INVESTIGATION INTO THE POSSIBLE USE OF *CRYPTOCOCCUS ALBIDUS* FOR LIPID ACCUMULATION USING VOLATILE FATTY ACIDS AS SOLE CARBON SOURCE FOR BIODIESEL PRODUCTION

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DECLARATION

I hereby declare that this submission is my own work towards my MSc degree and that, to the best of my knowledge, it contains no material published by another person or material which has been accepted in any other University for any degree except where due acknowledgement has been made in the text.

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ABSTRACT

Owing to rising global population and associated competition for food of which some serve as feedstock for biodiesel, especially the vegetable oils, it is necessary to identify alternative sources of lipids. The concept of using volatile fatty acids as sole carbon source for microbial lipids accumulation was investigated in flask cultures of *Crypotoccus albidus*. *C.albidus* was cultivated at a temperature of 25^{o}C, pH of 6.0 and pure VFA concentration of 5g/l.The hexane/ isopropanol alcohol/ distilled water solvent extraction method using a soxhlet apparatus was used for possible lipids extraction. Pure volatile fatty acids mixtures (acetic, propionic and butyric acids) of different ratios as well as volatile fatty acids from anaerobic digestion of faecal sludge were used. It was observed that *Crypotoccus albidus* could not grow well on volatile fatty acids as there were decreases in optical density from an initial 0.228 to 0.184 when pure VFAs were used with ammonium chloride as nitrogen source. When cells were grown on VFAs from faecal sludge also, there was no growth as a similar decrease in optical density was observed from 0.098 to -0.009. The effect of different nitrogen sources on the growth of *Crypotoccus albidus* was also investigated. It was observed that yeast extract which is an organic source of nitrogen gave the highest growth of cells with an optical density of 0.110 from 0.052. No lipid was extracted in this study as *C. albidus* could not grow on VFAs thereby not metabolizing the VFAs into lipids accumulated in its cells for extraction.

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LIST OF ACRONYMS

AIDS	Acquire Immune Deficiency Syndrome
ACL	Adenosine triphosphate-citrate lyase
ATP	Adenosine triphosphate
DHAP	Dihydroxyacetone phosphate
DW	Dry Weight
FA	Fatty acid
FAS	Fatty acid synthetase
MDH	Malate dehydrogenase
ME	Malic enzyme
NADPH	Nicotinamide adenine dinucleotide phosphate
PUFA	Polyunsaturated fatty acid production
SCO	Single Cell Oil
TAG	Triacylglycerols
TCA	Tricaborxylicacid
VFA	Volatile fatty acid

CHAPTER ONE

INTRODUCTION

1.1 Background

Majority of the world's energy needs are supplied through petrochemical sources, coal and natural gases. With the exception of hydroelectricity and nuclear energy, the other sources are finite and at current usage rates will be consumed shortly (Srivastava *et al*, 2004). Diesel fuels have an essential function in the industrial economy of a developing country and used for the transport of industrial and agricultural goods and operation of diesel tractor and pump sets in agricultural sector. However, there is a decline in the world oil reserves (1,342 billion barrels as of January 2009) and a rapid increase in the world fuel consumption (85 million barrels of liquid fuel per day in 2006 and projected to increase to 107 million barrels of liquid fuel per day in 2030), which have resulted in increasing price of petroleum-based fuels (Meng *et al*, 2008), (www.eia.doe.gov/oiaf/ieo/pdf/, May 20, 2009). In addition, the high energy demand in the industrialized world as well as in the domestic sector and pollution problems caused due to the widespread use of fossil fuels, make it increasingly necessary to develop the renewable energy sources of limitless duration (Meher *et al*, 2004).This has stimulated recent interest in alternative sources for petroleum-based fuels. An alternative fuel must be technically feasible, economically competitive, environmentally acceptable, and readily available. One possible alternative to fossil fuel is the use of lipids of plant origin like vegetable oils and tree borne oil seeds. This alternative diesel fuel can be termed as biodiesel (Meher *et al*, 2004). This fuel is biodegradable and non-toxic and has low emission profiles as compared to petroleum diesel. However using vegetable oils for biodiesel production has its own limitations because of competition for food which has the tendency of derailing the world food security, thus causing a shortage in food requirement. A lot of research

now, is into finding alternative sources of lipids for biodiesel production that will not affect the food security of man.

One of such research is the use of microbial lipids for biodiesel production. Microorganisms are receiving increasing attention for their potential applications to the oils and fats industry, either as a means of producing high quality fats, including some high speciality lipids, and also for being able to carry out selected biotransformation reactions which lead to higher value lipid products (Ratledge, 1991). Such microorganisms are called oleaginous organisms. They have the capacity for continual intake of carbon sources from a medium, converting the carbon source into lipid storage materials. The microbial lipids accumulated in oleaginous cells can be converted to biodiesel through a transesterification process. The major fatty acids in the lipids produced by oleaginous microorganisms are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), which are the major compounds of biodiesel (Fei *et al*, 2011). To date, most studies on lipid production by oleaginous microorganisms (microalgae, yeast, bacteria, etc.) have been carried out with glucose as the sole carbon source (Li *et al*., 2009; Papanikolaou *et al.*, 2010; Steen *et al*., 2010). However, the high cost of biodiesel from oleaginous microorganisms mainly stems from the high cost of glucose, which is estimated to be about 80% of the total medium cost. Therefore, considerable efforts have been directed toward minimizing the carbon source cost and finding new alternative carbon sources, including starch and ethanol (Hansson *et al*., 1986), pectin and lactose (Papanikolaou *et al.*, 2007), wastes (Xue et al., 2008; Fakas et al., 2008), and glycerol (Easterling et al., 2009; Fakas *et al*., 2009; Makri *et al*., 2010). Volatile fatty acids (VFAs), which can be produced from foodwastes, municipal sewage sludge, and a variety of biodegradable organic wastes via VFAs platform (Lim *et al.*, 2008a, Chang *et al.*, 2010), are promising

cheap alternative carbon sources for lipid accumulation by oleaginous microorganisms. One of such sources of VFAs in Ghana is faecal sludge which is in abundance and its management problematic. Anaerobic digestion of faecal sludge produces VFAs which is used as a sole carbon source in this work. In this study, the ability of *Cryptococcus albidus* (an oleaginous microorganism), to accumulate lipids was investigated. Fei *et al*, 2011, reported that *C. albidus* has a lipid content of 27.8%. This study also investigated how *C. albidus* accumulates lipids using fatty acids as the sole carbon source. The ability of *C. albidus* to utilize synthetic short chain fatty acids to produce higher molecular lipids that could be converted to biodiesel was studied. The ability of *C. albidus* to utilize volatile fatty acids in faecal sludge (dissolved) to produce lipids for further conversion to biodiesel was also investigated.

1.2 Problem Statement

Biodiesel is produced from lipids. Currently, the production of biodiesel is based mostly on plant oils, even though animal fats, and waste oils can also be used. In particular, soybean, rapeseed, and palm oils are adopted as the major feedstock for biodiesel production. They are produced on agricultural land, opening the debate on the impact of the expansion of bioenergy crop cultures, which displace land from food production. Furthermore, their price restricts the large-scale development of biodiesel to some extent. In order to meet the increasing demand of biodiesel production, other oil sources need to be explored. An alternative source for lipids for biodiesel is oleaginous organisms. Oleaginous species have the capacity for continual intake of carbon sources from a medium, converting the carbon source into lipid storage materials. Volatile fatty acids which are basically made up of carbon can be produced from food wastes, sewage sludge, faecal sludge and a variety of biodegradable organic wastes can be used as sole carbon source for oleaginous organism to metabolize into lipids for biodiesel. It is for this reason

that this research seeks to investigate the possibility of using *Cryptococcus albidus*, an oleaginous organism, to metabolize volatile fatty acids into lipids for biodiesel production. With the understanding of the abundance of organic waste which serves as source of volatile fatty acids, more biodiesel would be produced.

1.3 Justification

Owing to the abundance of organic waste in Ghana which has become a nuisance and a challenge with its management, there is a need to put waste to good use. Energy can be recovered from waste. An example of such a typical energy resource from waste is biodiesel. Biodiesel is drawing considerable attention on the basis of their nontoxic, sustainable, and energy efficient proprieties. Lipids are precursors for biodiesel production. Obtaining other sources of lipids other than the conversional source of vegetable oils, need to be investigated. Oleaginous organisms, which are abundant in nature, can serve as alternative sources for lipids for biodiesel production. *Cryptococcus albidus*, an example of an oleaginous organism has been reported by researchers to accumulate lipids in its cells using volatile fatty acids as sole carbon source.

1.4 Research Objectives

1.4.1 Main Objective

The main objective of this research was to investigate the possibility of using volatile fatty acids as a sole carbon source for lipid accumulation for biodiesel production by *Cryptococcus albidus.*

1.4.2 Specific Objective

The specific objectives of the study were

- to investigate the growth of *C. albidus* on volatile fatty acids

- to investigate the effect of different nitrogen source on lipid accumulation by *Cryptococcus albidus.*

1.5 Research Questions?

The main question this study seeks to address includes:

- Can volatile fatty acids serve as a sole carbon source for *C. albidus?*
- Can volatile fatty acids be converted to lipids by *C. albidus*?

1.6 Scope and Limitation of Study

Using microorganisms to produce lipids is an area of research that has not been given much attention. Obtaining substantial quantities of lipids from microorganism for biodiesel production is also a challenge. It is also not so easy to extract lipids from microorganisms. Lipid extraction methods for microorganisms which are mostly solvent extraction methods require large volumes of solvents which are cost intensive.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biodiesel

Biodiesel is an alternative renewable fuel that may be derived from a variety of feedstock (i.e. vegetable oils, animal fats, used frying oils, microbial oils) (Knothe et al, 2005), (Meng *et a*l, 2008). It is commonly produced by transesterification of pre-extracted oils with an alcohol (usually methanol or ethanol) in the presence of a catalyst (usually a base) to generate the fatty acid methyl/ethyl esters. It has received a lot of interest in recent years due to declining world oil reserves (1,342 billion barrels as of January 2009) and rapidly increasing world fuel consumption (85 million barrels of liquid fuel per dayin 2006 and projected to increase to 107 million barrels of liquid fuel per day in 2030), which have resulted in increasing price of petroleum-based fuels (Meng, 2004). In addition to being renewable and biodegradable, some other advantages of using biodiesel are, reduction in the importation of petroleum-based fuels, it has similar energy density to petroleum diesel and higher flash point, inherent lubricity, and reduction of most exhaust emissions (except NOx) (Knothe, 2005), (Liu *et al* , 2007). These advantages make biodiesel a promising alternative energy carrier.

Biodiesel's main economic challenge is the high feedstock/raw material cost, which for refined vegetable oil, accounts for 70–85% of the total biodiesel production cost (Haas *et al*, 2005). The growth of the biodiesel industry is limited by the availability of farmland and vegetable oil inventories, which result in high sensitivity of prices to oil demand from industry. These limitations also create a fuel versus food issue that requires urgent consideration of non-food related feed stock (Miao *et al*, 2008). It is for this reason that

this study seeks to investigate the use of microorganisms as lipid sources for biodiesel production using volatile fatty acids as sole carbon source.

2.2 Lipids

Lipids are a structurally diverse group of naturally occurring water-insoluble compounds that can, for convenience, be divided into the following eight categories: fatty acyls (e.g.,fatty acids), glycerolipids (e.g. monoacylglycerides, diacylglycerides, triacylglycerides), glycerophospholipids (e.g., phosphatidylcholine, phosphatidyl serine), sphingolipids, sterol lipids (e.g.,cholesterol, bile acids, vitamin D), prenol lipids (e.g., vitamins Eand K), saccharolipids, and polyketides (e.g., aflatoxin B1) ***(***Fahy et al, 2005***)***. Lipids also participate in a variety of biochemically and physiologically important roles such as acting as structural components of cell membranes, a metabolic fuel, energy stores, vitamins, antioxidants, as signal transduction molecules, lubricants, and waxes ***(***Watson, 2006;Stryer 2006**)**.

It has been estimated that the biological system lipidome is comprised of ~200,000 lipids ***(***Seppanen-Laakso, 2008***)***, while the cellular lipidome contains ~1,000 distinct lipids ***(***van Meer, 2005) differing in their chemical structure, location, and level, making their global measurement extremely challenging.

However, the categories of lipids of interest for biodiesel production are the glycerolipids (e.g., monoacylglycerides, diacylglycerides, triacylglycerides).

2.2.1 Sources of Lipids

Lipids are drawing considerable attention in relation to the production potential of biodiesel on the basis of their nontoxic, sustainable, and energy efficient proprieties (Ratledge *et al*., 2008). There are quite a number of sources of lipids in literature. Examples are

- oil producing crops such as palm fruit, sun flower, soya bean, rape,
- non edible sources such as jatropha
- animal fat such beef, lard, tallow
- recycled grease such as from trap grease
- oleaginous microorganisms such as *Cryptococcus species, Candida species*.

2.2.2 Lipids from Microorganism

Micro-organisms have often been considered for the production of oils and fats as an alternative to agricultural commodities. However, with the continuing low cost of agricultural production of oil-seeds, biotechnology has little to offer in the way of competition against such items as soybean oil, groundnut oil, and even polyunsaturated oils such as sunflower oil even though good facsimiles of these oils could be produced. It is now clear that if we are to use microorganisms to produce lipids, i.e. Single Cell Oil, then these must be highly specific ones which are currently expensive to obtain from agricultural sources (Ratledge, 1991).

Recently, the development of processes to produce single cell oil (SCO) by using heterotrophic oleaginous microorganisms has triggered significant attention (Azocar *et al.*, 2010). These organisms accumulate lipids, mostly consisting of triacylglycerols (TAG), that form the storage fraction of the cell. The occurrence of TAG as reserve compounds is widespread among all eukaryotic organisms such as fungi, plants and animals, whereas it has only rarely been described in bacteria (Meng *et al*., 2009). In fact, bacteria generally accumulate polyhydroxyalkanoates as storage compound and only few bacterial species, belonging to the actinobacterial genera *Mycobacterium*, *Streptomyces*, *Rhodococcus*and *Nocardia*produce relevant amounts of lipids (Alvarez &Steinbuchel,

2002). Among heterotrophic microorgansisms, oleaginous fungi, including both molds and yeasts, are increasingly been reported as good TAG producers.

2.2.3 Lipids extraction methods

Quantitative extraction of lipids from tissues or microorganisms of choice is key to their subsequent analysis. This section of the work looks at some techniques of lipid extraction from microorganisms.

Because lipids are water-insoluble, their extraction requires a combination of polar and nonpolar organic solvents. The goal of the extraction procedure generally is a quantitative recovery of all the different lipid classes. Three methods for the liquid–liquid extraction of lipids widely cited in literature are those of Folch, Lees, and Stanley, 1957; Bligh and Dyer, 1959 and Ways and Hanahan, 1964. All three methods use chloroform/methanol (2:1, v/v) as the extracting solvent. These protocols can be adapted for the extraction of lipids from whole yeast cells by including a step to break open the yeast cellwall, which is usually done by disintegrating the cells in the presence of glassbeads.

The preparation of a lipid extract includes the following basic steps:

1. Homogenization of the cells in the presence of organic solvents and glass beads.
2. Extraction of the lipids with chloroform/methanol (2:1; v/v).
3. Removal of non-lipid contaminants by washing the extract with aqueous salt solutions.
4. Drying of the extract by removal of the organic solvent (Xiao, 2000)

There is however another lipid extraction method from the cells of microorganisms. This is also a solvent extraction method which uses a combination of hexane, isopropanol alcohol and distilled water instead of chloroform and methanol (Gucket et al., 1988). It is

equivalent in terms of qualitative and quantitative lipids extracted. It is a low toxicity substitute for the chloroform/methanol/distilled water mixture. It also permits analysis of materials retained on nuclepore filters since polycarbonate is stable in these solvents (Nuclepore catalog, 1984).

In addition, isopropanol is an inhibitor of phospholipase D and has been used in previous work to keep plant lipids in native state (Christie, 1973). Other advantages hexane/isopropano alcohol/distilled water reported have been that it extracts less pigment and non-lipids (especially protoelipids) than chloroform/methanol (Hara et al, 1978).

In this work, the hexane/isopropanol alcohol/ distilled water extraction method was used for lipids extraction from yeast cells because of its advantages over the other extraction methods. A detailed description of the process is discussed in chapter 3.

2.3 Cryptococcus albidus

Cryptococcus albidus is an example of an oleaginous organism. That is, it has the ability to accumulate more than 20% of lipids in its cell by continuous intake of carbon source in a medium. There are seven recognized species of genus *Cryptoccocus*. These are *C. neofromans, C. laurentii, C.terreus, C. luteolus, C. albidus, C. gastricus, C. uniglutulatus* (Bennett, 1976, Bloomfield, Gordon, Elemdorf, 1963, Rippon, 1974).

C. albidus can be differentiated from other *Cryptococci* by features which include nitrate assimulation and viable growth at 37^{o}C (however *C. albidus* grows very well between 23^{o}C and 25^{o}C). It ferments maltose, and sucrose readily, galactose weakly, and demonstrates variable fermentation of melibiose and erytrithol (Pidcoe and Kaufman, 1968). It is also characterized by globose to elongate yeast-like cells orblastoconidia that reproduce by multilateral budding. Pseudohyphae are absent or rudimentary. On solid

media the cultures are generally mucoid or slimy in appearance. Colonies of *C. albidus* are usually non-pigmented, and are cream in color. Most strains have encapsulated cells with the extent of capsule formation depending on the medium. *C. albidus* is cosmopolitan, found on plants and in water and on skin of animals and humans. Although infections with *C. albidus* are rare it should be considered as a potential cause of ocular and systemic disease in immunoin competent patients and those with AIDS. Literature reports include: cutaneous infection, scleral ulceration of a 16-year-old girl with AIDS, eyes and blood of lymphoma patients, leukemia patients and in a rare case of mucormycosis empyema. Therefore care must be taken in handling *C.albidus* to avoid infections (http://www.interchg.ubc.ca/cmpt/pdf_mycology/0601_1).

Fig.2 1 *C.albidus* after subculturing

The sole carbon source for *C.albidus* in this work was pure volatile fatty acids (VFAs), a mixture of acetic acid, propionic acid and butyric acid in some ratios. From literature, *C.albidus* has a high affinity for acetic acid in mixtures of VFAs. Hence a ratio with a high proportion of acetic acid was used for this work as the sole source of carbon for lipids accumulation.

2.3.1 Biochemistry of microbial lipid accumulation

Lipid accumulation in oleaginous yeasts and molds has been demonstrated to occur when a nutrient in the medium (e.g. the nitrogen or the phosphorus source) becomes limited and

the carbon source is present in excess. Nitrogen limitation is the most efficient condition for inducing lipogenesis. During the growth phase, nitrogen is necessary for the synthesis of proteins and nucleic acids, while the carbon flux is distributed among energetic and anabolic processes yielding carbohydrates, lipids, nucleic acids and proteins. When nitrogen gets limited, the growth rate slows down and the synthesis of proteins and nucleic acids tends to cease. In non-oleaginous species, the carbon excess remains unutilized or is converted into storage polysaccharides, while, in oleaginous species, it is preferentially channeled toward lipid synthesis, leading to the accumulation of TAG within intracellular lipid bodies (Ratledge& Wynn, 2002; Granger *et al.*, 1993).

The biochemical pathway of lipid biosynthesis is not very different among eukaryotic organisms and does not differ in oleaginous and non-oleaginous fungi. The ability to accumulate high amounts of lipid depends mostly on the regulation the biosynthetic pathway and the supply of the precursors (i.e. acetyl-CoA, malonyl-CoA, and glycerol-3-phosphate) and the cofactor NADPH.

Most information were obtained from the model yeast *Saccharomyces cerevisiae* (Kohlwein, 2010), that does not accumulate lipids, and *Yarrowialipolytica*, that represent a model for biooil production and is suitable for genetic manipulation (Beopoulos *et al*., 2009b).

2.3.2 Fatty acids biosynthesis and modifications

De novo synthesis of fatty acids (FA), the first step of lipid accumulation, is carried out in the cytosol by fatty acids synthetase (FAS) complex. In yeasts, FAS bears phosphopantheteinetransferase activity to activate its acyl carrier protein (ACP) by loading the coenzyme pantothenate. FAS is a multimer of 6α and 6 βsubunits encoded by *fas2* and *fas1*, respectively, each subunit containing four functional domains. Therefore,

FAS consists in a α6β 6molecular complex of 2.6 MDa with 48 functional centers that catalyze all reactions required for synthesis of fatty acids through cycles of multistep reactions. FAS firstly loads acetyl-CoA on its β -ketoacyl-ACP synthase (KS), then it exherts β -ketoacyl-ACP reductase (KR), β -hydroxyacyl-ACP dehydratase (DH), and enoyl-ACP reductase (EAR) activities.

This set of reactions is repeated cyclically seven times to yield palmitoyl-ACP (Fig.3) (Tehlivets et al, 2007).

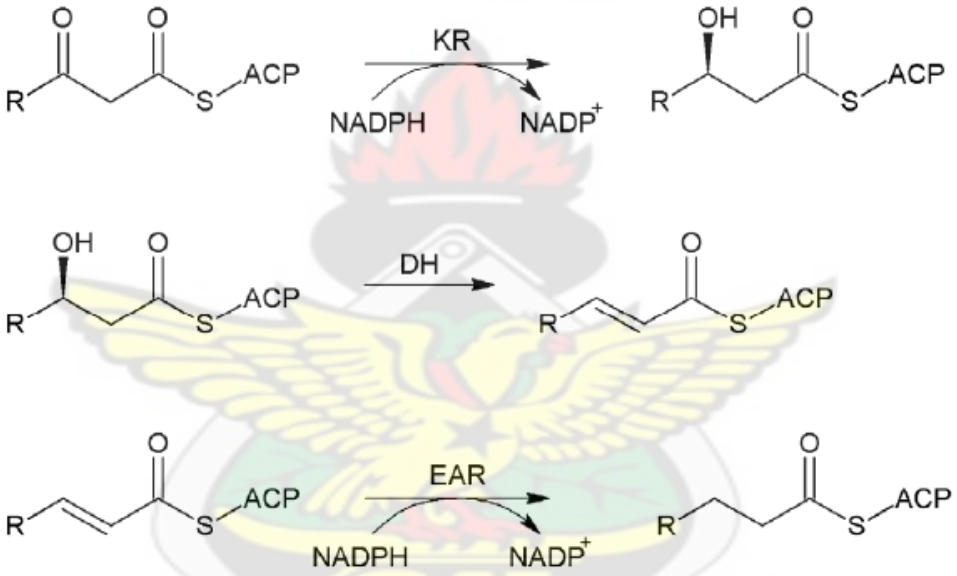


Fig 2.2 Reactions occurring sequentially in fatty acid synthetase: condensation of acyl-ACP and malonyl-ACP mediated by KS, NADPH-dependent reduction of the keto group to a hydroxyl group by means of KR, dehydration to create a double bond with DH and reduction of the double bond by means of EAR. R = H, $CH3(CH2)_{2n}$; n_{max}=7.

The biosynthesis of FA requires the constant supply of acetyl-CoA as initial biosynthetic unit and of malonyl-CoA as the elongation unit, supplying two carbons at each step. Nonoleaginous yeasts receive acetyl-CoA mostly from glycolysis. In oleaginous yeasts,

acetyl-CoA is mostly provided by the cleavage in the cytosol of citrate, which accumulated as aconsequence of nitrogen limitation (Ratledge, 2002) (Fig.2.3). In fact, lipid accumulation byoleaginous fungi does not occur under balanced nutrient conditions.In oleaginous yeasts, nitrogen limitation activates AMP-deaminase (Ratledge& Wynn, 2002), which supply ammonium to the nitrogen-starved cell. As a consequence, mitochondrial AMP concentration decreases, causing isocitrate dehydrogenase activity to drop. The TCA cycle is then blocked at the level of isocitrate, which accumulates and equilibrates with citrate through aconitase. Excess of citrate from TCA cycle is exported out of the mitochondrion via the malate/citrate antiport. Cytosolic ATP-citrate lyase (ACL) cleaves citrate to give oxaloacetate and acetyl-CoA (Fig.2.4).

ACL represents one of the key enzymes that contribute to the oleaginous trait of yeasts, whereas its activity is negligible in non-oleaginous species. ACL is composed of two subunits, encoded by *ACL1* and *ACL2* and is negatively regulated by exogenous FA.Malonyl-CoA is produced from acetyl-CoA by acetyl-CoA carboxylase (ACC) thatcondensate an acetyl-CoA unit with bicarbonate:

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \rightleftharpoons \text{malonyl-CoA} + \text{ADP} + \text{Pi}$$

ACC is also a key enzyme in *de novo* FA synthesis, since *ACC1* mutants became FA auxotrophs or maintain low levels of ACC activity (Tehlivets et al., 2007). ACC1 undergoes allosteric activation by citrate. Furthermore the transcription of *FAS1*, *FAS2*, and *ACC1* is coordinately regulated, being negatively regulated by FA.

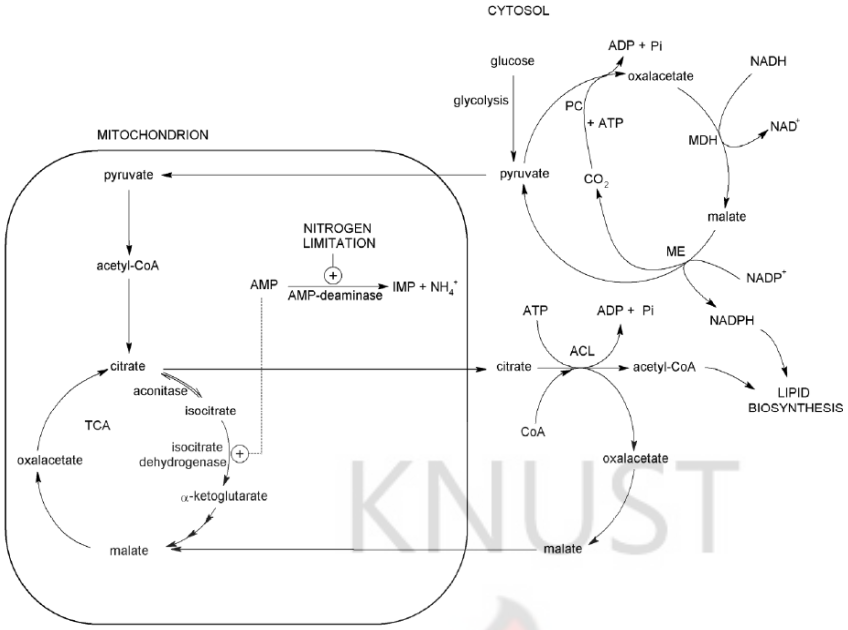


Fig 2.3 Lipid biosynthesis from excess of citrate as a consequence of nitrogen limitation.Adapted from Ratledge, 2004.

Cytosolic NADPH is required for KR and EAR functions of FAS. For each elongation step of the acyl chain, two molecules of NADPH are required. One of the major sources of cytosolic NADPH are the pentose phosphate pathway and the transhydrogenase cycle, which transforms NADH into NADPH through the activity of pyruvate carboxylase (PC), malate dehydrogenase (MDH), and malic enzyme (ME), catalyzing the following reactions:

$$\text{Pyruvate} + CO_2 + \text{ATP} \leftrightarrows \text{oxaloacetate} + \text{ADP} + \text{Pi} \quad \text{(PC)}$$

$$\text{Oxaloacetate} + \text{NADH} \leftrightarrows \text{Malate} + \text{NAD}^+ \quad \text{(MDH)}$$

$$\text{Malate} + \text{NADP}^+ \leftrightarrows \text{pyruvate} + CO_2 + \text{NADPH} \quad \text{(ME)}$$

$$\text{NADH} + \text{NADP}^+ \leftrightarrows \text{NADPH} + \text{NAD}^+$$

ME has been found in several oleaginous fungi and it has been regarded as a key enzyme involved in lipid accumulation (Ratledge, 2002). In *Mortierella circinelloides*, over

expression of ME enhanced lipid accumulation (Zhang *et al*., 2011), whereas over expression of the ME homologous in *Yarrowia lipolytica* did not result in yield improvement.

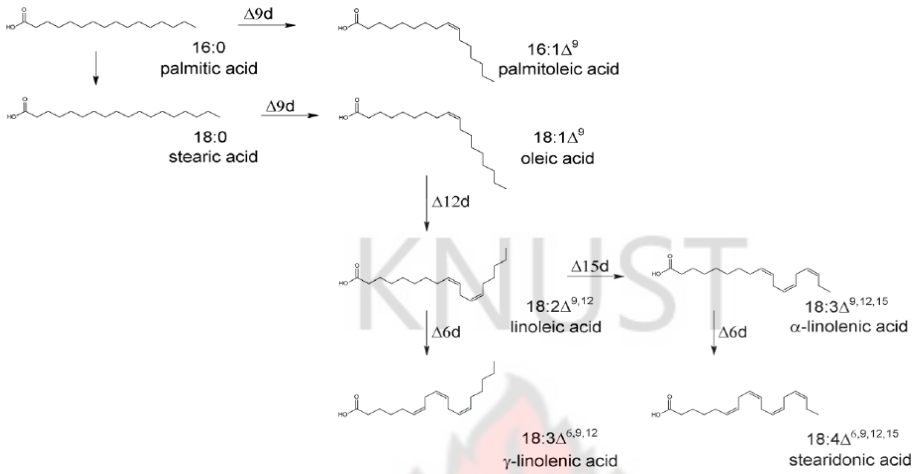


Fig.2.4 Biosynthesis of poly-unsaturated fatty acid.Δ9d, Δ12d and Δ15d are the most common desaturases which are present in the endoplasmic reticulum (Ratledge 2004).

The final products of FAS are myristic or palmitic acids, depending on the yeast species. Reactions resulting in further elongation or desaturation occur in the endoplasmic reticulum(ER). Elongation reactions are catalyzed by elongases (such as malonyl-palmitoiltransacylase, MPT) organized in a complex that requires malonyl-CoA provided by ACC. Desaturations areintroduced by ER desaturases, hydrophobic membrane-bound proteins. The most commondesaturases are Δ9, which inserts the first double bond onto palmitic and/or stearic acids, and Δ12, which catalyzes the insertion of the second unsaturation into oleic acid to produce linoleicacid. Δ6 and Δ15 desaturase activities have been recently described in in psychrophilic oleaginous yeasts, based on production of γand α-linolenic acids, respectively (Fig.2.4).

2.3.3 Biosynthesis of triacyl-glycerol

The fatty acyl-CoA produced by *de novo* synthesis is esterified with glycerol or sterols to produce triacyl-glycerol (TAG) and steryl-esters (SE), respectively. In oleaginous fungi, the neutral lipids SE and TAG are store inside the lipid bodies (LB). TAG are mostly formed by consecutive acylation of glycerol-3-phosphate (G3P), carried out by diverse acyl transferases. G3P is formed from glycerol by glycerol kinase or can be synthesized from dihydroxyacetone phosphate (DHAP) by G3P dehydrogenase, in a reversible reaction. *S.cerevisiae*can use both G3P and DHAP as acyl-group acceptor. The addition of the first acyl group leads to 1-acyl G3P, also named lysophosphatidic acid (LPA). LPA can also be formed by reduction of acyl-DHAP, carried out by a NADPH dependent reductase. A second acyltransferase loads another acyl group, producing 1,2-diacyl G3P (phosphatidic acid, PA).

Phosphate is removed from PA by phosphatidate phosphatase isoenzymes, generating diacylglycerol (DAG). DAG can be the direct precursor of TAG, or can be channeled toward phospholipids biosynthesis (Fig.2.5).

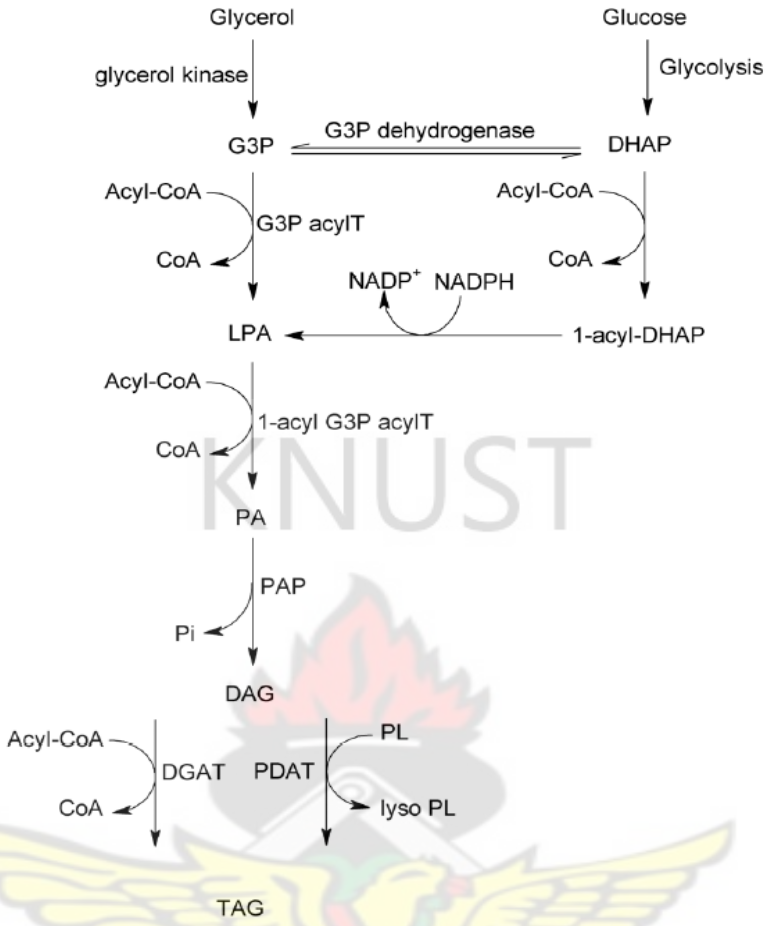


Fig.2 5.*De* novo synthesis of TAG (adapted from Czabany *et al.*, 2007)

The last step of *de novo* synthesis of TAG can be carried either by using diverse acyl donors, such as acyl-CoA or with phospholipids. In the former case, DAG acyl transferases (DGAT), which are integral proteins of the ER, can directly load the third Acyl-CoA. A DGAT enzyme is present in *S. cerevisiae*and *Y. lipolytica*and is mostly active during the stationary phase, although it is expressed also during the exponential phase. A second DGAT, more active during the exponential growth phase, has been identified in *Y. lipolytica.* In *S.cerevisiae,* the phospholipid:DAGacyltransferase (PDAT) is localized in the ER, whereas in *Y.lipolytica*it is present both in the ER and in the surface of LB (Fig.2.5).

2.3.4 Cultivation condition of oleaginous yeasts

Lipid accumulation by oleaginous yeasts depends mostly on nutrient limitation conditions when excess carbon is present in the medium. Nutrient limitation prevents cells from being generated, while the carbon excess is converted into storage TAG. Research reports that, phosphorus, magnesium, zinc, or iron limitation, lead to lipid accumulation in model oleaginous yeasts (Hall & Ratledge, 1977; Beopoulos *et al*., 2009; Wu *et al*., 2010). However, nitrogen limitation is the most efficient form of nutrient limitation for lipogenesis induction, leading to the highest values of substrate/lipid conversion yield and lipid content within biomass (Hall & Ratledge, 1977; Wynn *et al*., 2001). Thus, nitrogen limitation is commonly used to induce lipogenesis in oleaginous fungi and the utilization of cultural media with appropriate C/N ratio is crucial to maximize lipid production.

2.3.5 Batch, fed-batch and fermentation processes

Batch, fed batch, and continuous modes of culture have been developed to culture oleaginous microorganisms. Lipid production in batch cultures is carried out in a cultural medium with a high initial C/N ratio, the carbon source being present in an adequate excess with respect to the nitrogen source. In fact, in this condition, the flow of carbon utilization is limited only by the substrate uptake system of the cell, while the changes in nitrogen concentration determine the passage from a phase of balanced growth to a phase of lipid accumulation, causing the process to proceed through two phases. As nitrogen is consumed from the culture the C/N ratio tends to increase, but growth remains exponential and balanced until nitrogen is not the limiting substrate. During the growth phase, the carbon flow is mostly channeled to satisfy the growth need, therefore growth is balanced and lipid-free biomass is mostly produced (Fig.2.6 ii). As nitrogen concentration becomes limiting, the growth rate and the carbon flow toward biomass generation

decrease, while lipid production is triggered, resulting in a shift of microbial metabolism into the lipogenic phase (Fig.2.6 iii, Fig.2.7).

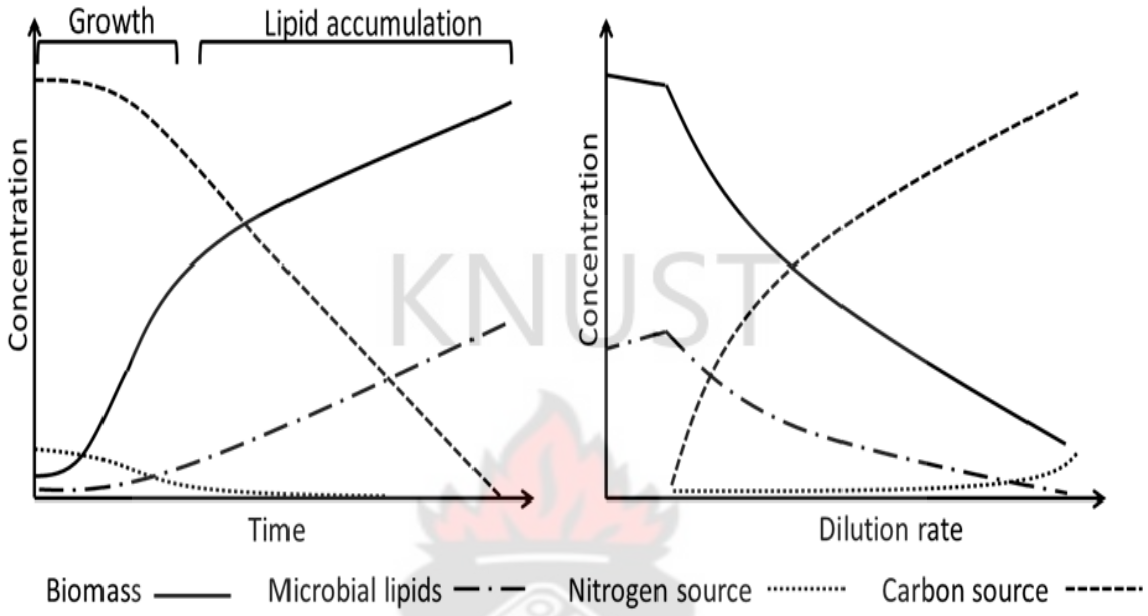


Fig 2.6 Modeling and prediction of the time-course of a batch fermentation (left) and the steady-state values of a continuous process (right) for microbial production of lipids. Axes are in arbitrary scales.

In batch cultures the initial C/N ratio of the cultural medium has a pivotal role in determining the bioprocess performance. In fact, both the rate and the yield of lipid production depend by the C/N ratio, which affects the duration of the exponential phase and the amount of biomass produced during growth. With a fixed carbon concentration, higher amounts of lipid-free biomass produced during the growth phase correspond to higher lipid production rates during the lipogenic phase, but to lower amounts of lipid content within cells and lipid/substrate conversion yields. Therefore, the initial C/N ratio needs to be optimized to maximize lipid productivity in batch cultures. The optimal C/N value is always high (e.g. in the range between 80 and 350 mol/mol) and strongly

depends on the microorganism, the medium composition, the carbon source (e.g. glucose, glycerol, etc.), and the nitrogen source (e.g. diverse organic or inorganic sources). The minimal C/N ratio suitable for lipid accumulation can be estimated as $(Y_{X/S} \cdot q)^{-1}$, where $Y_{X/S}$ is the biomass/carbon source yield coefficient under conditions of carbon limitations (C-mol/Cmol) and q is the nitrogen/carbon content of biomass (N-mol/C-mol) (Ykema *et al.*, 1986).

Unlike batch processes, in fed-batch mode, nutrients are fed into the bioreactor in a controlled manner, with the purpose to monitor and control the specific growth rate and the flows of nitrogen and carbon utilization. Through the judicious management of the feeding rate and composition, it is possible to control the C/N ratio within the culture and maintain the oleaginous microorganism in the optimal metabolic status, as appropriate, first for the growth phase, and later for the lipogenic phase. The lipogenic phase is the most extensive, corresponding to lipid production under nitrogen limitation, with constant C/N ratio, preventing loss of viability and acids production (Beopoulos et al., 2009a).

In continuous cultures, at the steady state, the assimilation of C and N sources and the microbial growth occur at constant rates, which ultimately depend by the dilution rate (D).

The concentration of the substrates within the bioreactor is steady and depends on the dilution rate as well, the actual C/N ratio of the culture remaining constant unlike in batch cultures. Likewise in batch cultures, in continuous cultures the C/N ratio of the fresh medium needs to be higher than $(Y_{X/S} \cdot q)^{-1}$ to obtain some lipid accumulation (Ykema *et al.*, 1986). However, at the steady-state with this medium, the C/N ratio within the culture is higher than in the fresh medium, due to nitrogen consumption.

2.3.6 Substrates and raw material

The demand for the inexpensive production of biofuels has intensified due to increasing concerns of climate change, depletion of petroleum-based fuels, and environmental problems. In a market economy, corporations aim to maximize profit, seeking the most competitive feedstock. To produce single-cell oils for biodiesel production, the carbon source has necessarily to be cheap and available in large quantities. Therefore, while the first investigations on oleaginous fungi most commonly employed glucose as carbon source, nowadays the production of single-cell oils is predominantly addressed to transformation of raw materials, by-products and surplus.

Glucose is the carbon source most commonly employed for growth of oleaginous fungi and lipid production (Boulton and Ratledge, 1984; Hansson and Dostalek, 1986; Hassan *et al.*, 1993; Heredia and Ratledge, 1988; Jacob, 1991; Jacob, 1992; Johnson et al., 1992; Li et al., 2007; Pan *et al*., 1986; Ratledge, 2004; Rau *et al*., 2005; Saxena *et al.*, 2008; Zhao *et al*., 2008). High glucose concentrations enhance the carbon flow that is directed toward TAG production, thus improving lipid production in several yeasts. However, growth of some yeast (e.g. *R. toruloides)* is inhibited by high concentration of glucose, (Li et al., 2007). Furthermore, in batch cultures, initial glucose concentration also affects the fatty acids composition of the lipids (Amaretti *et al.*, 2010).

Carbon sources other than glucose, such as xylose (Chistopher *et al.*, 1983; Heredia and Ratledge, 1988;), lactose (Christopher *et al.*, 1983; Daniel et al., 1999;), arabinose, mannose (Hansson and Dostalek, 1986), mannitol (Hansson and Dostalek, 1986), ethanol (Chistopher *et al*., 1983; Eroshin and Krylova, 1983), have been also investigated in the 80s and 90s for the production of microbial lipids.

Albeit glucose is a very good carbon source for lipid production with oleaginous fungi, molasses, which carbohydrate fraction is mainly composed of sucrose, glucose, and fructose, do not represent a promising raw material for lipid production, since they are characterized by a high nitrogen content which delays the unbalanced growth, where number of cells cannot augment anymore and lipids are accumulated (Johnson *et al*., 1995).

Carbons sources obtained from lignocellulosic biomasses represent one of the most important potential to produce biodiesel. In fact, several waste biomasses containing forest residues, agricultural residues, food wastes, municipal wastes, and animal wastes can be utilized for the production of lignocellulosic based microbial lipids. Microbial oil production from sulphuric acid treated rice straw hydrolysate (SARSH) by the yeast *Trichosporon fermentans* pointed out the difficulty to perform the process of lipid accumulation in presence of the inhibitory compounds released during hydrolysis, such as acetic acid, furfural, 5-hydroxymethylfurfural, and water soluble lignin (Huang *et al*., 2009). Selected strains were able to grow on xylose and glucose (Zhu et al., 2008), but the crude hydrolysate did not result an optimal substrate for a high yield process of lipid production. Cellulose and hemicellulose are generally hardly hydrolyzed and assimilated by yeasts, while they can be degraded and used as carbon source by filamentous fungi. A screening of endophytic fungi from the oleaginous plants was the selection of strains belonging to the genera *Microsphaeropsis, Phomopsis, Cephalosporium, Sclerocystis*and *Nigrospora*that simultaneously accumulated lipids (21.3 to 35.0% of dry weight) and produced cellulase (Peng and Chen, 2007). Albeit these strains could be exploited as microbial oil producers by utilizing straw as substrate, they have never been claimed again as a SCO producers on lingo-cellulosic biomass. Attempts to carry out lipid production in Solid State Fermentation (SSF) on wheat straw have been performed

exploiting a cellulolytic strain of *Aspergillus oryzae* (Lin *et al*., 2010). This strain is able to use cellulose as substrate and accumulate lipids in a low cost fermentation system on this abundant cellulosic by-product.

Other complex matrices have been used, such as solids from wheat bran fermentation (Jacob, 1991), sewage sludge (Angerbauer *et al*., 2008), wastewaters of animal fat treatment (Papanikolaou et al., 2002), whey derivatives (Ykema *et al*., 1989; Vamvakaki et al., 2010), olive oil mill wastewaters (Yousuf *et al*., 2010), and tomato waste hydrolysate (Fakas *et al*., 2008). Fei et al, 2011 have also used pure volatile fatty acids (VFAs) as substrate for lipid accumulation by oleaginous yeast for biodiesel production. The VFAs used were acetic acid, propionic acid and butyric acid in certain ratios. This work seeks to investigate this possibility of using pure VFAs for lipid accumulation by the oleaginous yeast C. albidus.

CHAPTER THREE

METHODOLOGY

3.1 Collection and Regeneration of *C.albidus*

A sample of *C. albidus* (ATCC 10672) was obtained from America Type Culture Collection (ATCC). *C. albidus* was rehydrated by adding 1ml sterile distilled water to the freeze- dried pellet, drawing up the entire contents into a 1000µl pipette and transferring to a test tube with 5ml sterile distilled water. *C. albidus* was allowed to rehydrate for 2hours 30minutes. *C. albidus* was revived and grown on yeast malt (YM) medium.

3.1.1 Preparation of YM medium

One litre YM medium was prepared by adding 3g yeast extract, 3g malt extract, 10g dextrose, 5g peptone and 20g agar to 1 litre of distilled water. The mixture formed was well stirred and the pH adjusted to 6 using 2M HCl and 2M NaOH solutions. The mixture was autoclaved at 121^{o}C for 15 minutes. The YM medium was allowed to cool after autoclaving and poured into petri dishes. This medium was used to grow *C. albidus.*

3.1.2 Growth of *C. albidus* on YM Medium

The revived *C. albidus* (100 µl) was discharged on to YM medium in petri dishes. With the aid of a sterilized L- shaped glass rod the suspension of *C. albidus* was spread on the surface of the medium. The plated organism was incubated at 25^{o}C for 72 hours. After 72 hours subculturing was done to obtain healthy growing *C. albidus*. These cultures were stored in a refrigerator and subcultured and used in experiments whenever required.

3.2 Investigation into the use of synthetic VFAs by *C. albidus*

One litre basal medium was prepared by adding 1g NH_4Cl , 3g KH_2PO_4 , 1g $MgSO_47H_2O$, 15mg $FeCl_36\ H_2O$, 7.5mg, $ZnSO_47H_2O$ to 1 lire of distilled water. The

mixture formed was well stirred and autoclaved at 121^{o}C for 15 minutes. The basal medium was allowed to cool after autoclaving and 5g synthetic VFAs (acetic acid, propionic acid, butyric acid) was added using a 0.2µm filter and the pH adjusted to 6 using 2M HCl and 2M NaOH solutions. To prevent the formation and precipitation of $MgPO_4$, $MgSO_4.7H_2O$ was autoclaved separately and added to the medium using a 0.2 µm filter. Experiments were performed in 500ml flasks containing 300ml of basal medium and VFAs. In all experiments, the ratio of VFAs (acetic acid: propionic acid: butyric acid) was 6:1:3. An experimental control was set up to help determine changes taking place in experimental flasks. The control contained all other constituents except *C. albidus* cells.

3.2.1 Inoculation

The medium was inoculated with $3*10^7$ of *C.albidus* cell and cultivated at 25^{o}C. Cell density was calculated using the formula adapted from Fuchs – Rosenthal Counting Chamber. Cell suspension was prepared and a haemocytometer and cover slip was prepared, ready for use. Approximately 9 micro liters of the cell suspension was pipette into counting chambers. The haemocytometer was mounted on a microscope and the microscope adjusted till cells could be clearly seen and cells counted from chamber to chamber. In order to determine the cell count, the total cells in each chamber was counted and the average number of cells in all the chambers calculated.

3.2.2 Harvesting of cells and lipid extraction

Optical density readings at 600 nm were taken to measure cell growth. Cells were harvested after 48 hours and centrifuged at 5300 rpm for 30 minutes. The resulting biomass pellets was rinsed twice with 0.9% NaCl for lipid extraction.

The biomass was homogenized with hexane isopropanol alcohol distilled water solvent mixture. The mixture was allowed to settle and stay overnight. A 0.45µm filter paper was used to filter homogenate with the help of vacuum pump, Buchner funnel and Erlenmeyer flask.

A heating mantle was set to a temperature of 50^{o}C. Filtrate was poured into a round bottom flask of soxhlet apparatus. The round bottom flask was placed on the heating mantle to recover solvent. After about 15 minutes the content of flask was poured into a crucible and further put in an oven set at 105^{o}C to dry off remaining solvent for 1 hour. The crucible and its content were cooled in a desiccator. After cooling, the weight of crucible and content were determined. The drying process was repeated until a constant weight for crucible and content was achieved. The mass of lipids is finally determined if any is collected in the crucible.

3.3 Investigation into the use of VFAs from faecal sludge by *C. albidus*

One litre basal medium was prepared by adding 1g NH_4Cl , 3g KH_2PO_4 , 1g $MgSO_47H_2O$, 15mg $FeCl_36\ H_2O$, 7.5mg, $ZnSO_47H_2O$ to 1 lire of distilled water. The mixture formed was well stirred and autoclaved at 121^{o}C for 15 minutes. The basal medium was allowed to cool after autoclaving and 5g of faecal sludge filtrate was added using a 0.2µm filter and the pH adjusted to 6 using 2M HCl and 2M NaOH solutions. To prevent the formation and precipitation of $MgPO_4$, $MgSO_4.7H_2O$ was autoclaved

separately and added to the medium using a 0.2 µm filter. Experiments were performed in 500ml flasks containing 300ml of basal medium and VFAs from faecal sludge. In all experiments a control was set up to help determine changes taking place in experimental flasks. The control contained all other constituents except *C. albidus* cells.

The medium was inoculated with $3*10^7$ of *C.albidus* cell and cultivated at 25^{o}C. Optical density readings at 600 nm were taken to measure cell growth. Cells were harvested after 48 hours and centrifuged at 5300 rpm for 30 minutes. The resulting biomass pellets was rinsed twice with 0.9% NaCl for lipid extraction. Lipid extraction was carried out as described in3.2.2 above.

3.3.1 Determination of concentration of VFAs in faecal sludge

Twenty five (25ml) of faecal sludge sample was filtered through 1.6µmglass fiber filter paper. Half a milliliter (0.5 ml) of the filtrate was pipette into a dry 25ml sample cell and 1.5ml ethyleneglycol and 0.2ml 19.2 N sulfuric acid standard solution added to filtrate. The mixture was swirled to mix. The sample cell with its content was placed in a water bath for 3 minutes and allowed to cool. 0.5mlof hydroxylamine hydrochloride solution, 2.0 ml of 4.5 N sodium hydroxide standard solution, 10 ml of ferric chloride sulfuric acid solution and 10 ml of distilled water was added to mixture in sample cell and mixed thoroughly by swirling. The mixture was allowed to settle for 3 minutes. Absorbance of mixture was taken at a wavelength of 495nm to determine the concentration of VFAs in faecal sludge.

3.4 Effect of nitrogen sources on *C. albidus* growth

Ammonium chloride, urea and yeast extract were used as nitrogen sources in separate experiments with an initial concentration of 1g per liter. These nitrogen sources formed part of basal medium prepared for the experiments

3.5 Counting Chamber

Cell density was calculated using the formula adapted from Fuchs – Rosenthal Counting Chamber. Cell suspension was prepared and a haemocytometer and cover slip was prepared, ready for use. Approximately 9 micro liters of the cell suspension was pipette into counting chambers. The haemocytometer was mounted on a microscope and the microscope adjusted till cells could be clearly seen and cells counted from chamber to chamber. In order to determine the cell count, the total cells in each chamber was counted and the average number of cells in all the chambers calculated. That is, the cell count was determined as follows:

Depth of haemocytometer = 0.1mm

Area of smallest square = $0.0025mm^2$

Volume of smallest square = $0.1mm \times 0.0025mm^2 = 0.00025mm^3$

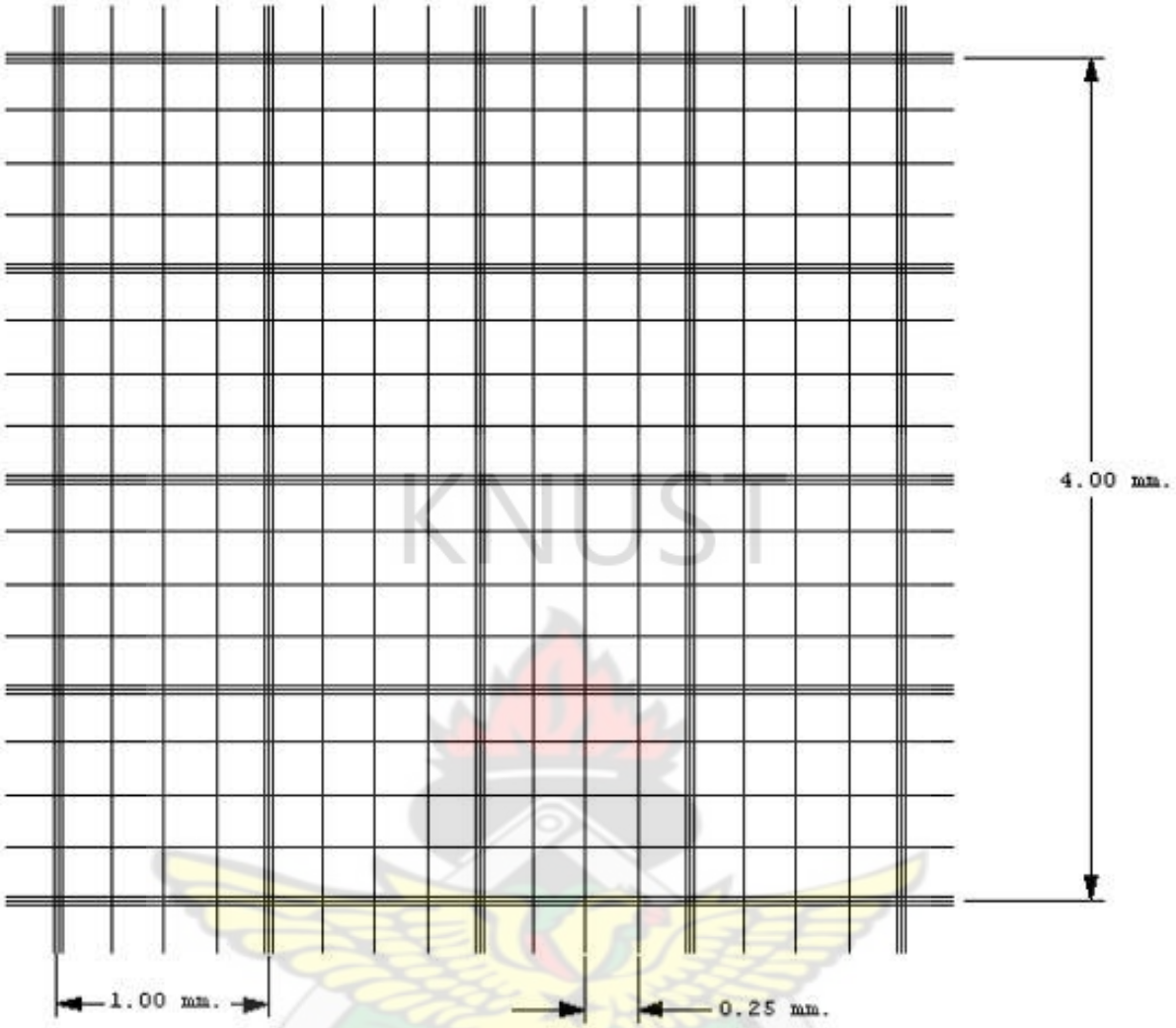
$1ml = 1000mm^3$

Volume of 80 squares (5×16) = $80 \times 0.00025mm^3$

Number of spores per ml = Number of cells in 80 squares × 10000

= 12.5 × 80 × 10000

= 1× 10^7 cells/ml



CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Investigation into the use of synthetic VFAs by *C. albidus*

Table 4.1 shows the optical density readings of *C. albidus* grown for 30 hours on synthetic VFAs. From Fig. 4.1, it was observed that the initial optical density of *C. abidus* which was 0.228 rose steadily to 0.381 at the 7th hour. This represents an increase of 0.153 in optical density units which is similar to work done by Chandran *et al* (2013) over the same period. However, the optical density began to decrease gradually to 0.098 till cells were harvested after 30 hours of growth. This was not the case in the work done by Chandran *et al*, who had an increase in optical density of 0.680 over the same period till cells reached stationary phase and harvested. The decrease in cell growth in this work can be attributed to the fact that *C. albidus* utilized the VFAs present in the early stage of its growth and there were no more carbon sources for *C. albidus* to feed on.

Table 4.1Growth of *C. albidus* using synthetic VFAs as carbon source

Time(hr)	Mean Absorbance	Standard Deviation	Absorbance(Optical Density) @ 95% Confidence level
0	0.228	0.014	0.228 ± 0.014
1	0.249	0.014	0.249 ± 0.014
3	0.251	0.016	0.251 ± 0.016
4	0.266	0.01	0.266 ± 0.010
5	0.277	0.014	0.277 ± 0.014
7	0.381	0.02	0.381 ± 0.020
8	0.289	0.022	0.289 ± 0.022
9	0.288	0.029	0.288 ± 0.029
24	0.122	0.093	0.122 ± 0.093
25	0.120	0.020	0.120 ± 0.012
26	0.110	0.022	0.110 ± 0.014
27	0.110	0.018	0.110 ± 0.011
28	0.099	0.012	0.099 ± 0.009
29	0.098	0.011	0.098 ± 0.009
30	0.098	0.021	0.098 ± 0.011

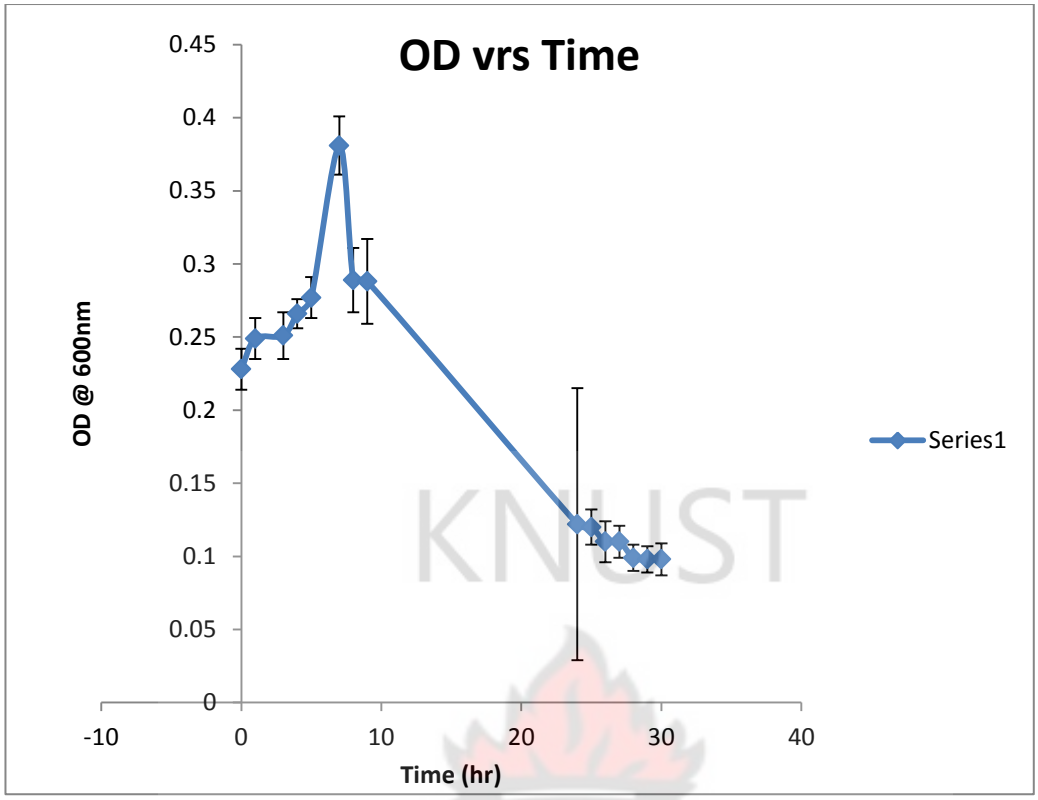


Figure 4.1 Cell growth of *C.albidus*. Cultivation conditions: 25^{o}C, pH 6.0 cultured for 30hrs. VFAs concentration was 5 g/l with ratio 6:1:3 in these cultures. Nitrogen source was NH_4Cl with concentration of 1 g/l

4.2 Investigation into the use of VFAs from faecal sludge by *C. albidus*

In Table 4.2a there was generally a decrease in optical density reading of *C. albidus* grown on faecal sludge with VFA concentration of 667mg/l. From an initial 0.071, optical density decreased to a low of -0.539 over 48 hours period of growth. A similar trend is observed in Table 4.2b that gives optical density readings of *C. albidus* on faecal sludge with VFA concentration of 334mg/l. These results could be attributed to the low concentration of VFAs in the faecal sludge used which could not support any significant growth *C. albidus*. It is also possible that the faecal sludge could also contain other easily available carbon sources that the organism may metabolize instead of the VFAs in the

faecal sludge. The faecal sludge medium is a very complex medium. The possible presence of inhibitors that can affect growth of *C. albidus* may explain the poor growth realized.

Table 4.2a Growth of *C. albidus* using faecal sludge with VFAs concentration of 667mg/l

Time(hr)	Average Absorbance	Standard Deviation	Absorbance(Optical Density) @ 95% Confidence level
0	0.071	0.012	0.071 ±0.012
1	0.031	0.003	0.032 ± 0.003
2	0.068	0.004	0.068 ± 0.004
3	0.063	0.004	0.063 ± 0.004
4	0.14	0.064	0.14 ± 0.064
24	-0.342	0.053	-0.342 ± 0.053
28	-0.460	0.078	-0.460 ± 0.078
29	-0.480	0.256	-0.480 ± 0.256
48	-0.539	0.204	-0.539 ± 0.204

Table 4.2b Growth of *C. albidus* using faecal sludge with VFA concentration of 334mg/l

Time(hr)	Average Absorbance	Standard Deviation	Absorbance(Optical Density) @ 95% Confidence level
0	0.098	0.061	0.098 ± 0.061
1	0.036	0.004	0.036 ±0.004
2	0.035	0.005	0.035 ±0.005
3	0.039	0.002	0.039 ± 0.002
4	0.066	0.043	0.066 ± 0.043
24	-0.307	0.006	-0.307 ±0.006
28	-0.341	0.047	-0.341 ± 0.122
29	-0.391	0.019	-0.391 ± 0.113
48	-0.409	0.031	-0.409 ± 0.078

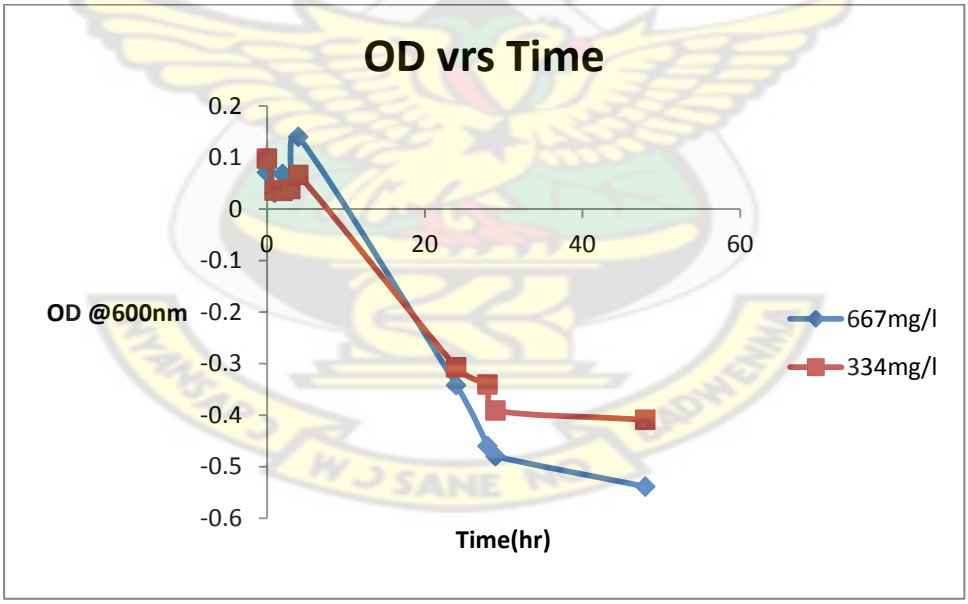


Figure 4.2Cell growth of *C.albidus* on faecal sludge withVFA concentration 667 mg/l and 334 mg/l. Cultivation conditions: 25°C, pH 6.0 cultured for 48hrs. Nitrogen source was NH_4Cl with concentration of 1 g/l

4.3 Effect of different nitrogen sources on *C. albidus* growth and lipid accumulation

The effect of different nitrogen sources on *C. albidus* growth was investigated. It was observed that out of the three nitrogen sources, yeast extract recorded the highest optical density from an initial reading of 0.052 to 0.104 which represent an increase of 0.052 as can be seen in Table 4.3a. Table 4.3c shows optical density readings when urea was used as nitrogen source with an overall increase of 0.025 from an initial reading of 0.042 to a high of 0.067. The lowest optical density was recorded in Table 4.3b when ammonium chloride was used as nitrogen source. From Table 4.3b there was a slight increase of 0.007 in optical density from an initial 0.067 to 0.074 which further decreased to 0.057. Figure 4.3 summarizes Table 4.3a,b,c on a chart. This finding is in agreement with a report by Fickers *et al*, 2004 who reported that organic nitrogen sources supports growth of oleaginous organisms better than inorganic nitrogen sources.

Though there was a slight growth of *C. albidus* in all the nitrogen sources used, no lipid was accumulated in the cells of *C. albidus* as can be seen in Table 4.4

Table 4.3a Effect of Yeast Extract on *C. albidus* growth

Time	Average Absorbance	Standard Deviation	Absorbance(Optical Density) @ 95% Confidence level
0	0.052	0.005	0.052 ± 0.005
1	0.066	0.009	0.066 ± 0.009
2	0.063	0.003	0.063 ± 0.003
3	0.062	0.003	0.062 ± 0.003
4	0.064	0.005	0.064 ± 0.005
5	0.066	0.008	0.066 ± 0.008
24	0.068	0.015	0.068 ± 0.015
26	0.067	0.017	0.067 ± 0.017
27	0.071	0.017	0.071 ± 0.017
28	0.079	0.019	0.079 ± 0.019
29	0.074	0.021	0.074 ± 0.021
48	0.104	0.032	0.104 ± 0.032
50	0.103	0.025	0.103 ± 0.025
52	0.103	0.018	0.103 ± 0.018

Table 4.3b Effect of Ammonium Chloride *C.albidus*growth

Time	Average Absorbance	Standard Deviation	Absorbance(Optical Density) @ 95% Confidence level
0	0.067	0.014	0.067 ± 0.014
1	0.074	0.003	0.074 ± 0.003
2	0.072	0.005	0.072 ± 0.005
3	0.068	0.004	0.068 ± 0.004
4	0.065	0.004	0.065 ± 0.004
5	0.066	0.005	0.066 ± 0.005
24	0.072	0.002	0.072 ± 0.002
26	0.063	0.001	0.063 ± 0.001
27	0.062	0.004	0.062 ± 0.004
28	0.060	0.009	0.060 ± 0.009
29	0.062	0.012	0.062 ± 0.012
48	0.060	0.011	0.060± 0.011
50	0.058	0.003	0.058 ± 0.003
52	0.057	0.008	0.057 ± 0.008

Table 4.3c Effect of Urea on *C. albidus* growth

Time	Average Absorbance	Standard Deviation	Absorbance(Optical Density) @ 95% Confidence level
0	0.042	0.003	0.042 ± 0.003
1	0.059	0.005	0.059 ± 0.005
2	0.061	0.003	0.061 ± 0.003
3	0.062	0.005	0.062 ± 0.005
4	0.053	0.002	0.053 ± 0.002
5	0.067	0.007	0.067 ± 0.007
24	0.056	0.006	0.056 ± 0.006
26	0.059	0.001	0.059 ± 0.001
27	0.061	0.005	0.061 ± 0.005
28	0.061	0.005	0.061 ± 0.005
29	0.062	0.005	0.062 ± 0.005
48	0.065	0.008	0.065 ± 0.008
50	0.064	0.006	0.064 ± 0.006
52	0.063	0.031	0.063 ± 0.031

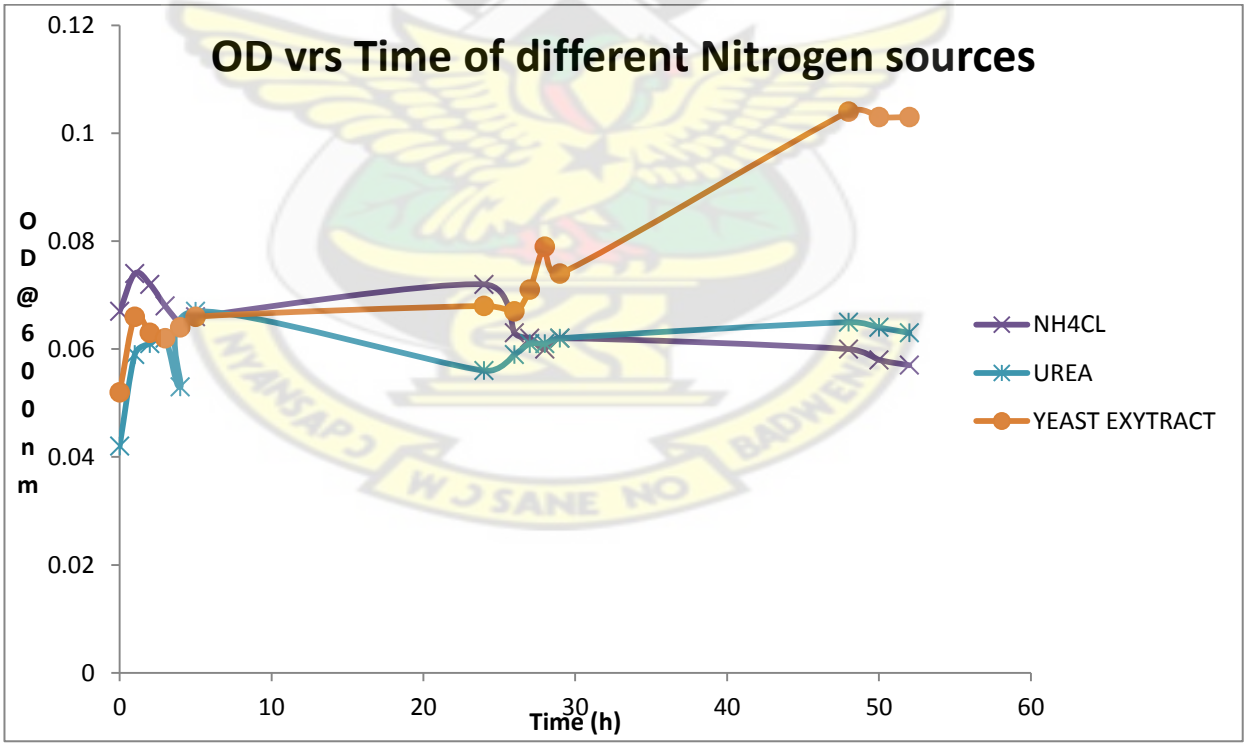


Figure 4.3 Cell growth of *C.albidus* with different nitrogen sources. Cultivation conditions: 25°C, pH 6.0 cultured for 48 h. VFAs concentration was 5 g/l with ratio 6:1:3 in these cultures. Nitrogen sources concentration 1 g/l

4.4 Lipid Extraction

Table 4.4Lipids Results

Sample (Different Nitrogen Source)	Weight of crucible (g)	Weight of crucible + solvent mixture + lipids (g)	Weight of crucible after solvent recovery (g)	Weight of lipids (g)
Ammonium Chloride	52.4204	120.8204	52.4204	0
Urea	55.3651	123.7651	55.3651	0
Yeast Extract	53.9226	122.3226	53.9226	0

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusions from Study Results

5.1.1 Investigation into the use of synthetic VFAs by *C. albidus*

From the results, it shows that *C. albidus* could not utilize synthetic VFAs as sole carbon source for growth and hence no lipid was accumulated.

5.1.2 Investigation into the use of VFAs from faecal sludge by *C. albidus*

C. albidus did not grow on faecal sludge. This could be due to the low concentration of VFAs in the faecal sludge (667mg/l and 334mg/l respectively). Other constituents of faecal sludge possibly could inhibit growth. It is also possible that *C. albidus* may not be the appropriate organism for the process.

5.1.3 Effect of different nitrogen sources on *C. albidus* growth and lipid accumulation

The results show that all three nitrogen sources did not have any effect on lipid accumulation as no lipid was extracted. However in terms of supporting growth, yeast extract recorded the highest growth though not very significant.

5.2 Recommendations for Future Studies

The results in this study are useful in understanding the possibility of using oleaginous microorganism for lipid accumulation for biodiesel production using VFAs as sole carbon sources. However it is worth noting that the right kind of organism, VFA source and composition and nitrogen source is used. Some suggestions for future research in this area are presented as follows:

1. Identifying, isolating and using indigenous oleaginous organism to save cost and time of importing genetically engineered organism like *C. albidus.* Also indigenous organisms can better adapt to the local environment and hence perform a better function of utilizing VFAs for lipid accumulation.
2. It will be necessary to track VFA consumption. This will justify claims as to whether organisms are feeding on VFAs or not.
3. Research on separate methods of determining the accumulation of lipids in cells while fermentation is ongoing. This will help give a clue as to what parameter needs to be varied. Rather than having to wait till the whole process is over.
4. Using VFAs from natural sources with high VFA concentration to save cost of using pure VFAs.
5. The composition of faecal sludge must be fully understood. It is very difficult to establish if a constant composition of faecal sludge exist.

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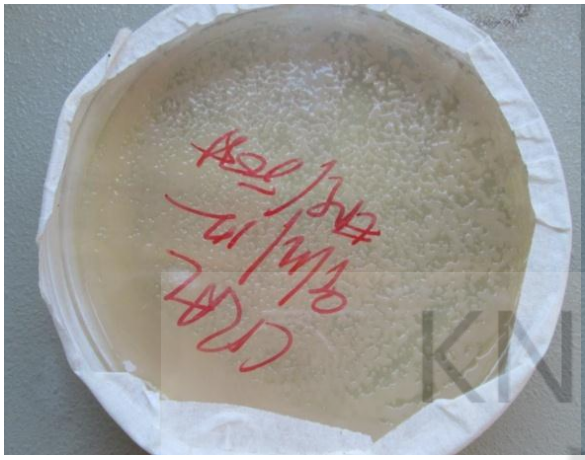
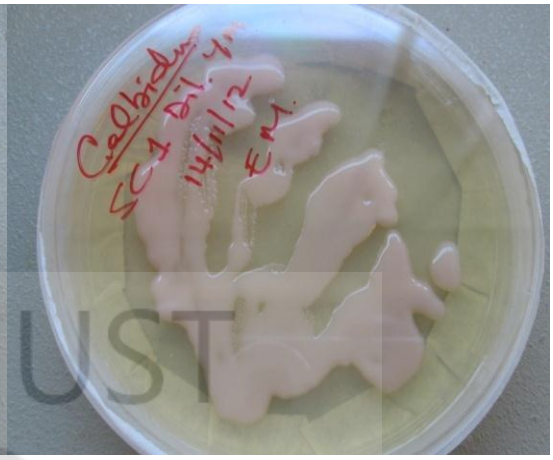
APPENDICES

APPENDIX A: PICTURES FROM STUDY



C. albidus **in petri dish after rehydration**



C. albidus **after subculturing**

Inoculation process

C. albidus **growing in medium**

APPENDIX B: RESULTS

Appendix B1Growth of *C. albidus* using synthetic VFAs as carbon source

Sample	Absorbance(Optical Density)														
	0h	1h	3h	4h	5h	7h	8h	9h	24h	25h	26h	27h	28h	29h	30h
A	0.212	0.233	0.234	0.254	0.262	0.361	0.264	0.255	0.153	0.14	0.135	0.13	0.1	0.11	0.12
B	0.237	0.258	0.264	0.272	0.279	0.4	0.308	0.3	0.196	0.1	0.1	0.105	0.11	0.09	0.078
C	0.234	0.257	0.256	0.271	0.289	0.383	0.294	0.308	0.018	0.12	0.095	0.095	0.087	0.094	0.096

Appendix B2 Growth of *C. albidus* using VFAs (667mg/l) from faecal sludge as carbon source

Sample	Absorbance(Optical Density)								
	0h	1h	2h	3h	4h	24h	28h	29h	48h
A	0.085	0.078	0.071	0.065	0.079	-0.281	-0.37	-0.413	-0.498
B	0.066	0.078	0.07	0.065	0.135	-0.377	-0.502	-0.51	-0.554
C	0.063	0.084	0.064	0.058	0.207	-0.367	-0.509	-0.517	-0.566

Appendix B3 Growth of *C. albidus* using VFAs (334mg/l) from faecal sludge as carbon source

Sample	Absorbance								
	0hr	1hr	2hr	3hr	4hr	24hr	28hr	29hr	48hr
A	0.147	0.04	0.03	0.04	0.035	-0.31	-0.38	-0.39	-0.4
B	0.118	0.03	0.04	0.04	0.048	-0.3	-0.36	-0.37	-0.39
C	0.03	0.04	0.04	0.04	0.115		-0.29	-0.41	-0.44

Appendix B4 Effect of Ammonium Chloride *C.albidus*growth

Sample	Absorbance													
	0h	1h	2h	3h	4h	5h	24h	26h	27h	28h	29h	48h	50h	52h
A	0.082	0.077	0.072	0.071	0.068	0.07	0.072	0.062	0.059	0.057	0.057	0.05	0.054	0.05
B	0.056	0.071	0.067	0.064	0.06	0.06	0.071	0.064	0.06	0.07	0.076	0.071	0.06	0.065
C	0.062	0.073	0.076	0.069	0.067	0.068	0.074	0.062	0.067	0.053	0.053	0.059	0.06	0.056

Appendix B5 Effect of Yeast Extract on *C. albidus* growth

Sample	Absorbance													
	0h	1h	2h	3h	4h	5h	24h	26h	27h	28h	29h	48h	50h	52h
A	0.053	0.069	0.06	0.062	0.068	0.066	0.069	0.07	0.072	0.076	0.074	0.131	0.126	0.12
B	0.056	0.073	0.065	0.065	0.066	0.074	0.082	0.083	0.088	0.1	0.095	0.113	0.106	0.104
C	0.046	0.056	0.064	0.059	0.058	0.059	0.053	0.049	0.054	0.062	0.054	0.068	0.077	0.085

Appendix B6 Effect of Urea on *C. albidus* growth

Sample	Absorbance													
	0h	1h	2h	3h	4h	5h	24h	26h	27h	28h	29h	48h	50h	52h
A	0.045	0.065	0.064	0.06	0.052	0.063	0.057	0.06	0.063	0.063	0.064	0.074	0.068	0.061
B	0.041	0.055	0.061	0.068	0.052	0.075	0.062	0.058	0.065	0.065	0.057	0.062	0.057	0.033
C	0.039	0.058	0.058	0.058	0.056	0.063	0.05	0.058	0.056	0.056	0.066	0.058	0.066	0.096