). All sequences of the studied strains were blasted against sequences in GenBank (http://blast. ncbi.nlm.nih.gov/Blast.cgi) and the CBS yeast database (http://www.cbs.knaw.nl/Collections/) in order to identify the oleaginous yeasts. Sequences of theD1/D2 of the 26S rRNA obtained during this study and related sequences from the GenBank (NCBI) database were aligned and phylogenetic analyses were done using MEGA 7 version (Kumar *et al.*, 2016). The phylogenetic relationship of these yeast strains is displayed in a distance based Neighbor-Joining tree.

3.2.9 Cultivation of oleaginous yeast in nitrogen-limited medium

Those yeast isolates tested positive for Sudan III stain were cultivated on nitrogen-limited media containing (g/L):glucose (70), $(NH_4)_2SO_4$ (0.30), yeast extract (0.30), $MgSO_4.7H_2O$ (1.5), $CaCl_2.2H_2O$ (0.1), KH_2PO_4 (2.0), $FeSO_4.7H_2O$ (0.035), $ZnSO_4.7H_2O$ (0.011), $MnSO_4.H_2O$ (0.007), $CoCl.6H_2O$ (0.002), $Na_2MoO_4.2H_2O$ (0.0013), and $CuSO_4.5H_2O$ (0.001). An inoculum level of 5% (v/v) was added. Cultures were performed in 250 mL conical flasks containing 50 mL media. The oleaginous yeasts were cultured in this nitrogen-limited media for 144 h at 30°C on a rotary shaker at 200 rpm. The initial pH of the medium was 5.5.

3.2.10 Analytical methods

3.2.10.1 Determination of yeast biomass

Yeast cells were harvested by centrifugation at 5000×*g* for 15 min. The supernatants were discarded. The pellet was harvested and washed twice with distilled water and frozen at -80^{o}C. The cells were then freeze dried over night to constant weight and the dry weight determined gravimetrically.

3.2.10.2 Determination of lipid content

Lipid extraction was done following Folch *et al*. (1957) with some modifications. Freeze dried biomass was ground with a pestle in a mortar. Then 1g of sample was homogenized with 3.75 mL solvent mixture of chloroform and methanol (2:1) and was left overnight. The following day the solvent mixture was transferred into clean separating funnel through Whatman No1 filter paper. Then 1.25 mL of solvent mixture was poured through filter paper into separating funnel. This was followed by washing with 0.75 mL of distilled water. The solvent/water mixture was left overnight to separate into two clear phases. The bottom phase was collected and the solvents evaporated under vacuum. Diethyl ether was used to transfer the extract into pre-weighed glass vials. The glass vials were left overnight in a fume hood to evaporate the diethyl ether. The following day, the vial with the sample was weighed. From this lipid concentration and lipid content were calculated as follows.

$$\text{Single cell oil content} = \frac{\text{Single Cell Oil Concentration (g/L)}}{\text{Cell Dry Weight(g/L)}} \times 100$$

3.2.10.3 Statistical analysis

All experiments were done in triplicate. One way-ANOVA was performed to calculate significant differences in treatment means. SPSS version 20.0 software was used for interpretation of data. Data with a p value < 0.05 were considered significantly different.

3.3 Results

3.3.1 Isolation and screening of yeasts from different sources

In this study, from a total of 200 samples collected from the various parts of Ethiopia, 340 yeast colonies were obtained.

Based on screening results using Sudan III staining technique, 18 yeast isolates were selected. When cells of these yeasts were stained with Sudan III stain and observed under oil immersion objectives, yellow intracellular inclusions were seen in each case (Figure 3.2) and based on the above observations 18 yeast isolates were selected as oleaginous and further investigations were undertaken.

3.3.2 Morphological and physiological identification of oleaginous yeasts

Despite advances in molecular identification techniques, morphological and physiological characteristics continue to be important in yeast systematics and identification. In the current study, the chosen yeast isolates were characterized and identified with different classical methods (morphological and physiological) before proceeding to molecular identification. The tested yeasts produced different looking colonies (Table 3.1). The yeasts differed in their colony color from orange, pink, salmon, red to creamy. The tested strains also varied in size from small sized colony with diameter of 2−3 mm, medium sized colony with diameter of 4−5 mm to large sized colony with diameter of 6−10 mm. The exhibited colony shapes were circular in some of the tested yeasts and were irregular in others. Furthermore, the isolated strains varied in their texture from smooth and dry, smooth and moist, wrinkled and moist to wrinkled and dry.

Microscopic observations were undertaken to examine shape of the cells and their budding patterns, whether they show unipolar, bipolar or multipolar budding. Such yeasts displayed

different budding pattern and cell shapes (Table 3.1). Some were observed to be reproduced by unipolar budding, others by bipolar budding. It was observed that the rest yeasts reproduced by multipolar budding. From these tested oleaginous yeasts, no yeast was able to be reproduced by fission. On the other hand, the 18 oleaginous yeasts showed different features of cell shape. The observed shapes of PY55, PY86, SY89 and SY94 were ovoidal. The rest of the yeast isolates displayed ovoidal to spherical shape.

Furthermore, other morphological tests were done for identification purposes. These were used as basis for partial identification of yeasts. These tests were investigation for filamentous growth (formation of pseudohyphae), formation of ballistoconidia and ascospore (Table 3.2). All the tested yeasts did not form ballistoconidia. All the tested yeasts failed to produce ascospore. The yeasts varied in their ability to form pseudohyphae. All the tested yeasts were able to form pseudohyphae except PY21, PY23, SY30, PY44 and PY61.

Glucose fermentation capacity of the tested yeasts was among the physiological tests investigated and all the tested yeasts did not ferment glucose. Thus, there was no need to undertake further fermentation tests on other carbon sources. This is because if a yeast fails to ferment glucose, by no means it is able to ferment other carbon sources. Assimilation tests of different carbon sources, i.e., glucose, sucrose, maltose, lactose, galactose, trehalose, melibiose and cellobiose were done. All the 18 oleaginous yeasts assimilated D-glucose, sucrose, maltose, D-galactose and trehalose. Lactose was assimilated only by PY39. Melibiose was assimilated only by PY39, SY89 and SY94. Cellobiose was assimilated only by SY09, PY32, SY89, SY94 and PY86. Nitrogen assimilation test was done using peptone as control, KNO3, L-lysine and creatine. From the 18 oleaginous yeasts, only SY89 and SY94 assimilated KNO_3. All the tested yeasts were able to assimilate L-lysine except SY89. None of the yeasts was able to assimilate creatine. Concerning growth in high

osmotic media containing 60%, none of the oleaginous yeasts were able to grow at this concentration. DBB color reaction and urea hydrolysis tests were done to distinguish whether the screened oleaginous yeasts are ascomycetes and basidiomycetes. In both tests, all the tested oleaginous yeasts gave a positive result. With respect to extracellular amyloid (starch) formation, from the 18 oleaginous yeasts, only PY39 was able to form extracellular amyloid or starch. The rest failed to form extracellular amyloid or starch. Furthermore, fat splitting test was done for the 18 oleaginous yeasts. All yeasts failed to split fat except PY39.

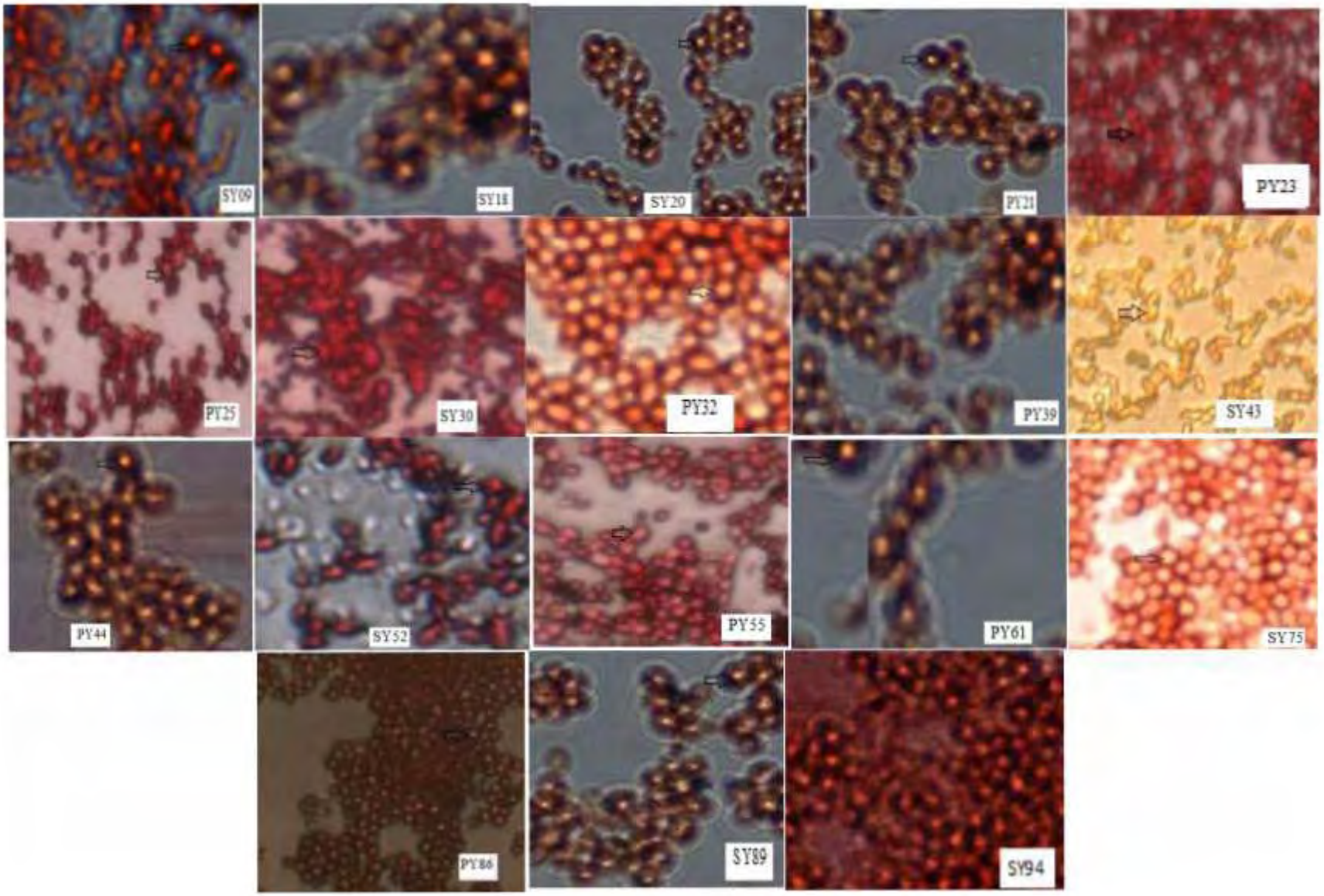

Figure 3.2: Phase contrast microscope of the isolated and identified yeasts stained with Sudan III on oil immersion (100X). Lipid bodies stained yellow.

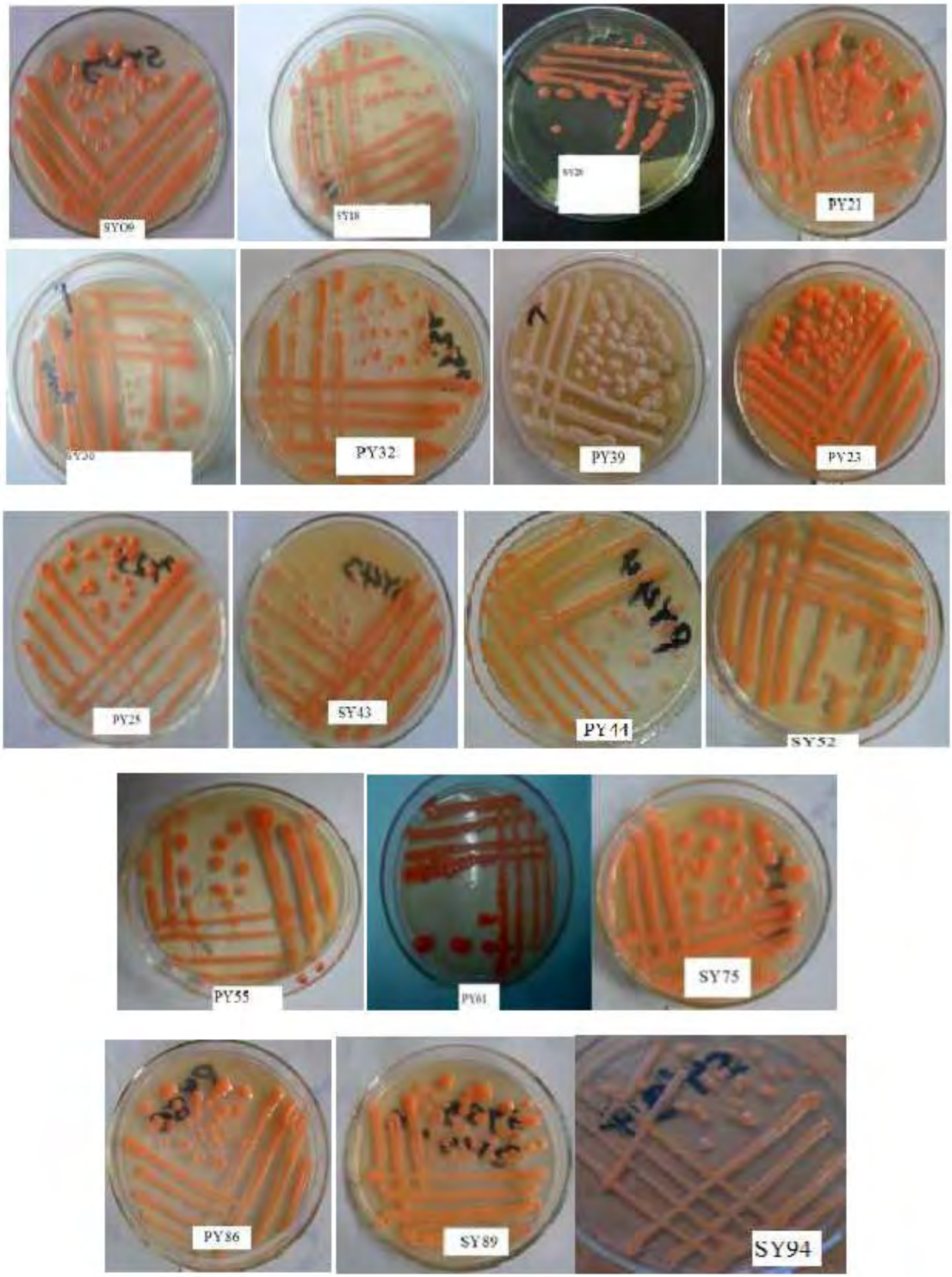

Figure 3. 3: Colony variability of selected oleaginous yeasts on agar medium.

Table 3.1: Colony characteristics, budding patterns and shape of potential oleaginous yeasts.

Strain	Source	Environment (site)	Colony morphology (colony color, size, shape , texture and diameter)	Budding pattern	Cell shape
SY09	soil	Yeka Terara	orange, medium, circular, smooth, dry colonies with diameter 4−5 mm	bipolar	ovoidal to spherical
SY18	soil	Intoto	pink, small, circular, smooth, dry colonies with diameter 2−3 mm	multipolar	ovoidal to spherical
SY20	soil	Intoto	orange, small, circular, smooth, moist colonies with diameter 2− 3 mm	multipolar	ovoidal to spherical
SY30	soil	Gurara	pink, medium, circular, smooth, moist colonies with diameter 3−5 mm	multipolar	ovoidal to spherical
SY43	soil	Gerbe Guracha	orange, irregular, large, smooth, moist colonies with diameter of 6−10 mm	multipolar	ovoidal to spherical
SY52	soil	Jimma	salmon, medium, irregular, smooth, dry colonies with diameter of 4−5 mm	bipolar	ovoidal to spherical
SY75	soil	Mota	orange, medium, irregular, wrinkled, moist colonies with diameter 4−5 mm	unipolar	ovoidal to spherical
SY89	soil	Dembecha	salmon, medium, circular, smooth, dry colonies with diameter of 4−5 mm	bipolar	ovoidal
SY94	soil	Finote Selam	orange, large, irregular, wrinkled , dry colonies with diameter of 6−10 mm	multipolar	ovoidal
PY21	fruit	Atikilit Tera	orange, small, circular, smooth, moist colonies with diameter of 2−3 mm	multipolar	ovoidal to spherical
PY23	fruit	Atikilit Tera	pink, circular, medium, smooth, moist colonies with diameter of 4−5 mm	multipolar	ovoidal to spherical
PY25	fruit	Atikilit Tera	orange, circular, medium, smooth, dry colonies with 4−5 mm in diameter	multipolar	ovoidal to spherical
PY32	flower	Atikilit Tera	orange, circular, medium, smooth, dry colonies with diameter of 4−6 mm	multipolar	ovoidal to spherical
PY39	flower	Arat Kilo	creamy, large, irregular, wrinkled, moist, colonies with diameter of 6−10 mm	multipolar	ovoidal to spherical
PY44	flower	Arat Kilo	pink, circular, medium, smooth, dry colonies with diameter of 4−5 mm	multipolar	ovoidal to spherical
PY55	flower	Ambo	pink, medium, circular, wrinkled, moist colonies with diameter of 4−5 mm	multipolar	ovoidal
PY61	leaf	Ambo	red, medium ,irregular, wrinkled, moist colonies with diameter of 4-5 mm	unipolar	ovoidal to spherical
PY86	leaf	Gondar Zurea	salmon, small, irregular, wrinkled, moist colonies with diameter of 2−3 mm	bipolar	ovoidal

Table 3.2: Other phenotypic characters of selected yeasts.

Yeast strain	Ballistoconidia	Ascospore formation	Pseudohyphae	DBB color reaction	Fat splitting	Urea hydrolysis	Growth in 60 % glucose	Starch formation	Glucose fermentation	Assimilation test											
										D-glucose	Sucrose	Maltose	Lactose	D-galactose	Trehalose	Melibiose	Celobiose	Peptone	KNO_3	L-lysine	Creatine
SY09	−	−	+	+	−	+	−		−	+	+	+	−	+	+	−	+	+	−	+	−
SY18	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
SY20	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
SY30	−	−	−	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
SY43	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
SY52	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
SY75	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
SY89	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	+	+	+	+	−	−
SY94	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	+	+	+	+	+	−
PY21	−	−	−	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
PY23	−	−	−	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
PY25	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
PY32	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	+	+	−	+	−
PY39	−	−	+	+	+	+	−	+	−	+	+	+	+	+	+	+	−	+	−	+	−
PY44	−	−	−	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
PY55	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
PY61	−	−	−	+	−	+	−	−	−	+	+	+	−	+	+	−	−	+	−	+	−
PY86	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	−	+	+	−	+	−

(-) negative test, (+) positive test

Morphological and physiological characterization test results led to the identification of isolated and screened oleaginous yeasts at the genus level. Based on this SY09, SY18, SY20, SY30, SY43, SY52, SY75, PY21, PY23, PY25, PY32, PY44, PY61, PY86 and SY94 were assigned as *Rhodotorula* species. So that *Rhodotorula* species can be the most frequently isolated. SY89 was assigned as *Rhodosporidium* species. Furthermore, PY39 was classified as *Cryptococcus* or *Trichosporon* species.

3.3.3 Molecular characterization of oleaginous yeasts

After screening yeasts with Sudan III, morphological and physiological characterization and identification of such potential oleaginous yeasts, they were further identified by sequence analysis of the ITS and the D1/D2 domains of the 26S rRNA gene and the sequences obtained from this study and that of closely related sequences from GenBank are displayed in Figure 3.4.

The sequences were blasted against sequences in GenBank (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the CBS database (http://www.cbs.knaw.nl/Collections/). The percentage of sequence similarity with data of additional strains and especially type strains were determined. The D1/D2 sequences were aligned and the phylogenetic relationship of these yeast strains is displayed in a distance based Neighbor-Joining tree (Figure 3.4). The tree obtained by analyzing ITS domain is not shown here since the same relationship among species was obtained. SY52 failed to be sequenced using D1/D2 domains of the 26S rRNA gene and that it is omitted in the phylogeny. However, it was identified as *Rhodotourla mucilaginosa* species based on morphological and physiological tests as well as by comparing its ITS sequences against sequences in GenBank (http://blast.ncbi. nlm.nih. gov/Blast.cgi) and the CBS yeast database (http:// www.cbs.knaw. nl/ Collections/) (data not shown). On the other hand, the D1/D2 domain of 26S rRNA gene of PY39 (693 nucleotides, ACC.NO. KX525704) showed highest genetic agreement

(99% similarity) with *Cryptococcus curvatus* (*C.curvatus)* CBS 570. The sequence analysis results of SY89 (776 nucleotides, ACC. NO.KX525703) also revealed that the amplified sequences were 100% identical to those corresponding to *Rhodosporidium kratochvilovae* (*R.kratochvilovae*) CBS 7436 and *R.kratochvilovae* CECT 11956,while 99% similarity was exhibited by this strain with *R.kratochvilovae* PYCC4778. In addition the sequence analysis result showed that the amplified sequences of SY94 (717 nucleotides, ACC.NO.KX525689) were 99% identical to those corresponding to *Rhodotorula dairenensis* (*R. dairenensis*) CAB2188, *R.dairenensis* CBG_16CCH, *R.glutinis var. dairenensis* IGC4897. Furthermore, PY21 (747 nucleotides, ACC.NO.KX525694), PY23 (765 nucleotides, ACC.NO.KX525690), PY25 (487 nucleotides, ACC.NO.KX525697), PY32 (803 nucleotides, ACC.NO.KX525705), PY44 (782 nucleotides, ACC.NO.KX525698), PY55 (770 nucleotides, ACC.NO.KX525699), PY61 (794 nucleotides, ACC.NO.KX525701), PY86 (748 nucleotides, ACC.NO.KX525691), SY09 (312 nucleotides, ACC.NO. KX525695), SY18 (777 nucleotides, ACC.NO. KX525702), SY20 (776 nucleotides, ACC.NO. KX525692), SY30 (737 nucleotides, ACC.NO. KX525700), SY43 (682 nucleotides, ACC.NO.KX525693) and SY75 (758 nucleotides, ACC.NO.KX525696) were 100% identical to *Rhodotorula mucilaginosa* (*R.mucilaginosa*) CAB2356, *R. mucilaginosa* ATCC 32763, *R.mucilaginosa* AFTOL-ID1548, *R.mucilaginosa* CECT11975, *R.mucilaginosa* CRUB 1441, *R.mucilaginosa* AF335987 and *R. mucilaginosa* CBS 9077.

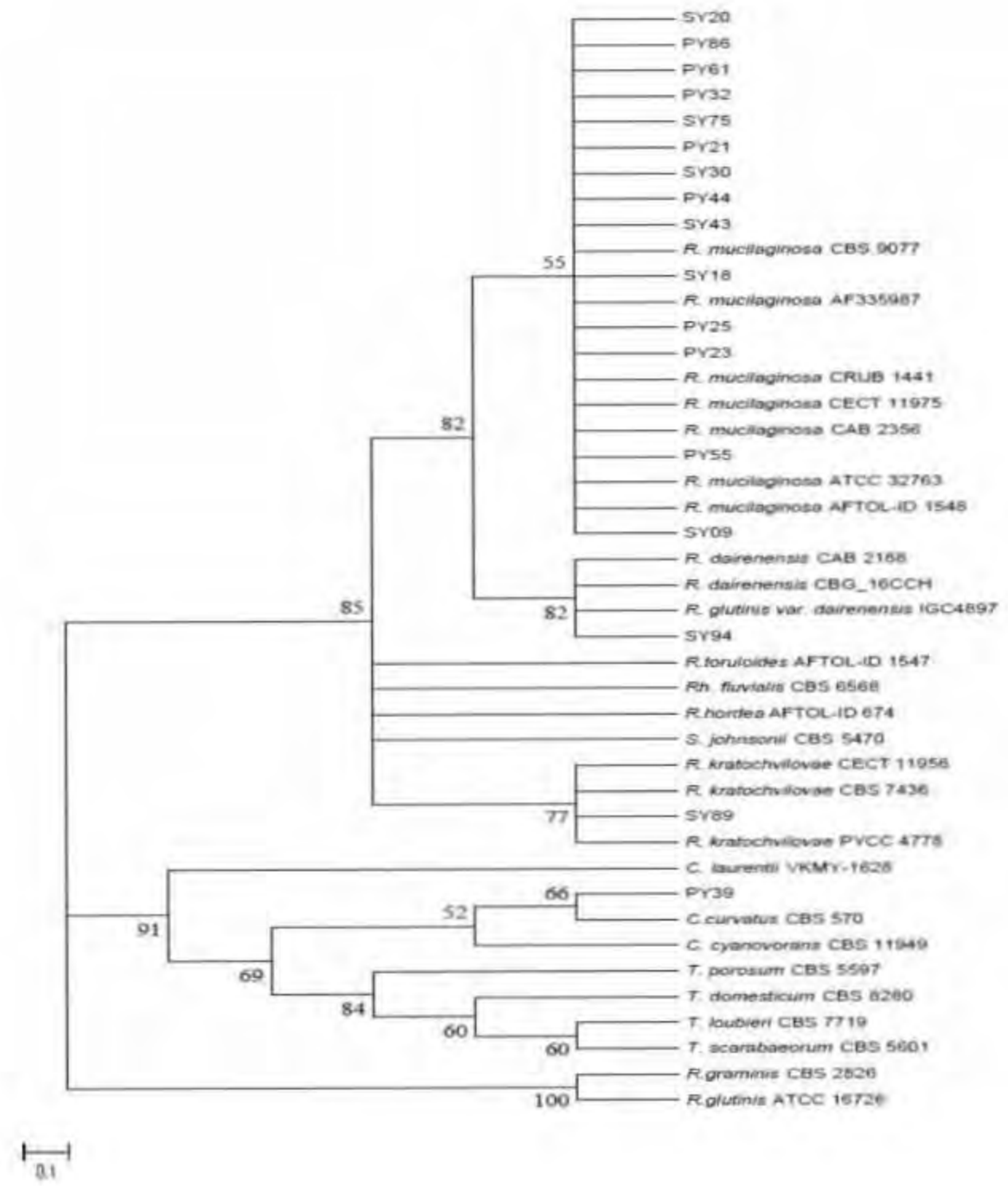

Figure 3.4: Phylogenetic tree of the D1/D2 domain of 26S rRNA gene sequences of oleaginous yeast strains with related yeast species in NCBI database. The tree was constructed using the Neighbor-Joining method of MEGA 7 software. The optimal tree with the sum of branch length = 1.81465379 is shown. Bootstrap values (1000 tree interactions) are indicated at the nodes.

3.3.4 Cultivation of oleaginous yeast in nitrogen-limited medium

In order to select the best oleaginous yeast from the screened 18 isolates, the lipid production yield and cellular lipid content were evaluated under nitrogen-limited condition at the end of 144 h of incubation for the 18 oleaginous yeasts (Table 3.3). The yeast strain PY44 gave the highest biomass (15.10±0.54 g/L), whereas PY32 produced the lowest biomass (10.32±0.18 g/L). Maximum lipid concentration was attained by PY39 (6.87±0.62 g/L) followed by SY89 **(**5.79±0.30 g/L), SY18 **(**5.27±0.27 g/L) and SY09 (4.97±0.27 g/L). PY61 gave the lowest lipid concentration (2.06±0.52 g/L). On the other hand, maximum cellular lipid content was attained by PY39 (46.51±0.70%) followed by SY89 (39.33±0.57%), SY18 (38.61±0.39%) and SY09 (36.15±0.41%). SY52 had the lowest lipid content, i.e., 16.99±0.85%. As it can be seen from Table 3.3, SY94 and PY21 gave both good lipid concentration and cellular lipid contents. On the basis of their substantial lipid production abilities, the four oleaginous yeast strains, namely PY39, SY09, SY89 and SY18 were selected and recommended for further optimization processes.

Table 3. 3: Biomass, lipid **concentration** and lipid content of oleaginous yeast strains.

Yeast strain	Dry biomass (g/L)	Lipid concentration (g/L)	Lipid content (%)
Rhodotorula mucilaginosa SY09	13.75 ±0.77^{abcd}	4.97±0.27^{bcd}	36.15±0.41^{c}
Rhodotorula mucilaginosa SY18	13.65±0.86^{abcd}	5.27±0.27^{abc}	38.61±0.39^{b}
Rhodotorula mucilaginosa SY20	10.79 ±1.24^{ef}	2.96±0.81^{def}	27.43±0.60^{e}
Rhodotorula mucilaginosa SY30	11.74 ±0.64^{def}	3.10±0.45^{def}	26.40±0.45^{f}
Rhodotorula mucilaginosa SY43	12.56 ±0.45^{cde}	3.39±0.29^{cde}	26.99±0.35^{e}
Rhodotorula mucilaginosa SY52	13.65±0.70^{abcd}	2.32±0.47^{fg}	16.99±0.85^{k}
Rhodotorula mucilaginosa SY75	12.60±0.66^{cde}	3.03±0.16^{def}	24.05±0.42^{fg}
Rhodosporidium kratochvilovae SY89	14.72±0.38^{abc}	5.79±0.30^{ab}	39.33±0.57^{b}
Rhodotorula dairenensis SY94	12.95±0.72^{bcd}	4.33±0.70^{bcde}	33.44±0.74^{d}
Rhodotorula mucilaginosa PY21	12.70±1.02^{bcde}	4.35±0.61^{bcde}	34.26 ± 0.55^{cd}
Rhodotorula mucilaginosa PY23	13.58 ±0.79^{abcd}	3.06±0.52d^{ef}	22.54±0.66^{h}
Rhodotorula mucilaginosa PY25	10.32 ±0.33^{f}	2.19±0.69^{fg}	21.22±0.51^{i}
Rhodotorula mucilaginosa PY32	10.02±0.18^{g}	2.34±0.42^{fg}	23.35±0.37^{g}
Cryptococcus curvatus PY39	14.77±0.65^{ab}	6.87±0.62^{a}	46.51±0.70^{a}
Rhodotorula mucilaginosa PY44	15.10 ±0.54^{a}	4.11±0.60^{cde}	27.23±0.55^{e}
Rhodotorula mucilaginosa PY55	12.66 ±1.21^{bcde}	2.49±0.48^{fg}	19.67±0.28^{j}
Rhodotorula mucilaginosa PY61	10.22 ±0.43^{f}	2.06±0.52^{g}	20.16±0.71^{j}
Rhodotorula mucilaginosa PY86	13.76 ±0.69^{abcd}	3.36±0.56^{cde}	24.42±0.71^{fg}

All parameters are expressed as mean ± SD values in the same column, n= 3, within each column, means are significantly different ($p < 0.05$), unless they have a common letter.

3.4 Discussion

In the current study, the identified high oil producing yeasts isolated from soil (9), flower (4), fruit (3) and leaf (2) (Table 3.1). Previous researchers also identified oleaginous yeasts from diverse habitats including soils (Pan *et al*., 2009; Schulze *et al*., 2014; Patel *et al*., 2014; Pereyra *et al*., 2014; Paserakung *et al*., 2015), flower surfaces (Dai *et al*., 2007; Srisuwan *et al*., 2016), leaf samples (Srisuwan *et al*., 2016) and fruit surfaces (Pereyra *et al*., 2014). So that soil (especially, forest soil) can be a best source (most important habitat) for isolation of oleaginous yeasts. As shown in Table 3.1, samples from Addis Ababa and surroundings were rich in oleaginous yeast diversity since about ten of them were isolated from Addis Ababa and its surroundings.

Conventionally, the presence of lipids from microorganisms is estimated using lysochromes such as Sudan Black B or Sudan III (Thakur *et al*., 1988) or Nile red (Kimura *et al*., 2004) staining. However, these techniques only provide preliminary information and do not allow precise insight into intracellular lipid content of the lipid accumulation ability of the tested microorganism. Therefore, there may be a high proportion of false-positive results with respect to lipid content (Pan *et al*., 2009).

High oil producing yeasts belong to the genus *Candida*, *Cryptococcus*, *Debaryomyces*, *Lipomyces*, *Rhodosporidium*, *Rhodotorula, Trichosporon and Yarrowia* (Pan *et al*., 2009). All except *Debaryomyces*, *Lipomyces* and *Yarrowia* are basidiomycetes. Many basidiomycetous yeast species including oleaginous ones have been included now in other existing or new genera (Liu *et al*., 2015; Wang *et al*., 2015). Accordingly, *Cryptococcus* genera have been transferred to *Cutaneotrichosporon*. The oleaginous yeast *Cryptococcus curvatus* is renamed as *Cutaneotrichosporon curvatus* (Liu *et al*., 2015). Similarly, *Rhodosporidium* has been transferred

to *Rhodotorula* and the oleaginous yeast *Rhodosporidium kratochvilovae* is renamed as *Rhodotorula kratochvilovae* (Wang *et al*., 2015). For this thesis, the older names will be used for convention.

Characterization of morphological and physiological features are important criteria in the taxonomy and identification of yeasts (Yarrow, 1998; Barnett *et al.*, 2000; Kurtzman *et al*., 2003; Kurtzman *et al*., 2011). Fermentation and/or assimilation of carbon compounds are important criteria in yeast identification which depend on various carbon sources (the details are listed in Table 3.2) for their energy supply and growth, the carbohydrates being the sources of greater importance. Yeasts are also able to assimilate different nitrogen sources. However, not all yeasts are capable of using all nitrogen sources equally (Yarrow, 1998; Barnett *et al.*, 2000; Kurtzman *et al*., 2003; Kurtzman *et al*., 2011).This is another basis for conventional identification of yeasts. Phenotypic features that are listed in Table 3.1 and 3.2 were important criteria in identifying the screened oleaginous yeasts. These conventional (traditional) identification methods however should be supplemented with highly sensitive and specific molecular identification methods (especially, sequencing both ITS domain and D1/D2 domains of the 26S rRNA gene) for confirmation. In the current study, the conventional methods helped in identifying the 18 oleaginous yeasts at the genus level. However, their correct identification at the genus level was confirmed by sequencing both ITS domain and D1/D2 domains of the 26S rRNA gene of the large subunits of each strain.

Naturally, oleaginous yeasts are dispersed throughout the entire phylogenetic tree of both basidiomycetes and ascomycetes (especially hemiascomycetes). In the current study, as it is seen from the phylogenetic tree in Figure 3.4, same species are clustered together. Different *Rhodotorula mucilaginosa* strains including the ones identified in this study cluster closer to

different strains of *Rhodotorula dairenensis* indicating that they are more related than *Rhodotorula glutinis* and *Rhodotorula graminis* (Fell and Statzell-Tallman, 1998). In addition, PY39 and *Cryptococcus curvatus* CBS 570 cluster together but they are a little bit far apart from *Cryptococcus cyanovorans* and *Cryptococcus laurentii* VKMY-1628 which signifies that, they are phylogenetically distinct. In this study, different strains of *Rhodosporidium kratochvilovae* including *Rhodosporidium kratochvilovae* SY89 clustered more closely with *Rhodotorula hordea* AFTOL-ID 674, *Rhodosporidium fluvialis* CBS 6568, *Rhodosporidium toruloides* AFTOL-ID 1547 and *Sporoblomyces johnsonii* indicating the relative relatedness of each.

Sequence analysis of ITS domain and/ or D1/D2 domain of the 26S rRNA gene techniques were applied in identifying yeasts for various purposes. For example, Pan *et al*. (2009) identified 20 oleaginous yeasts by sequencing the D1/D2 domains of 26S rDNA (rRNA gene). The nucleotide sequences of ITS 1 and 2 regions in the rRNA gene were determined by directly sequencing PCR-amplified fragments for species (17 species and five varieties) in the genus *Trichosporon* (Sugita *et al*., 1999). Leaw *et al*. (2006) also identified both medically important and oleaginous yeasts using ITS1 and ITS2 sequencing. They identified 373 strains (86 species). Various oleaginous species of genus *Cryptococcus*, *Rhodotorula* and *Trichosporon* were among the identified ones. Mohamed *et al*. (2014) identified three isolates of *Rhodotorula mucilaginosa* and three isolates of *Rhodotorula glutinis* using ITS.

Biomass production and lipid accumulation by oleaginous microorganisms are affected by the carbon source available during fermentation (Mamatha, 2009; Zhao *et al*., 2015). For the production of maximum cell biomass and lipid yield in oleaginous yeasts, medium with an excess of carbon source and limited amount of nitrogen sources has a significant influence (Ratledge, 2004). When nitrogen is low in the cultivation medium, the activity of nicotinamide adenine

dinucleotide isocitrate dehydrogenase decreases or even disappears from the mitochondria of the oleaginous yeasts. Then tricarboxylic acid cycle (TCA) is repressed, metabolism pathway altered, protein synthesis stopped and lipid accumulation activated (Pan *et al*., 2009) and. The excess carbon source is continued to be assimilated and converted into lipid. The process of lipid accumulation continues until the cells reach a personal limit of obesity. Some cells may continue to produce oil until they are physically unable to accumulate any more (Wynn and Ratledge, 2006). Such lipid biosynthetic activity doesn't occur in non-oleaginous microorganisms. In other words, non-oleaginous microorganisms do not accumulate lipid. When these non-oleaginous microorganisms placed in the same nitrogen limiting growth medium, non-oleaginous microorganisms either tend to cease further cell proliferation or, if they continue to assimilate the available carbohydrate substrate, then this is diverted into various polysaccharides, including glycogen and various glucans, mannans, etc. (Ratledge, 2004) or even produce large amounts of metabolites, such as citric acid (Wynn and Ratledge, 2006).

In this study, the 18 screened and identified oleaginous yeasts were tested for their lipid production capacity by growing them in a nitrogen-limited media (Table 3.3). It was observed that the yeasts varied in their lipid accumulating capacities with the maximum exhibited by *Cryptococcus curvatus* PY39 (46.51±0.70%) and minimum exhibited by *Rhodotorula mucilaginosa* PY61 (16.99±0.85%). The results of biomass and lipid content reported for *Cryptococcus curvatus* PY39 are better than the ones reported by Hassan *et al*. (1994) for another strain of *Cryptococcus curvatus*. However, such results reported for *Cryptococcus curvatus* PY39 are lower than the ones reported by Thiru *et al*. (2011) for a different strain of *Cryptococcus curvatus*. The different strains *Rhodotorula mucilaginosa* gave biomass, lipid concentration and lipid content, respectively in the range from 10.02±0.18 g/L, 2.06±0.52 g/L and 16.99±0.85% to 15.10 ±0.54 g/L, 5.27±0.27 g/L

and 38.61±0.39 %. From a different research work, a stain of *Rhodotorula mucilaginosa*, namely *Rhodotorula mucilaginosa* TJY15a was observed to accumulate about 47.9% w/w oil during batch cultivation, whereas 52.9% (w/w) of lipid was obtained during the fed-batch cultivation (Li *et al*., 2010). This yeast strain accumulated 55.4% (w/w) oil from the extract of Jerusalem artichoke tubers in its cells and cell dry weight reached 12.8 g/L within 48 h (Zhao *et al*., 2011). On the other hand, a strain of *Rhodosporidium kratochvilovae* HIMPA1 which was isolated from Himalayan permafrost soil showed boosted TAG accumulation in the lipid droplets (Patel *et al*., 2014). Patel *et al*. (2014) observed enhanced total lipid content (55.56%) and lipid concentration (8.39 ± 0.57 g/L), respectively when the yeast was grown in Hemp seeds aqueous extract. This yeast exhibited a lipid content of 41.92% and biomass of 6.2±0.8 g/L, respectively when it was grown in industrially used glucose synthetic medium. The results attained by this strain are better than the one exhibited in this study by *Rhodosporidium kratochvilovae* SY89. The differences in biomass and lipid accumulation of strains in this study and previous researches could be differences in the biochemistry and genetic constitution of the cells of oleaginous yeasts (Wynn and Ratledge, 2006; Nigam and Singh, 2014) and differences in culturing conditions (Li *et al*., 2008; Ageitos *et al*., 2011; Zhao *et al*., 2015).

3.5 Conclusion and recommendations

In conclusion, we have screened and identified 18 oleaginous yeasts that can accumulate microbial lipid with a higher yield when cultivated under nitrogen- limited conditions. It will be attractive to use them to produce microbial lipid as alternative sources for biodiesel production in future. *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were the most promising lipid accumulating oleaginous yeasts.

This research work also has shown that Ethiopian environment can be a rich source for oleaginous yeast diversity. Thus, many more and potentially promising oleaginous yeasts can be isolated from Ethiopian environment.

It is also recommended that researchers should focus on collecting samples from forest soils of the various parts of the country for the search for oleagionus yeasts.

Chapter 4: Optimization of Cultivation Conditions for the Production of Single Cell Oil by Oleaginous Yeasts

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Abstract

Optimizations of cultivation conditions are critical for increased biomass yield and lipid production by oleaginous yeasts. Moreover, the data generated is useful for scale up efforts aimed at biodiesel production. From a screening work for high oil production, 18 isolates were chosen. Among them, *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were selected for optimization of cultivation conditions for lipid production. To determine the optimal cultivation conditions for oleaginous yeasts, different carbon and nitrogen sources, C/N ratio, pH and inoculum size were investigated. Moreover, incubation temperature, shaking speed, culture volume (aeration rate) and duration of cultivation were investigated. Wide variations were recorded in the cultivation conditions that led to maximum lipid production by the yeasts under test. The maximum lipid production was attained within 120-144 h, using 50-70 g/L glucose as a carbon source, 0.50 g/L

yeast extract and 0.31- 0.85 g/L $(NH_4)_2SO_4$ as nitrogen sources, at C/N ratio of 100-140, pH range 5-6, 10% inoculum size as seed culture, 30^oC temperature, shaking speed of 200- 225 rpm and 50 mL culture medium. Lipid content was determined after extraction using solvent mixtures of chloroform and methanol (2:1). Under the optimized conditions, *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 accumulated lipids up to 7.22±0.26, 5.73±0.62, 6.47±0.05 and 7.65±0.77 g/L, respectively on cellular dry biomass basis. Such values correspond to lipid contents of 48.66±0.60, 38.38±3.90, 40.74±0.54 and 51.17±0.72%, respectively. These strains were further grown on media containing peel mixtures of papaya and mango. Under the optimized conditions, *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 exhibited a lipid yield and lipid content of 3.95±0.67 g/L and 35.02±1.63%, 2.66±0.49 g/L and 28.15±1.63%, 3.84±0.19 g/L and 36.76± 0.61%, and 4.31± 0.30 g/L and 35.18±1.40%, respectively. The fatty acid profiles were analyzed using gas chromatography coupled with flame ionization detector. Data revealed the presence of significant amount of oleic acid (47.44±2.14–54.40±1.15%), palmitic acid (10.69±0.66–24.04±0.39%), linoleic acid (6.34±0.64–21.24±0.36%) and low amount of stearic, linolenic, palmitoleic, arachidic, paullinic and behenic acids in the extracted yeast oils which indicate that the fatty acid profiles were very similar to that of conventional vegetable oils. The results of fatty acid profiles showed that the microbial lipids from the studied yeasts can be good feedstocks for biodiesel production.

Key words: Biomass, cultivation conditions, fatty acid, lipid content, oleaginous yeast

4.1 Introduction

Microorganisms capable of accumulating lipids above 20 to 70% of their dry biomass are termed oleaginous (Ratledge and Wynn, 2002). The oil produced from such microorganisms can be used as alternative sources of oils for human consumption (Ratledge, 2005), feedstocks for production of biodiesel (Beopoulos *et al*., 2008; Li *et al*., 2008), components in paints, coatings, detergents, cleaning products, cosmetics, plastics, rubber and intermediate products (Luque *et al*., 2010; Draaisma *et al*., 2013; Beopoulos *et al*., 2014).

Oil accumulation by oleaginous microorganisms depends on the cultivation conditions such optimizing carbon and nitrogen sources, carbon to nitrogen (C/N) ratio, inorganic salts and trace elements, incubation period and temperature, pH, agitation and aeration rate (Li *et al*., 2008; Ageitos *et al*., 2011; Zhao *et al*., 2015). Optimum C/N ratio of greater than 20 is needed for maximum production of lipids by oleaginous microorganisms (Papanikolaou and Aggelis, 2011). C/N ratio depends on the nature of microorganism, the medium composition, types of carbon and nitrogen sources (Ykema *et al*., 1986). Lipid accumulation in oleaginous yeasts occurs when the carbon source of the medium is depleted with nitrogen limitation (Wu *et al*., 2011).

The influence of initial pH of the culture medium also plays a critical role on lipid synthesis by oleaginous microorganisms. Different optimum pH values for maximum production of lipids by different oleaginous yeasts were found in previous studies (Tao *et al*., 2008; Zhu *et al*., 2008; El-Fadaly *et al*., 2009; Kraisintu *et al*., 2010). The optimum pH for a specific oleaginous yeast for biomass production and SCO is also influenced by the different carbon sources available in the growth medium (Angerbauer *et al*., 2008).

Cellular lipid accumulation is critically affected by temperature of the culture. Too high or too low temperature affects the cell growth and lipid accumulation (Sha, 2013). It is not only the type but also the composition of the lipid varies with different temperatures (Saxena *et al*., 2009).

Incubation period has also an effect on lipid production (Leesing *et al*., 2011). According to Beopoulos *et al*. (2008), lipid content reached highest value at stationary phase of growth. It is recommended that cells should be harvested at early stationary phase to prevent lipid degradation (Beopoulos *et al*., 2008).

It is well-known that agitation and aeration rates play significant roles in the production of SCO by oleaginous microorganisms. These parameters play a pivotal role in aerobic fermentation process and scale up of aerobic biosynthesis systems (Bandaiphet and Prasertsan, 2006). Optimization of cultivation parameters can result in higher lipid production by oleaginous yeasts and fosters their potential for industrial application. Optimization is an essential step of each industrial process because it can result in higher production and reduced cost of production (Enshaeieh *et al*., 2013b).

Certain species of algae, yeast, filamentous fungi and bacteria are capable of accumulating significant amount of oil in their lipid bodies and termed oleaginous microorganisms. These oleaginous microorganisms convert the carbon sources contained in various substrates into storage lipid under suitable cultivation conditions (Economou *et al*., 2011). In order to reduce the cost of microbial oil production, low-cost raw materials like lignocellulosic hydrolysates such rice straw (Huang *et al*., 2009) and rice hull (Economou *et al*., 20111), glycerol (Sriwongchai *et al*., 2013; Moustogianni *et al*., 2014), sugar industry by-product such as molasses (Almazan *et al*.,1981; Sabry *et al*.,1990; El-Fadaly *et al*., 2009; Karatay and Donmez, 2010; Gajdoš *et al*., 2015), effluent

from steam fish processing (Cheirsilp *et al*., 2011), whey (Akhtar *et al*.,1998), beer factory wastewater (Lutzu *et al*., 2016), starch wastewater (Xue *et al*., 2010), and products of fruit processing industry (Kulkarni *et al*., 2013; Park *et al*., 2014) were investigated.

Papaya and mango are produced in vast amount worldwide. Various juice producing industries and local juice sellers produce a vast amount of different fruit wastes including fruit peels. The peels from these fruits are disposed as wastes. The utilization of such wastes is needed to be emphasized to save the resources and protect the environment from pollution (Kulkarni *et al*., 2013). Fruit waste has high level of sugars, including sucrose, glucose and fructose (Choi *et al*., 2015). These fruit wastes are suitable for the production of SCO (Kulkarni *et al*., 2013; Park *et al*., 2014), ethanol, biogas, lactic acid, enzymes and single cell protein (Puligundla *et al*., 2014). Banana peel can also be used for the production of wine (Faturoti *et al*., 2006). To improve the economic competitiveness of the bioprocess microbial oil (TAGs), the use of wastes that have low or even negative value can be used as natural substrates (Hassan *et al*., 1994; Pan *et al*., 2009).These microbial oils can be used as feedstocks for the production of biodiesel (Li *et al*., 2008).

Gas chromatography (GC) is one of the most widely used techniques to analyze oils. A chromatographic analysis involves passing a mixture of the oils to be separated through a column that contains a matrix capable of selectively retarding the flow of fatty acids (Shahidi, 2001). GC can be used to determine the complete profile of molecules present in a lipid or oil. Lipids are usually derivitized prior to GC analysis to increase their volatility, so that fatty acid methyl esters will be formed from TAGs (Shahidi and Wanasundara, 2008). After being separated by the GC column, the fatty acid methyl esters are detected by suitable detectors commonly flame ionization detector. Some other commonly used detectors include UV-visible and fluorescence detectors.

This information can be used to calculate the amounts of saturated, unsaturated polyunsaturated fat and other components of the lipid such as cholesterol (Shahidi, 2001).

The objective of this study was to investigate the effect of different cultivation parameters on biomass production, lipid concentration and lipid content of *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89. In addition, the potential of lipid production capacity of such selected oleaginous yeasts was evaluated by cultivating them on mixtures of papaya and mango peels. Furthermore, the fatty acid profiles of these yeasts was analyzed using gas chromatography.

4.2 Materials and methods

4.2.1Yeast strains

As discussed in Chapter 3, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18, *Rhodosporidium kratochvilovae* SY89 and *Cryptococcus curvatus* PY39, in which the first three were isolated from soil and the last one was isolated from flower surface were studied. They were characterized and identified as described in Chapter 3. They were maintained using YM agar and kept at 4^{o}C with repeated sub culturing.

4.2.2 Optimization of cultivation conditions

4.2.2.1 Optimization of inoculum size

The influence of inoculum size was tested at 5,10,15,20 and 25% v/v concentrations in a nitrogen-limited media listed in Chapter 3. Dry biomass, lipid concentration and lipid content were evaluated. The shake speed was 200 rpm. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL media at a temperature of 30^{o}C for144 h. The experiments were done in triplicate.

4.2.2.2 Optimization of carbon sources

The influence of different carbon sources: glucose, sucrose, maltose, galactose, xylose and glycerol on the biomass yield and lipid content were investigated in a nitrogen-limited media. An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL media at a temperature of 30^{o}C for144 h. The shake speed was 200 rpm.The experiments were done in triplicate.

4.2.2.3 Optimization of glucose concentration

To know the optimum glucose concentration, various concentrations (10, 30, 50, 70 and 90 g/L) were tested in a nitrogen-limited media. An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL medium at a temperature of 30^{o}C for 144 h. The shake speed was 200 rpm. The experiments were done in triplicate.

4.2.2.4 Optimization of nitrogen sources

The effect nitrogen sources was tested by combining $(NH_4)_2SO_4$ and yeast extract, NH_4Cl and peptone, NH_4Cl and yeast extract, $(NH_4)_2SO_4$ and peptone, $(NH_4)_2SO_4$ and Urea, NH_4Cl and Urea]. These tests were evaluated in the presence of optimized glucose medium (50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for the rest yeasts). An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL medium for 144 h at 30^{o}C. The shake speed was 200 rpm. All experiments were done in triplicate.

4.2.2.5 Optimization of carbon to nitrogen (C/N) ratio

To study the effect of C/N ratio on oleaginous yeast biomass production, lipid concentration and lipid content, the best and optimized carbon source (glucose medium: 50 g/L for *Rhodosporidium*

kratochvilovae SY89 and 70 g/L for the rest yeasts) and the best and optimized nitrogen sources, i.e., 0.50 g/L of yeast extract with 0.47 g/L of $(NH_4)_2SO_4$ for *Cryptococccus curvatus* PY39; 0.50 g/L of yeast extract with 0.85 g/L of $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY09; 0.50 g/L yeast extract with 0.61 g/L $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY18; and 0.5 g/L yeast extract with 0.31 g/L $(NH_4)_2SO_4$ in the case of *Rhodosporidium kratochvilovae* SY89 were tested. The different C/N ratios in the medium ranging from 40 to 160 were tested. An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. The shake flask fermentations were incubated at 30ºC. The shake speed was 200 rpm. The experiments were done in triplicate. Prior to determination of C/N ratio, the total nitrogen content of yeast extract was determined.

4.2.2.5.1 Determination of total nitrogen content of yeast extract

Total nitrogen was determined using Kjeldahl method according to Krishna and Ranjhan (1980). Briefly, digestion flask containing about l g yeast extract sample, 6 mL of acid mixture (concentrated sulphuric acid and concentrated orthophosphoric acid,with ratio of 20:1) and about 3 g of catalyst mixture (K_2SO_4, selenium and $CuSO_4$ with the ratio of 10:0.1:1) were boiled to about 370ºC in order to allow digestion. Then distillation was performed by adding 25 mL of 40 % NaOH and using 25 mL of 2% boric acid and 10 drops of indicator solution. Finally, the distillate was titrated with standardized 0.1N sulphuric acid to a reddish color. The total nitrogen content (%) was determined using the following formula.

$$\text{Total nitrogen content (\%) by weight} = \frac{(V2 - V1)X\ N\ X0.14}{W} \times 100$$

Where:

V2 - volume in mL of the standard H_2SO_4 solution used in the titration of the yeast extract.

V 2 - volume in ml of the standard sulphuric acid used in the titration for the blank determination

N- Normality of standard sulphuric acid solution

W - Weight in grams of the test material in dry matter basis.

4.2.2.6 Optimization of intial pH

The effect of pH on yeast biomass yield and lipid accumulation was tested by varying the pH from 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. These tests were evaluated in the presence of optimized glucose medium (50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for the rest yeasts) and the best and optimized nitrogen sources, i.e., 0.50 g/L of yeast extract with 0.47 g/L of $(NH_4)_2SO_4$ for *Cryptococccus curvatus* PY39; 0.50 g/L of yeast extract with 0.85 g/L of $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY09; 0.50 g/L yeast extract with 0.61 g/L $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY18; and 0.5 g/L yeast extract with 0.31 g/L $(NH_4)_2SO_4$ in the case of *Rhodosporidium kratochvilovae* SY89. The pH was adjusted using 1N HCl and 1 N NaOH. pH adjustment was done prior to autoclaving the growth medium. An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. The C/N ratio was 140 for *Cryptococcus curvatus* PY39, 100 for *Rhodotorula mucilagionsa* SY09, and 120 for *mucilagionsa Rhodotorula* SY18 and *Rhodosporidium kratochvilovae* SY89. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL medium for144 h at 30^{o}C. The shake speed was 200 rpm. All tests were done in triplicate.

4.2.2.7 Optimization of temperature

The effect of temperature on biomass yield and lipid accumulation of the oleaginous yeast strains was tested. The cultivation process for biomass propagation and lipid accumulation were undertaken at temperatures of 20, 25, 30, 35 and 40^{o}C for 144 h. These tests were evaluated in the presence of optimized glucose medium (50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for the rest yeasts) and the best and optimized nitrogen sources, i.e., 0.50 g/L of yeast extract

with 0.47 g/L of $(NH_4)_2SO_4$ for *Cryptococccus curvatus* PY39; 0.50 g/L of yeast extract with 0.85 g/L of $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY09; 0.50 g/L yeast extract with 0.61 g/L $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY18; and 0.5 g/L yeast extract with 0.31 g/L $(NH_4)_2SO_4$ in the case of *Rhodosporidium kratochvilovae* SY89. The initial pH of the medium was adjusted at 5.0 for *Cryptococcus curvatus* PY39 and 5.5 for the rest yeasts. The C/N ratio was 140 for *Cryptococcus curvatus* PY39, 100 for *Rhodotorula mucilagionsa* SY09, and 120 for *mucilagionsa Rhodotorula* SY18 and *Rhodosporidium kratochvilovae* SY89. An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. The shake speed was 200 rpm. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL medium. The experiments were done in triplicate.

4.2.2.8 Optimization of agitation rate

To investigate the effects of agitation speed on biomass and lipid production, the selected oleaginous yeast strains were incubated for 144 h at varying agitation speed (100,125,150,175, 200 and 225 rpm). These tests were evaluated in the presence of optimized glucose medium (50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for the rest yeasts) and the best and optimized nitrogen sources, i.e., 0.50 g/L of yeast extract with 0.47 g/L of $(NH_4)_2SO_4$ for *Cryptococccus curvatus* PY39; 0.50 g/L of yeast extract with 0.85 g/L of $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY09; 0.50 g/L yeast extract with 0.61 g/L $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY18; and 0.5 g/L yeast extract with 0.31 g/L $(NH_4)_2SO_4$ in the case of *Rhodosporidium kratochvilovae* SY89.The C/N ratio was 140 for *Cryptococcus curvatus* PY39, 100 for *Rhodotorula mucilagionsa* SY09, and 120 for *mucilagionsa Rhodotorula* SY18 and *Rhodosporidium kratochvilovae* SY89. The initial pH of the medium was adjusted at 5.0 for *Cryptococcus curvatus* PY39 and 5.5 for the rest yeasts. An inoculum size of 10% v/v was added

to each nitrogen-limited cultivation medium. Biomass and lipid content were measured and recorded. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL media. The shake flask fermentations were incubated at 30^{o}C.The experiments were done in triplicate.

4.2.2.9 Optimization of working volume

To study the effect of aeration (oxygen supply) on biomass production and lipid content, the submerged culture was performed in 250 mL Erlenmeyer flasks containing 25, 50, 75,100 and 125 mL medium, respectively. These tests were evaluated in the presence of optimized glucose medium (50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for the rest yeasts) and the best and optimized nitrogen sources, i.e., 0.50 g/L of yeast extract with 0.47 g/L of $(NH_4)_2SO_4$ for *Cryptococccus curvatus* PY39; 0.50 g/L of yeast extract with 0.85 g/L of $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY09; 0.50 g/L yeast extract with 0.61 g/L $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY18; and 0.5 g/L yeast extract with 0.31 g/L $(NH_4)_2SO_4$ in the case of *Rhodosporidium kratochvilovae* SY89.The C/N ratio was 140 for *Cryptococcus curvatus* PY39, 100 for *Rhodotorula mucilagionsa* SY09, and 120 for *mucilagionsa Rhodotorula* SY18 and *Rhodosporidium kratochvilovae* SY89. The initial pH of the medium was adjusted at 5.0 for *Cryptococcus curvatus* PY39 and 5.5 for the rest yeasts. An inoculum size of 10% v/v was added to each nitrogen-limited cultivation medium. The flasks were kept at 30^{o}C with agitation speed of 225 rpm for *Cryptococcus curvatus* PY39 and *Rhodosporidium kratochvilovae* SY89 and 200 rpm for *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18 for 144 h.

4.2.2.10 Optimization of incubation period

Biomass, lipid concentration and lipid contents of the selected oleaginous yeasts were tested at 0, 24, 48, 72, 96,120,144 and 168 h of incubation. These tests were evaluated in the presence of optimized glucose medium (50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for the rest yeasts) and the best and optimized nitrogen sources, i.e., 0.50 g/L of yeast extract with 0.47

g/L of $(NH_4)_2SO_4$ for *Cryptococccus curvatus* PY39; 0.50 g/L of yeast extract with 0.85 g/L of $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY09; 0.50 g/L yeast extract with 0.61 g/L $(NH_4)_2SO_4$ in the case of *Rhodotorula mucilaginosa* SY18; and 0.5 g/L yeast extract with 0.31 g/L $(NH_4)_2SO_4$ in the case of *Rhodosporidium kratochvilovae* SY89. The C/N ratio was 140 for *Cryptococcus curvatus* PY39, 100 for *Rhodotorula mucilagionsa* SY09, and 120 for *mucilagionsa Rhodotorula* SY18 and *Rhodosporidium kratochvilovae* SY89. The initial pH of the medium was adjusted at 5.0 for *Cryptococcus curvatus* PY39 and 5.5 for the rest yeasts. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL media. The flasks were kept at 30^{o}C with agitation speed of 225 rpm for *Cryptococcus curvatus* PY39 and *Rhodosporidium kratochvilovae* SY89 and 200 rpm for *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18.

4.2.3 Production of single cell oil using peel mixture of papaya and mango

4.2.3.1 Substrate collection and preparation

Fresh papaya and mango peels were collected from fruit processors within the Arat Kilo area, Addis Ababa, Ethiopia. The peels were thoroughly washed with water to remove attached foreign materials. Then the peel mixture was homogenized and blended for 5 min. A 2% (v/v) sulphuric acid at a solid to liquid ratio of 1: 10 (w/v) was added in to the fruit peel mixture for hydrolysis. A 2 mm screen was used to sieve the resulting slurry before being stored at 20^{o}C for subsequent use. The diluted peel mixture then was boiled, allowed to cool and sedimentation of insoluble materials occurred. The sediments were removed by decantation. The resulting mixture was centrifuged with a high speed at 5000×*g* for 10 min for further removal of insoluble materials. The supernatant solution was separated from the pellet. The pellet was discarded and the supernatant was used for the cultivation purpose.

4.2.3.2 Determination of total sugar in papaya and mango peel mixtures

Sample preparation: For determination of total sugar, 100 mg of peel mixture of papaya and mango was homogenized and taken in boiling tube and hydrolyzed by adding 5mL of 96% sulphuric acid in it. Boiling tubes were kept in water bath for 3 h and then removed from water bath and cooled to room temperature. After cooling, it was neutralized by adding solid sodium carbonate until effervescence ceases. Then whole volume was made up to100 mL by adding distilled water and centrifuged. Then the supernatant was used for total sugar estimation.

Total sugar estimation: Total sugar contained in fruit peel mixture was analyzed by the method of DuBois *et al*. (1956) which is based on the phenol - sulphuric acid reaction. The standard curve for glucose was prepared by adjusting the final concentration to 0.2 mg/mL as 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, and 0.2. The final volume of each tube was made 2 mL by adding distilled water. The amount of fruit peel sample used was 0.2 mL. One mL of 5% phenol and 5mL of 96% sulphuric acid was added one by one in each tube and vortexed well so that the phenol and sulphuric acid get mixed thoroughly with working standard. After 10 min all the tubes were placed in water bath at 25°C for 15 min. At last the absorbance was read at 490 nm using spectrophotometer (JENWAY model 6405).The total sugar in the fruit peel mixture was calculated following the protocols developed by Sadasivam and Manickam (2005).

Absorbance corresponds to 0.2 mL of fruit peel sample test = X mg of glucose

$$100\,\text{mL of fruit peel sample solution contain} = \frac{X}{0.2} \times 100 = \%\ \text{of total sugar present}$$

4.2.3.3 Production of single cell oil from oleaginous yeasts peel wastes

Production of SCO was tested in a nitrogen-limited media as listed in the previous chapters except the carbon source. In this case the carbon source used was fruit peel mixture and the amount

used was estimated according to the method of DuBois *et al*. (1956) and Sadasivam and Manickam (2005). Fruit peel mixture of 53% (~70 g/L in the case of PY39, SY09, SY18 and SY18) and 37.90% (~50 g/L) was used. The fermentation medium was inoculated with 10% (v/v) of the liquid seed culture. In this case the number of cells in 10% v/v inoculum size was estimated using hemocytometer. The hemocytometer used was Neubaur Bright-Line Hemocytometer (made in Marienfled, Germany) with entire ruled central area measuring 1 mm on each side, with a total area of 1 mm^2. Each of the 25 squares measure 0.2 mm, with an area of 0.04 mm^2.

Number of cells/mL = Total Cells in Central 25-Square Ruled Area × Dilution Factor ×10^4 (ASBC, 1988).

All the optimized parameters were applied here for the production of SCO by each of the four oleaginous yeasts using peel mixtures of papaya and mango.

4.2.4 Analytical methods

4.2.4.1 Determination of yeast biomass

Yeast cells grown in a submerged culture were harvested by centrifugation at 5000×*g* for 15 min. The supernatants were discarded. Biomass (Pellet) was washed twice with distilled water, frozen at -80^{o}C and freeze dried over night to constant weight. Dry weight was determined gravimetrically.

4.2.4.2 Determination of lipid content

Lipid extraction was done following the protocol described by Folch *et al*. (1957), with some modifications. Dried biomass was ground with a pestle in a mortar and 1g of sample was extracted with 3.75 mL solvent mixture of chloroform and methanol (2:1) overnight. The solvent mixture was transferred into a clean separating funnel through Whatman No1 filter paper. Then 1.25 mL of the solvent mixture was added through filter paper into separating funnel. This was followed by

washing with 0.75 mL of distilled water. The solvent/water mixture was left overnight to separate into two clear phases. The bottom phase containing chloroform and lipid was collected and the solvents evaporated under vacuum. Diethyl ether was used to transfer the extract into pre-weighed glass vials and the solvent was evaporated. The SCO content was evaluated as:

$$\text{Single cell oil content} = \frac{\text{Single Cell Oil Concentration (g/L)}}{\text{Cell Dry Weight(g/L)}} \times 100$$

4.2.4.3 Analysis of fatty acid profiles using gas chromatography

To determine the fatty acid composition of the lipids, the extracted lipids were transferred to GC vials, dissolved in chloroform and methylated with trimethylsulphonium hydroxide (TMSOH) (Butte, 1983). The vials were then sealed and vortexed for approximately 5 sec. Fatty acid methyl esters were subsequently analyzed on a Shimadzu GC-2010 auto sampler gas chromatograph with a flame ionization detector. An injection volume of 0.5 µL of sample was added into a SGE-BPX-70 column (length= 50 m and inner diameter = 0.22 mm). The injection port had a temperature of 250^{o}C and a split ratio of 1:10. The column temperature was 200^{o}C. Hydrogen gas was used as a carrier gas at a flow rate of 40 mL/min. The total program time was 4.50 min per sample with a column flow rate of 1.37 mL/min. Peaks were identified by reference to authentic standards.

4.2.5 Statistical analysis

One way-ANOVA was performed to calculate significant differences in treatment means. SPSS version 20.0 software was used for interpretation of data. Data with a *p* value < 0.05 were considered significantly different. All experiments were done in triplicate.

4.3 Results

4.3.1 Effect of various parameters on biomass production and lipid content

4.3.1.1 Effect of inoculum size on biomass production and lipid accumulation

In this study, the effect of inoculum size on biomass production, lipid concentration and cellular lipid content by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 was tested under nitrogen-limited media. For this inoculum size test, 5, 10, 15, 20 and 25% v/v seed cultures were inoculated in the nitrogen-limited fermentation media. The results are shown in Figure 4.1. A maximum lipid concentration of 5.23±0.43 g/L which corresponds to lipid content of 39.53±1.93% per dry biomass of 13.23±1.21 g/L were observed at inoculum size of 10% v/v of *Cryptococcus curvatus* PY39, while lowest biomass (11.18±0.68 g/L), lipid concentration (3.34±0.20 g/L) and lipid content (29.87±1.03%) were recorded at inoculum size of 25% v/v. On the other hand, *Rhodotorula mucilaginosa* SY09 yielded maximum lipid content of 35.49±1.88% per biomass (13.50±1.16 g/L) at the same inoculum size (10% v/v), while lowest biomass (11.80±1.04 g/L) was exhibited when 5% v/v seed culture was inoculated. Minimum lipid content (21.36±2.50%) was attained by this strain at inoculum size of 25% v/v. Furthermore, the effect of inoculum size was also tested for *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 similar to the above mentioned two strains. Inoculation volume of 10% v/v was still the favorable for both strains in giving maximum biomass and lipid content. *Rhodotorula mucilaginosa* SY18 gave 34.71±2.29% as its maximum cellular lipid content per dry biomass of 12.39±0.61 g/L, whereas *Rhodosporidium kratochvilovae* SY89 yielded 37.25± 0.75% as its highest cellular lipid content per dry biomass of 14.28±0.73 g/L. Lowest biomass (8.98±0.72 g/L) and lipid content (19.93±2.01%) were observed at inoculum size of 5% v/v by *Rhodotorula mucilaginosa* SY18.

Rhodosporidium kratochvilovae SY89 yielded lowest biomass (11.52±0.50 g/L) at inoculum size of 5% v/v, while this strain gave minimum lipid content (26.67±0.85%) at inoculum size of 25% v/v. Intermediate values were recorded in the remaining inoculum sizes by the four strains.

Based on the results in Figure 4.1, biomass, lipid concentration and lipid content at different inoculum levels, one can conclude that 10% v/v is the optimum inoculum size for all the yeasts investigated.

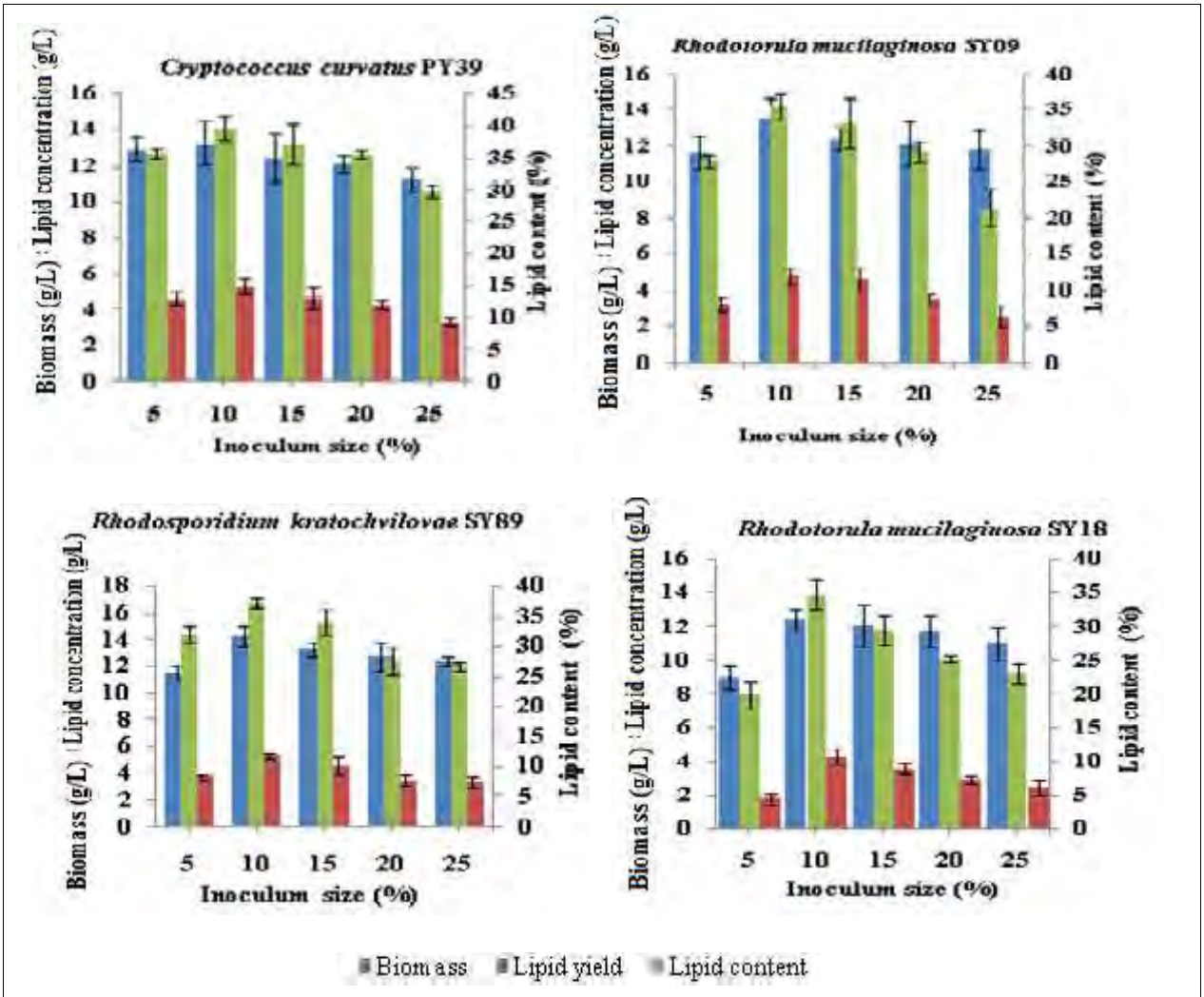

Figure 4. 1: Effects of inoculum size on biomass production, lipid concentration and lipid content by selected oleaginous yeasts. Error bars in the figures represent standard deviation.

4.3.1.2 Effect of various carbon sources on biomass production and lipid content

The effects of various carbon sources (glucose, sucrose, maltose, lactose, galactose, xylose and glycerol) on biomass yield and lipid content by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were tested using nitrogen-limited media. The results are displayed in Table 4.1. In all the cases the highest biomass and lipid content were exhibited when glucose was used as a sole source of carbon. Based on this maximum biomass (14.53±0.41 g/L) and lipid content (48.66±0.60%) were attained by *Cryptococcus curvatus* PY39 when glucose was supplied as a sole source of carbon. *Rhodotorula mucilaginosa* SY09 gave maximum biomass of 15.32±0.87 g/L and lipid content of 32.38±0.85%, respectively. Highest biomass (14.80±1.20 g/L) and lipid content (40.74±0.54%) were exhibited by *Rhodotorula mucilaginosa* SY18 when the yeast was still grown on glucose. Furthermore, *Rhodosporidium kratochvilovae* SY89 gave the maximum biomass (14.62±0.65 g/L) and lipid content (42.68±0.95%) when glucose was used as a carbon source.

On the other hand, the four selected yeasts gave the least biomass and lipid content when grown in nitrogen-limited media containing xylose except that of *Rhodotorula mucilaginosa* SY09 which yielded a minimum biomass of 9.11±1.45 g/L when glycerol was used as the carbon source (Table 4.1). Biomass of 8.38±0.26 g/L and lipid content of 24.70±0.48% were exhibited by *Cryptococcus curvatus* PY39 when grown in a medium containing xylose. *Rhodotorula mucilaginosa* SY09 gave its lowest lipid content (21.10±0.94%) when xylose was used as a carbon source in a growing medium. The lowest biomass and lipid contents attained by *Rhodotorula mucilaginosa* SY18 were 9.78±2.14 g/L and 25.56±0.75%, respectively when xylose was used. Similarly, biomass (10.38±2.14 g/L) and lipid content (26.01±2.11%) were attained by *Rhodosporidium kratochvilovae* SY89 when grown in medium containing xylose.

In terms of maximum lipid producing capacity, *Cryptococcus curvatus* PY39 gave the highest lipid content (48.66±0.60%) followed by *Rhodosporidium kratochvilovae* SY89 which exhibited a lipid content of 42.68±0.95%. *Rhodotorula mucilaginosa* SY09 gave the lowest lipid content, which is 32.38±0.87% when glucose was used as a carbon source.

In all isolates the results recorded were maximum when glucose was used as the carbon source.

4.3.1.3 Optimization of glucose concentration

To know the optimum glucose concentration for maximum lipid content per dry biomass of *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89, various concentrations, i.e., 10, 30, 50, 70 and 90 g/L glucose concentrations were investigated. Results indicate that the optimum glucose concentration that led to maximum biomass was 30 g/L for *Rhodotorula mucilaginosa* SY18, 50 g/L for *Rhodosporidium kratochvilovae* SY89, 70 g/L for *Cryptococcus curvatus* PY39 and 90 g/L for *Rhodotorula mucilaginosa* SY09 leading to a biomass production of 15.94±1.16, 16.37±1.57, 16.82±2.27 and 14.22±1.00 g/L (Table 4.1), respectively. On the other hand, the best glucose concentration that led to maximum lipid content was 50 g/L for *Rhodosporidium kratochvilovae* SY89 and 70 g/L for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18 leading to a lipid content of 42.46±2.31, 40.43±1.73,34.55±1.75 and 36.26±2.13% (Table 4.1), respectively.

On the other hand, the least amount of biomass and lipid content were attained by the four oleaginous yeast strains, when the media contained 10 g/L glucose (Table 4.1). The lowest biomass and lipid contents were exhibited by *Cryptococcus curvatus* PY39 in a nitrogen-limited medium with 10 g/L glucose viz., 11.88±1.10 g/L and 28.81±2.02%, respectively. *Rhodotorula mucilaginosa* SY09 yielded lowest content (19.11±2.11%) per minimum dry biomass of 9.40±1.42

g/L. *Rhodotorula mucilaginosa* SY18 gave minimum biomass (11.28±1.74 g/L) and cellular lipid content (23.49±1.38%) at the same glucose concentration. Finally, the minimum biomass and lipid contents exhibited by *Rhodosporidium kratochvilovae* SY89 were10.79±1.75 g/L and 20.76 ±1.86 %, respectively.

Table 4.1 shows that, 70 g/L glucose was the most favorable concentration for maximum lipid production by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18 and hence was selected for further activities for the three yeast species and a concentration of 50 g/L of glucose was the most favorable for maximum lipid content by *Rhodosporidium kratochvilovae* SY89 and this concentration of glucose was selected for further activities.

Table 4. 1: Effect of various parameters on biomass production and lipid content by selected oleaginous yeasts.

Condition	***Cryptococcus curvatus* PY39**		***Rhodotorula mucilaginosa* SY09**		***Rhodotorula mucilaginosa* SY18**		***Rhodosporidium kratochvilovae* SY89**	
	Biomass (g/L)	**Lipid content (%)**	**Biomass (g/L)**	**Lipid content (%)**	**Biomass (g/L)**	**Lipid content (%)**	**Biomass (g/L)**	**Lipid content (%)**
Carbon source (70 g/L)								
Glucose	14.53±0.41^{a}	48.66±0.60^{a}	15.32±0 .87^{a}	32.44±0.87^{a}	14.80±1.20^{a}	40.74±0.54^{a}	14.62±0.65^{a}	44.66±0.95^{a}
Sucrose	12.75 ±0.46^{b}	36.87±0.66^{c}	13.56±0.98^{ab}	30.23±0.76^{b}	12.77±0.93^{b}	36.96±1.03^{b}	12.95±0.08^{c}	38.30±0.67^{b}
Maltose	11.67± 0.41^{c}	39.93±1.36^{b}	12.16±0.58^{bc}	28.90±2.02^{c}	12.16± 0.80^{b}	35.77±3.46^{b}	12.78±0.16^{c}	35.22±1.00^{c}
Galactose	11.78± 0.99^{c}	32.26±0.89^{e}	10.88±0.76^{c}	24.78±0.69^{d}	11.56±1.22^{bc}	33.23±0.65^{C}	11.59±0.85^{d}	33.13±0.99^{d}
Xylose	8.38±0.26^{d}	24.70±0.48^{d}	10.47±0.69^{c}	21.10±0.94^{e}	9.78± 2.14^{d}	25.56±0.75^{e}	10.38±2.14^{e}	26.01±2.11^{e}
Glycerol	11.21±0.89^{c}	36.76±1.43^{c}	9.11±1.45^{c}	28.45±1.82^{c}	10.55±1.13^{c}	31.68±2.18^{d}	13.67±0.13^{b}	32.42±1.00^{d}
Glucose concentration (g/L)								
10	11.88±1.10^{b}	28.81±2.02^{d}	9.40±1.42^{b}	19.11±2.11^{c}	11.28±1.74^{c}	23.49±1.38^{c}	10.79±1.75^{c}	20.76±1.86^{d}
30	13.40±0.42^{b}	34.78±2.64^{b}	11.77±1.64^{ab}	19.37±1.34^{c}	15.94±1.16^{a}	28.29±2.46^{ab}	13.23±1.44^{b}	32.88±2.98^{ab}
50	14.35±1.08^{ab}	30.52±1.59^{c}	13.04±1.36^{ab}	31.08±1.85^{ab}	14.91±2.23^{ab}	33.92±0.87^{ab}	16.37±1.57^{a}	42.46±2.31^{a}
70	16.82±2.27^{a}	40.43±1.73^{a}	13.75±1.83^{ab}	34.55±1.75^{a}	14.56±1.05^{ab}	39.69±2.13^{a}	15.01±0.95^{ab}	35.78±2.29^{ab}
90	15.04±0.75^{ab}	35.84±1.56^{b}	14.22±1.00^{a}	26.90±2.88^{b}	13.24±1.39^{b}	27.87±3.40^{b}	14.66±2.18^{ab}	27.89±1.46^{c}
Nitrogen source (0.30 g/L each)								
$(NH_4)_2SO_4$,YE	15.75± 0.25^{b}	40.31±0.79^{a}	15.74±1.00^{a}	35.07±0.63^{a}	16.77±0.26^{a}	38.58±0.07^{a}	16.95±0.73^{a}	42.89±2.05^{a}
NH_4Cl, peptone	16.89±0.23^{a}	36.76±0.36^{b}	15.52±1.08^{ab}	27.54±0.65^{cd}	16.08±0.45^{b}	36.69±0.17^{b}	15.37±0.07^{b}	39.49±0.42^{b}

Table 4.1 Continued

NH_4Cl,YE	16.91±0.40^{a}	36.01±0.26^{b}	13.79±0.98^{bc}	28.92±1.14^{c}	13.86±0.89^{d}	32.54±3.24^{c}	14.72±0.92^{bc}	35.73±0.28^{c}
$(NH_4)_2SO_4$, peptone	16.25±0.12^{b}	37.84±0.41^{b}	14.77±0.65^{b}	34.55±0.88^{b}	15.25±0.24^{c}	37.18±0.85^{b}	16.79±1.20^{a}	39.75±3.22^{b}
$(NH_4)_2SO_4$, urea	15.10±0.15^{c}	35.70±0.19^{c}	14.31±0.95^{b}	25.69±1.24^{d}	15.91±0.14^{b}	36.20±0.43^{b}	14.56±0.77^{c}	29.73±0.65^{d}
NH_4Cl, urea	14.96±1.21^{d}	34.63±0.56^{d}	12.15±1.12^{c}	18.37±0.92^{e}	11.76±0.79^{e}	26.78±2.33^{d}	13.15±0.12^{d}	28.75±0.75^{e}
pH								
4.0	13.30±0.28 ^{c}	29.70±1.14^{e}	12.32±1.11^{bc}	24.68±1.23^{c}	11.55±0.55^{c}	26.49±2.34^{f}	13.30±0.08^{d}	31.28±0.27^{e}
4.5	14.48±0.34^{b}	37.65±1.54^{c}	14.08±1.00^{ab}	30.65±0.87^{b}	13.49±0.43^{b}	27.80±0.17^{e}	14.48±0.34^{c}	33.49±1.56^{d}
5.0	16.71±0.11^{a}	42.19±0.50^{a}	14.67±1.02^{ab}	34.28±1.17^{a}	14.38±0.80^{ab}	30.68±0.92^{c}	15.12±0.09^{b}	36.77±2.04^{b}
5.5	16.32±0.14^{ab}	40.67±0.27^{b}	16.45±1.12^{a}	35.45±1.34^{a}	14.11±1.68^{ab}	35.67±0.67^{a}	16.32±0.78^{a}	38.38±0.67^{a}
6.0	14.97±0.07^{b}	37.82±1.05^{c}	15.12±0.78^{ab}	33.63±1.51^{a}	14.86±0.44^{a}	31.89±0.45^{b}	15.23±0.28^{b}	34.74±1.30^{b}
6.5	14.94 ±0.37^{b}	32.26±0.60^{d}	14.57±1.57^{ab}	32.49±1.26^{b}	13.64±0.57^{b}	28.52±0.38^{d}	13.10±0.54^{d}	30.23±0.04^{c}
7.0	12.15±0.05^{e}	27.98±2.23^{f}	11.23±1.09^{c}	22.50±1.18^{c}	10.58±0.11^{c}	24.86±0.05^{g}	10.21±1.52^{e}	26.74±1.44^{f}
Temperature (^{o}C)								
20	12.87 ±0.64^{a}	30.22±1.02^{d}	12.32±1.00^{bc}	26.89±1.19^{c}	11.55±0.02^{c}	31.00±0.20^{d}	11.85± 0.30^{c}	32.15±0.20^{d}
25	13.05 ±0.37^{a}	35.32±0.73^{b}	13.87±1.11^{b}	33.29±1.10^{b}	14.05±0.03^{a}	36.73±0.45^{b}	13.25±0.05^{b}	35.55±0.98^{b}
30	14.97±0.03^{a}	45.49±0.67^{a}	16.23±0.69^{a}	36.06±0.96^{a}	13.99 ±0.29^{a}	37.19±0.77^{a}	15.06± 1.03^{a}	47.54±1.34^{a}
35	11.66 ±0.62^{b}	32.33±0.34^{c}	13.14±0.80^{b}	28.44±1.44^{c}	12.57±0.01^{b}	34.52±1.60^{c}	12.13±0.12^{c}	34.87±0.39^{c}
40	8.17 ±0.13^{c}	23.52±0.36^{e}	7.45±1.10^{c}	18.00±0.97^{d}	9.05±0.03^{d}	26.52±0.31^{e}	8.79± 0.02^{d}	19.45±0.56^{e}

YE- Yeast Extract

All parameters are expressed as mean±SD values in the same column, n= 3, within each column, means are significantly different ($p< 0.05$), unless they have a common letter.

4.3.1.4 Effect of nitrogen sources on biomass production and lipid content

The s of combined organic nitrogen (yeast extract, peptone and urea at 0.30 g/L concentration) and inorganic nitrogen sources $(NH_4)_2SO_4$ and NH_4Cl at 0.30 g/L concentration by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were determined in the nitrogen-limited media containing optimized glucose concentrations for each oleaginous yeast. As shown in Table 4.1, *Cryptococcus curvatus* PY39 gave maximum biomass when it was grown on medium containing NH_4Cl and peptone in combinations as well as in the medium containing NH_4Cl and yeast extract. The biomass exhibited by *Cryptococcus curvatus* PY39 when grown on a medium containing NH_4Cl and peptone was 16.89±0.23 g/L, while a biomass of 16.91±0.40 g/L was attained by this yeast when grown on medium containing NH_4Cl and peptone. This yeast gave maximum lipid content (40.31±0.79%) when $(NH_4)_2SO_4$ and yeast extract were used in combinations as nitrogen sources. In the presence of $(NH_4)_2SO_4$ and yeast extract in a medium, *Rhodotorula mucilaginosa* SY09 gave maximum biomass of 15.74±1.00 g/L and lipid content of 35.07±0.63%. Combinations of $(NH_4)_2SO_4$ and yeast extract in a culture medium also supported *Rhodotorula mucilaginosa* SY18 to give maximum biomass and lipid content viz., 16.77±0.26 g/L and 38.58± 0.07%, respectively. A higher lipid content, i.e., 36.69±0.17% was attained by this strain when combinations of NH_4Cl with peptone were provided as nitrogen sources. This strain gave lipid content of 37.18±0.85% when grown on media containing combinations of $(NH_4)_2SO_4$ and peptone. Furthermore, *Rhodosporidium kratochvilovae* SY89 also exhibited maximum biomass when grown on medium containing combinations of $(NH_4)_2SO_4$ with yeast extract and $(NH_4)_2SO_4$ and peptone viz., 16.95±0.73 g/L and 16.79±1.20 g/L, respectively. This yeast gave maximum lipid content (42.89±2.05%) when $(NH_4)_2SO_4$ and yeast extract were used as nitrogen sources in combinations

too. Higher lipid content was also recorded when *Rhodosporidium kratochvilovae* SY89 grew on media containing combinations of NH_4Cl with peptone and $(NH_4)_2SO_4$ and peptone. The lipid content that was recorded when this yeast grew on media containing combinations of NH_4Cl and peptone was 39.49±0.42%. A lipid content of 39.75±3.22% was exhibited by this strain when $(NH_4)_2SO_4$ and peptone were used in combinations.

Results also showed that for all oleaginous yeasts investigated, the combined nitrogen sources that gave lowest biomass and lipid content were NH_4Cl and urea (Table 4.1) for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 leading to biomass and lipid contents of 14.96±1.21 g/L and 34.63±0.56%, 11.76±0.79 g/L and 26.75±2.33%, 11.76±0.79 g/L and 26.78±2.33%, and 13.15±0.12 g/L and 28.75±0.75%, respectively.

Based on the results in Table 4.1, the lipid content at different combinations of different nitrogen sources, one can understand that $(NH_4)_2SO_4$ and yeast extract are the optimum nitrogen sources for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89. Accordingly, $(NH_4)_2SO_4$ and yeast extract were selected as optima nitrogen sources for the four strains for further investigations.

4.3.1.5 Effect of C/N ratio on biomass production and lipid content

The effect of C/N ratio on biomass production and lipid content of selected oleaginous yeasts were studied by varying C/N ratios from 40 to 160 in nitrogen-limited media composed of 70 g/L of glucose for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18 and 50 g/L of glucose for *Rhodosporidium kratochvilovae* SY89, 0.5 g/L of yeast extract and various concentrations of $(NH_4)_2SO_4$ as shown in Table 4.2 and adjusted pH of

5.5. To know the total nitrogen content of yeast extract, Kjeldahl method was used and the approximate result obtained was 20%.

Table 4.2: The C/N ratio in nitrogen-limited medium.

C/N ratio	**Carbon source (glucose)**		**Nitrogen sources**	
	PY39,SY09,SY18	**SY89**	**PY39, SY09,SY18**	**SY89**
40	70 g/L	50 g/L	0.5 g/LYE and 2.83 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 1.89 g/L$(NH_4)_2SO_4$
60	70 g/L	50 g/L	0.5 g/LYE and 1.74 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 1.08 g/L $(NH_4)_2SO_4$
80	70 g/L	50 g/L	0.5 g/L YE and 1.18 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 0.71 g/L$(NH_4)_2SO_4$
100	70 g/L	50 g/L	0.5 g/L YE and 0.85 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 0.47 g/L $(NH_4)_2SO_4$
120	70 g/L	50 g/L	0.5 g/L YE and 0.61 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 0.31 g/L $(NH_4)_2SO_4$
140	70 g/L	50 g/L	0.5 g/L YE and 0.47 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 0.20 g/L $(NH_4)_2SO_4$
160	70 g/L	50 g/L	0.5 g/L YE and 0.35 g/L $(NH_4)_2SO_4$	0.5 g/L YE and 0.12 g/L $(NH_4)_2SO_4$

YE- yeast extract

The results of the effect of C/N ratio on biomass, lipid concentration and lipid content by the four oleaginous yeasts are presented in Figure 4.2. At C/N ratio of 140, *Cryptococcus curvatus* PY39 exhibited its maximum lipid concentration and lipid contents, i.e., 6.20±0.33 g/L and 41.75±2.31%, respectively per dry biomass of 14.85±0.90 g/L. *Rhodotorula mucilaginosa* SY09 also gave maximum biomass (14.79±1.11 g/L) and lipid content (36.65±2.55%) at C/N ratio of 100. Similarly, the maximum biomass (14.30 ±1.32 g/L) was obtained by *Rhodotorula mucilaginosa* SY18 at C/N ratio of 100, whereas maximum lipid concentration (5.72±0.13 g/L) and lipid content (40.05± 1.50%) were observed at C/N ratio of 120. On the other hand, a maximum lipid concentration of 5.87±0.53 g/L which corresponds to a lipid content of 45.54±1.32% per biomass of 12.89±1.37 g/L exhibited by *Rhodosporidium kratochvilovae* SY89 at C/N ratio of 120.

On the contrary, the lowest biomass and lipid content were exhibited by the four stains at C/N ratio of 40 except that of *Rhodotorula mucilaginosa* SY18 which gave minimum biomass at C/N ratio of 160. The lowest biomass, lipid concentration and lipid content was exhibited by *Cryptococcus curvatus* PY39 in a nitrogen-limited media with C/N ratio of 40 viz., 10.24±1.13 g/L, 2.22±0.29 g/L and 21.67±1.80%, respectively. *Rhodotorula mucilaginosa* SY09 gave lowest biomass (10.04 ±1.22 g/L), lipid concentration (2.00±0.27 g/L) and lipid content (19.32± 2.31%) at the same C/N ratio, i.e., 40. *Rhodotorula mucilaginosa* SY18 gave the lowest biomass (10.13±0.71 g/L) at C/N ratio of 160, whereas it gave the lowest lipid concentration (2.98±0.34 g/L) and lipid content (25.14±2.14%) at C/N ratio of 40. At C/N ratio of 40, *Rhodosporidium kratochvilovae* SY89 showed its minimum biomass, lipid concentration and lipid content as 9.70±0.88 g/L, lipid concentration 1.96±0.22 g/L and 20.21±1.46%, respectively.

Based on the above result, C/N ratio of 140 was the optimum and selected value for *Cryptococcus curvatus* PY39 and hence was used for further studies. C/N ratio of 100 was the optimum and selected value for *Rhodotorula mucilaginosa* SY09. C/N ratio of 120 was the optimum and selected values for *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 and hence was used for further studies.

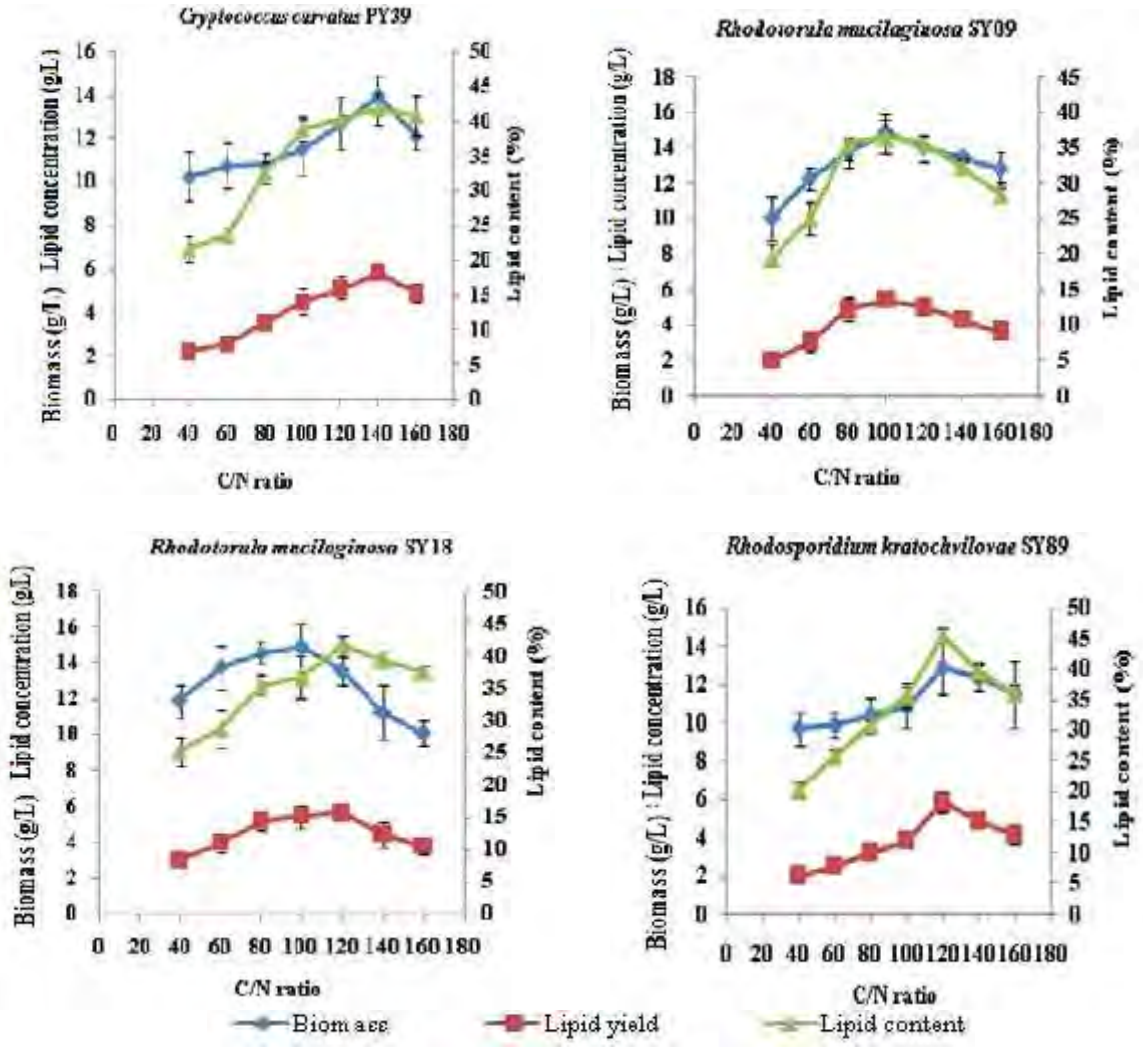

Figure 4.2: Effect of C/N ratios on cell growth and lipid accumulation by selected oleaginous yeasts. Error bars in the figures represent standard deviation.

4.3.1.6 Effects of initial pH on cell growth and lipid content

To know the best initial pH for maximum production of cell biomass and cellular lipid content in *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 a nitrogen-limited medium with pH ranging from 4.0 to 7.0 were tested. Results indicate that the optimum pH that lead to maximum lipid content lied between 5.0 and 6.0. It was found that initial of 5.0 was optimal for *Cryptococcus curvatus* PY39

and 5.5 for *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 which lead to a lipid content of 42.19±0.50, 35.45±1.34, 35.67±0.67, and 38.38±0.67% (Table 4.1), respectively. On the other hand, the best pH that leads to maximum biomass was also intial pH of 5.0 for *Cryptococcus curvatus* PY39 and *Rhodotorula mucilaginosa* SY09, 6.0 for *Rhodotorula mucilaginosa* SY18 and 5.5 for *Rhodosporidium kratochvilovae* SY89.They lead to a biomass yield of 16.71±0.11, 16.45±1.02,14.86±0.44 and 16.32±0.78 g/L (Table 4.1), respectively.

Results also showed that the pH that lead to the lowest biomass and lipid content was 7.0 for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 leading to biomass and lipid contents of 12.15±0.05 g/L and 27.98±2.23%, 11.23±1.09 g/L and 22.50±1.18%, 10.58±0.11 g/L and 24.86±0.05% and 10.21±1.52 g/L and 26.74±1.44% (Table 4.1), respectively.

The lipid concentration and cellular lipid percentage content the dry biomass at different initial pH values gave a clear picture for the optimum pH values for each oleaginous yeast strain. Accordingly, pH 5.0 was selected as the optimum pH in the medium for *Cryptococcus curvatus* PY39. On the other hand, pH 5.5 was the best pH in the growth medium for the rest of the oleaginous yeasts for further activities.

4.3.1.7 Effect of temperature on biomass and lipid production

Biomass and cellular lipid content of oleaginous yeasts, namely *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 was tested by growing each at temperatures of 20, 25, 30, 35 and 40^{o}C in a nitrogen-limited culture media. As it is presented in Table 4.1, the best temperature for maximum biomass yield was 30^{o}C for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, and

Rhodosporidium kratochvilovae SY89 and 25ºC for *Rhodotorula mucilaginosa* SY18 that led to biomass production of 14.97±0.03, 16.23±0.69, 15.06±1.03, and 14.05±0.03 g/L, respectively. Results also indicated that the optimum temperature for maximum lipid content by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 was 30ºC that led to lipid content of 45.49±0.67, 36.06± 0.96,37.19±0.77 and 47.54±1.34% (Table 4.1), respectively.

On the other hand, all strains gave the least amount of biomass, lipid concentration and lipid content at temperature of 40ºC. Based on this, *Cryptococcus curvatus* PY39 gave biomass (8.17±0.13 g/L) and lipid content (23.50±0.36%). Biomass (7.45±1.10 g/L) and lipid content (18.00±0.97%) were exhibited by *Rhodotorula mucilaginosa* SY09. The Lowest biomass and lipid content were exhibited by *Rhodotorula mucilaginosa* SY18 at temperature of 40ºC viz., 9.05±0.03 g/L and 26.52±0.31%, respectively. At temperature of 40ºC, *Rhodosporidium kratochvilovae* SY89 showed its minimum biomass and lipid content as 8.79±0.83 g/L and 19.45±0.56%, respectively. Since the maximum lipid contents were observed at the temperature of 30ºC by the four oleaginous yeasts, this temperature was selected for further experiments.

4.3.1.8 Effect of agitation rate on biomass production and lipid content

The effects of agitation rate on cell biomass production, lipid concentration and lipid content by the four oleaginous yeasts were determined under nitrogen-limited conditions by varying the agitation speed from 100 to 225 rpm is depicted in Figure 4.3. A sharp increase in biomass and lipid concentration as well as lipid content was exhibited by *Cryptococcus curvatus* PY39 when the agitation speed was increased from 100 to 225 rpm. This strain gave maximum lipid concentration (5.50±0.66 g/L) and lipid content (40.00±2.36%) per dry biomass of 13.75±0.77 g/L at the agitation speed of 225 rpm. Similarly, maximum lipid concentration of 5.14±0.68 g/L was

exhibited by *Rhodotorula mucilaginosa* SY09 at the agitation speed of 200 rpm which corresponds to 38.21±3.31% lipid content per dry biomass of 13.45±1.50 g/L. On the other hand, *Rhodotorula mucilaginosa* SY18 exhibited maximum biomass as 15.12±1.09 g/L in nitrogen-limited medium at 225 rpm. This strain had maximum lipid yield (5.66±0.66 g/L) and lipid content (37.81±2.33%) at agitation speed of 200 rpm. A lipid content of 36.22±1.87% were exhibited by this yeast strain at agitation speed of 225 rpm, which are still good results. Maximum biomass of 14.25±0.35 g/L was attained by *Rhodosporidium kratochvilovae* SY89 when the agitation rate was held at 175 rpm, while the highest lipid concentration (6.24±0.66 g/L) and lipid content (46.37±2.36%) were obtained by this strain when the agitation speed was controlled at 225 rpm.

Results from Table 4.1 also indicate that the agitation speed that led to the lowest biomass and lipid content was 100 rpm for *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 leading to biomass production, lipid concentration and lipid contents of 11.44±0.41 g/L, 2.96±0.25 g/L and 25.87±0.77%; 8.55±1.21 g/L,1.66±0.59 g/L and 19.42±0.78%; 9.44±0.59 g/L,1.78±0.41 g/L and 18.86±2.55% and 10.52±0.78 g/L, 2.94±0.48 g/L and 27.95±0.75%, respectively.

Based on the facts generated, all further experiments were carried out in nitrogen-limited media at agitation speed of 200 rpm for *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18 and 225 rpm for *Cryptococcus curvatus* PY39 and *Rhodosporidium kratochvilovae* SY89.

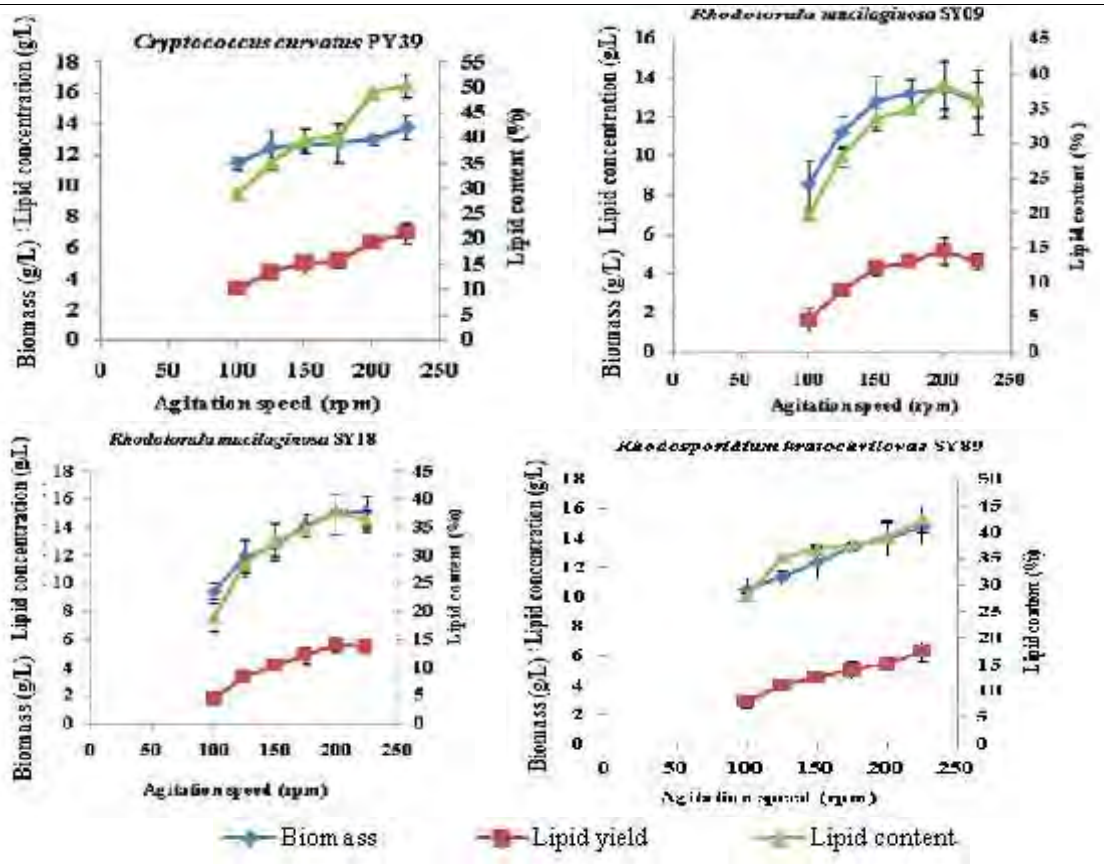

Figure 4. 3: Effect of agitation speed on yeast biomass production and lipid content by selected oleaginous yeasts. Error bars in the figures represent standard deviation.

4.3.1.9 Optimization of culture volume

To study the effect of aeration (oxygen supply) on biomass production, lipid concentration and cellular lipid content by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89, the submerged cultures were performed by varying of the volume of the medium (25, 50, 75, 100 and 125 mL). In this investigation the maximum biomass, lipid concentration and lipid content were exhibited by *Cryptococccus curvatus* PY39 when the culture volume was 50 mL (14.48±0.24 g/L, 6.61±0.66 g/L and 45.65±1.54%, respectively). Maximum biomass, lipid concentration and lipid content was exhibited by *Rhodotorula mucilaginosa* SY09 when the volume of the medium was held at 50 mL as 15.98±0.39 g/L,6.02±0.65 g/L and 37.68±0.54%, respectively. Maximum biomass of

13.62±1.55 g/L was attained by *Rhodotorula mucilaginosa* SY18 when the culture volume was 25 mL, while maximum lipid concentration (5.23±0.17 g/L) and lipid content (38.8±1.17%) were achieved at culture volume of 50 mL by this strain. As shown in Figure 4.4, one can observe that there is a decrease in biomass, lipid concentration and lipid content as the volume of the culture medium increased. Similarly, maximum biomass, lipid concentration and lipid content was exhibited by *Rhodosporidium kratochvilovae* SY89 when the volume of culture medium was held at 50 mL as 15.22±0.34 g/L, 6.47±0.08 g/L and 42.51±1.43%, respectively.

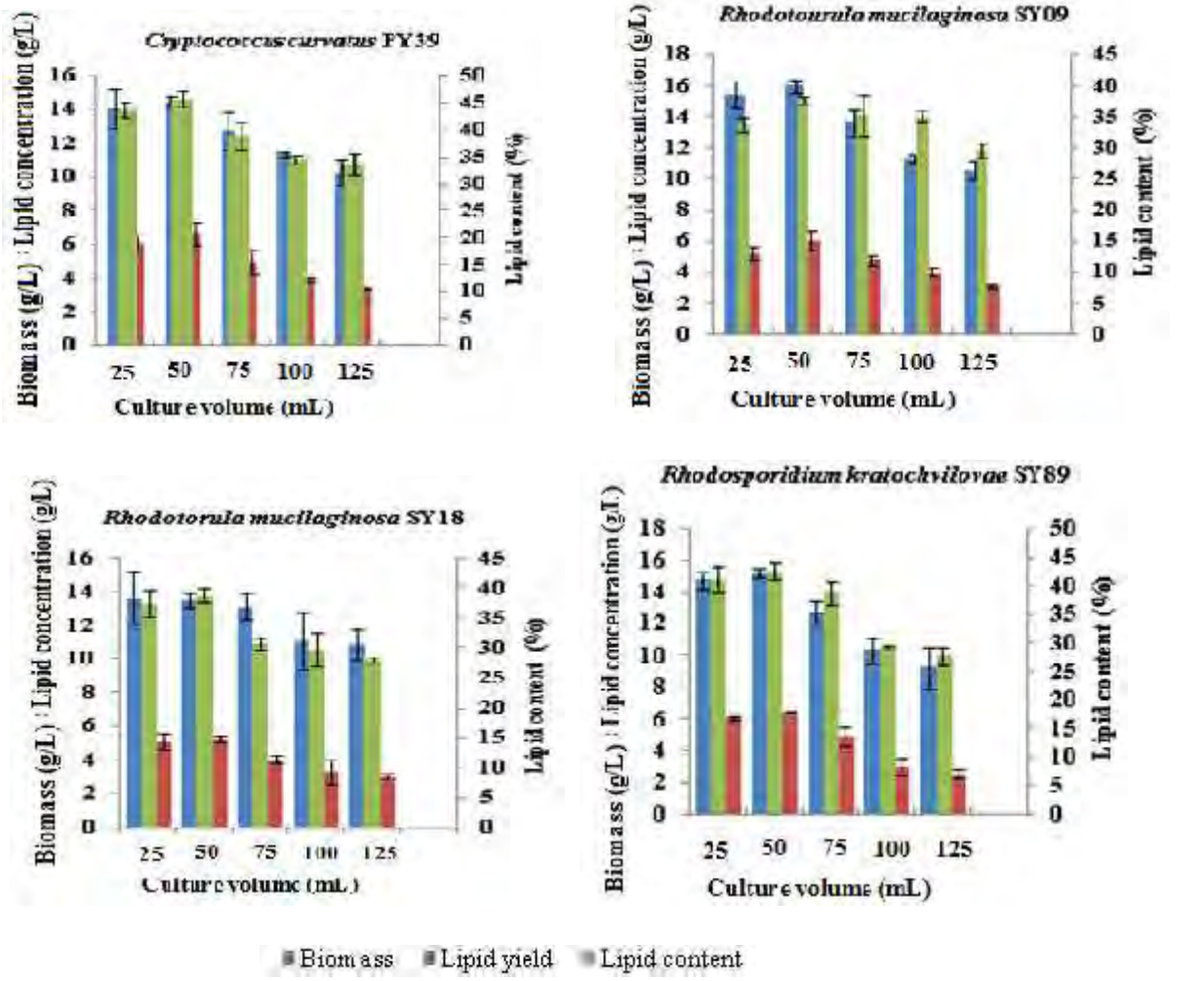

Figure 4. 4: The effect of culture volume on biomass production and lipid content by selected oleaginous yeasts. Error bars in the figures represent standard deviation.

4.3.1.10 Effect of incubation period on biomass production and lipid production

Biomass production, lipid concentration and cellular lipid content of *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were tested at 0, 24,48,72,96, 120,144 and 168 h in nitrogen-limited media separately. It was apparent that, there was a sharp increase in biomass production, lipid concentration and lipid content from 0 to 144 h by *Cryptococcus curvatus* PY39. Maximum lipid concentration of 7.22±0.26 g/L was attained by *Cryptococcus curvatus* PY39 which corresponds to a lipid content of 46.01±0.50% per biomass of 15.69±0.86 g/L at 144 h incubation (Figure 4.5). *Rhodotorula mucilaginosa* SY09 gave maximum biomass, 14.93± 1.45 g/L and lipid content 38.38±3.90% at 120 h growth (Figure 4.5).

At 120 h incubation, *Rhodotorula mucilaginosa* SY18 also gave maximum biomass, lipid concentration and lipid content as 14.67±0.93 g/L,5.42±0.73 g/L and 36.94±0.51%, respectively (Figure 4.5). On the other hand, *Rhodosporidium kratochvilovae* SY89 gave maximum biomass (15.48±0.78 g/L) at 168 h incubation. However, this yeast gave maximum lipid content (51.17±1.72%) after growth period of 144 h (Figure 4.5). A decrease in both lipid concentration and lipid content were exhibited by this oleaginous yeast after growth of 144 h.

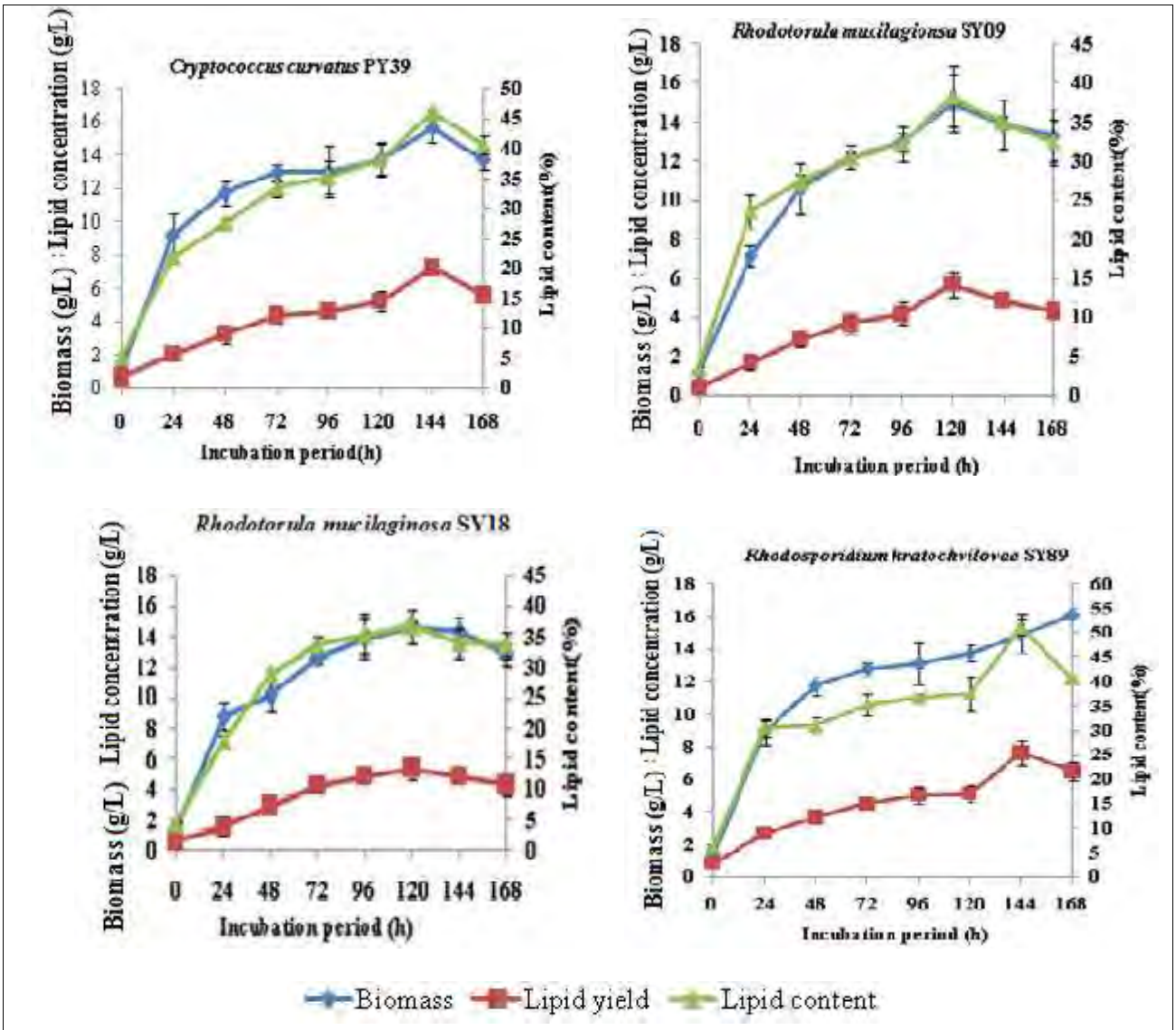

Figure 4. 5: Time course of biomass and lipid concentration as well as cellular lipid content by selected oleaginous yeasts. Error bars in the figures represent standard deviation.

4.3.2 Estimation of total sugar in peel mixtures of papaya and mango

Total sugar present in fruit peel mixture was estimated at absorbance of 490 nm with different concentration of working standard of glucose solution presented in the Figure 4.6.

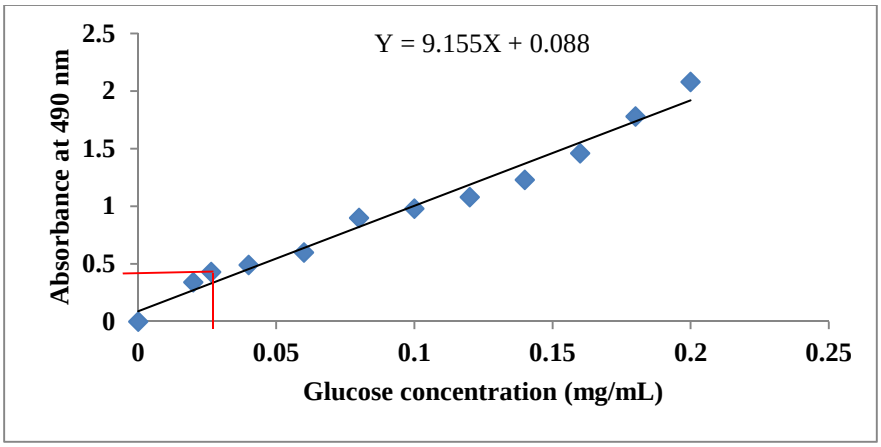

Figure 4. 6: Standard curve of glucose for the determination of total sugar in peel mixtures of papaya and mango.

Percentage of total sugar in fruit the peel sample mixture was 13.20%.

4.3.3 Production of single cell oil using peel mixtures of papaya and mango

Biomass, lipid concentration and cellular lipid content of the selected oleaginous yeasts was tested by growing them on peel mixture of papaya and mango hydrolysate that were inoculated 10% v/v (~7.94×10^8 cells/mL) seed cultures. The results obtained in this study were considerable and showed high potential of biomass, lipid concentration and lipid content by the investigated yeast strains. *Cryptococcus curvatus* PY39 when grown on peel mixtures gave biomass, lipid concentration and lipid content of 11.28±0.61 g/L,3.95±0.67 g/L and 35.02±1.63%, respectively. Similarly, *Rhodotorula mucilaginosa* SY09 gave biomass, lipid concentration and lipid content of 9.45±0.64 g/L, 2.66±0.49 g/L and 28.15±1.63%, respectively when it was cultivated under the same conditions as above. On the other hand, *Rhodotorula mucilaginosa* SY18 gave a lipid content of 36.76±0.61% which corresponds to a lipid concentration of 3.84±0.19 g/L per biomass of 10.44±0.79 g/L when grown under the same condition as above. Furthermore, the biomass (12.25±1.36 g/L), lipid concentration (4.31±0.30 g/L) and lipid content (35.18±1.40%) were exhibited by *Rhodosporidium kratochvilovae* SY89 in a nitrogen-limited peel mixture medium.

4.3.4 Fatty acid profiles

The fatty acid composition (profiles) of lipids, produced by the four yeast strains were examined and is shown in Table 4.3. The fatty acid compositions are qualitatively similar but slightly differ quantitatively among the four oleaginous yeasts. The fatty acids exhibited include palmitic acid, linolenic acid, oleic acid, linoleic acid, linolenic acid, paulinic acid (C20:1) and behenic acid (C22:0). Arachidic acid (C20:0) was produced by the four strains except *Rhodosporidium kratochvilovae* SY89. The major fatty acid in all the isolates was oleic acid with content ranging between 47.44±2.14% and 54.40±1.15 % of the total lipid. Relatively high yields of palmitic acid ranging from 10.69±0.66 % to 24.04±0.39% of the total lipid were obtained in all four isolates. In addition to oleic and palmitic acids, linoleic acid with 21.24±0.36 % was one of the main products in *Rhodotorula mucilaginosa* SY18. In addition, *Cryptococcus curvatus* PY39 contained relatively considerable amounts of stearic acid (13.16±0.93%), while *Rhodosporidium kratochvilovae* SY89 had 13.34±1.15% linoleic acid content.

Table 4. 3: Fatty acid profiles of selected oleaginous yeasts.

Strain	**Relative abundance of fatty acid residues (% w/w) of the total lipid**						
	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Lenolenic acid (C18:3)	Trace fatty acids
C. curvatus PY39	17.39±0.47	0.29±0.08	13.16±0.93	54.40±1.15	7.78±0.78	1.92±0.29	0.69±0.1
R. mucilaginosa SY09	24.04±0.39	0.75±0.08	6.08±0.72	47.44±2.14	6.34±0.64	3.91±0.43	0.57±0.09
R.mucilaginosa SY18	10.69±0.66	0.45±0.05	3.11±0.26	52.86±1.72	21.24±0.36	6.65±0.50	0.73±0.17
Rh.kratochvilovae SY89	18.63±0.53	0.50±0.09	5.68±0.32	53.82±1.25	13.34±1.15	5.47±0.60	0.34±0.13

All percentage values are expressed as mean ±SD.

4.4 Discussion

It is known that cultivation conditions are crucial for high lipid accumulation by oleaginous yeasts. Optimization of cultivation conditions is necessary to raise the efficiency of SCO production and reduce the cost. Optimization helps in the reduction cultivation time and also reduce the cost of production, so that it becomes valuable from economical point of view (Enshaeieh *et al*., 2013b). Inoculum size, availability of carbon and nitrogen sources, C/N ratio, availability of inorganic salts, temperature, agitation and aeration rates, pH, incubation period (culturing time) etc., are also known to influence the growth rate and lipid production (Ageitos *et al*., 2011; Li *et al*., 2008; Enshaeieh *et al*., 2013b; Zhao *et al*., 2015). However, there are some differences across species and strains.

Size/volume of inoculum as a starter culture in the growing medium plays a key role on yeast SCO production. Inoculum size is needed to shorten the lag phase and makes microbial growth directly go into the logarithmic phase in the larger culture medium (Kitcha, 2012). Naturally, the optimum amount of inoculum size depends on the type of species and strain to be cultured. For example, in this study, from the tested inoculum sizes as seed cultures (initial cell densities), the highest biomass and lipid contents were obtained by each of the four oleaginous yeast strains when 10% v/v inoculum sizes were inoculated (Figure 4.1). According to previous studies inoculum size of 10% v/v was also optimum for higher biomass and lipid concentration as well as lipid content for *Rhodosporidium toruloides* AS2.1389 (Hong *et al*., 2006) and *Rhodotorula glacialis* DBVPG4875 (Amaretti *et al*., 2010a). Chen *et al*. (2013), reported 5% v/v inoculation volume as an initial cell density for maximum biomass and lipid accumulation in *Trichosporon cutaneum*. On the other hand, Sankh *et al*. (2013) reported 20% v/v inoculum size as an initial cell density for maximum

biomass and lipid accumulation by *Pichia kudriavzevii*. Lowest biomass and lipid contents exhibited at an inoculum size of 25% v/v by *Cryptococcus curvatus* PY39 could be due to the transferring of too much nutrient depleted media or it could be due to the transferring of too much metabolic waste to the culture medium. On the other hand, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 yielded lowest biomass when 5% v/v inoculum size was inoculated into the larger culture media in each case. This may be due to the transferring of lowest initial cell density to the larger culture media.

Biomass production, lipid accumulation and fatty acid profile of oleaginous microorganisms are affected by the carbon source available during fermentation (Mamatha, 2009; Zhao *et al*., 2015). For the production of maximum cell biomass and lipid in oleaginous yeasts, medium with an excess of carbon source and limited amount of nitrogen sources has a significant influence (Ratledge, 2004). In this study, glucose, sucrose, maltose, galactose, xylose and glycerol were tested as carbon sources for the production of cell biomass and cellular lipid content. As it can be seen in Table 4.1, glucose as a carbon source gave maximum biomass and cellular lipid content by all the four oleaginous yeast strains. Considerable biomasses and lipid contents were also attained by each of the four strains when grown both on sucrose and maltose. Ratledge (2002), Saxena *et al.* (2008), Zhao *et al.* (2008), Ahmad *et al*. (2015) and Enshaeieh *et al*. (2015b) also reported glucose as the best carbon source for production of SCO. The effects of different carbon sources (glucose, fructose and sucrose) on lipid production in *Rhodotorula glutinis*, *Rhodosporidium toruloides* and *Lipomyces starkeyi* were also evaluated by Vijayakumar *et al*. (2010). Among the various carbon sources, glucose produced higher biomass and lipid contents.

On the other hand, lowest biomass and cellular lipid content were exhibited by all the strains when xylose was used as a carbon source (Table 4.1). For economic reasons however glucose is not

recommended for industrial production of SCO. Even though the four oleaginous gave lowest biomass and lipid content when xylose was supplied as a carbon source, it is still recommended for industrial production of SCO because it is obtained from cheap cellulosic wastes.

Glucose is often used as a comparison basis to evaluate the performance of other carbon substrates including wastes. Therefore, in this study we investigated the effect of glucose concentration, ranging from 10 to 90 g /L, on biomass and SCO yields by strain the selected oleaginous yeasts. From the evaluated glucose concentrations, higher lipid production was exhibited by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09 and *Rhodotorula mucilaginosa* SY18 when each of these oleaginous yeasts were grown in nitrogen-limited media containing 70 g/L of glucose, while a medium containing 50 g/L of glucose was found to be optimum for *Rhodosporidium kratochvilovae* SY89 for maximum production of biomass and lipid content (Table 4.1). When the tested yeasts grew on media containing lower concentrations of glucose (especially 10 g/L and 30 g/L glucose), they yielded considerable biomass and lipid. Thus, it is recommended that such lower glocuse concentrations should be used for industrial production of SCO. This saves cost of production. Previous researchers also reported different glucose concentrations for maximum production of biomass, lipid concentration and lipid content by various oleaginous yeasts. Accordingly, Enshaeieh *et al*. (2013a) reported 75 g/L for maximum production of biomass (12.7 g/L), lipid concentration (7.13 g/L) and lipid content by *Rhodotorula* sp.110, while Kraisintu *et al*.(2010) reported 70 g/L was the optimum glucose concentration for *Rhodosporidium toruloides* DMKU3-TK16 for maximum production of biomass, lipid concentration and lipid content. Media containing concentrations of 80 and 90 g/L glucose were the best for maximum production of biomass, lipid concentration and lipid content by *Torulaspora globosa* YU5/2

(Leesing and Baojungharn, 2011) and *Rhodotorula mucilaginosa* F (Enshaeieh *et al*., 2015a), respectively.

In this study, the effect of combined organic and inorganic nitrogen sources on biomass production, lipid concentration and cellular lipid content by selected yeasts was determined in nitrogen-limited media. The results showed that combinations of $(NH_4)_2SO_4$ and yeast extract were the most favorable nitrogen sources for the maximum lipid production by the selected oleaginous yeasts among the different combinations tested as nitrogen compounds (Table 4.1). However, the above four oleaginous yeasts gave lowest biomass and cellular lipid content when they were grown in a media containing NH_4Cl and urea as nitrogen sources. This differences may be due to the assimilation preferences of some nitrogen sources over the others (Evans and Ratledge, 1984). According to previous researchers, different nitrogen sources supported different oleaginous yeasts either in combination or alone. *Trichosporon fermentans* gave maximum biomass and lipid content when it was grown in media containing urea and peptone, respectively (Zhu *et al*., 2008). On the other hand, Kraisintu *et al*. (2010) reported that *Rhodosporidium toruloides* DMKU3-TK16 gave the highest biomass when the yeast was grown in medium containing yeast extract and $(NH_4)_2SO_4$ as nitrogen sources, while a higher cellular lipid contents of 53.71 and 53.10% were produced, when peptone was used with NH_4Cl and with $(NH_4)_2SO_4$. Maximum biomass (17.57 g/L), lipid concentration and lipid content were exhibited by *Rhodotorula* sp. 110 when yeast extract and $(NH_4)_2SO_4$ were used in combination (Enshaeieh *et al*., 2013a).

For the production of lipids by oleaginous microorganisms, there must be nutrient imbalance culture medium and specifically the C/N ratio has to be high (Li *et al*., 2007; Beopoulos *et al*., 2009; Ageitos *et al*., 2011). In the current study, the selected oleaginous yeasts exhibited maximum

biomass, lipid concentration and lipid content at the C/N ratio ranging from 100 – 140 (Figure 4.2). Such optimum C/N ratios that enable the four oleaginous yeasts to accumulate maximum lipid were found to be high. This means that in a high C/N ratio medium, the depletion of the nitrogen levels is rapid and that sugar would selectively go in for lipid synthesis, thereby accounting for high lipid yields (Moreton, 1988; Fakas *et al*., 2008). Other researchers also reported C/N ratio as an important parameter for maximum lipid production and found optimum C/N ratio for the yeasts they tested and hence 77, 163 and 140 were found to be the optimum C/N ratios for maximum biomass and lipid production by *Rhodosporidium toruloides* ATCC 10788 (Turcotte and Kosaric (1989), *Trichosporon fermentans* (Zhu *et al*., 2008) and *Rhodosporidium toruloides* DMAKU3-TK16 (Kraisintu *et al*., 2010), respectively.

pH is one of the most important physiological factors affecting cell growth and lipid production (Hong *et al*., 2006). In this study, highest biomass and lipid contents were attained by the selected oleaginous yeasts within the pH values of 5.0 – 6.0 (Table 4.1), indicating that the tested oleaginous yeasts prefer acidic pH to grow and accumulate lipids (Subramaniam *et al.*, 2010; Ageitos *et al.*, 2011; Zhao *et al*., 2015). When these four oleaginous yeasts were compared in terms of their lipid content with respect to pH, *Cryptococcus curvatus* PY39 is a good candidate followed by *Rhodosporidium kratochvilovae* SY89. *Rhodotorula mucilaginosa* SY18 was also competent. *Rhodotorula mucilaginosa* SY09 was poor. Previous studies also reported similar pH values for different oleaginous yeasts. Accordingly, 5.0 was the optimum and selected pH for *Lipomyces starkeyi* (Angerbauer *et al*., 2008) and *Rhodotorula glutinis* EF081370 (Tao *et al*., 2008) for maximum production of biomass and lipid, while 5.5 was the best pH for *Rhodosporidium toruloides* DMKU3-TK16 (Kraisintu *et al*., 2010), *Cryptococcus curvatus* NRRLY−1511 (El-Fadaly *et al*., 2009) for maximum production of biomass and lipid. pH of 6.0 was the optimum

value for maximum production of biomass and lipid yield by *Trichosporon fermentans* (Wang *et al*., 2005) and *Trichosporon cutaneum* (Chen *et al*., 2013).

Cellular lipid accumulation is critically affected by the culture temperature. Temperature affects all living organisms and controls the growth rate, lipid synthesis and alters the composition of fatty acid at cellular level (Mamatha, 2009; Zhao *et al.*, 2015). In the current study, *Cryptococcus curvatus* PY39 exhibited maximum biomasses at temperatures of 20, 25 and 30^{o}C, the biomasses exhibited at these temperatures were significantly the same. On the other hand, *Rhodotorula mucilaginosa* SY18 yielded significantly the same maximum biomasses at temperatures of 25 and 30^{o}C, while the rest yeasts yielded maximum biomasses at the temperature of 30^{o}C. However, maximum lipid contents were attained by each of the four oleaginous yeasts at the growth temperature of 30^{o}C. Such results show that temperature of lipid synthesis and cell growth temperature are not consistent (Zhao *et al*., 2015). Such maximum results obtained at 30^{o}C in line with the results reported by Hong *et al*. (2006), Syed *et al*. (2006) and Mamatha (2009). It was evident in this work that at a temperature of 40^{o}C, the least biomass, lipid concentration and lipid content were exhibited by all the four strains. An increase in temperature will increase enzyme activity. If the temperature is too high, enzyme activity will diminish (enzymes may be denatured). Lowering temperature will decrease enzyme activity. Such effects in turn might influence on biomass production and lipid yield.

Agitation and aeration have great importance in aerobic culture to increase biomass and hence lipid production (Saad *et al*., 2014). In the current study, the optimum agitation speed for the maximum biomass production and lipid content by the four oleaginous yeasts was in the range 175 – 225 rpm. This is because the higher the agitation speed, the oxygen that dissolve in the medium become higher and it increases growth and lipid content as energy metabolism and

synthesis of lipid components need oxygen (Enshaeieh *et al*., 2013b). Too low shaking speed of the flask culture would cause low dissolved oxygen in the medium, limits cell growth and synthesis of oil, whereas too high rotation speed could cause the shear stress to become larger and reduces cell growth and synthesis of oil (Zhao *et al*., 2015). Other researchers also investigated optimum agitation speeds for different oleaginous yeasts. Accordingly, 180 and 200 rpm were optimum agitation speeds for *Rhodotorula glutinis* (Dai *et al*., 2007) and *Cryptococcus curvatus* NRRLY−1511 (El-Fadaly *et al*., 2009), respectively.

Aeration has a critical effect on production of biomass and hence on SCO yield by oleaginous microorganisms. Aeration positively increases consumption of fermentable sugars by lipid producing microorganisms resulting in an increased yield of SCO produced per unit of sugar consumed (Ratledge and Wynn, 2002). It is suggested that oleaginous yeasts require substantial oxygen supply for energy and biosynthesis (Hong *et al*., 2006). In this study, the effect of aeration on biomass production, lipid concentration and cellular lipid content by selected oleaginous yeasts in submerged cultures were performed by changing the culture volume in a 250 mL Erlenmeyer flask. Maximum biomass and lipid content were exhibited when the culture volume was held at 50 mL. As shown in Figure 4.4, one can observe that there is a decrease in biomass, lipid concentration and lipid content as the volume of the culture medium increased. This is a confirmation of the increase of oxygen solubility in lowering percentage of medium volume per flask volume increased (Malaiwong *et al*., 2016).The results obtained in this study were in line with that studied by Hong *et al.* (2006) and Mamatha (2009).

Different oleaginous microorganisms require different culture time (incubation period) for maximum biomass production and lipid accumulation (Hong *et al*., 2006; Dai *et al*., 2007; El-Fadaly *et al*., 2009; Kraisintu *et al*., 2010). In the current study, the selected oleaginous yeasts for

optimization purposes exhibited highest biomasses and lipid contents towards the latter incubation periods (120- 168 h). In the previous studies, researchers have found that there is a close relationship between the length of incubation period and the best time for the synthesis of lipids (Zhao *et al*., 2015). There were reports of oleaginous yeasts during the whole growth period, the accumulation of oil was very little in the early stage because of the nutrients and cell growth, most oil (grease) was used to the synthesis of cell membrane, the lipid would have increased significantly and reach the peak in the stable phase (Zhao *et al*., 2015). Other researchers also reported different optimum incubation periods for maximum production of lipid by different oleaginous yeasts. Incubation period of 72 h was the optimum for maximum biomass and lipid production both in *Rhodotorula glutinis* (Dai *et al*., 2007) and *Cryptococcus curvatus* NRRLY−1511 El-Fadaly *et al*. (2009). On the other hand, *Rhodosporidium toruloides* DMKU3-TK16 exhibited maximal lipid concentration and lipid cellular lipid content after 168 h of cultivation (Kraisintu *et al*., 2010).

Fruit based industries produce large volume of wastes, these wastes pose increasing disposal and pollution problems and represent a loss of valuable biomass and nutrients (Umesh and Preethi, 2014). However, these carbohydrate rich wastes can be tuned into valuable substrates for the commercial production of lipids and thus can be regarded as available option for meeting the growing demand for lipids. For economic reasons, low-cost feedstock substrates such as peels of orange, banana, potato, sugarcane bagasse, corn stalk, tomato waste, rice bran, rice straw and rice hull was used as a carbon source for the production of SCO (Huang *et al*., 2009; Economou *et al*., 2011; Enshaeieh *et al*., 2015a; Shalma *et al*., 2016). On the other hand, such fruit wastes have limitations such as their seasonal availability, substrate preparation cost, high water content and spoilage problems. Still with limitations they can be alternative feedstocks.

Microbial lipids have long been considered as an alternative to sources of conventional oils. To produce such lipids, oleaginous microorganisms, especially oleaginous yeasts have to be grown on cheap substrates for economic reasons. Taking this into account, the four oleaginous yeasts were cultivated under nitrogen-limited media containing papaya and mango peel mixtures for the production of lipid. Peel mixture of papaya and mango hydrolysate were found to be rich in carbohydrate content and supported the growth of oleaginous yeasts. The results obtained in this study are in agreement with the research works by Tinoi and Rakariyatham (2015) on utilization of pineapple waste hydrolysate for lipid production by *Rhodoturula glutinis* and Rattanapoltee and Kaewkannetra, (2014) on hetrotrophic growth of a microalgae, namely *Scenedesmus acutus* that cultivated on pineapple peels.

The type of species and growth conditions such as temperature, pH, type of substrate (medium of composition), variation in C/N ratio, and oxygen availability influence the fatty acid profiles and the efficiency of lipid accumulation (Jacob, 1993; Amaretti *et al*., 2010a). In this study, the fatty acid profiles of *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89 were evaluated using GC. It was observed that the fatty acid compositions are qualitatively similar but slightly differ quantitatively among the four oleaginous yeasts.

All the four oleaginous yeasts exhibited a predominant amount of oleic acid and low amounts of other fatty acids (linoleic acid, palmitic acid, linolenic acid, palmitoleic acid, paulinic acid, arachidic acid and behenic acid). The fatty acid profile determined in the current study displayed preferential synthesis of high percentage of saturated and monounsaturated fatty acids in the four oleaginous yeast strains. The concentration of saturated and monounsaturated fatty acids of *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18

and *Rhodosporidium kratochvilovae* SY89 added up 85.24±2.63, 78.31±3.33, 67.11±1.69 and 78.63±2.19%, respectively. In previous studies, researchers have also reported oleic acid as the major fatty acid in SCOs followed by palmitic acid and low amount of other fatty acids (Zhao *et al*., 2011; Seo *et al*., 2013; Ahmad *et al*., 2015). The fatty acids that were exhibited by the four strains were not only similar to fatty acid profiles of other oleaginous yeasts but also they fit well with the fatty acid profiles of different vegetable oils such as rapeseed, soybean, palm, and sunflower (Ma and Hanna, 1999; Christophe *et al*., 2012). From these comparisons, one can see that there are similarities with the relative abundance of fatty acid profiles with slight differences. This again confirmed that the lipids from these oleaginous yeasts are good oil feedstock for biodiesel production. In addition, the main components of fatty acid profiles of the four oleaginous yeasts are C16:0, C18:0, C18:1 and C18:2 which is similar to cacao-butter (Schulze *et al*., 2014). Thus, such fatty acids can be used for human consumption.

4.5 Conclusion and recommendations

Cultivation conditions such as inoculum size, availability of carbon and nitrogen sources, C/N ratio, incubation temperature and time, agitation and culture volume (aeration) and pH played a critical role on the growth rate and lipid accumulation by *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89. Comparing the results with previous data, it is clear that there are some differences across species and strains and it is, therefore, important to investigate the optimal parameters for each strain tested.

This study also showed the capacity of the four oleaginous yeasts to utilize peel mixtures of papaya and mango hydolysate as a media for the production of biomass and lipid. Thus, peel mixtures of papaya and mango hydolysate could be used as cheap substrates for the cost-effective production

of SCO which is used as a feedstock for the preparation of biodiesel. The ability to utilize fruit peel hydrolysate is very important from an environmental point of view. This research work provides an insight to cultivate oleaginous yeasts on other lignocellulosic wastes.

All the studied oleaginous yeast species (*Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89) have been observed to realize their ability to produce higher concentrations of saturated and monounsaturated fatty acids (with oleic acid the most dominant) than those of unsaturated fatty acids. This make these oleaginous yeasts the most promising candidates for biodiesel production.

Chapter 5: Transesterification of Single Cell Oil from *Rhodosporidium kratochvilovae* SY89 into Biodiesel

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Abstract

Single cell oils have long been considered as an alternative to conventional oil sources. The oil produced can also be used as feedstocks for biodiesel production. Oleaginous yeasts have relatively high growth and lipid production rates, can utilize a wide variety of cheap agro-industrial wastes, such as molasses, and can accumulate lipid above 20% of their biomass when they are grown in a bioreactor under conditions of controlled excess carbon and nitrogen limitation. In this study, *Rhodosporidium kratochvilovae* SY89 was cultivated in nitrogen-limited medium containing cane molasses as a carbon source. It accumulated lipids up to 38.25±1.10% on a cellular biomass basis which corresponds to a lipid concentration of 4.82±0.27 g/L. The fatty acid profiles of extracted yeast lipids were analyzed using gas chromatography coupled with flame ionization detector. A significant amount of oleic acid (58.51±0.76%), palmitic acid (15.70±1.27%), linoleic acid (13.29±1.18%) and low amount of other fatty acids were detected in the extracted yeast lipids. The lipids were used to prepare biodiesel and the yield was 85.30%. The properties of this biodiesel were determined and found to be comparable to the specifications established by ASTM D6751 and EN14214 related to biodiesel quality. Based on the results obtained, the biodiesel from

Rhodosporidium kratochvilovae SY89 oil could be a competitive alternative to conventional diesel fuel.

Key words: Biodiesel, cane molasses, oleaginous yeast, *Rhodosporidium kratochvilovae*, single cell oil, transesterification

5.1 Introduction

Oleaginous microorganisms, including yeasts, which are capable of accumulating lipids, have long been considered as an alternative to conventional oil sources. Oleaginous yeasts have high growth and lipid production rates, can utilize a variety of waste carbon sources (including cheap agro-industrial residues such as molasses) and can accumulate lipids from 20 to 70% of their dry cell biomass when grown in a bioreactor under conditions of controlled carbon excess and nitrogen limitation (Hassan *et al.*, 1993; Meesters *et al.*, 1996).

Biodiesel is a biodegradable, nontoxic, environmentally friendly and cleaner fuel alternative to petroleum-derived diesel fuel (Tyson, 2001; Dai *et al*., 2009; Kraistinu *et al*., 2010). It has attracted much attention recently because it is made from renewable resources (Li *et al*., 2008) and may reduce net carbon dioxide emissions by 78% on a life cycle basis (Tyson, 2001) and hence contributes to the reduction in emissions to global warming (Ahmad *et al*., 2013).

Currently, biodiesel (FAME) is produced from plant oils (canola, rape seed, and soya bean etc.) and /or animal fats by transesterification with short chain alcohols such as methanol, ethanol, propanol and buthanol (Vicente *et al.*, 2004; Pan *et al*., 2009; Liu *et al*., 2010). Methanol utilized most frequently because of its low-cost and its physical and chemical advantages (Fukuda *et al*., 2001). During transesterification reaction the oil or fat reacts with an alcohol to form esters and glycerol in the presence of a suitable catalyst (Ma and Hanna, 1999). Transesterification is the

displacement of an alcohol from an ester by another alcohol in a process similar to hydrolysis except that an alcohol is employed instead of water.

Transesterification process is a sequential reversible reaction (Figure 2.4). TAGs are first reduced to diacylglycerols. Diacylglycerols then reduced to monoacylglycerols. Lastly, monoacylglycerols are reduced to fattyacyl esters. Glycerol is released as a by-product (Fukuda *et al*., 2001).

However, producing biodiesel from vegetable oils or animal fats has many limitations. Firstly, it competes with the food market, since these oils and fats are also used for human consumption. Secondly, using oils, especially vegetable oils, as raw materials have high costs. Thirdly, more time and man power are needed for their production (Vicente *et al.*, 2009; Kraistinu *et al*., 2010). To compensate this cost, oleaginous microorganisms have to be grown on low-cost feedstocks (agro-industrial wastes) to replace the above fats and oil sources. These agro-industrial wastes include molasses, wheat bran, sugar cane bagasse, corn stover, wheat straw, saw mill and paper mill waste (Yousuf, 2012). From the many substrates proposed for the economic conversion to lipids, molasses is considered as one of the best feedstock for the cultivation of lipid producing microorganisms (Sabry *et al*., 1990).

Molasses is a dark brown viscous liquid obtained as a by-product in the processing of cane or beet sugar. Molasses contains uncrystallized sugar and some lost sucrose. It is used in the production of bio-polymer (Berwanger *et al.*, 2007), bio-surfactant (Nitschke *et al*., 2004), lactic acid (Coelho *et al.*, 2011), bioethanol **(**Periyasamy and Venkatachalam, 2009; Fadel *et al*., 2013; Arshad *et al*., 2014) and biodiesel (Almazan *et al*., 1981; Sabry *et al*., 1990; Karatay and Donmez, 2010; Gajdoš *et al*., 2015).

The sugar contents of molasses can be determined using phenol-sulphuric acid (DuBois *et al*., 1956), DNS (Miller, 1959), Lane-Eynon (Ranganna, 1977) or HPLC (Kakita *et al*., 2002) methods. Due to its universality, time efficiency, accuracy and selectivity for the quantification of carbohydrates, HPLC is the best method for sugar analysis (Kakita *et al*., 2002).

The objective of this study was to transesterify single cell oil that was extracted from *Rhodosporidium kratochvilovae* SY89 grown on molasses in a bioreactor into biodiesel.

5.2 Materials and methods

5.2.1 Yeast strain

The research work in this chapter was conducted at the University of the Free State, South Africa. Due to time shortage I was unable to work on all the four selected oleaginous yeasts. Thus, *Rhodosporidium kratochvilovae* SY89 (the details are discussed in the previous chapters) was selected among the four oleaginous yeasts. This strain was maintained using YM agar and was kept at 4^{o}C with sub culturing for further activities.

5.2.2 Inoculum preparation

A loopful of yeast cells growing on YM agar slants was inoculated into a sterilized nitrogen-limited medium. The culture was allowed to grow for 24 h at 30^{o}C, pH 5.5 and shake speed of 225 rpm. Inoculum size of 10% v/v (~7.94×10^{8} cells/mL) was added to the nitrogen-limited medium.

5.2.3 Cultivation of *Rhodosporidium kratochvilovae* SY89 in bioreactor

5.2.3.1 Cultivation of *Rhodosporidium kratochvilovae* SY89 in bioreactor using glucose

Prior to bioreactor cultivation of *Rhodosporidium kratochvilovae* SY89 using cane molasses as a substrate, this yeast was grown using glucose as a carbon source under nitrogen-limited medium. This nitrogen-limited medium was prepared as discribed in Chapter 3 except few modifications.

In this case glucose (50 g/L), $(NH_4)_2SO_4$ (0.31 g/L) and yeast extract (0.50 g/L) were used. The medium was sterilized by autoclaving at 121^oC for 15 min. The sterilized and labeled solutions were kept for the cultivation of *Rhodosporidium kratochvilovae* SY89. The fermentation medium was inoculated with 10% (v/v) of the liquid seed culture and then cultivated in a FerMac320, 0.8 L stirred-tank bioreactor. Fermentations were performed under the following conditions: work volume: 600 mL, stirring rate: 500 rpm, culture temperature, 30°C, initial pH, 5.5 and aeration rate: 1.25 vvm.

5.2.3.2 Cultivation of *Rhodosporidium kratochvilovae* SY89 in bioreactor using cane molasses

After cultivating *Rhodosporidium kratochvilovae* SY89 using glucose as a carbon source, cultivation of this yeast was undertaken using cane molasses. The cane molasses used as a cultivation medium for *Rhodosporidium kratochvilovae* SY89 was obtained from Wonji Factory, Wonji, Ethiopia. The molasses was diluted to 50% (v/v). The diluted molasses then was boiled, allowed to cool and sedimentation of insoluble materials occurred. The sediments were removed by decantation. The resulting molasses was centrifuged with a high speed at 5000×*g* for 10 min for further removal of insoluble materials. The supernatant solution was separated from the pellet. The pellet was discarded and the supernatant was used for the cultivation purpose. Glucose, fructose and sucrose contents of the molasses were determined by Waters HPLC(Waters Corp., Milford, MA,USA) using an Aminex HPX-87P column (300 X7.8 mm) at 85°C with MilliQ water at a flow rate of 0.6 mL/ min as eluent. The injection volume was 10µL. Peak identification of each sugar was based on the retention times (tR) of each sugar [(sucrose (tR = 17.45 min), glucose (tR = 21.98 min) and fructose (tR =25.96 min)]. Before the quantitative determination of sugars in the molasses, standard solutions of sucrose, glucose and fructose were prepared and used to

prepare calibration curves for each sugar. The concentrations of the different sugars in the molasses were determined using these curves.

The nitrogen-limited fermentation medium contained all the above ingredients except the carbon source in this case is 13.10% v/v (~ 50 g/L total sugar) cane molasses was used. The 13.10% v/v concentration is based on the total sugar concentrations of the molasses (38%). The ingredients were sterilized by autoclaving at 121^{o}C for 15 min. The bioreactor fermentation conditions were kept similar to those used when glucose was used as carbon source for bioreactor cultivation.

5.2.4 Analytical methods

5.2.4.1 Determination of yeast biomass and lipid content

Yeast cells were harvested by centrifugation at 5000×*g* for 15 min. The supernatants were discarded. Biomass (Pellet) was washed twice with distilled water, frozen at -80^{o}C and freeze dried over night to constant weight. Dry weight was determined gravimetrically. Lipid extraction was done following Folch *et al*. (1957) with some modifications (the details are discussed in Chapter 3). The SCO content was calculated as follows.

$$\text{Single cell oil content} = \frac{\text{Single Cell Oil Concentration (g/L)}}{\text{Cell Dry Weight(g/L)}} \times 100$$

5.2.4.2 Analysis of fatty acids profiles using gas chromatography

To determine the fatty acid composition of the lipids, the extracted lipids were transferred to GC vials, dissolved in chloroform and methylated with trimethylsulphonium hydroxide (TMSOH) (Butte, 1983). The vials were then sealed and vortexed for approximately 5 sec. Fatty acid methyl esters were subsequently analyzed on a Shimadzu GC-2010 auto sampler gas chromatograph with a flame ionization detector. An injection volume of 0.5 µL of sample was added into a SGE-BPX-70 column (length =50 m and inner diameter = 0.22 mm). The injection port had a temperature of

250°C and a split ratio of 1:10. The column temperature was 200°C. Hydrogen gas was used as a carrier gas at a flow rate of 40 mL/min. The total program time was 4.50 min per sample with a column flow rate of 1.37 mL/min. Peaks were identified by reference to authentic standards (library of fatty acid profiles).

5.2.5 Statistical analysis

One way-ANOVA was performed to calculate significant differences in treatment means. SPSS version 20.0 software was used for interpretation of the data. Mean separations were performed by Tukey *post hoc* tests. All experiments were done in triplicate.

5.2.6 Transesterification of single cell oil into biodiesel

After the extraction of SCO from *Rhodosporidium kratochvilovae* SY89 (the details of extraction process are discussed in Chapter 3, Section 3.2.10), sulphuric acid catalyzed transesterification reaction was carried out in a 100 mL round bottom flask under the following conditions: reaction time, 7 h; rotation speed, 200 rpm; temperature, 55°C; molar ratio, 12:1 (25 mL oil and 7.5 mL methanol) and catalyst, 0.25 mL of 80% H_2SO_4. Petroleum ether was used to separate the biodiesel (upper) layer. The reaction mixture was cooled undisturbed and set aside for phase separation. The final product biodiesel was obtained after evaporating the ether solution. Biodiesel yield (%) relative to the weight of the yeast oil was estimated following the method of Miao and Wu (2006). The number of moles of biodiesel helped in calculating the theoretical mass of biodiesel. Theoretical Mass = Moles of Biodiesel (= 3 x Number of Moles of Yeast Oil) x Molar Weight of Biodiesel.

$$\text{Biodieselyield}(\%) = \frac{\text{Massof biodiesel}}{\text{Theoretical mass}} \times 100$$

5.2.7 Characterization of biodiesel properties

The different properties of biodiesel produced from oil extracted from *Rhodosporidium kratochviolovae* SY89 was calculated directly from the FAME profiles using the online version of Biodiesel Analyzer Software (Biodiesel Analyzer© Version, 2.2.,2016, http://www. brteam.ir/ analysis/).The fuel properties of biodiesel analyzed include saponification value (SV), iodine value (IV), cetane number (CN), cloud point (CP), density (ρ),viscosity (v),oxidation stability (OS), pour point (PP), cold filter plugging point(CFPP), long chain saturated factor (LCSF), high heating value (HHV), saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), degree of unsaturation (DU), allylic position equivalent (APE) and bis-allylic position equivalent (BAPE).

5.3 Results

5.3.1Cultivation of *Rhodosporidium kratochvilovae* SY89 in bioreactors

5.3.1.1 Cultivation of *Rhodosporidium kratochvilovae* SY89 in bioreactor using glucose

Prior to bioreactor cultivation of *Rhodosporidium kratochvilovae* SY89 using cane molasses, the yeast was grown in nitrogen-limited medium using glucose as a carbon source. The fermentation was undertaken in a 0.8 L stirred tank bioreactor. Set-up of the fermentation process is shown in Figure 5.1. As depicted in Figure 5.2, this yeast gave a maximum lipid concentration of 8.60±0.81 g/L which corresponds to a lipid content of 56.06±1.70% per dry biomass of 15.34±1.47 g/L.

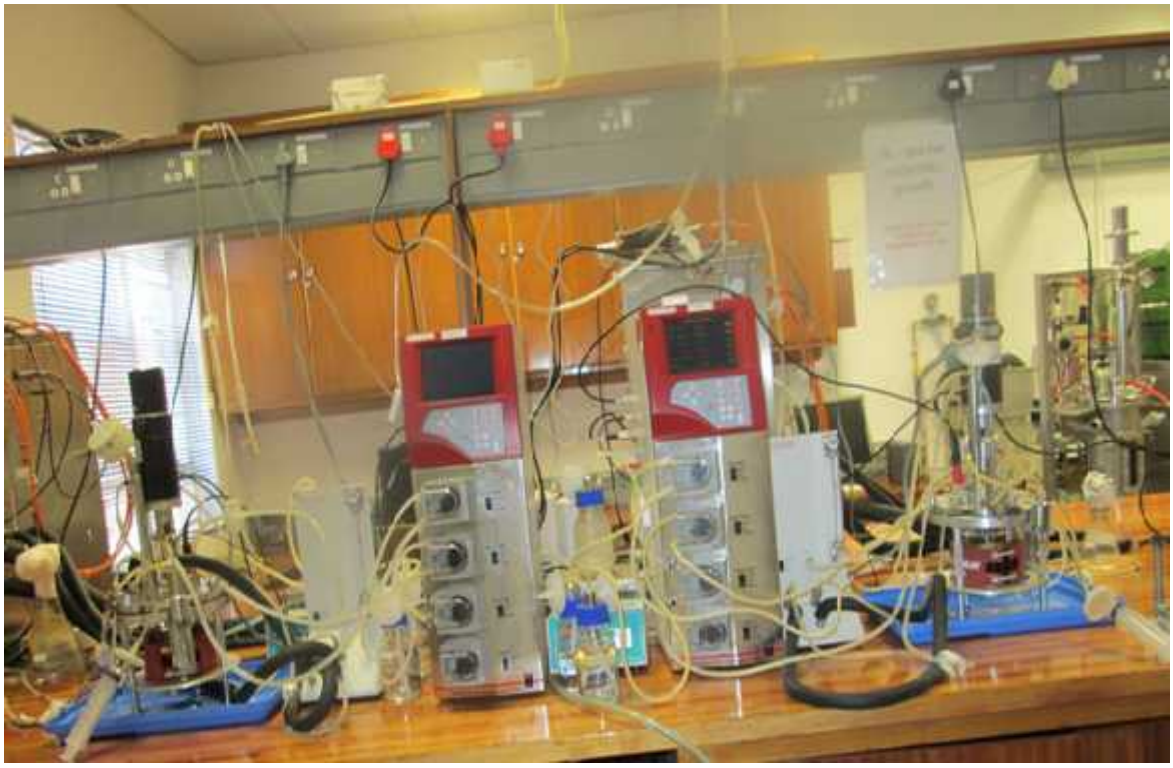


Figure 5.1: Experimental set-up during cultivation of *Rhodosporidium kratochvilovae* SY89 in bioreactor.

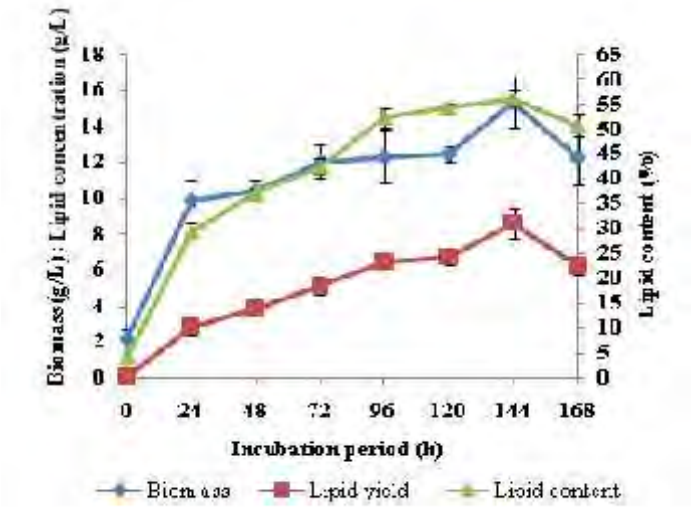
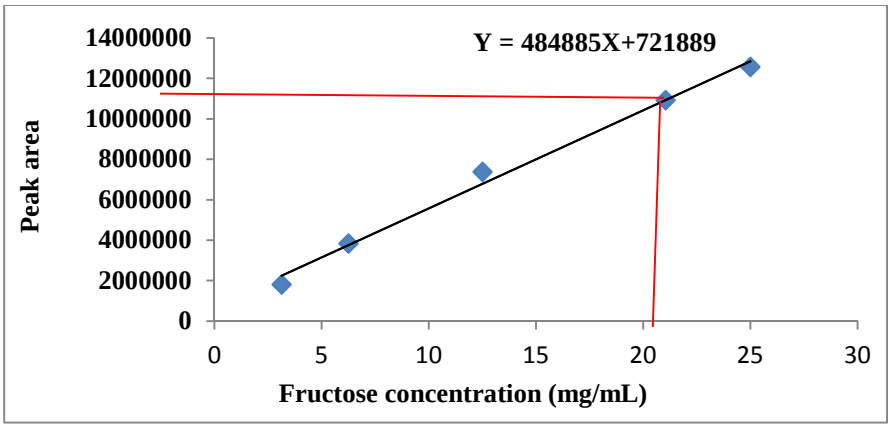


Figure 5.2: Time course of cell growth and lipid accumulation by *Rhodosporidium kratochvilovae* SY89 using glucose as a substrate in stirred tank bioreactor. Error bars in the figure represent standard deviation.

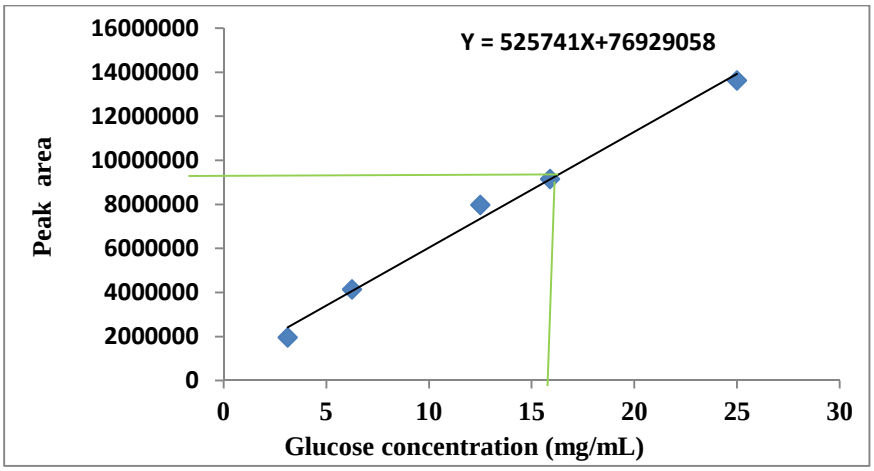
5.3.1.2 Bioreactor cultivation of *Rhodosporidium kratochvilovae* SY89 using cane molasses

In this study, SCO production from cane molasses by *Rhodosporidium kratochvilovae* SY89 was developed for the first time. Prior to cultivation of *Rhodosporidium kratochvilovae* SY89, the three sugar concentrations of cane molasses were analyzed using HPLC and standard curves were constructed for each sugar. Figures 5.3(a-c) show standard curves of glucose, fructose and sucrose. The correlation coefficients (R^2) were glucose, 0.991077; fructose, 0.990834 and sucrose, 0.990018. These standard curves were used to determine the concentration fructose, glucose and fructose. The concentrations of glucose, fructose and sucrose in molasses are presented in Table 5.1. The estimated total sugar, calculated as the sum of the three sugars, was 38.28%. As shown in Table 5.1, the amount of sucrose recorded was very low.

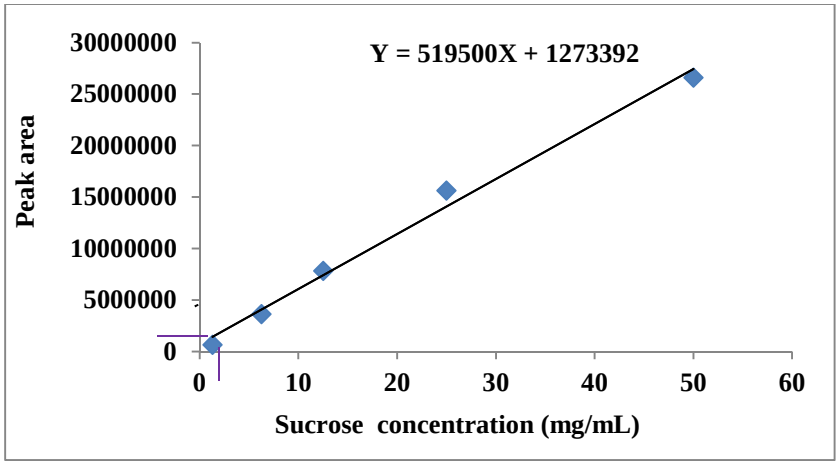
After cultivation of *Rhodosporidium kratochvilovae* SY89 using glucose in a bioreactor and determination of sugar content in cane molasses, the yeast was then grown on the cane molasses for 168 h. The results of biomass, lipid concentration and lipid content are shown in Figure 5.4. The results obtained in this study were meaningful and showed high potential of biomass, lipid concentration and lipid content by this strain. When *Rhodosporidium kratochvilovae* SY89 was grown on cane molasses medium in a bioreactor, it accumulated lipids up to 38.25±1.10% on a cellular dry biomass basis. This result corresponds to a lipid concentration of 4.82±0.27 g/L. Maximum biomass of 13.25±1.36 g/L achieved at 120 h of incubation.



(1)



(2)



(3)

Figure 5.3: Calibration curves for fructose (1), glucose (2, and sucrose (3).

Table 5.1: Composition of sugars in cane molasses.

Sugars	Composition in cane molasses (%)
Fructose	21.05
Glucose	15.94
Sucrose	1.29
Total	38.28

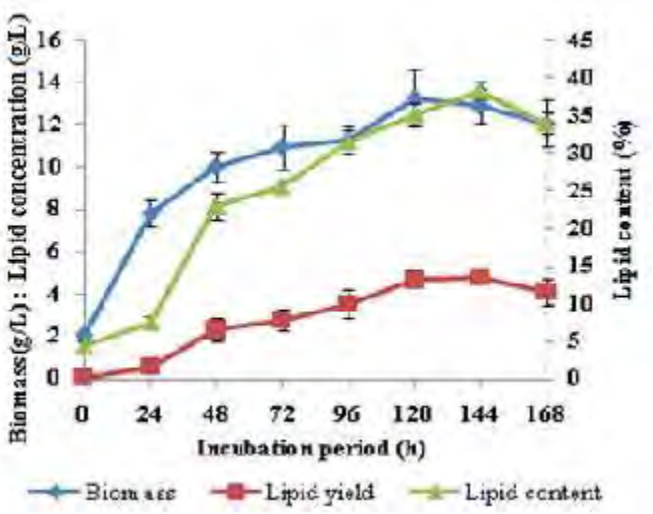


Figure 5. 4: Time course of cell growth and lipid accumulation with *Rhodosporidium kratochvilovae* SY89 using molasses as a substrate in stirred tank bioreactor. Error bars in the figure represent standard deviation.

5.3.2 Fatty acid profiles (compositions)

Fatty acid compositions of *Rhodosporidium kratochvilovae* SY89 grown on both glucose and cane molasses in a bioreactor was analyzed by GC coupled with flame ionization detector and was found that there were remarkable differences in terms of percentage composition of the fatty acid profiles. So that the yeast appeared to produce oleic acid as the largest lipid component (42.89±1.90%), followed by linoleic acid (21.98±1.80%), palmitc acid (14.99±1.10%), stearic acid

(14.36±1.38%), linolenic acid(0.31±0.10%) and palmitoleic acid (0.12±0.02%) and trace fatty acids (arachidic acid, paulinic acid and behenic acid (0.91±0.12%) when it was grown on glucose. Similarly, this yeast produced oleic acid as its largest lipid component, followed by palmitc acid, linoleic acid, strearic acid, linolenic acid and palmitoleic acid when it was cultured on molasses. In general, the fatty acid profiles exhibited were oleic acid (58.51±0.76%), palmitc acid (15.70±1.27%), linoleic acid (13.29±1.18%), stearic acid (4.38±0.36%), linolenic acid (2.76±0.97%), palmitoleic acid (0.59±0.17%) and trace fatty acids (1.70±0.23%).

5.3.3 Production of biodiesel

To produce microbial biodiesel, the extracted oil from *Rhodosporidium kratochvilovae* SY89 was transesterified using methanol and a yield of 85.30% was obtained.

5.3.4 Characterization of biodiesel properties

To evaluate the potential of biodiesel produced from *Rhodosporidium kratochvilovae* SY89 as a substitute of diesel fuel, the different physico-chemical properties of biodiesel including saponification value (SV), iodine value (IV), cetane number (CN), long chain saturated factor (LCSF), cold filter plugging point (CFPP), cloud point (CP), pour point (PP), oxidation stability (OS), higher heating value (HHV), kinematic viscosity (υ), density (ρ), saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), degree of unsaturation (DU), allylic position equivalent (APE) and bis-allylic position equivalent (BAPE) were determined. The results are presented in Table 5.2.

Table 5.2: Selected physico- chemical properties of biodiesel produced from *Rhodosporidium kratochvilovae* SY89 grown on cane molasses compared to standard biodiesel specifications.

Biodiesel property	**Biodiesel from SY89**	**US biodiesel standard**	**EU biodiesel standard**
SV(mg/g)	192.29	NS	NS
IV mg I/100 g oil	84.83	NS	< 120
CN	55.59	> 47	> 51
CP (^oC)	3.27	3 − 15	-4
ρ (g/cm^3)	0.83	NS	0.86− 0.9
υ (mm^2/S)	3.66	1.6 − 9.0	3.5− 5.0
OS (h)	9.94	3 min	6 min
PP (^oC)	-3.28	-15 − 10	NS
CFPP (^oC)	-4.66	Summer max 0; winter max < -15	NS
LCSF	3.76	NS	NS
HHV(^oC)	37.63	NS	NS
SFA (%)	20.08	NS	NS
MUFA (%)	59.10	NS	NS
PUFA (%)	16.05	NS	NS
DU	91.20	NS	NS
APE	90.61	NS	NS
BAPE	18.81	NS	NS

SV - saponification value, IV- iodine value, CN- cetane number, LCSF- long chain saturated factor, CFPP- cold filter plugging point , CP- cloud point , PP - pour point, OS- oxidation stability, HHV- higher heating value, υ - kinematic viscosity, ρ – density, SFA - saturated fatty acid, MUFA - monounsaturated fatty acid, PUFA - polyunsaturated fatty acid , DU - degree of unsaturation, APE - allylic position equivalent and BAPE- bis-allylic position equivalent

NS- Not specified, min-minimum.

5.4 Discussion

SCOs, also known as microbial oils have long been considered as alternative sources to traditional oils. Oleaginous yeasts have a high specific growth rate, can utilize a variety of cheap agro-industrial wastes as carbon sources and can accumulate lipid from 20 to 70% (on a dry cell

biomass) when grown in a bioreactor under conditions of controlled carbon excess and nitrogen limitation (Hassan *et al.*, 1993; Meesters *et al.*, 1996). These agro-industrial residues are not only low in cost but also abundant to use them as a substrate for microbial lipid production (Khot *et al*., 2012; Zheng *et al*., 2012). Among these wastes, molasses is widely used for various biotechnological purposes (Karatay and Donmez, 2010; Coelho *et al.*, 2011; Arshad *et al*., 2014; He *et al*., 2014).

In the present study, cultivation of a yeast, *Rhodosporidium kratochvilovae* SY89 in a bioreactor was undertaken in a nitrogen-limited medium containing glucose for the production of biomass, lipid concentration and lipid content. As shown in Figure 5.2, when grown in a medium containing glucose as a carbon source in a bioreactor, *Rhodosporidium kratochvilovae* SY89 exhibited a maximum biomass of 15.34±1.47 g/L, lipid concentration of 8.60±0.81 g/L and lipid content of 56.06 ±1.70%. The lipid content obtained in a bioreactor (56.06 ±1.70%) was higher than that of the traditional shake flask culture (51.17±1.72%), which is approximately 5% increment. This could be due to better aeration and mixing in a bioreactor; all parameters were controlled (temperature, pH, foam, oxygen delivery system etc.) in a bioreactor. Such value suggest that *Rhodosporidium kratochvilovae* SY89 could be considered as an attractive yeast for the production of SCO if cultivated under fed-batch or continuous bioreactor. According to a report by Amaretti *et al*. (2010b), *Rhodotorula glacialis* AS 4.7 was cultivated in a bioreactor with 2 L working volume with glucose as carbon source. Based on the report a maximum biomass of 28 g/L, lipid concentration of 19 g/L as well as lipid content of 68% were obtained.

This oleaginous yeast was then grown in a medium containing cane molasses in a bioreactor. The results obtained in this study were considerable and showed high potential of biomass and lipid production by *Rhodosporidium kratochvilovae* SY89. However, maxium results of biomass, lipid

concentration and lipid content were lower than when this yeast strained was cultivated in the same bioreactor using glucose as a carbon source (Figure 5.4). This could be due to the difficulty of metabolizing sucrose by this strain. Other researchers also reported the potential of cane molasses as a substrate for oleaginous yeasts including *Rhodotorula glutinis* (Almazan *et al*., 1981), *Trichosporon fermentans* (Zhu *et al*., 2008), *Candida lipolytica*, *Candida tropicalis* and *Rhodotorula mucilaginosa* (Karatay and Dönmez, 2010), *Cryptococcus laurentii* 11 (Castanha *et al*., 2014) and *Yarrowia lipolytica* (Gajdoš *et al*., 2015) for biomass and lipid production.

Biodiesel is a clean burning attractive alternative second generation biofuel derived from lignocelluosic (renewable) biomass by transesterification of TAGs. It has received much attention because of its technical, environmental and strategic advantages. Biodiesel has less CO_2 and sulphur emissions to the atmosphere and hence contributes to the reduction in emissions to greenhouse gases (Ahmad *et al*., 2013). The quality of biodiesel depends upon the fatty acid composition of the oil feedstock. In this study, the fatty acid profiles (compositions) of lipid obtained from bioreactor cultivation of *Rhodosporidium kratochvilovae* SY89 using glucose as a substrate were analyzed using GC and was found that there were remarkable differences in terms of percentage composition of the fatty acid profiles. Data revealed the presence of high content of oleic acid (42.89±1.90%). Similarly, fatty acid composition of the lipid fraction of this yeast when grown in a medium containing cane molasses in a stirred tank fermenter, consisted of oleic acid as the largest lipid component (58.51±0.76%) and low amount of other fatty acids in the extracted yeast oils. The concentration of saturated and monounsaturated fatty acids of *Rhodosporidium kratochvilovae* SY89 adds up to 79.18±2.56% which makes the lipids from this strain as good oil feedstock for biodiesel production (Byreddy *et al*., 2015).

A high quality biodiesel is made mainly from methyl esters with C16 and C18 fatty acids in saturated or monounsaturated form (Ramos *et al.*, 2009; Christophe *et al*., 2012).On the other hand, highly unsaturated fatty acid methyl esters are oxidized easily during long term storage and could have negative influence on the engine motor. They are not recommended for biodiesel production (Metzger and Bornscheuer, 2006).

The relative percentages of fatty acid profiles exhibited by *Rhodosporidium kratochvilovae* SY89 are in concurrent with the fatty acid profiles reported by Johnson *et al*. (1995), Li *et al*. (2010), Vieira *et al*. (2014) and Tchakouteu *et al*. (2015) for other oleaginous yeasts. These researchers reported that lipids from these oleaginous yeasts contained mainly oleic acid, palmitic acid and to a lesser extent linoleic acid and linolenic acid. The fatty acid profiles that were exhibited by *Rhodosporidium kratochvilovae* SY89 were not only similar to fatty acid profiles of other oleaginous yeasts but also they fit well to the fatty acid profiles of different vegetable oils such as rapeseed, soybean, palm, and sunflower oils (Ma and Hanna, 1999; Christophe *et al*., 2012).

To produce microbial biodiesel, the extracted oil from *Rhodosporidium kratochvilovae* SY89 was transesterified using methanol and a yield of 85.30% was obtained. Dai *et al*. (2007) also obtained a biodiesel yield of 81.7% from *Rhodotorula glutinis* by growing the yeast on lignocellulosic materials. Miao and Wu (2006) obtained high yields of biodiesel from heterotrophic growth of *Chlorella protothecoides* at molar ratio levels of 45:1 and 56:1 which accounted for 68% and 63%, respectively. From this one can see that the biodiesel yield obtained in this study is better than previous works in terms of biodiesel yield.

To evaluate the potential of the produced biodiesel from *Rhodosporidium kratochvilovae* SY89 as a substitute of diesel fuel, the different physico-chemical properties of biodiesel such as

saponification value (SV), iodine value (IV), cetane number (CN), long chain saturated factor (LCSF), cold filter plugging point (CFPP), cloud point (CP), pour point (PP), oxidation stability (OS), higher heating value (HHV), kinematic viscosity (υ), density (ρ), saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), degree of unsaturation (DU), allylic position equivalent (APE) and bis-allylic position equivalent(BAPE) were determined.

As shown in Table 5.2, the results were compared with US biodiesel standard, ASTM D6751 (ASTM, 2002) and EU biodiesel standard, EN14214 (ACEA, 2009). The recorded iodine value for the produced yeast biodiesel is 84.83 mg I/100 g oil, which is below the maximum value of 120 mgI/100 g oil standard of EN 14214. Iodine value is a measure of degree of unsaturation of a lipidic material.The higher the IV, more double bonds present in the lipid. The degree of unsaturation greatly influences fuel oxidation tendency. This represents the highest quality of biodiesel.

Cetane number which is dimensionless descriptor and indicator of the combustion speed of diesel fuel and is required for good engine performance (Knothe, 2006). It determines the combustion behavior of the biodiesel, i.e., ignition delay time, which is the time between the injection and ignition (Islam *et al*., 2013). The CN recorded in this study was 55.60. This value is in agreement with the standards for biodiesel (ASTM D6751), which recommend a minimum CN of 47 and the CN set by EU biodiesel standard EN14214 is above 51(Minnesota Statutes, 2008). Higher cetane number helps to ensure good cold start properties and minimize the formation of white smoke.

The oxidation stability value of FAME of *Rhodosporidium kratochvilovae* SY89 was 9.94, which is an important feature related to the stability and performance of biodiesel. This shows that the biodiesel produced from *Rhodosporidium kratochvilovae* SY89 oil is stable.

The kinematic viscosity value recorded for the biodiesel produced in this study was 3.66 mm^2/S. Such result falls in the ranges set by both US biodiesel standard ASTM D6751 (1.6−9.0 mm^2/S) and EU biodiesel standard EN14214 (3.5−5.0 mm^2/S).

A value of -3-28^oC of pour point was obtained in this research work. This value also falls in the range set by US biodiesel standard ASTM D6751 (-15−10^oC). Cloud point of 3.27^oC was exhibited in this study. The value 3−15^oC is set by US biodiesel standard, ASTM D6751.

The density recorded by the biodiesel produced from the yeast *Rhodosporidium kratochvilovae* SY89 was 0.83 g/cm^3 which is approximated to the biodiesel standard of EN14214 (0.86−0.9 g/cm^3).

Saponification value is used in checking adulteration. A high saponification value of fats and oils are due to high proportion of shorter carbon chain lengths of the fatty acids (Barku *et al*., 2012). The high saponification value in a given biodiesel suggests it has low level of impurities (Barku *et al*., 2012).The saponification value recorded was 192.29 mg/g which comply with the microalgal oil with the value of 189.30 mg/g (Miao and Wu, 2006).

The value recorded for long chain saturated factor is used to calculate the cold filter plugging point (CFPP), which is based on the amount of long chain saturated fatty acids (from C16:0) in the oil was -4.66^oC. The CFPP value is related to the minimum temperature at which the biodiesel can generate clogging and problems in the motor (Souza *et al*.,2016).The heating value of fatty acid

esters increases with molecular chain length (with the number of carbon atoms) and decreases with their degree of unsaturation (the number of double bonds). The heating value recorded for the biodiesel from SY89 was 37.63ºC.The biodiesel from *Rhodosporidium kratochvilovae* SY89 oil could therefore be a competitive alternative to conventional diesel fuel.

Other chemical and physical values were analyzed, including SFA (saturated fatty acid), MUFA (monounsaturated fatty acid), degree of unsaturation (DU), long chain saturated factor (LCSF), allylic position equivalent (APE) and bis-allylic position equivalent (BAPE (summarized in f 5.2). These characteristics are also important in determining the quality of a given biodiesel. Most of these properties are in agreement with the specifications established by ASTM D6751 and EN14214 related to biodiesel quality.

5.5Conclusion and recommendations

This study demonstrated that *Rhodosporidium kratochvilovae* SY89 is able to utilize molasses as a carbon source for the production of biomass and lipid because it has high contents of sugar. The biomass and lipid exhibited by this strain when it was grown on molasses as a carbon source was lower than the biomass and lipid production attained when it was grown on molasses, probably due to the difficulty of metabolizing sucrose.

Production of microbial oil using cheap substrates such as molasses is good for countries like Ethiopia because cost for purchasing and transportation of petroleum oil can be reduced at least partially.

Oil from yeast including *Rhodosporidium kratochvilovae* SY89 (if genetically modified) could replace biodiesel from fossil fuel in the future.

Chapter 6: General Conclusion and Recommendations

The results in this research work suggest that many more oleaginous yeasts can be isolated from Ethiopian environment. The 18 yeast strains that we isolated have the potential to be used for the production of single cell oil and hence biodiesel at the industrial level.

Cultivation parameters like inoculum size, availability of carbon and nitrogen sources, C/N ratio, incubation temperature, culture time, agitation and aeration rates, and pH had an influence on the biomass production, lipid accumulation and lipid profile of *Cryptococcus curvatus* PY39, *Rhodotorula mucilaginosa* SY09, *Rhodotorula mucilaginosa* SY18 and *Rhodosporidium kratochvilovae* SY89. Comparing it to previous data it is clear that there are some differences across species and strains and it is, therefore, important to investigate the optimal parameters for each strain tested. Such cultivation parameters could influence efficiency of biodiesel production from the yeast oil.

SCO from the four oleaginous yeasts is high in oleic acid; an important feature for biodiesel production.

To improve the production of lipid in oleaginous yeasts, cultivation conditions should be optimized, fermentation conditions should be improved (i.e., either batch, fed-batch or continuous fermenters or bioreactors should be used).

Cheap substrates like lignocellulosic wastes and sugar factory by-products (e.g., peel mixtures of papaya and mango and molasses) should be used for cultivation purpose to minimize cost of production.

Key areas of future research also include enhanced fermentation systems, improved downstream extraction and separation, searching for promising, robust and efficient oleaginous yeast strains and improved optimization of substrates.

Strain improvement (mutagenesis, protoplast fusion, genetic engineering etc.) could give yeasts for commercial production of biodiesel and gradual replacement of fossil fuels in the future.

7. References

Abreham Berta and Belay Zerga (2015). Biofuel energy for mitigation of climate change in Ethiopia. *J.Energ. Natural Resour*.**4**: 62–72.

ACEA (European Automobile manufacturers′ Association). (2009). Biodiesel Guidelines, European Automobile Manufacturers Association, Brussels, Belgium. http://www. acea.be/ uploads/publications/20090423_B100_Guideline.pdf.

Adachi, D., Hama, S., Numara, T., Nakashima, K., Ogino, C., Fukuda, H. and Kondo, A. (2011). Development of an *Aspergillus oryzae* whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production. *Bioresour*.*Technol*. **102**: 6723–6729.

Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P. and Villa, T.G. (2011). Oily yeasts as oleaginous cell factories. *Appl. Microbiol. Biotechnol*. **90**:1219–1227.

Ahmad, M., Khan, M.A., Zafar, M. and Sultana, S. (2013). *Practical Handbook on Biodiesel Production and Properties*. CRC Press, Florida.

Ahmad, F.B., Zhang, Z., Doherty, W.O.S. and O'Hara, I.M. (2015). A multi-criteria analysis approach for ranking and selection of microorganisms for the production of oils for biodiesel production. *Bioresour.Technol.* **190**: 264–273.

Ainscough, S. and Kibbler, C.C. (1998). An evaluation of the cost-effectiveness of using CHROM agar for yeast identification in a routine microbiology laboratory. *J.Med. Microbiol*. **47**: 623 −628.

Akhtar, P., Gray, I. and Asghar, A. (1998). Synthesis of lipids by certain yeast strains grown on whey permeate. *J.Food Lipids*. **5**:283–297.

Almazan, O., Klibansky, M. and Otero, M.A. (1981). Microbial fat synthesis by *Rhodotorula glutinis* from blackstrap molasses in continuous culture. *Biotechnol. Lett*. **3**:663−666.

Allen, L. A., Barnard, N.H., Fleming, M. and Hollis, B. (1964). Factors influencing the growth and fat formation of *Rhodotorula gracilis*. *J. Appl.Bacteriol*. **27**:27−40.

Alper, H. and Stephanopoulos, G. (2009). Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? *Nature Rev. Microbiol*. **7**:715−723.

Alvarez, H.M, Kalscheuer, R. and Steinbuchel, A. (1997). Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. *Fett/Lipid.* **99**:239–246.

Alvarez, H.M. and Steinbuchel, A. (2002). Triacylglycerols in prokaryotic microorganisms. *Appl. Microbiol. Biotechnol.* **60**:367–376.

Amaretti, A., Raimondi, S., Sala, M., Roncaglia, L., Lucia, M.D., Leonardi, A. and Rossi, M. (2010a). Single cell oils of the cold adapted oleaginous yeast *Rhodotorula glacialis* DBVPG4875. *Microb.Cell Fact*. **23**: 59−73.

Amaretti, A., Raimondi, S., Sala, M., Roncaglia, L., Lucia, M.D., Leonardy, A. and Rossi, M. (2010b). Production of single cell oil by the cold adapted oleaginous yeast *Rhodotorula glacialis* AS 4.7: effects of the growth temperature and the C: N ratio. *Chem.Eng.Trans.* **20**: 109–114.

Amaretti, A., Raimondi, S., Leonardi, A. and Rossi, M. (2011). Lipid production from glycerol by *Candida Freyschusii*. Proceedings of FEMS of 14th Congress of European Microbiologists, Geneva, Switzerland, Geneva, Switzerland, June 26-30, 2011.

Anderson, A.J. and Wynn, J.P. (2001). Microbial poly-hydroxyalkanoates, polysaccharides and lipids. **In**: *Basic Biotechnology*, (Ratledge, C. and Kristiansen, B., eds), 2nd ed., Cambridge University Press, Cambridge.

Angerbauer, C., Siebenhofer, M., Mittelbach, M. and Guebitz, G.M. (2008). Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. *Bioresour.Technol.* **99**: 3051−3056.

AOAC (Association of Official Analytical Chemists). (1995). Official Methods of Analysis, 16thed, AOAC International, Gaithersburg, MD.

Arshad, M., Ahmed, S., Zia, M.A. and Rajoka, M.I. (2014). Kinetics and thermodynamics of ethanol production by *Saccharomyces cerevisiae* MLD10 using molasses. *Appl. Biochem. Biotechnol.* **172**:2455–2464.

Arroyo-López, F.N., Durán-Quintana, M.C., Ruiz-Barba, J.L.Querol, A. and Garrido-Fernández, A. (2006). Use of molecular methods for the identification of yeast associated with table olives. *Food Microbiol*. **23**:791–796.

ASBC (American Society of Brewing Chemists). (1988). Report of Subcommittee on Improved Microscopic Yeast Cell Counting Journal. **46**:123.

ASTM. (2002). Standard Specification for Biodiesel Fuel (B100) Blend Stock for Distillate Fuels, American Society for Testing and Materials, D6751–02.

Bandaiphet, C. and Prasertsan, P. (2006). Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, kLa in exopolysaccharide production from *Enterobacter cloacae* WD7. *Carbohyd. Polym*. **66**:216–228.

Bankar, A.V., Kumar, A.R. and Zinjarde, S.S. (2009). Environmental and industrial applications of *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol*. **84**:847−865.

Barnett, J.A., Payne, R.W. and Yarrow, D. (2000). *Yeast-Characteristics and Identification*, 3rded, Cambridge University Press, Cambridge.

Barku, V.Y.A, Nyarko, H.D. and Dordunu, P. (2012). Studies on the physicochemical characteristics, microbial load and storage stability of oil from Indian almond nut (*Terminalia catappa l*.). *Food Sci. Quality Manag.* **8**:9− 17.

Bartlett, J. M. S. and Stirling, D. (2003). A short history of the polymerase chain reaction. *PCR Protocols*. **226**: 3−6.

Bekatorou, A., Psarianos,C. and Koutinas, A.A. (2006). Production of food grade yeasts. *Food Technol.Biotechnol.* **44:**407−415.

Beopoulos, A., Mrozova, Z., Thevenieau, F., Le-Dall, M.T., Hapala, I., Papanikolaou, S., Chardot, T. and Nicaud, J.M. (2008). Control of lipid accumulation in the yeast *Yarrowia lipolytica. Appl.Environ.Microbiol.* **74**:7779−7789.

Beopoulos, A., Chardot, T. and Nicaud, J.M. (2009). *Yarrowia lipolytica*: a model and a tool to understand the mechanisms implicated in lipid accumulation. *Biochimie*. **91**:692−696.

Beopoulos, A., Verbeke, J., Bordes, F., Guicherd, M., Bressy, M., Marty, A. and Nicaud, J.M. (2014). Metabolic engineering for ricinoleic acid production in the oleaginous yeast *Yarrowia lipolytica*. *Appl .Microbiol. Biotechnol*. **98**:251–262.

Berwanger, A.L.D., Scamparini, A.R.P., Domingues, N.M., Vanzo, L.T., Treichel, H. and Padilha, F.F. (2007). Biopolymer productionsynthetized by *Sphingomonas capsulata*, using industrial media. *Cienc. Agrotec*. **31**:177–183.

Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J.Biochem.Physiol.* **37**: 911–917.

Breuer, U. and Harms, H. (2006). *Debaryomyces hansenii:* an extremophilic yeast with biotechnological potential. *Yeast*. **23**: 415−437.

Butinar, L., Spencer-Martins, I. and Gunde-Cimerman, N. (2007). Yeasts in high Arctic glaciers: the discovery of a new habitat for eukaryotic microorganisms. *Antonie Van Leeuwenhoek*. **91**:277−289.

Butte, W. (1983). Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. *J.Chromatog. A*. **261**: 142–145.

Caggia, C., Restuccia, C., Pulvirenti, A. and Giudici, P. (2001). Identification of *Pichia anomala* isolated from yoghurt by RFLP of the ITS region. *Int. J. Food Microbiol.* **1**:71−73.

Calderone, R. A. (2002). *Candida and Candidiasis*. *American Society of Microbiology Press.* ISSN 1058-4838, Washington, DC.

Castanha, R.F., Mariano, A.P., Morais, L.A.S., Scramin, S. and Monteiro, R.T.R. (2014).Optimization of lipids production by *Cryptococcus laurentii* 11 using cheese whey with molasses. *Braz. J. Microbiol.* **45**:379-387.

Certik, M. and Shimizu, S. (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *J.Biosci. Bioeng*. **87**:1−14.

Chen, Y.C., Eisner, J.D., Kattar, M.M., Rassoulian-Barrett, S.L., Lafe, K., Limaye, A. P. and Cookson, B.T. (2001). Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. *J.Clin.Microbiol.* **39**: 4042–4051.

Chen, X.F., Huang, C., Yang, X.Y., Xiong, L., Chen, X.D. and Ma, L.L. (2013). Evaluating the effect of medium composition and fermentation condition on the microbial oil production by *Trichosporon cutaneum* on corncob acid hydrolysate. *Bioresour. Technol*. **143**:18–24.

Cheisilp, B., Suwannarat, W. and Niyomdecha, R. (2011). Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production. *New Biotechnol.* **28**:362–368.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnol.Adv.* **25**:294−306.

Choi, S.Y., Ryu, D.D.Y. and Rhee, J.S. (1982). Production of microbial lipid: Effects of growth rate and oxygen on lipid synthesis and fatty acid composition of *Rhodotorula gracilis*. *Biotechnol. Bioeng*. **24**: 1165−1172.

Choi, I.S., Lee, Y.G., Khanal, S.K., Park, B.J. and Bae, H.J. (2015). A low-energy, cost-effective approach to fruit and citrus peel waste processing for bioethanol production. *Appl.Energ*. **140**: 65–74.

Christophe, G., Kumar, V., Nouaille, R., Gaudet, G., Fontanille, P., Pandey, A. and Larroche, C. (2012). Recent developments in microbial oils production: a possible alternative to vegetable oils for biodiesel without competition with human food? *Braz.Arch.Biol. Technol*. **55**:29−46.

Cleasby, T.G. (1963). The feeding value of molasses. *Proceedings of the South African Sugar Technologists' Association.*113−117.

Coelho, L.F., De Lima, C.J.B., Rodovalho, C.M., Bernardo, M.P. and Contiero, J. (2011). Lactic acid production by new *Lactobacillus plantarum* LMISM6 grown in molasses: Optimization of medium composition. *Braz.J.Chem.Eng*. **28**:27–36.

Cook, A.H. (1958). *The Chemistry and Biology of Yeasts*. Academic press, New York.

Crueger, W. and Crueger, A. (1990). *Biotechnology: A Textbook of Industrial Microbiology*. Sinauer associates, Sunderland.

Curtin, D. (1983). Molasses general considerations. National Feed Ingredient Association West Desmoines, 3−10.

Cutler, J.E., Glee, P.M. and Horn, H.L. (1988). *Candida albicans* and *Candida stellatoidea*-specific DNA fragment. *J. Clin. Microbiol*. **26**: 1720−1724.

Dai, C., Tao, J., Xie, F., Dai, Y. and Zhao, M. (2007). Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity. *Afri.J.Biotechnol*. **6**:2130−2134.

de Hoog,G.S. and Gerrits van den Ende,A. H. G. (1998). Molecular diagnostics of clinical strains of filamentous basidiomycetes. *Mycoses*. **41**: 183–189.

Deak, T. and Beuchat, L.R. (1996). *Handbook of Food Spoilage Yeasts*. CRC Press, Florida.

Deak, T. (2008). *Handbook of Food Spoilage Yeasts*. 2nd ed., CRC press, Florida.

Dlauchy, D., Tornai−Lehoczki, J. and Peter, G. (1999). Restriction enzyme analysis of PCR amplified rDNA as taxonomic tool in yeast identification. *Syst. Appl.Microbiol.* **22**: 445−453.

Draaisma, R.B., Wijffels, R.H., Slegers, P.M., Brentner, L.B., Roy, A. and Barbosa, M.J. (2013). Food commodities from microalgae. *Curr .Opin. Biotechnol*. **24**:167–177.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal.Chem.* **28**: 350 –356.

Dunn, R.O. (2011). Improving the cold flow properties of biodiesel by fractionation. **In**: *Soybean Applications and Technology* (Ng, T.B., ed), InTech, Europe.

Drucken, Z. (2008). Triacylglycerol synthesis in the oleaginous yeast *Yarrowia lipolytica*. *Bioresour.Technol*. **90**: 309−319.

Economou, Ch.N. Aggelis, G., Pavlou, S. and Vayenas, D.V. (2011). Single cell oil production from rice hulls hydrolysate. *Bioresour. Technol*. **102**: 9737–9742.

El−Fadaly, H.A., El−Naggar, N.A. and Marwan, S.M. (2009). Single cell oil production by an oleaginous yeast strain in a low cultivation medium. *Resear.J.Microbiol*. **4**:301−313.

Enshaeieh, M., Abdoli, A., Nahvi, I. and Madani, M. (2013a). Selection and optimization of single cell oil production from *Rodotorula* 110 using environmental waste as substrate. *J.Cell Mol. Resear.* **4**: 68−75.

Enshaeieh, M., Abdoli, A. and Nahvi, I. (2013b). Medium optimization for biotechnological production of single cell oil using *Yarrowia lipolytica* M7 and *Candida* sp. *J.Cell Molec. Resear*. **5**: 17−23.

Enshaeieh, M., Abdoli, A., Madani, M. and Bayat, M. (2015a). Recycling of lignocellulosic waste materials to produce high-value products: single cell oil and xylitol. *Int.J.Environ.Sci. Technol*. **12**:837–846.

Enshaeieh, M., Abdoli, A. and Madani, M. (2015b). Single cell oil (SCO) production by *Rhodotorula mucilaginosa* and its environmental benefits. *J.Agr.Sci.Tech.* **17**: 387−400.

EPSE (Ethiopian Petroleum Supply Enterprise). (2016). A report. Addis Ababa, Ethiopia.

Esteve−Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. (1999). Identification of yeast by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int.J.Syst. Bacteriol.* **49**: 329–337.

Evans, C.T. and Ratledge, C. (1984). Effect of nitrogen source on lipid accumulation in oleaginous yeasts. *J Gen. Microbiol.* **130**: 1693-1704.

Fadel, M., Keera, A.A., Mouafi, F.E. and Kahil, T. (2013). High level ethanol from sugar cane molasses by a new thermotolerant *saccharomyces cerevisiae* strain in industrial scale. *Biotechnol. Resear. Int.* Hindawi Publishing Corporation. http:// dx.doi.org/ 10.1155/ 2013/ 253286.

Fahy, E., Subramaniam, S., Murphy, R., Nishijima, M., Raetz, C., Shimizu, T., Spener, F., Van-Meer, G., Wakelam, M. and Dennis, E.A. (2009). Update of the lipid maps comprehensive classification system for lipids. *J.Lipid Resear.* **50**: S9–S14.

Fakas, S., Papanikolaou, S., Galiotou-Panayotou, M., Komaitis, M. and Aggelis, G. (2006). Lipids of *Cunninghamella echinulata* with emphasis to γ-linolenic acid distribution among lipid classes. *Appl. Microbiol. Biotechnol*. **73**: 676−683.

Fakas, S., Papanikolaou, S., Galiotou-Panayotou, M., Komaitis, M. and Aggelis, G. (2008). Organic nitrogen of tomato waste hydrolysate enhances glucose uptake and lipid accumulation in *Cunninghamella echinulata*. *J.Appl.Microbiol*. **105**: 1062−1070.

Faturoti, B. O., Emah, G. N., Isife, B. I., Tenkouano, A. and Lemchi, J. (2006). Prospects and determinants of adoption of IITA plantain and banana based technologies in three Niger Delta States of Nigeria. *Afric.J.Biotechnol*. **5**:1319–1323.

Fell, J.W. and Statzell-Tallman, A. (1998). *Rhodotorula* F.C. Harrison. *The Yeasts: A Taxonomic Study* (Kurtzman, C.P. and Fell, J.W., eds), Elsevier, Science, Amsterdam.

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G. and Statzell−Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large subunit rD1/D2 domain sequence analysis. *Int.J.Syst. Evol. Microbiol*. **50**:1351−1371.

Fernandez−Espinar, M. T., Esteve-Zarzoso, B., Querol, A. and Barrio, E. (2000). RFLP analysis of the ribosomal internal transcribed spacers and the5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and differentiation of flour yeasts. *Anton Van Leeuwenhoek.* **78**:87−97.

Findley, K., Rodriguez-Carres, M., Metin, B., Kroiss, J., Fonseca, A., Vilgalys, R., Heitman, J. (2009). Phylogeny and phenotypic characterization of pathogenic *Cryptococcus* species and closely related saprobic taxa in the Tremellales. *Eukary. Cell*. **8**:353−361.

Flórez, A.J., Morales, M.P., Roth, E.P., Laich, F., Pérez, B.R. and Álvarez, S.M. (2007).Yeast molecular identification and typing. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, pp535−546.

Folch, J., Lees, M. and Sloane−Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J.Biol. Chem.* **226**:497−509.

Fukuda, H., Konodo, A. and Noda, H. (2001). Biodiesel production by tranesterifcation of oils. *J.Biosci. Bioeng.* **92**:405−416.

Gajdoš, P., Nicaud, J-M., Rossigno, T. and Čertík, M. (2015). Single cell oil production on molasses by *Yarrowia lipolytica* strains overexpressing *DGA2* in multicopy. *Appl.Microbiol. Biotechnol.* **99**:8065–8074.

Garner, C.D., Starr, J.K., Patrick, L., McDonough, P.L. and Altier, A.C. (2010). Molecular identification of veterinary yeast isolates sequence−based analysis of the D1/D2 region of the large ribosomal subunit. *J.Clin.Microbiol.* **48**: 2140–2146.

Gill, C.O., Hall, M.J. and Ratledge, C. (1977). Lipid accumulation in oleaginous yeast (*Candida*107) growing on glucose in single-stage continuous culture. *Appl.Environ.Microbiol.* **33**:231−239.

Glick, B.R., Pasternak, J.J. and Patten, C.L. (2010). *Molecular Biotechnology: Principles and Application of Recombinant DNA Technology*, 4th ed., ASM Press, Washington DC.

Goksungur, Y. and Zorlu, N. (2001). Production of ethanol from beet molasses by calcium alginate immobilized yeast cells in a packed bioreactor. *Turk J.Biol.* **25**:265−275.

Groenewald, M., Robert, V. and Smith, M.T. (2011). The value of the D1/D2 and internal transcribed spacers (ITS) domains for the identification of yeast species belonging to the genus *Yamadazyma. Persoonia.* **26**: 40–46.

Guffogg, S.P., Thomas−Hall, S., Holloway, P. and Watson, K. (2004). A novel psychrotolerant member of the hymenomycetous yeasts from Antarctica: *Cryptococcus watticus* sp. nov. *Int. J.Syst.Evo.Microbiol*. **54**: 275−277.

Guillamo´n, J.M., Sa´nchez, I. and Huerta, T. (1997). Rapid characterization of wild and collection strains of the genus *Zygosaccharomyces* according to mitochondrial DNA patterns. *FEMS Microbiol. Lett*. **147**:267−272.

Guillamòn, J.M., Sabaté, J., Barrio, E., Cano, J. and Querol, A. (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal ITS region. *Arch. Microbiol*. **169**: 387–392.

Gujjari, P., Suh, S.O., Coumes, K. and Zhou, J.J. (2011). Characterization of oleaginous yeast revealed two novel species: *Trichosporon cacaoliposimilis* sp. nov. and *Trichosporon oleaginosus* sp. nov. *Mycologia.* **103**:1110−1118.

Hagler, A. N. and Ahearn, D. G. (1981). A rapid DBB test to detect basidiomycetous affinity of yeasts. *Int.J.Syst.Bacteriol*. **31**:204–208.

Hall, M.J. and Ratledge, C. (1977). Lipid accumulation in an oleaginous yeast (*Candida*107) growing on glucose, under various conditions in a one- and two-Stage continuous culture. *Appl.Environ.Microbiol*. **33**:577−584.

Hassan, M., Blanc, P.J., Granger, L.M., Pareilleux, A. and Goma, G. (1993). Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast *Apiotrichum Curvatum* grown in single stage continuous culture. *Appl. Microbiol. Biotechnol*. **40**:483–488.

Hassan, M., Blanc, PJ., Pareilleux, A. and Goma, G.(1994). Production of single cell oil from prickly-pear juice fermentation by *Cryptococcus curvatus* grown in batch culture. *World J.Microbiol. Biotechnol.***10**:534–537.

He, J., Wu, A.M., Chen, D., Yu, B., Mao, X., Zheng, P., Yu, J. and Tian, G. (2014). Cost effective lignocellulolytic enzyme production by *Trichoderma reesei* on a cane molasses medium. *Biotechnol. Biofuels*.**7**:43.

Hierro, N., Gonza´lez, A., Mas, A. and Guillamon, J.M. (2004). New PCR-based methods for yeast identification. *J.Appl.Microbiol*. **97**:792–801.

Higashiyama, K., Fujikawa, S., Park, E.Y. and Shimizu, S. (2002). Production of arachidonic acid by *Mortierella* Fungi. *Biotechnol. Bioprocess Eng*.**7**: 252−262.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006). Environmental, economic and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of National Academy of Sciences of the United States of America.* **103**:11206−11210.

Holdsworth, J.E. and Ratledge, C. (1988). Lipid turnover in oleaginous yeasts. *J.Gen. Microbiol.* **134**: 339−346.

Holdsworth, J. E. and Ratledge, C. (1991). Triacylglycerol synthesis in the oleaginous yeast *Candida curvata* D. *Lipids*. **26**: 111–118.

Hong, S. G., Chun, J., Oh, H.W. and Bae, K. S. (2001). *Metschnikowia koreensis* sp. nov. a novel yeast species isolated from flowers in Korea. *Int.J.Syst. Evolu.Microbiol.* **51**: 1927−1931.

Hong, L.Y., Bo, L., Bao, Z.Z. and Wu, B.F. (2006). Optimization of culture conditions for lipid production by *Rhodosporidium toruloides. Chinese J.Biotechnol.* **4**: 650−656.

Hu, C., Zhao, X., Zhao, J., Wu, S. and Zhao, Z.K. (2009). Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. *Bioresour.Technol.* **100**:4843−4847.

Hu,C.,Wu,S.,Wang,Q.,Jin,G.,Shen,H. and Zhao,Z.K. (2011). Simultaneous utilization of glucose and xylose for lipid production by *Trichosporon cutaneum*. *Biotechnol. Biofuels*. **4**:1−8.

Huang, C., Zong, M., Wu, H. and Liu, Q. (2009). Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. *Bioresour. Technol*. **100**: 4535–4538.

Islam, M., Magnusson, M., Brown, R., Ayoko, G., Nabi, M., and Heimann, K. (2013). Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles. *Energ.* **6**:5676–5702.

Jacob, Z. (1993). Yeast lipid biotechnology. *Adv. Appl. Microbiol*. **39**: 185–212.

Johnson, V., Singh, M., Saini, V.S., Sista, V.R. and Yadove, N.K. (1992). Effect of pH on lipid accumulation by oleaginous yeast: *Rhodotorula glutinis* IIP−30. *World J.Microbiol. Biotechnol.* **8**:382−384.

Johnson, V.W., Singh, M., Saini, V.S., Adhikari, D.K., Sista, V. and Yadav, N.K. (1995). Utilization of molasses for the production of fat by an oleaginous yeast, *Rhodotorula glutinis* IIP−30. *J.Indust. Microbiol*. **14**: 1−4.

Johnson, R.B. and Barnett, H.J. (2003). Determination of fat content in fish feed by supercritical fluid extraction and subsequent lipid classification of extract by thin layer chromatography - flame ionization detection. *Aquaculture*. **216**: 263−282.

Jyothi, D., Khanam, S. and Sultana, R. (2010). Optimization of microwave assisted solvent extraction of withanolides from leave of ashwagandha. *Inter.J.Compressive Pharma.* **4**:1−5.

Kalscheuer, R., Stölting, T and Steinbüchel, A. (2006). Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiol*. **152**:2529–2536.

Karatay, S.E. and Donmez, G. (2010). Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. *Bioresour.Technol*. **101**:7988−7990.

Kakita, H., Kamishima, H., Komiya, K. and Kato, Y. (2002). Simultaneous analysis of monosaccharides and oligosaccharides by high-performance liquid chromatography with post column fluorescence derivatization. *J.Chromatog. A*. **961**:77–82.

Khot, M., Kamat, S., Zinjarde, S., Pant, A., Chopade, B. and Ravikumar, A. (2012). Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. *Microb. Cell Fact.* **11**: 1−13.

Kimura, K., Yamaoka, M. and Kamisaka, Y. (2004). Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *J.Microbiol. Methods*. **56**:331–338.

King, J.W. (2000). Advances in critical fluid technology for food processing. *Food Sci. Technol.Today*. **14**:186−191.

Knothe, G. (2006). Analyzing biodiesel: Standards and other methods. *JAOCS*. **83**:823 – 833.

Knutsen, A.K., Robert, V., Poot, G. A., Epping, W., Figge, M., Holst-Jensen, A., Skaar, I. and Smith, M.T. (2007). Polyphasic re-examination of *Yarrowia lipolytica* strains and the description of three novel *Candida* species: *Candida osloensis* sp.nov, *Candida alimentaria* sp.nov. and *Candida hollandica* sp.nov. *Int.J.Syst. Evol. Microbiol*. **57**:2426–2435.

Kosa, M. and Ragauskas, A.J. (2010). Lipids from heterotrophic microbes: advances in metabolism research. *Trends Biotechnol*. **29**: 53−61.

Kraisintu, P., Yongmanitchai, W. and Limtong, S. (2010). Selection and optimization for lipid production of a newly isolated oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. *Kasetsart J.* (*Nat. Sci.*). **44**: 436 – 445.

Krishna, and Ranjhan, S.K. (1980). *Laboratory Manual for Nutrition Research*. Vicas Publishing House, New Delhi.

Kulkarni, A., Singh, A., Kumbhar, B.K. and Sahgal, M. (2013). Optimization of pomace and banana peel fermentation for production of single cell oil. *FMFI*. **2**:170–178.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol*. **33**:1870–1874.

Kurtzman, C.P. and Robnett C.J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek*. **73**: 331–371.

Kurtzman, C.P. (2000). Four new yeasts in the *Pichia anomala* clade. *Interna.J.System.Evolu. Microbiol*.**50**:395–404.

Kurtzman, C. P. (2001). Six new anamorphic ascomycetous yeasts near *Candida tanzawaensis*. *FEMS Yeast Res.* **1**:177−185.

Kurtzman, C.P., Boekhout, T., Robert, V., Fell, J.W. and Deak, T. (2003). Methods to identify yeasts. **In**: *Yeasts in food: Beneficial and detrimental aspects*, (Boekhout, T. and Robert, V., eds), CRC Press, England.

Kurtzman, C.P. and Fell, J.W. (2004). Yeasts. **In**: *Biodiversity of Fungi- Inventory and* Monitoring *Methods* (Mueller, G. M., Bills, G.F. and Foster, M.S., eds), Elsevier Academic Press, London.

Kurtzman, C.P. and Piškur, J.(2006).Taxonomy and phylogenetic diversity among the yeasts. **In**: *Comparative Genomics: Using Fungi as Models* (Sunnerhagen, P. and Piskur, J., eds), Vol. 15, Springer-Verlag, Berlin.

Kurtzman, C.P., Fell, J.W., Boekhout, T. and Robert, V. (2011). Methods for isolation, phenotypic characterization and maintenance of yeasts. **In**: *The Yeasts: A Taxonomic Study* (Kurtzman, C.P., Fell, J.W. and Boekhout, T., eds), 5th ed., Elsevier Science, Amsterdam.

Kutty, S.N. and Philip, R.(2008). Marine yeasts: A review. *Yeast.* **25**:465–483.

Lachance, M. A. and Starmer, W. T. (1998). *Ecology and Yeasts*. **In**: The Yeasts: A Taxonomy Study (Kurtzman, C. P. and Fell,J. W.,eds), 4thed., Elsevier Science, Amsterdam.

Lamers, D., Van Biezen, N., Martens, D., Peters, L., Van de Zilver, E., Dreumel1, N.J., René H., Wijffels, R.H. and Lokman, C. (2016). Selection of oleaginous yeasts for fatty acid production. *BMC Biotechnol*. **16**:45.

Leaw, S.N., Chang, H.C., Sun, H.F. Barton, R., Bouchara, J.P. and Chang, T.C. (2006). Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. *J.Clini. Microbiol*. **44**: 693–699.

Ledesma-Amaro, R., Santos, M.A., Jimenez, A. and Revuelta, J.L. (2014). Strain design of *Ashbya gossypii* for single-cell oil production. *Appl. Environ.Microbiol*. **80**:1237−1244.

Leesing, R., Kookkhunthod, S. and Nontaso, N. (2011). Microalgal lipid production by microalgae *Chlorella* sp. KKU-S2. *World Academy Sci.Eng.Technol.* **76**:499−502.

Leesing, R. and Baojungharn, R. (2011). Microbial Oil production by isolated oleaginous yeast *Torulaspora globosa* YU5/2. *Eng.Technol*.**76**: 799−803.

Leman, J. (1997). Oleaginous microorganisms: An assessment of the potential. *Adv.Appl. Microbiol.* **43**:195−243.

Li, M., Liu, G.L., Chi, Z. and Chi, Z.M. (2010). Single cell oil production from hydrolysate of cassava starch by marine-derived yeast *Rhodotorula mucilaginosa* TJY15a. *Biomass Bioenerg*. **34**:101−107.

Li, N., Deng, Z.N., Qin, Y.L., Chen, C.L. and Liang, Z.Q. (2008). Production of polyunsaturated fatty acids by *Mucor recurvus* sp. with sugarcane molasses as the carbon source. *Food Technol. Biotechnol.* **46**:73–79.

Li, Y., Zhao, Z. and Bai, F. (2007). High density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microb.Technol*. **41**:312−317.

Li, Q., Du, W. and Liu, D. (2008). Perspectives of microbial oils for biodiesel production. *Appl. Microbiol. Biotechnol*. **80**: 749−756.

Li, M., Liu, G.M., Chi, Z. and Chi, Z.M. (2010). Single cell oil production from hydrolysate of cassava starch by marine−derived yeast *Rhodotorula mucilaginosa* TJY15a. *Biomass Bioenerg*. **3**4:101−107.

Liang, X.A., Dong, Y.B., Miao, X.J. and Dai, C.J. (2006). Production technology and influencing factors of microorganisms grease. *Food Res.Dev*. **27**:46−47.

Liu, G.Q., Lin, Q.L., Jin, X.C., Wang, X.L. and Zhao, Y. (2010). Screening and fermentation optimization of microbial lipid-producing molds from forest soils. *Afri.J.Microbiol.Resear*. **4**:1462−1468.

Liu, X.Z., Wang, Q.M., Göker, M., Groenewald, M., Kachalkin, A.V., Lumbsch, H.T., Millanes, A.M., Wedin, M., Yurkov, A.M., Boekhout, T. and Bai, F.Y. (2015). Towards an integrated phylogenetic classification of the Tremellomycetes. *Stud. Mycol*. **81**: 85–147.

Lopes, M.B, Soden, A., Martens, A., Henschke, P.A. and Langridg, P. (1998). Differentiation and species identification of yeasts using PCR. *Inter. J.Syst. Bacteriol.* **48**: 279−286.

Luque, R., Lovett, J.C., Datta, B., Clancy, J., Campelo, J.M. and Romero, A.A. (2010). Biodiesel as feasible petrol fuel replacement: a multidisciplinary overview. *Energ. Enivron. Sci*. **3**:1706–1721.

Lutzu, G.A., Zhang, W. and Liu, T. (2016). Feasibility of using brewery wastewater for biodiesel production and nutrient removal by *Scenedesmus dimorphus. Environ.Technol*. **37**:1568–158.

Ma, F. and Hanna, M.A. (1999). Biodiesel production: a review. *Bioresour.Technol.* **70**:1−15.

Magee, P.T., Bowdin, L. and Staudinger, J. (1992). Comparison of molecular typing methods for *Candida albicans*. *J.Clin.Microbiol.* **30**: 2674−2679.

Malaiwong, N., Yongmanitchai, W. and Chonudomkul, D. (2016). Optimization of arachidonic acid production from *Mortierella alpina* PRAO7-10 by response surface methodology. *Agric. Natural Resour*. **50**:162 −172.

Maldonade, I.R., Scamparini, A.R.P. and Rodriguez-Amaya, D.B. (2007). Selection and characterization of carotenoid-producing yeasts from Campinas region, *Brazil. Braz.J. Microbiol.* **38**: 65−70.

Martini, A. (1992). Biodiversity and conservation of yeasts. *Biodivers. Conserv*. **1**:324– 333.

Mamatha, S.S. (2009). Polyunsaturated fatty acids of *Mucor* sp. with special reference to gamma linolenic acid, thesis submitted to the University of Mysore for the award of degree of doctor of philosophy in microbiology, pp123−169.

McGinnis, M.R. (1980). *Laboratory Hand Book of Medical Mycology*, Academic Press, New York.

Meesters, P.A. E.P., Huijberts, G.N.M. and Eggink, G. (1996). High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl. Microbiol. Biotechnol*. **45**:575−579.

Meeuwse, P., Tramper, J. and Rinzema, A. (2011). Modeling lipid accumulation in oleaginous fungi in chemostat cultures I: Development and validation of chemostat model for *Umbelopsis isabellina*. *Bioprocess Biosyst. Eng*. **34**:939–949.

Merz, W.G. (1990). *Candida albicans* strain delineation. *Clin. Microbiol. Rev*.**3**: 321−334.

Metzger, J.O. and Bornscheuer, U. (2006). Lipids as renewable resources: current state of chemical and biotechnological conversion and diversification. *Appl.Microbiol.Biotechnol*. **71**:13−22.

Miao, X.L. and Wu, Q.Y. (2006). Biodiesel production from heterotrophic microalgae. *Bioresour.Technol*. **97**:841−846.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. **31**:426–428.

Minnesota Statutes. (2008). Petroleum Diesel Fuel and Biodiesel Technical Cold Weather Issues. Minnisota Department of Agriculture, Report to the Legislature.1−16.

Mohamed, M.A.E., Abdel-Razik, A.B. and Ibrahim, S.A. (2014). Identification of different species of *Rhodotorula* using internal transcribed spacers. *Open repository agriculture*. e45011822.doi: 10.7392/openaccess.45011822.

Molina, F.I., Inoue, T. and Jong, S.C. (1992). Restriction polymorphisms in the internal transcribed spacers and 5.8S rDNA of *Saccharomyces. Curr. Microbiol*. **25**: 251–255.

Moser, B.R. (2011). Biodiesel production, properties, and feedstocks. **In**: *Biofuels*: *global impact on renewable energy*, *production agriculture*, *and technological advancements* (Tomes, D., Lakshmanan, P. and Songstad, D., eds), Springer, New York.

Moreton, R.S. (1988). Physiology of lipid accumulation. **In**: *Single cell oil* (Moreton, R.S., ed), Harlow: Longmans Scientfic and Technical, New York.

Moustogianni, A., Bellou, S., Triantaphyllidou, I.E. and Aggelis, G. (2015). Feasibility of raw glycerol conversion into single cell oil by *zygomycetes* under non-aseptic conditions. *Biotechnol*. *Bioeng*. **112**:827–831.

Musters, W., Boon, K., Van der Sande, A.F.M., Van Heerikhuizen, H. and Planta, R.J. (1990). Functional analysis of transcribed spacers of yeast ribosomal DNA. *EMBO.J.* **9**:3989−3996.

Neiman, A.M. (2005). Ascospore formation in the yeast *Saccharomyces cerevisiae. Microbiol Mol. Biol. Rev.* **69**:565−584.

Ness, F., Lavallee, F, Dubourdieu, D., Aigle, M. and Dulaub, L. (1993). Identification of yeast strains using the polymerase chain reaction. *J.Sci. Food Agric.* **62**:89−94.

Nigam, P.S. and Singh, A. (2014). Fermentation (industrial) | production of oils and fatty acids. **In**: *Encyclopedia of Food Microbiology* (Bat, C.A. and Tortorello, M.L., eds), 2nd ed., Elsevier Academic Press, Amsterdam.

Nitschke, M., Ferraz, C. and Pastore, G.M. (2004). Selection of microorganisms for biosurfactant production using agro-industrial wastes. *Braz.J.Microbiol.* **35**:81−85.

Oda, Y., Yabuki, M., Tonomura, K. and Fukunaga, M. (1997). A phylogenetic analysis of *Saccharomyces* species by the sequence of 18S−28S rRNA spacer regions. *Yeast.* **13**: 1243−1250.

Pahl, G. (2005). Biodiesel: growing a new green economy. White river junction Vermont. Chelses Green publishers. ISBN−10: 1931498652, ISBN−13: 978−1931498654.

Pan, L.X., Yang, D.F., Shao, L., Li, W., Chen, G.G and Liang, Z.Q. (2009). Isolation of the oleaginous yeasts from the soil and studies of their lipid producing capacities. *Food Technol. Biotechnol*. **47**: 215−220.

Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I. and Aggelis, G. (2002). Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. *Appl.Microbiol.Biotechnol*. **58**:308–312.

Papanikolaou, S., Komaitis, M and Aggelis, G. (2004). Single cell oil production by *Mortierella isabellina* grown on high-sugar content media. *Bioresour.Technol*. **95**: 287−291.

Papanikolaou, S. and Aggelis, G. (2011). Lipids of oleaginous yeasts. Part II: Technology and potential applications: A review. *Eur. J. Lipid Sci. Technol*. **113**: 1052–1073.

Park, W.K., Moon, M., Kwak, M.S., Jeon, S., Choi, G.G. and Lee, B. (2014). Use of orange peel extract for mixotrophic cultivation of *Chlorella vulgaris*: Increased production of biomass and FAMEs. *Bioresour.Technol*. **171**: 343–349.

Paserakung, A., Pattarajinda, V., Vichitphan, K. and Froetsche, M.A. (2015). Selection and identification of oleaginous yeast isolated from soil, animal feed and ruminal fluid for use as feed supplement in dairy cattle. *Lett. Appl. Microbiol*. **61**: 325–332.

Patel, A., Pravez, M., Deeba, F., Pruthi, V., Singh, R.P. and Pruthi, P.A. (2014). Boosting accumulation of neutral lipids in *Rhodosporidium kratochvilovae* HIMPA1 grown on hemp (*Cannabis sativa* Linn) seed aqueous extract as feedstock for biodiesel production. *Bioresour. Technol*. **165**: 214–222.

Periyasamy, S. and Venkatachalam, S. (2009). Production of bioethanol from sugar molasses using *Saccharomyces Cerevisiae. Modern Appl.Sci.* **3**:32−37.

Pereyra, V., Martinez, A., Rufo, C. and Vero, S. (2014). Oleaginous yeasts form Uruguay and Antarctica as renewable raw material for biodiesel production. *American J.BioSci.* **2**:251−257.

Phaff, H.J. (1986). Ecology of yeasts with actual and potential value in biotechnology. *Microbiol. Ecol.* **12:**31− 42.

Phaff, H.P. (2001). *Yeasts*. Encyclopedia of Life Sciences, Nature Publishing Group, University of California, Davis, California.

Pöggeler, S. (2001). Mating-type genes for classical strain improvements in ascomycetes. *Appl. Microbiol. Biotechnol.* **56**:589−601.

Poli, J.S., Dallé, P., Senter, L., Mendes, S., Ramirez, M., Vainstein, M.H. and Valente, P. (2013). Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils. *Braz.J. Biosci*. **11**: 203−208.

Puligundla, P., Obulam, V.S.R., Oh, S.E. and Mok, C. (2014). Biotechnological potentialities and valorization of mango peel waste: A Review. *Sains Malaysiana*.**43**: 1901–1906.

Raimondi, S., Rossi, M., Leonardi, A., Bianchi, M.M., Rinaldi, T. and Amaretti, A. (2014). Getting lipids from glycerol: new perspectives on biotechnological exploitation of *Candida freyschussii*. *Microb. Cell Fact*. **13**:83.

Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L. and Perez, Á. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresour. Technol*. **100**:261–268.

Ranganna, S. (1977). *Manual of Analysis of Fruit and Vegetable Products*, Tata McGraw-Hill Publishing, New Delhi.

Ratledge, C. (2002). Regulation of lipid accumulation in oleaginous microorganisms. *Biochem. soci.Trans*. **6**:1047−1050.

Ratledge, C. and Wynn, J.P. (2002).The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* **51**: 1−51.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochemie*. **86**:807−815.

Ratledge, C. (2005). Single Cell Oils for the 21stCentury. **In**: Single cell oils (Cohen, Z. and Ratledge, C., eds) AOCS Press, Champaign, Illinois.

Rattanapoltee, P. and Kaewkannetra, P. (2014). Utilization of agricultural residues of pineapple peels and sugarcane bagasse as cost-saving raw materials in *Scenedesmus acutus* for lipid accumulation and biodiesel production. *Appl. Biochem.Biotechnol*. **173**:1495–1510.

Rossi, M., Buzzini, P., Cordisco, L., Amaretti, A., Sala, M., Raimondi, S., Ponzoni, C., Pagnoni, U.M. and Matteuzzi, D. (2009). Growth, lipid accumulation, and fatty acid composition in

obligate psychrophilic, facultative psychrophilic and mesophilic yeasts. *FEMS microbial. Ecol*. **69**: 363−372.

Rossi, M., Amaretti, A., Raimondi, S. and Leonardi, A. (2011). Getting Lipids for Biodiesel Production from Oleaginous Fungi. **In**: *Biodiesel - Feedstocks and Processing Technologies* (Stoytcheva, M., ed), InTech, Europe.

Ruangudom, C. and Punpeng, B. (2011). Effect of C/N ratio and temperature on lipid accumulation of *Rhodosporidium toruloides* TISTR 5123 using sugar cane juice. The 12th Asean Food Conference, 16−18 June, 2011, BITEC Bangna, Bangkok, Thailand. Psychrophilic, and mesophilic yeasts. *FEMS microbial Ecol*. **69**: 363−372.

Saad, N., Abdeshahian, P., Kalil, M.S., Yusoff, W.M. and Hamid, A.A. (2014). Optimization of aeration and agitation rate for lipid and gamma linolenic acid production by *Cunninghamella bainieri* 2a1 in submerged fermentation using response surface methodology. *Scientific World J.*http://dx.doi.org/10.1155/2014/280146,1−12.

Sabry, S.A., Ghanem, K.M. and Yusef, H.H. (1990). Production of microbial lipids from beet molasses. *J. Islamic Acad. Sci*. **3**:310−313.

Sadasivam, S. and Manickam, A. (2005). *Biochemical Methods*. New Age International (P) Ltd, New Delhi.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K. and Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Sci*. **239**: 487–491.

Saito, T., Kato, A, Ochiai, H. and Morita, N. (2005).Temperature adaptation in *Dictyostelium*: role of D5 fatty acid desaturase. *Microbiol*. **151**: 113–119.

Sakuradani, E., Ando, A., Ogawa, J. and Shimizu, S. (2009). Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding. *Appl.Microbiol.Biotechnol.* **84**:1−10.

Sankh, S., Thiru, M., Saran, S. and Rangaswamy, V. (2013). Biodiesel production from a newly isolated *Pichia kudriavzevii* strain. *Fuel*. **106**: 690-696.

Santala, S., Efimova, E., Kivinen, V., Larjo, A., Aho, T., Karp, M. and Santala, V. (2011). Improved triacylglycerol production in *Acinetobacter baylyi* ADP1 by metabolic engineering. *Microb. Cell Fact.* **10**:1−10.

Santamauro, F., Whiffin, F.M., Scott R, J. and Chuck, C.J. (2014). Low-cost lipid production by oleaginous yeast cultured in non-sterile conditions using model waste resources. *Biotechnol. Biofuels.* **7**:34.

Saxena, R.K., Anand, P., Saran, S. and Isar, J. (2009). Microbial production of 1, 3-propanediol: recent developments and emerging opportunities. *Biotechnol. Advances*. **27**: 895−913.

Schafer, K. (1998). Accelerated solvent extraction of lipids for determining the fatty acid composition of biological material. *Analytica. Chimica. Acta.* **358**: 69−77.

Schober, S. and Mittelbach, M. (2007). Iodine value and biodiesel: Is limitation still appropriate? *Lipid Technol*. **19**:281−284.

Schulze, I., Hansen, S., Großhans, S., Rudszuck, T., Ochsenreither, K., Syldatk, C. and Neumann, A. (2014). Characterization of newly isolated oleaginous yeasts - *Cryptococcus podzolicus*, *Trichosporon porosum* and *Pichia segobiensis. AMB Exp*.**4**: 24.

Scorzetti, G., Fell, J.W., Fonseca, A. and Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res.* **1497**:1−23.

Seo, Y.H., Lee, I.G. and Han, J.I. (2013).Cultivation and lipid production of yeast *Cryptococcus curvatus* using pretreated waste active sludge supernatant. *Bioresour. Technol*. **135**: 304–308.

Sha, Q. (2013). A comparative study on four oleaginous yeasts on their lipid accumulating Capacity. Swedish University of Agricultural Sciences, Department of Microbiology, Master'sthesis.

Shahidi, F. (2001). *Current Protocols in Food Analytical Chemistry*. John Wiley and Sons, Hobeken, New Jersy.

Shahidi, F. and Wanasundara, P.J. (2008). Extraction and analysis of lipids. **In**: *Food Lipids: Chemistry, Nutrition and Biotechnology* (Akoh, C.C. and Min, D.B., eds), Taylor and Francis, CRC Press, London.

Shalaby, E.A. (2011). Algal biomass and biodiesel production. **In**: *Biodiesel − Feedstocks and processing technologies* (Stoytcheva, M., ed), InTech, Europe.

Shalma, S.M., Ranjitha, J. and Vijayalakshmi, S. (2016). Utilization of agro waste as carbon sources for high lipid production by *Aspergillus niger*. *Inter.J.Chem.Tech.Resear*. **9**:635−639.

Sijtsma, L., Springer, J., Meesters, P.A.E.P., de Swaaf, M.E. and Eggink, G. (1998). Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. *Recent Res. Devl.Microbiol*. **2**:219– 232.

Sláviková, E. and Vadkertiová, R. (2000). The occurrence of yeasts in the forest soils. *J. Basic Microbiol*. **40**: 207–212.

Sláviková, E. and Vadkertiová, R. (2003). The diversity of yeasts in the agricultural soil. *J.Basic Microbiol*. **43**: 430–436.

Souza, L.T.A, Mendes, A.A. and de Castro, H.F. (2016). Selection of lipases for the synthesis of biodiesel from *Jatropha* oil and the potential of microwave irradiation to enhance the reaction

rate. BioMed Research International. Hindawi Publishing Corporation. http://dx.doi.org/ 10.1155/2016/1404567.

Spotholz, C.H., Litchfield, J.H. and Ordal, Z.J. (1956). The effect of pH, temperature and composition of the medium on growth characteristics of *Rhodotorula gracilis*. *Appl. Microbiol.* **4**:285−288.

Srisuwan, W., Techapun, C., Seesuriyachan, P., Watanabe, M. and Chaiyaso, T. (2016). Screening of oleaginous yeast for lipid production using rice residue from food waste as a carbon source. *KKU. Res.J.* **22**:116−126.

Sriwongchai, S., Pokethitiyook, P., Kruatrachue, M., Bajwa, P.K. and Lee, H. (2013). Screening of selected oleaginous yeasts for lipid production from glycerol and some factors which affect lipid production by *Yarrowia lipolytica* strains. *J.Microbiol.Biotechnol.Food Sci*. **2**: 2344−2348.

Starmer, W.T., Phaff, H. J., Ganter, P.F. and Lachance, M.A. (2001). *Candidaorba* sp. nov. a new cactus-specific yeast species from Queensland, Australia. *Int.J.Syst.Bacteriol.* **51**: 699−705.

Subramaniam, R., Dufreche, S., Zappi, M. and Bajpai, R. (2010). Microbial lipids from renewable resources: production and characterization. *J.Ind.Microbiol.Biotechnol.* **37**:1271–1287.

Subramaniam, S., Fahy, E., Gupta, S., Sud, M., Robert, W. Byrnes, R.W., Cotter, D., Dinasarapu, A.R. and Maurya, M.R. (2011). Bioinformatics and systems biology of the lipidome: A review. *Chemical Reviews.* **111**: 6452−6490.

Sugita, T., Nishikawa, A., Ikeda, R. and Shinoda, T. (1999). Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. *J.Clin.Microbiol.Jun*. **37**:1985–1993.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour.Technol.* **83**: 1−11.

Syed, M. A., Singh, S. K., Pandey, A., Kanjilal, S. and Prasad, R. B. N. (2006). Effects of various process parameters on the production of α-linolenic acid in submerged fermentation. *Food Technol. Biotechnol.* **44**:282−287.

Tao, J., Dai, C., Li, R., Chen, J. and Zhang, B. (2008).The changes of microdiesel composition with the culture conditions by fallen leaves of *Populus euramevicana* cv. *Int.J.Sustain. Energ.* **27**:73–79.

Tat, M.E. and Van Gerpen, J.H. (1999). The Kinematic viscosity of biodiesel and its blends with diesel fuel. *AOCS.* **76***:* 1511–1513.

Tchakouteu, S.S., Kalantzi, O., Gardeli, C., Koutinas, A.A., Aggelis, G. and Papanikolaou, S. (2015). Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. *J.Appl. Microbiol.* **118**: 911–927.

Thakur, M.S, Prapulla, S.G. and Karanth, N. G. (1988). Microscopic observation of Sudan Black B staining to monitor lipid production by microbes. *J.Chem.Technol. Biotechnol*. **42**:129−134.

Thiru, M., Sankh, S. and Rangaswamy, V. (2011). Process for biodiesel production from *Cryptococcus curvatus*. *Bioresour.Technol*. **4**:10436–10440.

Tinoi, J. and Rakariyatham, N. (2015). Utilization of pineapple waste hydrolysate for lipid production by oleaginous yeast *Rhodoturula glutinis. Int.J.Adv.Resear*. **3**:1233−1243.

Trost, A., Graf, B., Eucker, J., Sezer, O., Possinger, K., Gobel, U. B. and Thomas, A. (2004). Identification of clinically relevant yeasts by PCR/RFLP. *J.Microbiol.Meth*. **56**:201−211.

Turcotte, I.G. and Kosaric, N. (1989). Effect of C/N ratio on lipid production by *Rhodosporidium toruloides* ATCC 10788. *Biotechnol. Lett*. **II**: 637−642.

Tyson, K.S. (2001). Biodiesel handling and use guidelines. NREL publication NREL/TP-580−30.

Umesh, M. and Preethi, K. (2014). Fermentative utilization of fruit peel waste for lactic acid production by *Lactobacillus plantarum*. *Indian J. Appl. Resear*. **4**:449− 451.

Vieira, J.P.F., Ienczak, J. L., Rossell, C. E.V., Pradella, J. G. C. and Franco, T.T. (2014). Microbial lipid production: screening with yeasts grown on Brazilian molasses. *Biotechnol Lett.* **36**:2433–2442.

Van Gerpen, J., Shanks, B., Pruszko, R., Clements, D. and Knothe, G. (2004). Biodiesel Analytical Methods.National Renewable Energy Laboratory, NREL/SR-510-36240.

Versavaud, A. and Hallet, J.N. (1995). Pulsed-field gel electrophoresis combined with rare-cutting endonucleases for strain identification of *Candida famata*, *Kloeckera apiculata* and *Schizosaccharomyces pombe* with chromosome number and size estimation of the two former. *Syst. Appl. Microbiol*. **18**:303−309.

Verweij, P. E., Breuker, I.M. Rijs, A. J.M. M. and Meis, J.F.G. M. (1999). Comparative study of seven commercial yeast identification systems. *J.Clin.Pathol*. **52**:271–273.

Vicente, G., Martinez, M. and Aracil, J. (2004). Integrated biodiesel production: a comparison of different homogenous catalysts systems. *Bioresour. Technol*. **92**:297−305.

Vicente, G., Bautista, L., Rodriguez, R., Gutierrez, F.J., Sadaba, I., Ruiz-Vazquez, R.M., Torres-Martinez, S. and Garre, V. (2009). Biodiesel production from biomass of an oleaginous fungus. *Biochem. Eng.J.* **48**:22–27.

Vijayakumar, S., Kumutha, K., Krishnan, P.S. and Gopal, H. (2010). Effect of carbon sources on lipid and biomass production by oleaginous yeast cultures. *Madras Agric.J*. **97**:62–64.

Vilgalys, R. and Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* **172**:4238–4246.

Virot, M., Tomao, V., Colnagui, G., Visinoni, F. and Chemat, F. (2007). New microwave−integrated Soxhlet extraction an advantageous tool for the extraction of lipids from food products. *J. Chromatog.* **1174**:138−144.

Wang, L., Sun, Y.M., Wang, P.Z. and Zhao, Z.B. (2005). Effects of metal ions on lipid production by fermentation with *Trichosporon fermentans*. *J.Dalian Inst.Light Industry.* **24**: 259–263.

Wang, Q.M., Yurkov, A.M., Göker, M., Lumbsch, H.T., Leavitt, S.D., Groenewald, M., Theelen, B., Liu, X.Z., Boekhout, T. and Bai, F.Y. (2015). Phylogenetic classification of yeasts and related taxa within *Pucciniomycotina. Stud. Mycol*. **81**: 149–189.

Ward, O.P. and Singh, A. (2005). Omega-3/6 fatty acids: Alternative sources of production. *Process Biochem*. **40**:3627−3652.

Wesselink, J., Iglesia, B., James, S. A., Dicks, J. L., Roberts, I. N. and Rayward−Smith, V.J. (2002). Determining a unique defining sequence for yeast species using hashing techniques. *Bioinform*. **18**: 1004−1010.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. **In**: *PCR Protocols: A Guide to Methods and Applications* (Innis, N., Gelfand, D., Sninsky, J. and White, T., eds), Academic Press, New York.

Willemsen, M., Breynnaert, J. and Lauwers, S. (1997). Comparison of Auxacolor with API 20C Aux in yeast identification. *Clin. Microbiol.Infect*. **3**:369−375.

www.mycobank.org

Wynn, J.P., Hamid, A.A., Li, Y. and Ratledge, C. (2001). Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*. *Microbiol*. **147**: 2857−2864.

Wynn, J.P. and Ratledge, C. (2006). Microbial production of oils and fats. **In**: *Food Biotechnology* (Shetty, K., Paliyath, G., Pometto, A. and Levin, R.E., eds), 2nded, Taylor and Francis, New York.

Xiao, L. (2010). Evaluation of extraction methods for recovery of fatty acids from marine products. Master thesis of EMQAL project.

Xing, D., Wang, H., Pan, A., Wang, J. and Xue, D. (2012). Assimilation of corn fiber hydrolysates and lipid accumulation by *Mortierella isabellina. Biomass Bioenerg*. **39**: 494 −501.

Xue, F., Gao, B., Zhu, Y., Zhang, X., Feng, W., and Tan, T. (2010). Pilot-scale production of microbial lipid using starch wastewater as raw material. *Bioresour. Technol*. **101**: 6092–6095.

Yamauchi, H., Mori, H., Kobayashi, T. and Shimizu, S. (1983). Mass production of lipids by *Lipomyces starkeyi* in microcomputer aided fed-batch culture. *J.Ferment.Technol*. **61**: 275−280.

Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. **In**: *The Yeasts: A Taxonomic Study* (Kurtzman, C.P. and Fell, J.W., eds), Elsevier, Science, Amsterdam.

Ykema, A., Verbree, E.C., Verseveld, H.W. and Smit, H. (1986). Mathematical modeling of lipid production by oleaginous yeasts in continuous cultures. *Antonie van Leeuwenhoek*. **52**:491−506.

Yi, S.J. and Zheng, Y.P. (2006). Research and applications of oleaginous microorganisms. *China Foreign Energ*. **11**:90−94.

Yu, X., Zheng, Y., Dorgan, K.M. and Chen, S. (2011). Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulphuric acid. *Bioresour. Technol*. **102**:6134–6140.

Zhao, X., Kong, X., Hua, Y., Feng, B. and Zhao, Z.K. (2008). Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. *European J.Lipid Sci. Technol.* **110**: 405–412.

Zhao, C.H., Chi, Z., Zhang, F., Guo, F.J., Li, M., Song, W.B. and Chi, Z.M. (2011). Direct conversion of inulin and extract of tubers of Jerusalem artichoke into single cell oil by co-cultures of *Rhodotorula mucilaginosa* TJY15a and immobilized inulinase-producing yeast cells. *Bioresour.Technol*. **102**: 6128–6133.

Zhao, C., Liu, Z.G., Qingli, Z., Sun, G., Tang, X., Shi, W. and Lu, X. (2015). Effects of culture conditions on single cell oil accumulation. *J.Chem.Pharm.Res.* **7**:2189−2191.

Zheng, Y., Yu, X., Zeng, J. and Chen, S. (2012). Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulphuric acid pretreatment of wheat straw. *Biotechnol. Biofuels.* **5**:1750−54.

Zhu, Y., Yanagihara, K., Guo, F. and Qing, X. L. (2000). Pressurized fluid extraction for quantitative recovery of chloroacetanilide and nitrogen heterocyclic herbicides in Soil. *J. Agric. Food Chem.* **48**:4097−4102.

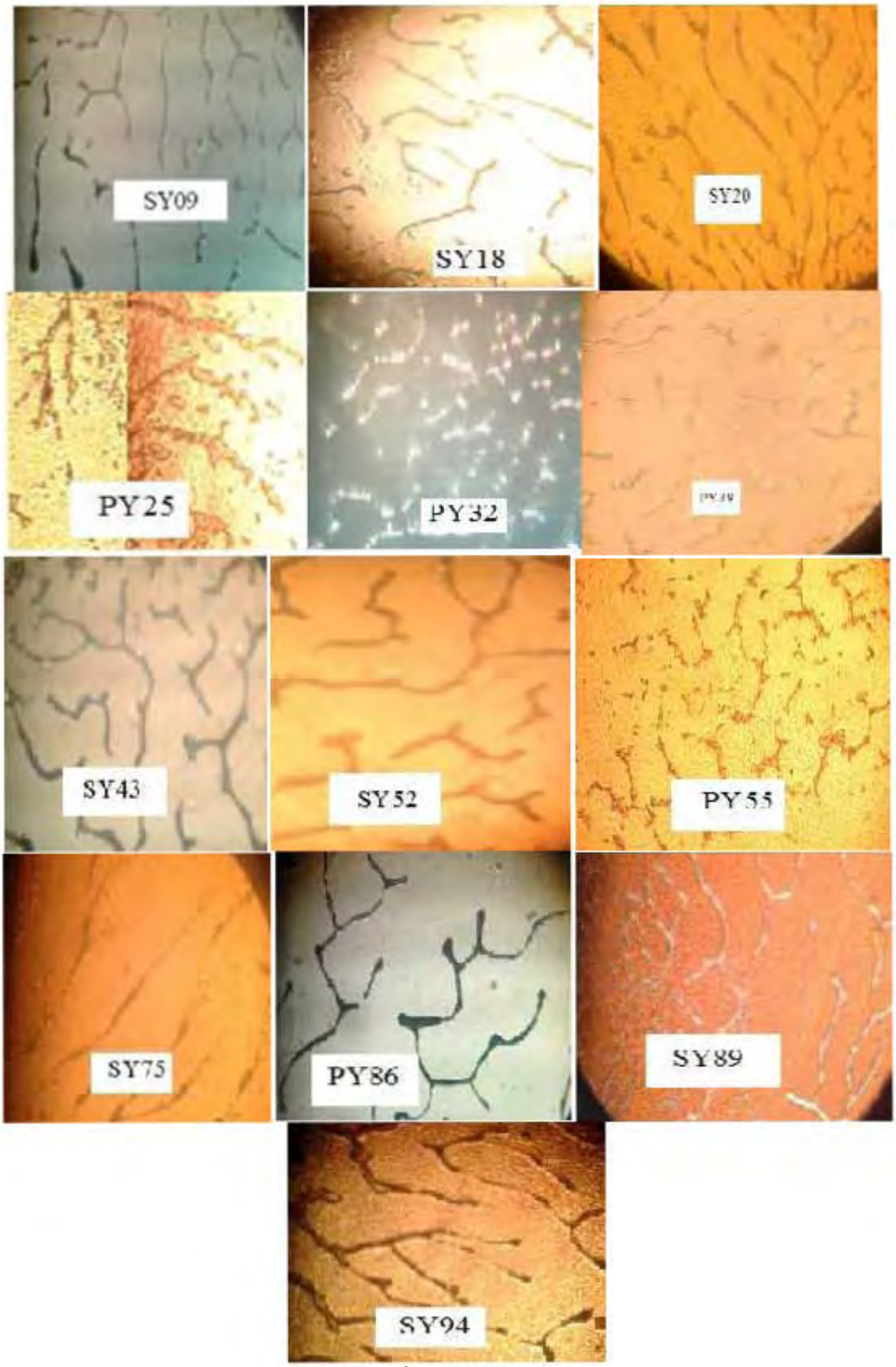
Zhu, L.Y., Zong, M.H. and Wu, H. (2008). Efficient lipid production with *Trichosporon fermentans* and its use for biodiesel preparation. *Bioresour. Technol*. **99**: 7881–7885.

Zuleta, E.C., Baena, L., Rios, L.A. and Calderón, J.A. (2012). The oxidative stability of biodiesel and its impact on the deterioration of metallic and polymeric materials: a review. *J. Braz. Chem.Soc*. **23**: 2159−2175.

8. Appendices

Appendix1: Urea hydrolysis test result.

Appendix 2: Sample fermentation test.



Appendix 3: Pseudohyphae of the yeasts characterized in the study.

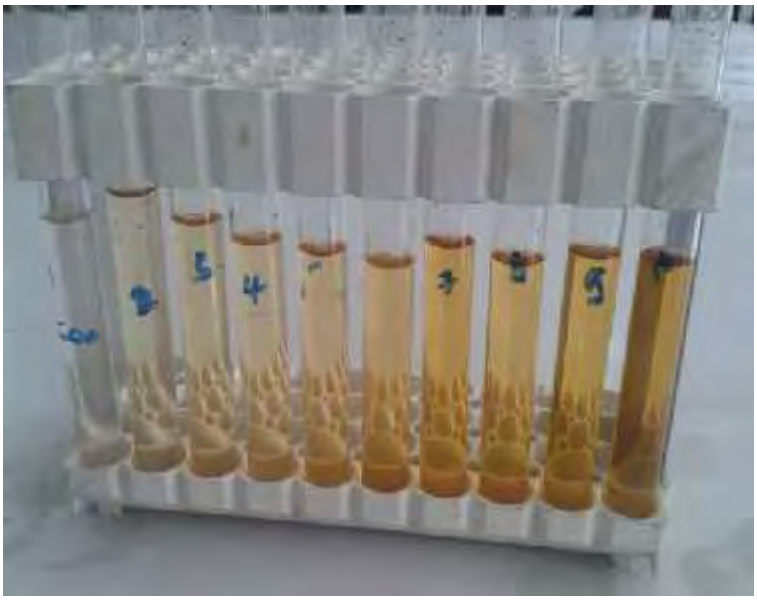
Appendix 4: Submerged cultivation of oleaginous yeast in nitrogen-limited medium.

Appendix 5: Yeast biomass produced after centrifugation.

Appendix 6: Ground yeast cells.

Appendix 7: Extraction of oil from yeast biomass.

Appendix 8: Rota vapor used in extraction.



Appendix 9: Preparation of standard glucose for analysis of total sugar in peel mixtures of papaya and mango.

Appendix10: Flask cultivation of selected oleaginous yeasts using peel mixtures of papaya and mango.

Appendix 11: Molasses used bioreactor for cultivation.

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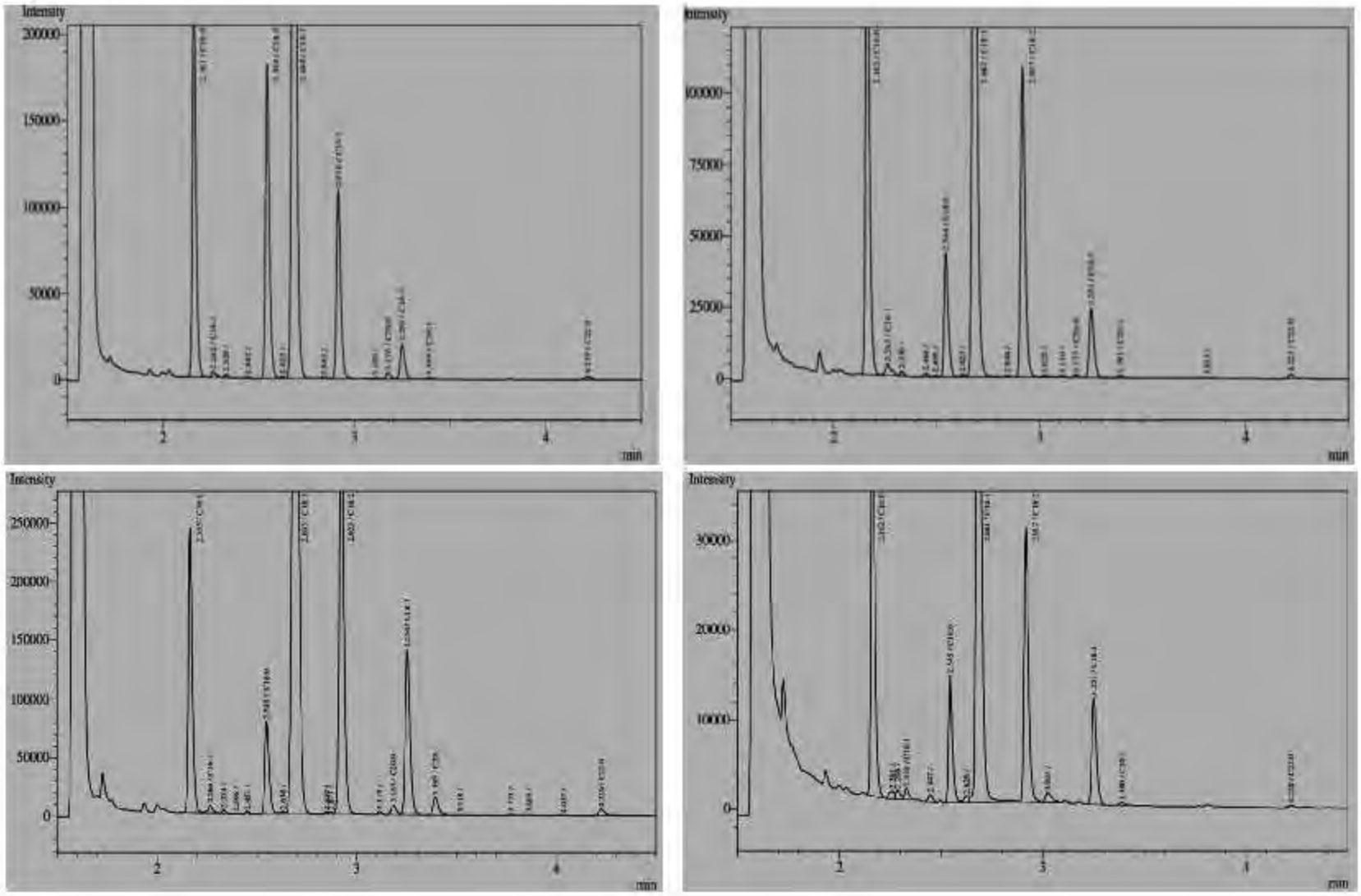
Appendix 12: Sample of SCO dissolved in diethyl ether.

Appendix 13: Methyl esters with upper biodiesel layer and lower glycerol layer.



Appendix14: Chromatogram showing the fatty acid profiles of PY39 (1), SY09 (2), SY18 (3), and SY89 (4**).**

8.1 Preparation of TMSOH

Day1

- Pour 100 mL of methanol into Erlenmeyerflask (washed with methanol and covered with foil).
- Add 4 g of 3-methyl sulfonium iodide or tri- methyl sulfonium iodide to the flask.
- Add 4 g of silver oxide(no contact with metal) to the mixture
- Stirr the mixture over night at 15 w speed.

Day2

- Wash the funnel with methanol.
- Filter the mixture through filter paper.
- Test with standard oil.
- Store in freezer and use it when necessary.

Declaration

I, the undersigned, here honestly declare that this PhD dissertation is my own original work and has not been submitted by me or any other body elsewhere at another university or other academic institution to fulfill a similar purpose. All materials used for the thesis has been dully acknowledged.

PhD candidate: Tamene Milkessa Signature_____ Date_____