Growth Kinetics and Lipid Accumulation in

Rhodosporidium toruloides

2010

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Growth kinetics and lipid accumulation in *Rhodosporidium toruloides*

ABSTRACT

The current interest in oleaginous yeasts as a viable source for microbial oil production has led to increasing research in understanding the growth conditions and kinetics of oleaginous yeasts with the aim to optimise productivity. Microbial oils can be used as food supplements and feedstock for biodiesel production, with the current increase in prices of fossil fuel and food, microbial oil is undoubtedly a perfect renewable feedstock alternative for biodiesel production. However the set back in using microbial oil is the high cost of production especial the cost of fermentation. Recently, an oleaginous yeast *Rhodosporidium toruloides* has attracted great interest in microbial oil production because of its high lipid content and cell density. In this study, growth of *Rhodosporidium toruloides* on glucose, pure glycerol and crude glycerol were studied with the aim to understand its ability to utilize a by-product from biodiesel processing plant as a cheap carbon source.

The result of this study showed that *Rhodosporidium toruloides* could use glucose, pure glycerol and crude glycerol as carbon source however; while it can grow on high concentration of 100 g/L of glucose and pure glycerol; there was growth inhibition at concentrations of crude glycerol higher than 20g/L as a result of toxic and inhibitory effects of impurities in crude glycerol. *R. toruloides* accumulated a Lipid content of 47.61% and biomass of 10.55 g/L when grown on glucose. Likewise, a lipid content of 41% and 13.09 g/l biomass in pure glycerol were achieved.

R. toruloides can utilize crude glycerol as carbon source and therefore further research in optimizing lipid productivity using crude glycerol as carbon source is recommended.

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CHAPTER ONE INTRODUCTION

The unsustainable nature and increase in prices of fossil fuels have led to current increase in research on alternatives to petroleum-based fuels (Ratledge, 1991; Knothe, 2010). Renewable alternative fuel is needed because fossil fuels have depleting supplies; in addition, combustion of fossil fuels emits carbon dioxide which contributes to global warming. Biomass promises a better future in development of alternatives to fossil fuels because of its sustainable nature. Biomass is not only been explored for biofuel production but also for possibilities of producing alternative petroleum-based chemicals such as polyhydroxybutyrate (Koutinas *et al*., 2007).

Oleaginous microorganisms could be used for production of microbial oil as an alternative feedstock for biodiesel production due to the fact that they grow faster than plants, require no arable land for its cultivation, and are sustainable (Rittman, 2008; Chisti, 2007). Among all the oleaginous microorganisms, oleaginous yeast is the subject of this research because of its high growth rate, lipid content and ability to accumulate high cell density (Li *et al*., 2007). However, a challenge to producing oil from yeast is the high fermentation cost; consequently, wastes such as Jerusalem artichoke (Zhao *et al*., 2007) and sewage sludge (Angerbauer *et al.,* 2008) have been used as a cheap carbon source to grow oleaginous yeasts.

Cost of microbial oil production from yeast can also be reduced through improve productivity; culture conditions has been optimised (Li *et al*., 2006) to improve lipid productivity of *Rhodosporidium toruloides*.

Different oleaginous yeast species have different growth conditions; therefore an intensive study of any oleaginous yeast is needed in order to maximize its productivity.

Knowledge of the growth kinetics is important for the prediction of growth rate, productivity and lipid yield under different growth conditions. In oleaginous yeast, the prediction of these parameters will help in deciding whether oil production from yeast will be commercially feasible.

The oleaginous yeast investigated was *R. toruloides*. This oleaginous yeast is known for its high lipid content and high cell density when grown on glucose (Li *et al*., 2007).

In this research, the possibilities of producing oil from *R. toruloides* were explored through the study of its growth kinetics. The growth of yeast on glucose and pure glycerol were compared; in addition, influence of organic and inorganic nitrogen sources on biomass accumulation were investigated.

The results of this study answered the following questions;

i.

ould there be any different in biomass accumulation of oleaginous yeast grown under the same carbon to nitrogen ratio but different nitrogen source?

ii.

ould the presence of impurities in a non conventional carbon source affect its utilization as carbon source by oleaginous yeast?

iii.

an by-product from transesterifcation process be use as carbon source for oleaginous yeast?

iv.

oes oleaginous yeast grow better in carbon source containing six carbons (C_6) or three carbons (C_3)?

CHAPTER TWO LITERATURE REVIEW
Oleaginous yeasts prospect in microbial oil production.

2.1 Introduction

The quest for renewable fuel source has led to increase in research for alternative fuel source to fossil fuel. Fossil fuels are non-renewable and have depleting reserves, in addition, fossil fuel produces large amount of carbon dioxide on combustion. Carbon dioxide is a green house gas and it contributes to global warming, therefore, there is need for alternative fuel source that will not increase the rate of global warming. Biofuels such as bioethanol, biodiesel, dimethyl ether (Semelsberger *et al*., 2006) and biogas (Kamm & Kamm, 2004) are good alternative to fossil fuel due to their sustainability and low carbon dioxide emission. Biodiesel is more environmental friendly and biodegradable than gasoline; in addition, Demirbas (2007) reported that biodiesel has higher combustion efficiency than conventional diesel. The properties and advantages of biodiesel as an alternative to conventional diesel are reported by several authors (Moser, 2009; Demirbas, 2007; Gerpen, 2005). Biodiesel is produced using renewable feedstock such as crops (rape seed, palm oils) and oleaginous microorganisms (algae, yeast). Canakci and Sanli (2008) noted that the quality of biodiesel is affected by the source of feedstock. However, the increase in demand for food has made the use of food crops for production of biofuels an unwise wise choice and has resulted in the current food versus fuel debate. Oleaginous microorganisms do not compete with food crops for arable land and therefore do not affect the demand for food; this makes oleaginous microorganism a very good alternative feedstock for biodiesel production.

Current research interest on oleaginous microorganisms as a potential feedstock for biodiesel production (Meng *et al*., 2009) is due to the sustainability of oleaginous microorganisms. Microalgae have a higher growth rate than crops and it was reported to be a good alternative feedstock for biodiesel production due to its high biomass production and photosynthetic

efficiency compared to crops (Meng *et al*., 2009). The disadvantage of microalgae algae is that it requires more land space for its cultivation than yeast.

Lipid accumulation in oleaginous yeast is known to occur when a nutrient is limiting and limiting nitrogen in the medium was reported in Ratledge (2002) to be more effective in accumulating lipid than limiting other nutrients.

2.2 Oleaginous microorganisms - an overview

Oleaginous microorganisms are microorganisms that can accumulate lipid up to twenty percent of their body weight (Ratledge, 2002). Although all living organism synthesize a minimum amount of lipid, only oleaginous microorganism can accumulate lipid more than twenty percent of their body weight and they include some species of algae, moulds and yeast (Meng *et al*., 2009). Lipid content of oleaginous microorganisms are much higher than that of energy crops, therefore oleaginous microorganisms are a close substitute for energy crops such as jatropha, sun flower and palm oil (Vicente, *et al*., 2010). A comparative table showing oil content of different microorganisms and crops is given in Table 2.1

Table 2.1: Lipid content of some crops, yeast, mould, bacteria, and algae

Microorganism	Species	Oil content (% dry weight)	Reference
Yeast	*Rhodosporidium toruloides*	66	(Ratledge, 1991, p. 430)
	Candida curvata	58	
	Lipomyces starkeyi	63	
	Cryptococcus albidus	65	
Mould	*Mucor mucedo*	51	(Ratledge, 1991, p.431)
	Aspergillus nidulans	51	
	Aspergillus oryzae	57	
Algae	*Botrycoccus braunii*	25-75	(Chisti, 2007, p. 296)
	Neochloris oleabundans	35-54	
	Tetraselmis sueica	15-23	
Crop	Jathropha seed	30-50	(Pramanik, 2003, p. 240)

From Table 2.1, oleaginous microorganisms have high lipid content than most energy crops that are currently used for biodiesel production. Oleaginous yeasts have higher lipid content compared to moulds and most algae.

2.2.1 Oleaginous algae

Algae have a higher oil production and biomass accumulation rate than crops and can also grow in arid land and brackish water (Griffiths & Harrison, 2009), in addition, algae are more photosynthetically efficient than conventional energy crops (Patil *et al*., 2010). These make algae an alternative to energy crops.

Microalgae can be cultivated phototrophically or heterotrophically. In phototrophic cultivation, microalgae utilize carbon dioxide and light energy to photosynthesize chemical energy. Closed photo-bioreactors and open ponds such as race way ponds can be used to grow microalgae phototrophically, in addition, they are the practical ways for large scale production of microalgae (Chisti, 2007). It is economically to cultivate algae in open ponds; however, algae productivity is affected by high risk of bacterial contamination and temperature fluctuation (Brennan and Owende, 2009; Chisti, 2007). Although lipids yield are optimised in closed system, Brennan and Owende (2009) reported that closed systems are very costly.
Heterotrophic cultivation of algae involves the utilization of organic compounds such as glucose and acetate as carbon source by the microalgae. Huang *et al*. (2008) reported that glucose is the best carbon source for growing algae but due to high cost of glucose, cheaper carbon source such as corn powder hydrolysate (Xu *et al*., 2006) has been used to grow algae. In addition to carbon source, Chisti (2007) emphasized that nitrogen, phosphorus, iron, and silicon is essential for algae growth. Nitrogen composition of growth medium is very important in lipid accumulation when algae are cultivated in heterotrophic mode. Huang *et al*. (2008) reported that microalgae prefer organic nitrogen to inorganic nitrogen. Lipid content and fatty acid composition of microalgae is affected when nitrogen is limiting in the growth medium (Huang *et al*., 2008). In support of variation of lipid content and fatty acid

composition under nitrogen limitation, Erila *et al*. (2005) reported that saturated fatty acid composition of *Botryococcus braunii* increased when it was grown under nitrogen limitation. Temperature is also reported in literature (Erila *et al*., 2005) to affect growth and fatty acid composition of microalgae. Erila *et al*. (2005) explained that high poly unsaturated fatty acids (PUFA) produced at low temperature by microalgae is due to the fact that algae maintains its cell fluidity by producing more PUFA, in addition, the activity of desaturase and elongase involved in biosynthesis of PUFA is improved due to high level of intercellular molecular oxygen at low temperature (Erila *et al*., 2005).

Algae lipids are mostly neutral lipids such as triglyceride, and polar lipids such as phospholipids (Huang *et al*., 2008). Algae can biosynthesize their own triglyceride using acetyl co A and L-α phosphoglycerol as two major primers for triglyceride biosynthesis (Huang *et al*., 2008). The steps in triglyceride biosynthesis are shown in Figure 2.1 below. L-α phosphoglycerol which is a product of glycolysis reacts with coenzyme A in a reaction catalysed by glycerol phosphate acyl transferase to form lysophosphatic acid (Huang *et al*., 2008). Phosphatic acid formed from the reaction between lysophosphatic acid and coenzyme A undergoes hydrolysis to form diglyceride. The hydrolysis reaction is catalysed by phosphatidate phosphatise. The final step in triglyceride biosynthesis in microalgae is the reaction of diglyceride with coenzyme A to form triglyceride.

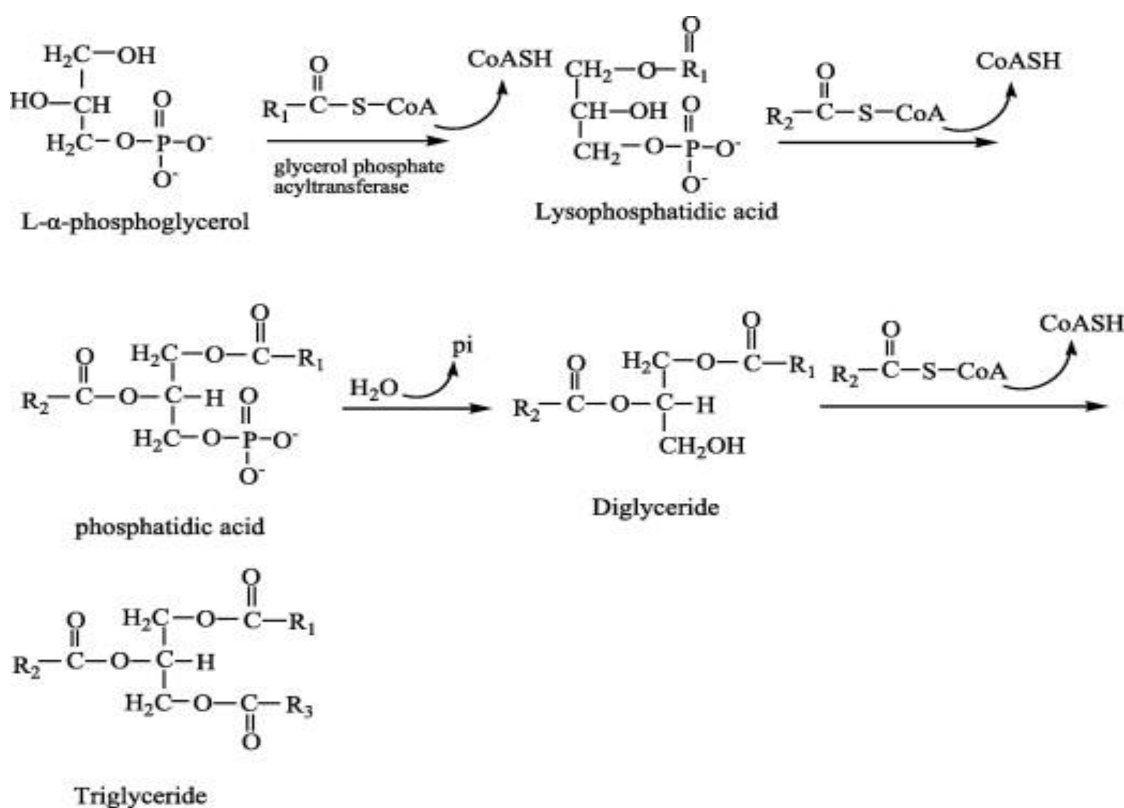


Figure 2.1: Triglyceride biosynthesis in microalgae.

(Huang *et al*., 2008, p.41)

Algae as an alternative oleaginous organism for oil production is faced with technical challenge for cultivation of high oil content algae. According to Griffiths and Harrison (2009), the bottleneck in using algae for oil production is the algae productivity. Algae have been genetically (Huang *et al*., 2008) and metabolically engineered (Chisti, 2007) to improve lipid productivity. Metabolic engineering has been used to improve algae productivity through improved photosynthetic efficiency, increased growth rate and enhanced lipid content

2.2.2 Oleaginous fungi

Fungi are eukaryotic organisms and include yeast and moulds. Oleaginous fungi are those moulds and yeast that can accumulate lipid in quantity more than 20% of their weight.

Moulds are filamentous organisms and it has been reported in literature (Papanikolaou *et al*., 2007) to be an edible source of edible oil. Just like yeast and algae, lipid accumulation in moulds is triggered when nitrogen is exhausted from the medium, however, unlike yeast and algae, nitrogen exhaustion in medium only triggers lipid accumulation only when sugar-based carbon sources are used (Papanikolaou *et al*., 2007). However, when hydrophobic carbon sources are used such as free fatty acids, lipid accumulation is independent of nitrogen exhaustion (Papanikolaou *et al*., 2007).

Temperature is an important factor in regulating the degree of unsaturation in moulds (Kendrick & Ratledge, 1992), in addition, decrease in temperature from 20ºC to 30ºC decreased the percentage of polyunsaturated fatty acid from 18% to 27% w/w of total fatty acid. Kendrick and Ratledge (1992) also reported that thermophilic moulds produce more saturated fatty acid than mesophilic moulds. The reason for production of more saturated fatty acid by thermophiles at low temperature is due to the fact that thermophilic moulds cannot produce unsaturated fatty acid in quantity enough to maintain cell membrane in crystalline state (Kendrick & Ratledge, 1992).

Oleaginous moulds were reported in Papanikolaou *et al*. (2004) to have different lipid yields when grown under batch, fed-batch, and continuous mode of cultivation. Lipid yields of some moulds are shown in Table 2.2 below.

Table 2.2: Lipid yields of some moulds grown under different mode of cultivation (Papanikolaou *et al*., 2004, p. 290)

Mould	Mode of cultivation	Carbon source	Lipid (g/l)	Lipid yields on cells $Y_{x/s}$ (g/g)
Mucor circinelloides	Fed-batch	Acetic acid	2.6	0.11
Cunninghamella echinulata	Batch	Starch	11.5	0.30
Cunninghamella echinulata	Batch	Glucose	4.4	0.49
Mortierella isabellina	Batch	Glucose	18.1	0.50

Oleaginous yeast is the microorganism of interest in this study; detailed review on its growth and lipid accumulation are discussed in subsequent sections.

2.2.3 Oleaginous bacteria

Like other oleaginous microorganisms, some bacteria are considered to be oleaginous because of their ability to accumulate more than 20% of body weight as lipids.

Leman (1997) reported that phospholipids and glycolipids are the lipids accumulated by oleaginous bacteria and these lipids are found in the cell envelopes thereby poses difficulties in the extraction. Recently, triglycerol has been detected in aerobic heterotrophic bacteria and cyanobacteria. Alvarez and Steinbuchel (2002) reported that triglycerol occurs in group of actinomycetes such as *Streptomyces*, *Norcardia* and *Rhodococcus*.

Like other oleaginous microrganisms, lipid accumulation in oleaginous bacteria occurs when nitrogen is limited in the growth medium during the stationary phase (Alvarez & Steinbuchel, 2002). During the exponential growth phase of oleaginous microrganisms, triglycerol is not

accumulated rather it is used in the biosynthesis of phospholipids needed for cell growth and proliferation (Alvarez & Steinbuchel, 2002).

Diacylglycerol acyltransferase (DGAT) was reported to be responsible for the switch from phospholipid formation during the exponential phase to triglycerol biosynthesis during the stationary growth phase (Alvarez & Steinbuchel, 2002).

2.3 Oleaginous yeasts: growth and lipid accumulation

2.3.1 Growth kinetics and models

Growth phase of oleaginous yeast is the same with other microorganisms. The growth phase includes; lag phase, exponential phase and stationary phase. During lag phase, oleaginous yeast adapt to the medium. Lag phase is usually long when the process medium is different from the inoculum medium. Maturity of yeast cells also occur in the lag phase. Doubling of yeast cells occurs during the exponential phase and yeast doubling will continue until a growth substrate in the medium is limiting. In the stationary phase, the rate of cell growth is proportional to the rate of cell death. Lipid accumulation in oleaginous yeast occurs during the stationary phase.

Monod kinetics is used to explain the growth of microorganisms in a limiting substrate medium. Maximum specific growth rate and saturation constant are used in Monod's model to described microbial growth kinetics (Kovarova-Kovar & Egli, 1998) and the relationship between growth rate and substrate consumption is given in the equation below

$$\mu = \mu_{max} \frac{s}{K_s + s} \quad (2.1)$$

Where

μ= specific growth rate (h^{-1})

μ_{max} = Maximum specific growth rate (h^{-1})

s = Limiting substrate concentration (g/L)

K_s= Saturation constant (g/L)

Different models have been proposed for studying lipid accumulation in oleaginous yeast, Karanth and Sattur (1991) proposed a model for predicting lipid accumulation and rate of sustrate consumption in oleaginous yeast.

2.3.1 Biochemistry of lipid accumulation in oleaginous yeasts

Lipid accumulation occurs when a medium nutrient especially nitrogen is exhausted (Ratledge, 2002). According to Ratledge (2002), when nitrogen is limited, the carbon source is assimilated and converted to triacylglycerols but because the microorganisms cease to proliferate due to limited nitrogen, the lipids are then stored within the cells.

Many papers (Ratledge, 2002; (Botham & Ratledge, 1979); Liu, *et al.*, 2009; Beopoulos *et al.*, 2008) have been written on the biochemical explanation of lipid accumulation in oleaginous microorganisms and the explanation is summarised in Figure 2.2. Nitrogen depletion in the medium leads to depletion of Adenosine mono phosphate (AMP). Adenosine monophosphate is needed for the activity of NAD^+ dependent Isocitrate dehydrogenase (Botham and Ratledge, 1979). Therefore metabolism of citrate during the Krebs cycle is arrested making citrate to accumulate in the mitochondria and enters the cytoplasma where it is converted to Acetyl Co A and Oxaloacetate by ATP citrate lyase (Botham and Ratledge, 1979). Oxalo acetate is converted to pyruvate and NADPH is releases in the process. Acetyl Co A is then used for biosynthesis of fatty acid. Botham and Ratledge, (1979) noted that fatty acid biosynthesis is activated by high ATP. In addition, Ratledge, (2002) noted that malic enzyme influences the regulation of lipid biosynthesis because the enzyme provides NADPH needed for lipid biosynthesis.

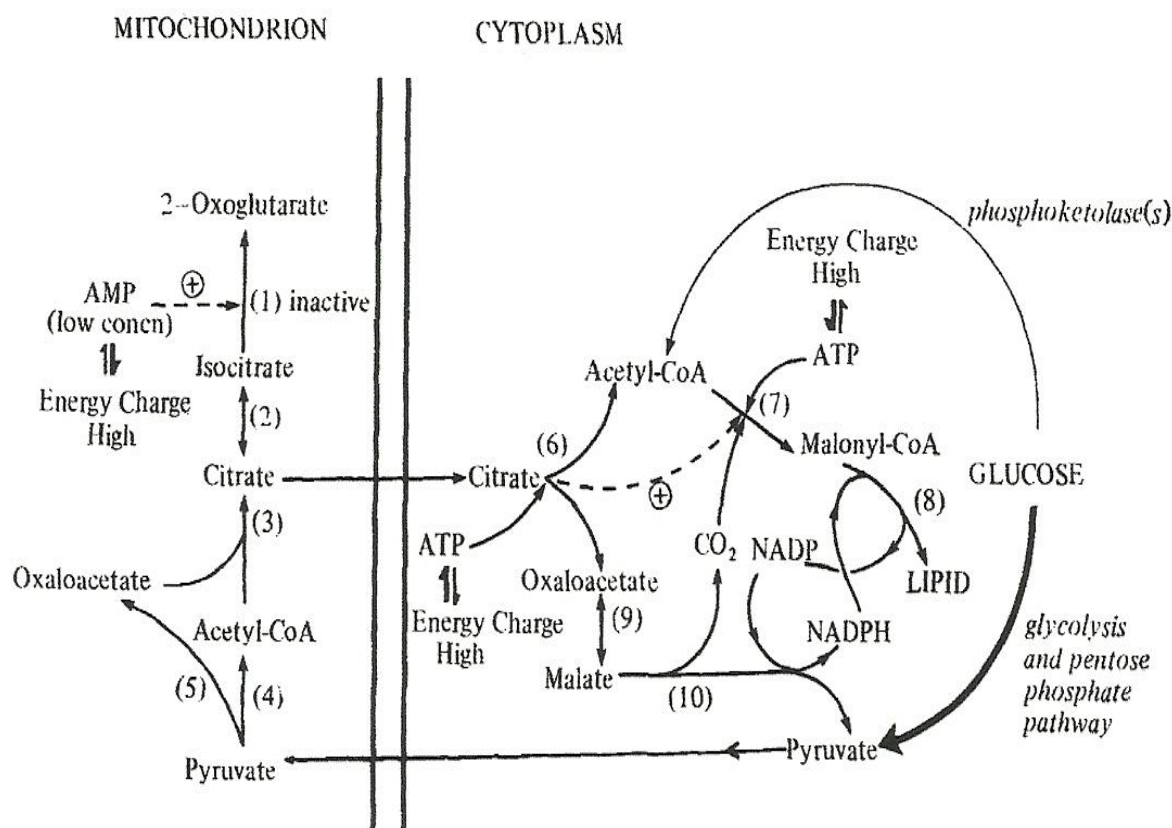


Figure 2.2: Pathway for lipid biosynthesis in oleaginous yeast

Source: (Botham & Ratledge, 1979)

2.4 Factors affecting growth, lipid accumulation and fatty acid profiles of oleaginous yeasts

Kimura *et al*. (2006) pointed out that composition of the medium and culture conditions are factors affecting lipid accumulation in oleaginous yeast. In addition, Hassan *et al*. (1993) reported that cultivation technique affects fatty acid production. According to Hassan *et al.,* (1993), fatty acids are produced more in continuous cultivation than in batch cultivation; however, Li *et al*. (2007) reported high cell density and lipid content in *Rhodosporidium toruloides* grown under fed-batch.

2.4.1 Medium composition

Medium composition has varying effect on different species and strain of oleaginous yeast. *Rhodotorula gracilis* accumulated more triacylglycerols in nitrogen limited medium than in carbon limited medium (Rolph *et al*., 1989). Limitation of medium nutrients such as nitrogen, magnesium and iron was showed to have different effect on lipid yield and lipid content

(Beopoulos *et al*., 2009). However, Beopoulos *et al*., (2009) confirmed nitrogen limitation as the most efficient nutrient limitation for the accumulation of lipids in oleaginous yeast. Carbon to nitrogen ratio is an important factor for lipid accumulation because different oleaginous yeast has different optimal carbon to nitrogen ratio. The effect of carbon to nitrogen ratio is reported in Saxena *et al*., (1998); *Rhodotorula minuta* accumulated 48% w/w lipid with 22.5% glucose conversion when grown under carbon to nitrogen ratio of 30.

Type of carbon source used for the growth of oleaginous yeast is another factor that affects growth rate and lipid content. Specific growth rate of microorganism is reported in Saxena *et al*., (1998) as an indication of microorganism's affinity to carbon substrate, this was observed in *R. minuta* which had a high specific growth rate of 0.34 h^{-1} when grown on glucose and was more than 0.30 h^{-1}, 0.36 h^{-1}, and 0.11 h^{-1} when grown on fructose, sucrose and galactose respectively..

Oxygen uptake of oleaginous yeast has been reported to affects the growth and fatty acid profiles of oleaginous yeast (Davies *et al*., 1990). Davies *et al*. (1990) reported that oxygen uptake rate lower than 7mmol $L^{-1}h^{-1}$ during the oil accumulation phase of *Apiotrichum curvatum* decreased the total unsaturated fatty acid. The decreased in unsaturated fatty acid was observed in oleic and linoleic acids which decreased from 50% to 41% and 8% to 3% respectively when *Apiotrichum curvatum* was grown in oxygen uptake rate lower than 3mmol $L^{-1}h^{-1}$ (Davies *et al*., 1990). Oxygen also affects cell growth since oxygen is needed for cell proliferation and limitation of oxygen will lead to cell death (Davies *et al*., 1990).

2.4.2 Culture condition

Temperature of the growth medium is one of the factors that affect the growth and poly unsaturated fatty acid synthesis in oleaginous yeast (Granger *et al*., 1992). Low temperature has little effect on lipid accumulation, however; an increase in level of polyunsaturated fatty acid was observed when *Rhodotorula gracilis* is grown under low temperature as reported by Rolph *et al*. (1989). Saxena *et al*. (1998) confirmed that temperature affects fatty acid profiles in oleaginous yeast. According to Saxena *et al*. (1998), Rhodotorula *minuta* produced long fatty acid chains such as palmitoleic and oleic acids at 32°C whereas short fatty acid chains of such as caprylic, enathic and perlargonic acids were produced at temperature of 38°C. Saxena *et al*. (1998) attributed the change in fatty acid chain to the temperature sensitive acyl-carrier protein which is part of the enzyme that carries out fatty acid elongation. In addition, Saxena *et al*. (1998) noted that degree of unsaturation decreases with increase in

temperature, this was attributed to decrease in the activity of desaturase enzyme with increase in temperature (Saxena *et al*., 1998). Granger *et al*. (1992) also confirmed that a decrease in cultivation temperature decreases the specific growth rate and product synthesis of *Rhodotorula glutinis*.

There is an optimal pH for growth and lipid accumulation in different oleaginous yeast, therefore, pH of the medium is one of the factors that affect the biomass yield and lipid content in oleaginous yeast. Johnson *et al*. (1992) reported a significant variation in lipid content and biomass yield in *Rhodotorula glutinis* grown under varied pH. According to Johnson *et al*. (1992), biomass concentration decreased from 22.50 g/L to 15.20 g/L while the lipid content decreased from 66% to 44% as pH increases from 4 to 6.

2.5 *Rhodosporidium toruloides* a model yeast for microbial oil production

Rhodosporidium toruloides also known as *Rhodotorula gracillis* (Hsieh *et al*., 2009), is a teleomorph of *Rhodotorula glutinis*, and is produced together with two other species namely *R. sphaerocarpum*, and *R. diobovatum,* from *R. glutinis*, due to plasmogamy and karyogamy (Oloke, 2006).

Current interest on *R. toruloides* for microbial oil production is due to the fact that it can grow to a high cell density and lipid content (Li *et al*., 2007). Lipid accumulation can be achieved in *R. toruloides* under nitrogen limitation as well as phosphorus limitation (Wu *et al*., 2010).

When grown under nitrogen limiting condition, Kraisintu *et al*. (2010) observed that it has lipid bodies of diameter of about 0.5 µm to 2 µm; however, Holdsworth and Ratledge (1987) observed that *R. toruloides* degrades accumulated lipids under carbon limitation to produce more cells.

R. toruloides have been grown under batch and fed batch mode of cultivation under which it accumulated lipids at different rates as shown in Table 2.3

Table 2.3: Dry cell weight, lipid content, and lipid of *R. toruloides* grown under different culture conditions

Limiting Substrate	Carbon source	Mode of cultivation	Dry cell weight (g/L)	Lipid (g/L)	Lipid content (w/w%)	Lipid coefficient (g/g)	Reference
Phosphate	Glucose	batch (shake flask)	19.40	12.10	62.10	0.22	(Wu *et al.*, 2010, p. 6127)
Nitrogen	Glucose	batch (shake flask)	19.90	12.60	63.10	0.22	(Wu *et al.*, 2010, p.6127)
	Glucose	batch (shake flask)	6.78	4.23	62.30	Not determined	(Kraisintu *et al.*, 2010)
	Glucose	fed-batch (bioreactor)	106.50	71.90	67.50	0.23	(Li *et al.*, 2007, p.314)
			151.50	72.72	48.00	Not determined	
	Glucose	repeated fed-batch (bioreactor)	101.65	61.40	60.40	0.24	(Zhao *et al.*, 2010)
	Pure glycerol	batch	13.09	5.37	41.00	Not determined	This study
	Jerusalem Artichoke	batch (Shake flask)	25.50	10.07	39.5	Not determined	(Zhao *et al.*, 2010)

2.6 Process of biodiesel production from oleaginous microorganism

Biodiesel is a alkyl monoesters of fatty acids from vegetable or animal origin (Canakci and Gerpen, 2001). The biodiesel process is shown is Figure 2.2. The main step in biodiesel production is the transesterfication step.

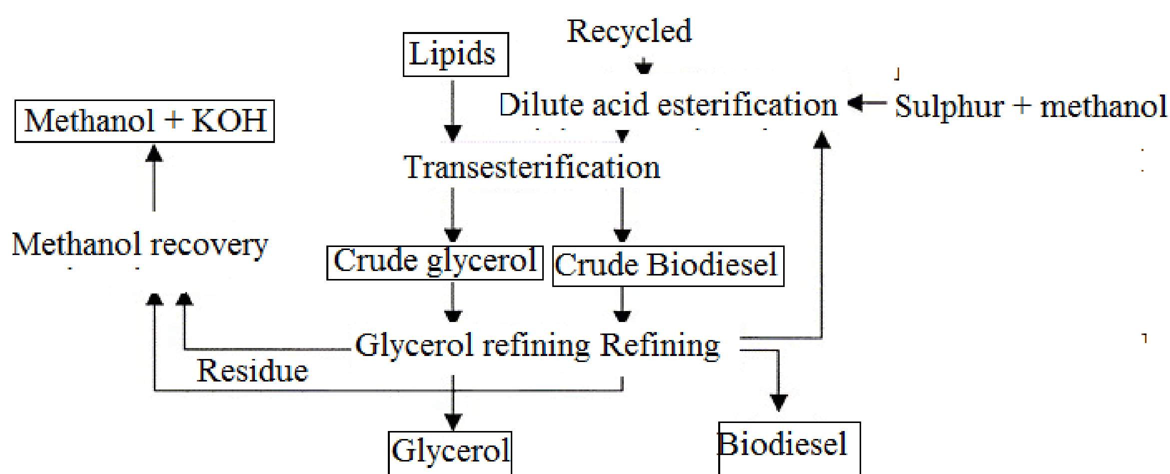


Figure 2.3: Biodiesel production process

(Huang, et al., 2010)

Transesterification is a three step reversible reaction that involves the reaction of alcohol with triglyceride to form glycerol and esters (Marchetti *et al*., 2007).

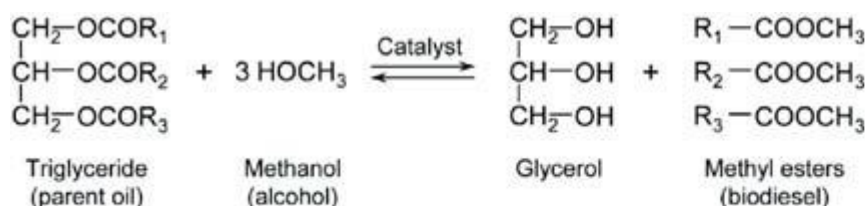


Figure 2.4: Transesterification reaction

(Marchetti *et al*., 2007)

The reaction steps depend on temperature, mixing intensity, presence of free fatty acids and moisture (Meher *et al*., 2006), nature and amount catalyst (Marchetti *et al*., 2007). Catalysts are always used in the transesterification step to speed up the reaction, Marchetti *et al*., (2007) reported using alkali, acids and enzymes as catalyst for transesterification. The mechanisms of alkali, acid and lipase catalysed transesterifications are explained in Meher *et al*. (2006). Alkali catalysts such as potassium hydroxide and sodium hydroxide are normally reacted with alcohol especially methanol or ethanol to form alkoxy in order to enhance the efficiency of the process (Marchetti *et al*., 2007). Alkali catalysts are added in amounts

ranging from 0.005% to 0.35% while the optimum process temperature is 60ºC, however, depending on the type of catalyst used, the temperature range of 25ºC to 120ºC are used (Marchetti *et al*., 2007). When cheaper raw materials that have high free fatty acid and water such as waste cooking oil are used for biodiesel production, Liu and Zhao (2007) reported that alkaline catalysed transesterification is not the ideal method to use because alkali forms soap with free fatty acid. Soap formation during transesterification reduces biodiesel yield and makes purification of biodiesel difficult, however this limitation is overcome by using strong acid catalysts (Liu and Zhao, 2007).

Acidic catalyst such as sulphuric acids added in amount ranging from 0.5% to 1% has been used to speed up transesterfication reactions; in addition, Marchetti *et al*. (2007) noted that acid catalyst has high ester yield than other catalysts but slower reaction rate.

Enzyme such as lipase has been reported (Marchetti *et al*., 2007) to be a viable method for producing alkyl esters from vegetable oil and animal fats. Lipase makes separation of the end product easier and offers the ability for enzyme reuse; however, lipase is very expensive.

2.9 Summary

From the literature survey, it is obvious that the study of difference species of oleaginous yeast is very important in order to enhance lipid productivity since different species have different growth requirements. An important factor in maximizing growth and lipid production is the source of carbon and nitrogen because some species of oleaginous preferred organic nitrogen to in-organic nitrogen as discussed in previous sections. Oxygen supply, carbon to nitrogen ratio and temperature are also factors discussed in the previous section that affect growth and lipid production in oleaginous yeast.

CHAPTER THREE RESEARCH OBJECTIVES

If the prices of fossil fuels and edible oil continue to increase with the same rate as it does in the last five years, then microbial oil will be indispensible as source of edible oil and raw

material for biodiesel process. Interest in microbial oil is not only driven by price, the depleting reserve of fossil fuel and the environmental issues also make microbial oil indispensible

From the literature review, oleaginous microorganisms have been exploited on their ability to produce microbial oil. Interest on oleaginous yeasts for microbial oil production is due to high growth rate and lipid contents of oleaginous yeast.

However, the drawback to commercialization of microbial oil production using oleaginous yeasts is the high cost of fermentation. Upstream process cost contributes most to the high cost of production. Upstream process involves all the activities that geared towards growing oleaginous yeast, growing the oleaginous yeast, and harvesting the cells. Costs of raw materials contribute most to upstream process cost; therefore, any aspect of research that gears towards making microbial oil production using oleaginous yeast more economical is very valuable.

Improving oleaginous yeast lipid productivity, growing oleaginous yeast on less expensive raw materials and utilizing effective cell disruption techniques and extraction methods are all aspects of research currently done to improve the economics of microbial oil production using oleaginous yeast.

From the literature review, oleaginous yeasts have different response to growth conditions. With the exception of glucose, not all other carbon sources can be utilized by all oleaginous yeasts to produce lipids. Some oleaginous yeasts prefers inorganic nitrogen to organic nitrogen, others prefer organic nitrogen to inorganic nitrogen. Different carbon to nitrogen ratios are optimum for different oleaginous yeasts. Therefore, thorough understanding of different oleaginous yeast is pertinent to understand how to improve the productivity.

Among all the oleaginous yeast studied in the literature, *R. toruloides* is known for accumulating high cell density and high lipid content when grown on glucose using fed-batch mode of cultivation. However, not much work has been done on this yeast.

From literature survey, only few studies have been published on using cheap carbon source to grow *R. toruloides*, In this research, utilization of crude glycerol as carbon source to grow *R. toruloides* was investigated. The outcome of the investigation gave a clear understanding on ability to *R. toruloides* to utilize by-product from biodiesel processing industries.

Crude glycerol is a by-product from transesterification process. Large quantities of crude glycerol are expected as by-product from large scale production of biodiesel. An option would be to use crude glycerol as carbon source in fermentation. This will not only reduce the cost of raw material, in addition, it will remove the cost of disposal.

This research aim was to explore the possibility of producing oil from *R. toruloides* through the study of its growth kinetics using various carbon sources. The objectives was based on the hypothesis that the growth rate, biomass accumulation and lipid content in oleaginous yeast are affected by the type of carbon source the yeast is grown on and the growth limiting substrate. Interest was on specific growth rate and lipid content of *R. toruloides*.

This research was carried out to achieve the following objectives;

- tudy the growth of *R. toruloides* on glucose, and pure glycerol.
- nvestigate the ability of *R. toruloides* to use crude glycerol as carbon source.
- Investigate the effect of organic and inorganic nitrogen sources on biomass yield

Studying the growth of *R. toruloides* on pure glycerol was very important because it gave a clear understanding on whether *R. toruloides* can as well grow on the impurities in crude glycerol since the only difference between pure glycerol and crude glycerol is the impurities (methanol, catalyst such as sodium hydroxide or potassium hydroxide)in crude glycerol. Studying the growth on glucose was carried out to compare the lipid content when *R. toruloides* was grown on expensive carbon source (glucose) with less expensive carbon source (crude glycerol). Effects of nitrogen sources were investigated to compare *R. toruloides* on expensive inorganic ammonium sulphate and cheap corn-steep liquor.

These objectives were carried out through these experiments.

1. Investigative studies on growth of *R. toruloides* on glucose and pure glycerol were carried out by growing *R. toruloides* on different concentrations of glucose and pure glycerol respectively. *R. toruloides* growth in shake flasks was compared with its growth in bioreactor when grown on glucose, and glycerol as carbon sources.

2. Studies on the utilization of crude glycerol as carbon source was carried out in shake flask by growing *R. toruloides* on different concentration of crude glycerol. Dry cell weight and optical density of *R. toruloides* was only determined at the end of fermentation period.

3. Investigative studies on the effect of nitrogen on biomass yield were carried out by growing *R. toruloides* using ammonium sulphate, corn-steep liquor, peptone, and yeast extract as nitrogen sources. The amount of nitrogen added in the medium was identical for all the sources.

4. Error is inevitable in any experiment. In this research, although systematic errors were minimized through calibration of equipments, random errors were identified through triplicates experiments. Variations in results are stated in standard deviation.

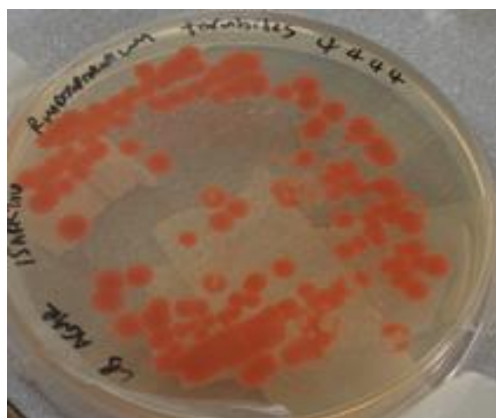
CHAPTER FOUR MATERIALS AND METHODS

4.1 Introduction

This chapter gives detailed description of all the materials used and the principle of operation of equipments used. Pictures are given for clear understanding of most equipment.

4.2 Microorganism

Master culture of *R. toruloides* in YMY slant (compositions of YMY is stated in section 4.71) were obtained from Shell Company and stored at 4ºC. Working cell bank was made from the master culture. Below is the picture of *R. toruloides* on slant (Figure 4.1) and under microscope (Figure 4.2).



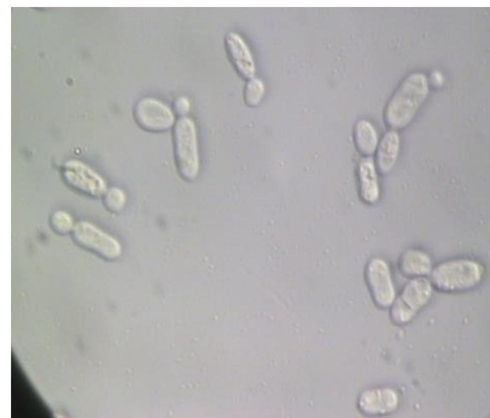


Figure 4.1 Figure 4.2

Figure 4.1: Picture of Master slant of R. toruloides

Figure 4.2: Picture of *R. toruloides* cells from master slant viewed under microscope using 100X lens

4.2.1 Working cell bank

Colony from the slant was used to inoculate 100 mL of sterilized YMY medium. The inoculum was incubated at 30ºC for 20 h using a rotary shaker at 200 rpm. The medium was centrifuged at 1000 rpm for 5 minutes and the supernatant discarded aseptically. The cells were re-suspended in a chilled 100 mL of sterilized YMY medium containing 15% glycerol. 1mL each of the cell suspension was transferred aseptically using a 1mL micropipette to 100 cryopreservation tubes, the tubes were sealed with parafilm and the tubes were stored at -4ºC freezer. One of the vials was used for cell counting.

4.2.2 Cell counting

Cell count of inoculum was carried out to ascertain the number of cells used for the fermentation. Dilution of the cells was made in order to have fewer cells to count. The well suspended cells was dropped on the haemocytometer and covered with a microscope cover slide. The cells were counted using 10X objective lens.

4.3 Chemicals

All chemicals used were bought from Fisher Scientific chemicals with the exception of the following;

I. Magnesium sulphate and Potassium iodate which were bought from Analar.
II. Malt extract, yeast extract, glycerol, corn steep liquor and agar which were bought from Sigma-Aldrich.
III. Glycine which was bought from Fissons

4.4 Medium composition and preparation

The composition of different medium used in the fermentation is summarised below. All media were sterilized in the autoclave for 20 minutes at 121 ^{o}C and allowed to cool before inoculation in the microflow cabinet.

4.4.1 Inoculum medium and preparation

Inoculum medium used was YMY. The nutrient composition of YMY is summarised in Table 4.1. The volume of inoculum medium used depended on the volume of process medium. When 1.5 L of process medium was used, the volume of YMY was 150 mL (10% v/v of inoculum) and the compositions of YMY added was calculated in proportion with the composition in Table 4.1

Table 4.1: Composition of YMY

Nutrient	Composition (g/L)
Glucose	10
Malt extract	3
Yeast extract	3
Peptone	5

All inocula were made from the working cell bank vials. The vials were thawed quickly by running it over warm water. Two vials containing 1mL each of the cells were used to inoculate 100 mL of the YMY medium. The inoculum was incubated for 48 h at 30°C using rotary shaker at 200 rpm.

4.4.2 Process media using Glucose as carbon source

R. toruloides was grown on medium GIN1, GIN2, and GIN3 in order to study its growth on different concentration of glucose. The composition of these medium is stated in Table 4.2. Medium GIN1, GIN2, and GIN3 contained 60 g/L, 70 g/L and 100 g/L of glucose respectively. *R. toruloides* was grown in bioreactor using medium GIN3.

Medium GIN4, GPEP, GSCL, and GYES contained ammonium sulphate, peptone, corn steep liquor and yeast extract as nitrogen sources, these media were used to investigate the effect of nitrogen source on biomass accumulation. The compositions of these media is summarised in table 4.2 below.

Table 4.2 Process media using glucose as carbon source

Nutrient	Medium name and composition (g/L)						
	GIN1	GIN2	GIN3	GIN4	GPEP	GCSL	GYES
Glucose	60	70	100	70	70	70	70
$(NH_4)_2SO_4$	12	0.1	0.1	4.71	-	-	-
Peptone	-	-	-	-	0.46	-	-
Yeast extract	0.5	0.5	0.5	-	-	-	0.73
Corn steep liquor	-	-	-	-	-	0.82	-
$MgSO_4$	1.5	1.5	1.5	1.5	1.5	1.5	1.5
$CaCl_2$	0.2	0.2	0.2	0.2	0.2	0.2	0.2
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

4.4.3 Process media using Pure Glycerol as carbon source

Growth of *R. toruloides* in shake flask using pure glycerol as carbon source was investigated using medium PIN1, PIN2, PIN3, PIN4, PIN5, and PIN6 containing different concentrations of pure glycerol ranging from 10 g/L to 100 g/L. The compositions of these media is summarised in Table 4.3.

Growth of *R. toruloides* in bioreactor was studied using only medium PIN6. Effects of nitrogen sources on *R. toruloides* when grown using pure glycerol as carbon source was studied using medium PIN7, PPEP, PCSL, and PYES containing ammonium sulphate,

peptone, corn steep liquor and yeast extract respectively as nitrogen sources. The compositions of these media is summarised in Table 4.4

Table 4.3 Process media using pure glycerol as carbon source

Nutrient	Medium name and composition (g/L)					
	PIN1	PIN2	PIN3	PIN4	PIN5	PIN6
Pure Glycerol	10	20	30	70	80	100
$(NH_4)_2SO_4$	0.1	0.1	0.1	0.1	0.1	0.1
Peptone	-	-	-	-	-	-
Yeast extract	0.5	0.5	0.5	0.5	0.5	0.5
Corn steep liquor	-			-		
$MgSO_4$	1.5	1.5	1.5	1.5	1.5	1.5
$CaCl_2$	0.2	0.2	0.2	0.2	0.2	0.2
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4

Table 4.4 Process media for investigating effect of nitrogen source using pure glycerol as carbon source.

Nutrient	Medium name and composition (g/L)			
	PNH4	PPEP	PCSL	PYES
Pure Glycerol	72	72	72	72
$(NH_4)_2SO_4$	4.7	-	-	-
Peptone	-	0.46	-	-
Yeast extract	-	-	-	0.73
Corn steep liquor	-	-	0.82	-
$MgSO_4$	1.5	1.5	1.5	1.5
$CaCl_2$	0.2	0.2	0.2	0.2
KH_2PO_4	0.4	0.4	0.4	0.4

4.4.4 Process media using Crude glycerol as carbon source

Studies on the utilization of crude glycerol as carbon source by *R. toruloides* was carried out using CIN1, CIN2, CIN3, CIN4, CIN5, CIN6, CIN7, and CIN8, these media contained crude

glycerol concentration ranging from 3 g/L to 160 g/L. Compositions of the media is summarised in Table 4.5 below.

Table 4.5 Process media using crude glycerol as carbon source.

Nutrient	Medium name and composition (g/L)							
	CIN1	CIN2	CIN3	CIN4	CIN5	CIN6	CIN7	CIN8
Crude Glycerol	3	6	8	10	20	40	130	160
$(NH_4)_2SO_4$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Yeast extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$MgSO_4$	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
$CaCl_2$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

4.5 Experiment Set up and design

4.5.1 Shake flask experiment set up and design

All shake flask experiments were carried out using 250 mL conical flasks containing 50 mL of process media. The flasks were incubated at 200 rpm in 30^{0}C incubator and cultivated for 72 hours or in few occasion although it will be stated, cultivated for 144 hours.

4.5.2 Bioreactor experiment set up and design

R. toruloides was grown in 2 L bioreactor with a working volume of 1 L. The picture of the bioreactor is shown in Figure 4.3 below.

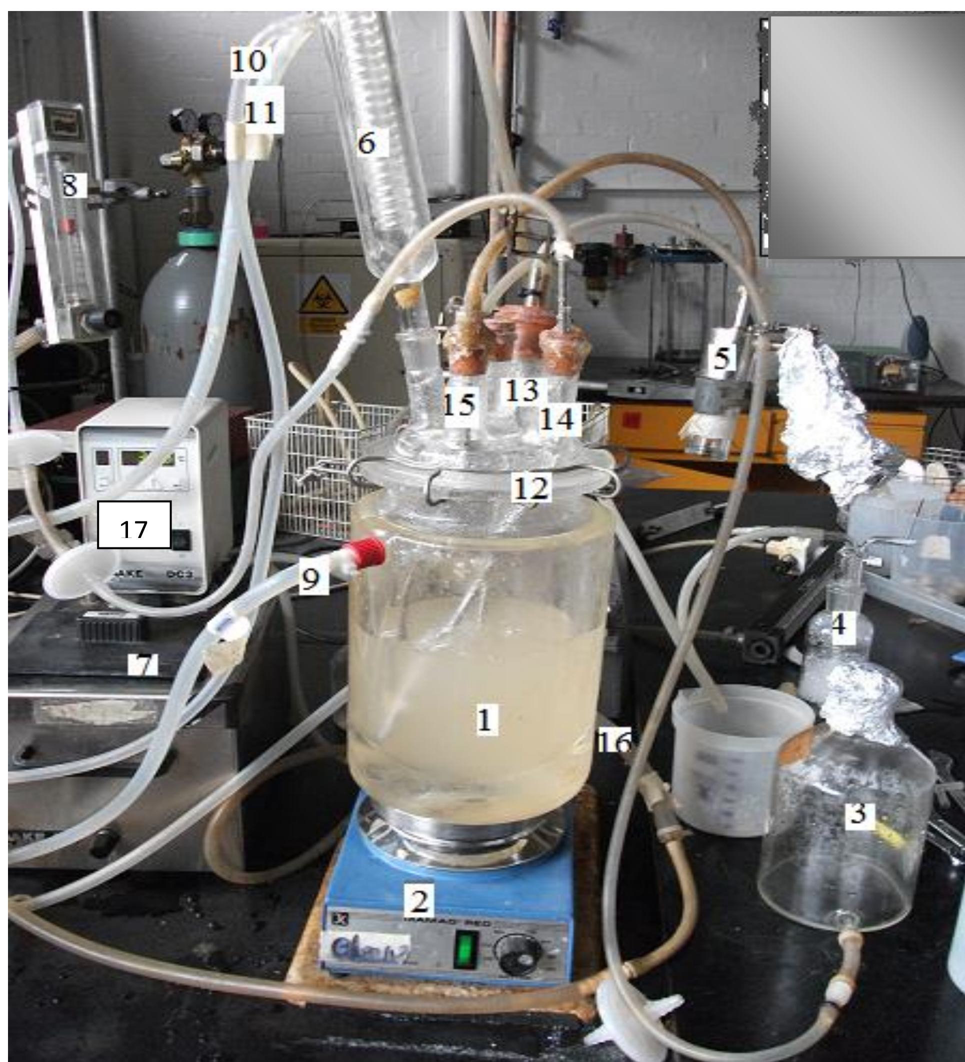


Figure 4.3: Set-up of I L working volume Bioreactor for batch fermentation

Bioreactor (1), magnetic stirrer (2), sampling port (5), Inoculum reservoir (3), water bath (7), air flow meter (8), condenser (6), condenser water inlet pipe (11), condenser outlet pipe (10), bioreactor water inlet pipe (9), bioreactor water outlet pipe (16), water pump (17), Sampling and anti-foaming port (13),

Inoculum in (3) was introduced through (15). The bioreactor was maintained at 30°C using the water from the water bath (7). The warm water was pumped to the bioreactor using water pump (17). The warm water flows in bioreactor water jacket through (9) and cold water flows back to water bath through (16). The bioreactor was stirred using the magnetic stirrer (2) at 200 rpm. Air (0.9 vvm) was introduced through (14) and measured using the air flow meter (8). Water was introduced to the condenser through (11) and water let out through (10). Samples were taken at interval through (5). The cover of the bioreactor (12) was sealed with

grease to be air tight. Pressure inside the bioreactor was reduced by dipping a pipe from the condenser into disinfectant (4).

R. toruloides was grown in two identical bioreactors using glucose and pure glycerol as carbon source.

4.6 Sample collection and handling

Most analyses such as optical density, dry cell weight, Sudan test, and pH were carried out soon the samples were collected. In situations when analyses were not done immediately such as in glucose determination, the fermentation broth was centrifuged at 6000 rpm and the supernatant was poured into 2 mL centrifuge tube and stored at 4ºC.

Since all shake flask experiments were carried out in triplicates, samples were taken from the triplicate flasks. The flasks were shaken to ensure uniformity of medium before samples were taken.

4.7 Analytical methods

4.7.1 Optical density determination

Cell growth was measured by measuring the optical density of the cells. The optical density is directly related to cell mass, therefore is used to measure the cell growth. Cell growth was measured at 600 nm using a Shimadzu spectrophotometer. 4 mL of the fermentation broth was centrifuged at 6000 rpm after which the supernatant was discarded. The cells were washed three times with distilled water before re-suspended into 4 mL of distilled water. Optical density of the cells suspension was measured at 600nm using distilled water as the blank. Serial dilutions were made when the optical density was greater than 1.500. A plot of optical density with time gives the growth curve from which the specific growth rate and doubling time were obtained.

4.7.2 Dry cell weight (DCW) measurement

Dry cell weight was carried out to measure the biomass yield with time. The unit of the measurement is in g/L. 10 mL of well suspended fermentation broth was filtered under a vacuum using a 0.022 µm filter. The filters with the cells were dried at 70ºC overnight and allowed to cool in the desiccator before the final weights were measured. The weights of the cells were obtained by subtracting the weight of the filter without cells from the weight of filter with cells.

DCW = (WF2 – WF1) x 100 (4.1)

DCW= Dry cell weight (g/L)

WF2= Weight of the filter with cells after drying (g)

WF1= Weight of the filter without cells (before filtration) (g)

4.7.3 Glucose determination

Residual glucose in the fermentation broth was determined to know how much glucose that was consumed in order to calculate the substrate consumption. The glucose concentration of the fermentation broth was measured using glucose analyser (Analox Instrument, UK).

The operating principle of glucose analyser is based on the measurement of oxygen uptake of the sample and the oxygen uptake is directly proportional to the glucose concentration of the sample.. β-D glucose reacts with oxygen abstracted from the sample to form gluconic acid and hydrogen peroxide. The glucose concentration from the analox instrument was given in g/L

B –D glucose + Oxygen → Gluconic acid + Hydrogen peroxide (4.2)

The instrument was calibrated using a standard glucose solution of 4.5 g/L. Serial dilutions of the samples were made in order to obtain a concentration in the range 0 to 4.5 g/L

4.7.4 pH measurement

The pH of the fermentation medium and broth were measured. The pH was calibrated with buffer 4.0 and 7.0 before use.

4.8 Lipid extraction

Lipid extraction was carried out using Soxhlet extractor. The diagram of the soxhlet extractor is shown in Figure 4.4. Soxhlet extractor was invented by Franz Von Soxhlet in 1879 for extraction of lipids from solid material.

A known weight of grounded (disrupted) cells of *R. toruloides* were placed in thimble (2), the thimble was then put in the extraction chamber (5). The condenser (4) was fitted above the extraction chamber to condense the evaporated hexane. Cool water was introduced to the condenser through (6) and let out through (7). The flask (3) containing hexane was fixed below the extraction chamber but on top of a heat source (1). When hexane was heated above its boiling point, it evaporated and flows through (9) to the condenser and finally condensed

into the extractive chamber. This continued until the extraction chamber was full, the mixture of hexane and extracted lipid automatically siphoned into the flask through (8).

Extraction was carried out for six hours after which hexane was evaporated from the mixture of hexane and extracted lipids in the flask using a rotatory evaporator.

The lipid content was calculated from the formula below

$$\% \text{ Lipid content} = \frac{WF_d - WF_b}{Ws} \times 100 \quad (4.4)$$

Where

WF_d = weight of rotatory evaporator flask with lipids (g)

WF_b = weight of empty rotator evaporator flask (g)

Ws = weight of sample used for extraction (g)

Figure 4.4 Soxhlet extractor using hexane as solvent

4.9 Statistical method

Standard deviation is a measure of variation or dispersion in a distribution. The fomula used to calculate standard deviation is shown in equation 4.3. Results in this research are stated in relation to the standard deviation.

$$\sigma_x = \sqrt{\left(\frac{1}{N-1}\sum(x_1 -)\right)^2} \qquad (4.3)$$

Where

σ_x = standard deviation

N = sample size

X_1 = observed values of samples

$\bar{x}$ = mean of the observed values

CHAPTER FIVE RESULTS AND DISCUSSIONS

5.1 Introduction

This chapter deals with explanation of all experimental results.

The growth kinetics of *R. toruloides* was studied in shake flask and bioreactor using glucose and pure glycerol as carbon sources. The ability of *R. toruloides* to utilize crude glycerol as carbon is discussed.

For a better understanding of the growth kinetics of *R. toruloides,* experiments such as carbon substrate consumption and lipid yield were carried out.

Although that some experiments failed due to contamination; some experiments that are devoid of contamination were obtained and the results of those good experiments are discussed below.

5.2 Growth of *Rhodosporidium toruloides* using glucose as carbon source

Glucose is a monosaccharide sugar that cells used as carbon source. Glucose has been used in literatures Saxena *et al*. (1998), and Li *et al*. (2007) to grow oleaginous yeast. Glucose was used to grow *R. toruloides* in Li *et al*., (2007).

5.2.1 *Rhodosporidium toruloides* growth in shake flask

Shake flask experiment is a very convenient way of carrying out preliminary growth test on aerobic microorganisms. Shaking aids the transfer of oxygen. For effective oxygen transfer, the volume of the medium inside the flask should be a minimum of 20% of the flask volume. In all experiments in this research, 250 mL Erlenmeyer flasks were used and 50 mL of medium was put in all the flasks. The flasks were incubated at 30ºC using a 200 rpm rotary shaker incubator. A 10% v/v volume of inoculum containing 4 x10^6 cells/mL was used to inoculate all the flasks.

The oleaginous yeast, *R. toruloides* was grown on different media containing varying concentration of glucose and ammonium sulphate in order to obtain the best growth medium and understand the growth kinetics. A preliminary test on growth of *R. toruloides* was carried out using medium GIN1 which was used by Li *et al*. (2007). Medium GIN1 contained glucose and ammonium sulphate concentrations of 60 g/L and 12 g/L respectively.

From Figure 5.1, in the preliminary experiment, it was observed that *R. toruloides* entered the log phase within approximately 15 h from time of inoculation. Final biomass accumulation was 4.5 ± 0.05 g/L. This experiment gave a rough idea on the growth of *R. toruloides.* This preliminary experiment was improved whereby the inoculum was incubated for 48 h instead of 24 h that was used in the preliminary experiment. In the preliminary experiment the inoculum was prepared from a freshly prepared slant and was grown for 24 h but in all other experiments, inoculum was prepared from working cell bank and grown for 48 h because it was observed that *R. toruloides* experienced a long lag phase when prepared from working cell bank. This might be due to the fact that *R. toruloides* requires more time to become active since it has been preserved at -4°C.

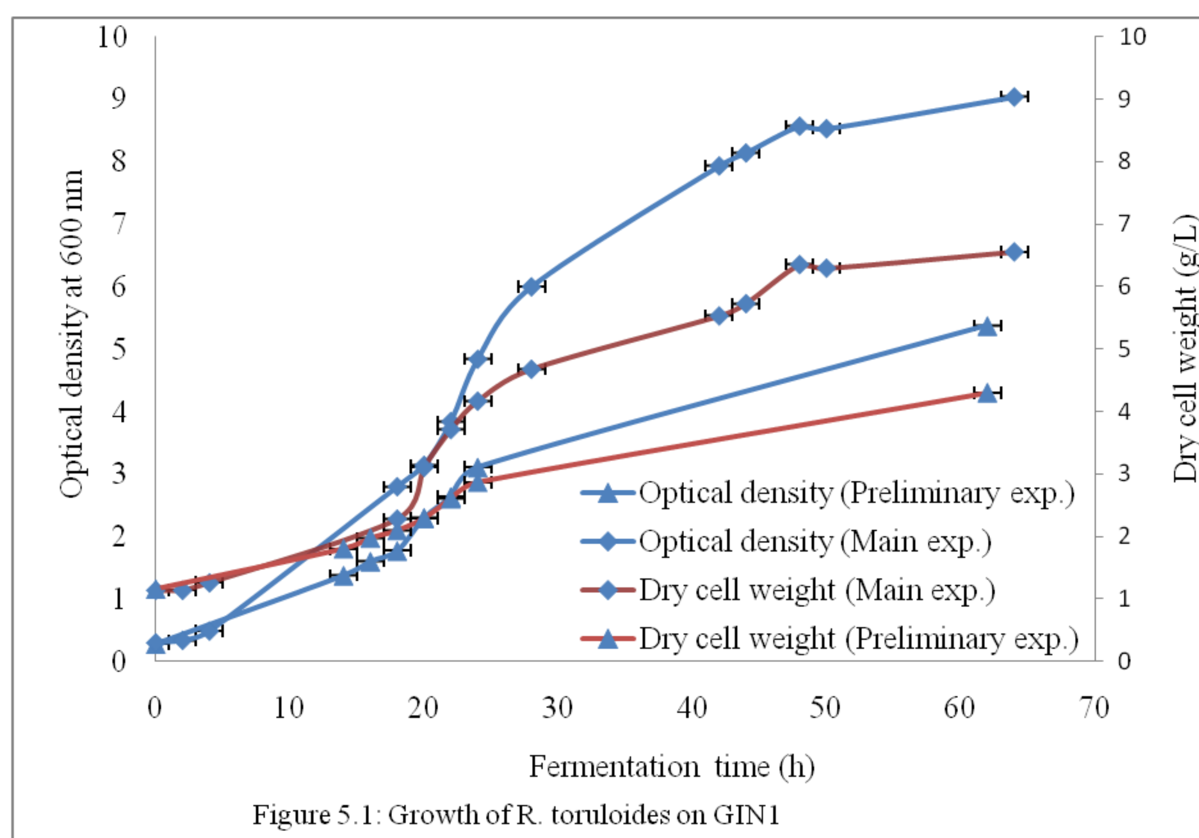


Figure 5.1: Growth of R. toruloides on GIN1

From the optical density of main experiment in Figure 5.1, it was observed that yeast *R. toruloides* grows rapidly in medium GIN1 having a lag phase of about 6 h. The dry cell weight at the end of experiment was 6.56 ± 0.18 g/L compared to preliminary experiment

which was 4.28 ± 0.03 g/L. Since the culture condition for the preliminary and main experiment was the same except the number of days the inoculum was cultivated, it can be concluded that the difference in dry cell weight was due to the fact that more active cells were used in the main experiment than in the preliminary experiment.

To understand the growth kinetics, parameters such as specific growth rate, yield of cells on substrate, yield of cells on product, and maximum specific growth rate were determined

Specific growth rate is the change in biomass concentration with time, mathematically this is written as

$$\frac{dX}{dt} = \mu X \qquad (5.1)$$

Solving equation (5.1) gives growth equation at the log phase

$$X = X_0 \, e^{\mu_m t} \qquad (5.2)$$

Where

X = the cell concentration at time t (Dry cell weight in g/L), X_0 = Initial cell concentration (Dry cell weight in g/L), μ_m = maximum specific growth rate (h^{-1})

The maximum specific growth rate is obvious in a semi-log plot of optical density with fermentation time. The semi-log plot is shown in Figure 5.2 below.

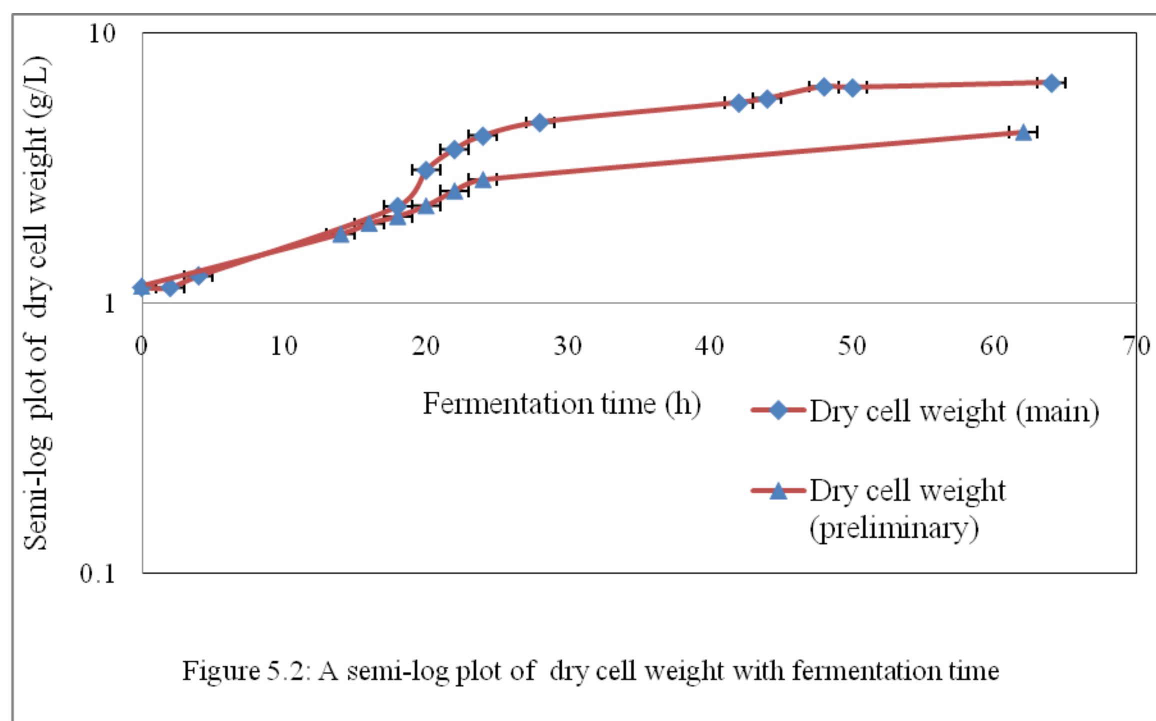


Figure 5.2: A semi-log plot of dry cell weight with fermentation time

From Figure 5.2, the maximum specific growth rate of *R. toruloides* was 0.25 h^{-1} from the main experiment whereas it was 0.21 h^{-1} from the preliminary experiment.

However, *R. toruloides* accumulated a lipid content of 4.14 ± 0.01 % w/w when grown on GIN1. *R. toruloides* had a high specific growth rate because there was sufficient nitrogen and carbon source in the medium, whereas the low lipid content was due to the fact the carbon to nitrogen ratio was below 20. This result is in support of Saxena *et al*. (1998) that carbon to nitrogen ratio affect lipid accumulation.

Another experiment which aimed to optimized the lipid yield and productivity was carried out by growing *R. toruloides* on medium GIN2 and GIN3.

R. toruloides was grown in two medium GIN2 and GIN3 which only differed in the glucose concentration, the glucose concentrations were 70 g/L, and 100 g/L in GIN2, and GIN3 respectively. The experiments were designed to ascertain whether there is a significant difference in specific growth rate and biomass accumulation when the yeast was grown in the two medium. The growth of *R. toruloides* in GIN1 and GIN2 is shown in Figure 5.3.

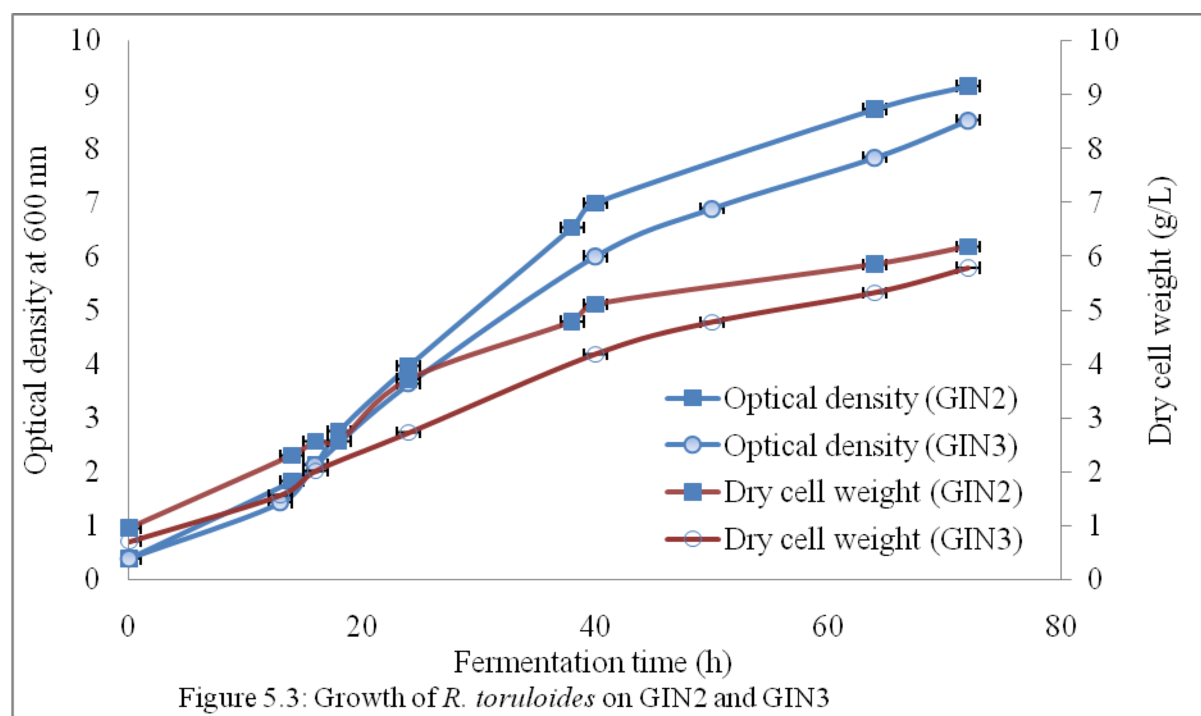


Figure 5.3: Growth of *R. toruloides* on GIN2 and GIN3

It was observed in Figure 5.3, that there was no significant decrease in biomass accumulation when *R. toruloides* was grown on medium GIN2 and GIN3 containing 70 g/L and 100 g/L glucose respectively since the dry cell weight decreased from 6.18 ± 0.20 g/L to 5.91± 0.07 g/L. This is in support of Li *et al*. (2007) that *R. toruloides* can grow on glucose up to 120 g/L without substrate inhibition.

From Figure 5.4 below, maximum specific growth rate decreases with increasing glucose concentration. The maximum growth rates in GIN2 and GIN3 were 0.14 h^{-1} and 0.16 h^{-1} respectively.

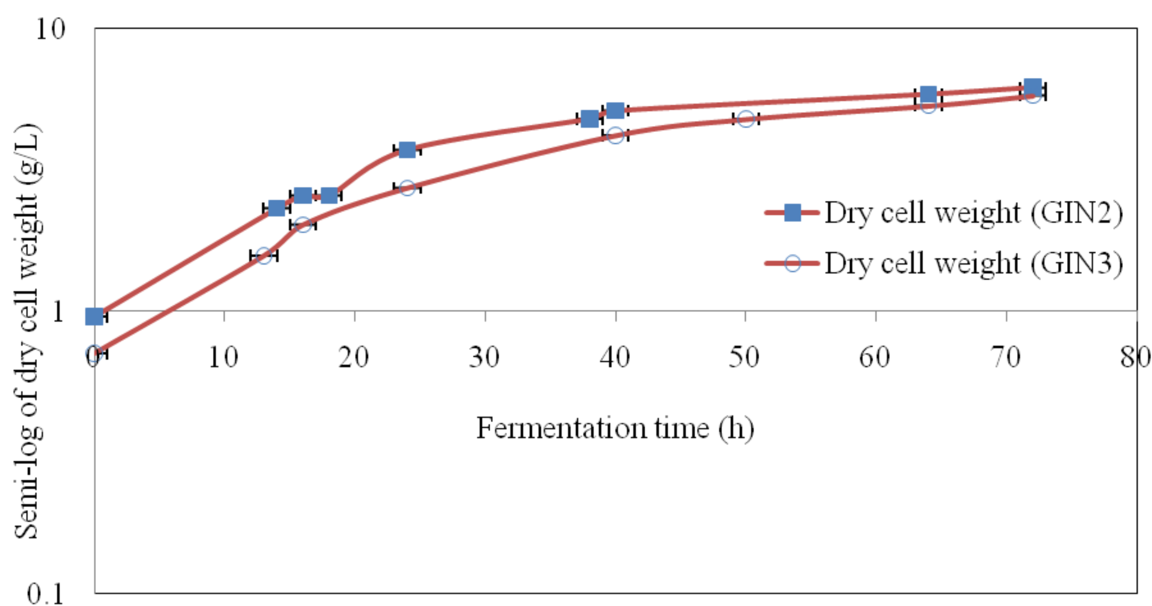


Figure 5.4: A semi-log plot of dry cell with time for GIN2 and GIN3

Since there was no significant difference in specific growth rate of *R. toruloides* in GIN2, and GIN3, therefore; medium GIN3 was used to grow the yeast in a bioreactor.

5.2.2 *Rhodosporidium toruloides* growth in bioreactor

The oleaginous yeast was gown in 2 L bioreactor of working volume of 1 L. The temperature was maintained at 30°C using the water batch, however, the pH was not controlled. During fermentation, there was no foam formation therefore anti-foam was not used. The fermentation was run for 144 h during which the pH, optical density, and glucose consumption were measured.

From Figure 5.5 shown below, since the pH was not controlled, the pH of the fermentation broth decreased from 5.50 ± 0.05 to 3.50 ± 0.03, the decrease in pH was due to formation of organic acids such as citric acid. Papanikolaou *et al*. (2006) reported that citric acid is formed from sugar catabolism and it is one of the products of glucose fermentation by oleaginous yeast. Therefore, *R. toruloides* must have produced many products as well as citric acid that resulted in the pH decreased. Biomass increased from 0.54 ± 0.04 g/L to 10.55 ± 0.04 g/L. The residual sugar decreased from 101 ± 0.07 g/L to 60 ± 0.04g/L.

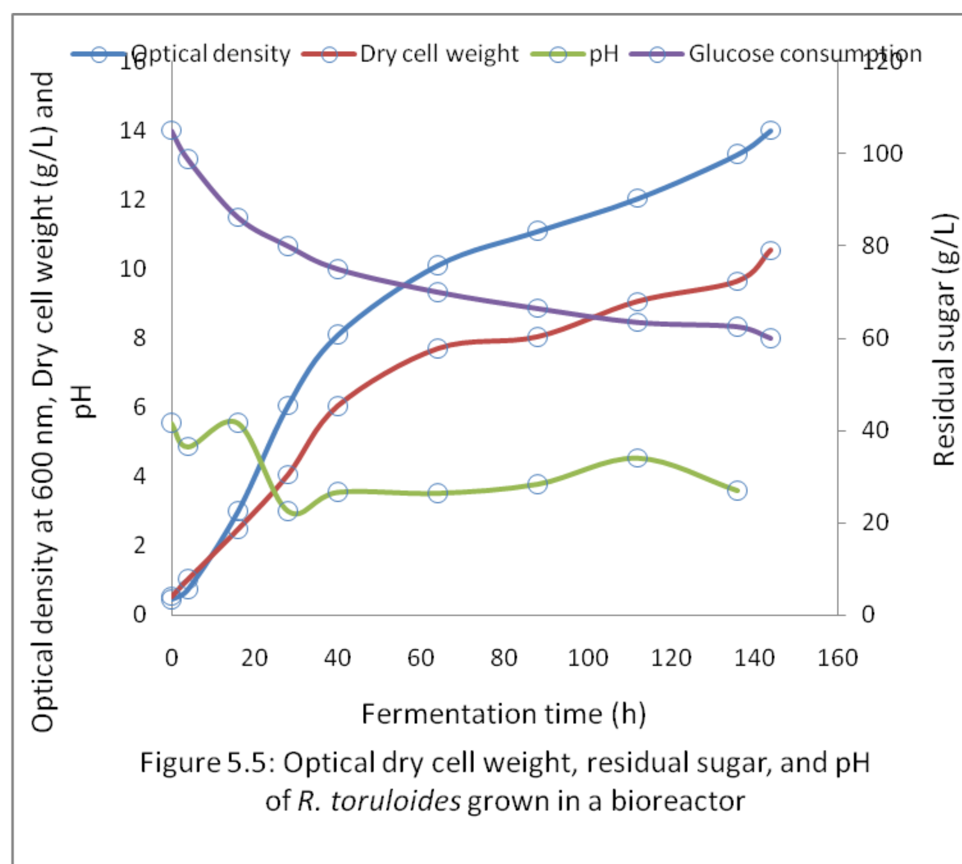


Figure 5.5: Optical dry cell weight, residual sugar, and pH of *R. toruloides* grown in a bioreactor

Lipid content and lipids of 47.61± 0.02 % w/w and 5.00 ± 0.02 g/L were got respectively.

5.3 Growth of *R. toruloides* on pure glycerol

Glycerol is the backbone of triglycerides and it is polyhydroxy alcohol that can be utilised by cells as carbon and energy source (Tamas and Hohmann, 2003).

Glycerol enters the cell through passive diffusion, facilitated diffusion and active uptake, however, facilitated diffusion is the mechanism mostly used (Tamas and Hohmann, 2003). Once inside the cell, Tamas and Hohmann (2003) reported that glycerol is phosphorylated by cytosolic glycerol kinase and oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate. The dihydroxyacetone phosphate formed enters the glycolytic pathway (Tamas and Hohmann, 2003).

Using pure glycerol to grow microorganisms is not new. Pure glycerol has been used to grow bacteria such as *Actinobacillus succinogenes* (Vlysidis, *et al*., 2009) and oleaginous yeasts (Easterling *et al*., 2009; Meesters *et al*., 1996). However, the literature on growing *R. toruloides* on pure glycerol is scare.

5.3.1 Shake flasks growth studies

In this research, *R. toruloides* was grown in PIN1, PIN3, PIN4, PIN5, PIN6, and PIN7 containing 10 g/L, 20 g/L, 30 g/L, 70 g/L, 80 g/L and 100 g/L respectively. These experiments were designed to ascertain the effect of glycerol concentrations on biomass accumulation.

The optical densities and dry cell weights at the different concentrations of pure glycerol are shown in Figure 5.6. *R. toruloides* grew in 100 g/L of pure glycerol without substrate inhibition. Optical densities and dry cell weights increased from 3.22 ± 0.02, and 1.97 ± 0.01 g/L to 11.06 ± 0.09, and 6.93 ± 0.15 g/L respectively as pure glycerol concentration was increased from 10 g/L to 100 g/L.

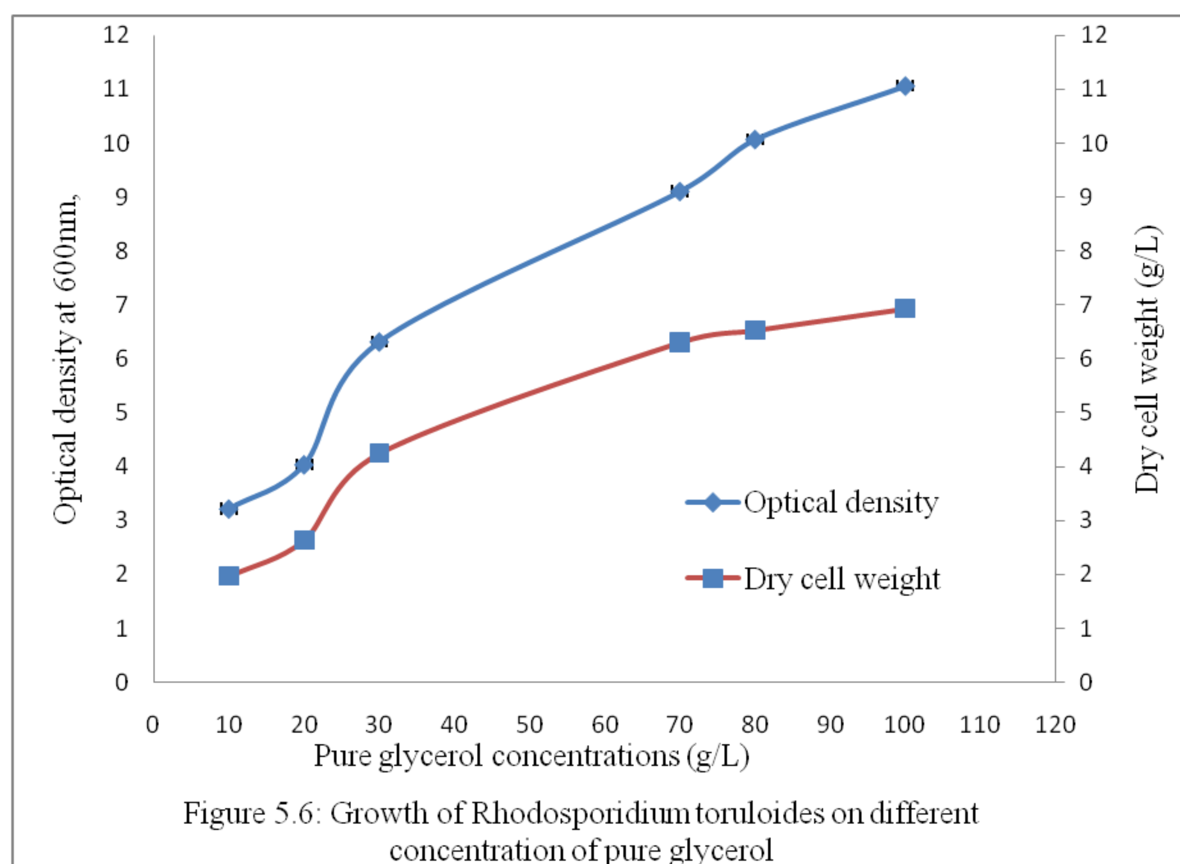


Figure 5.6: Growth of Rhodosporidium toruloides on different concentration of pure glycerol

Cryptococcus curvatus (Meesters, *et al.*, 1996) showed growth inhibition at glycerol concentration higher than 64 g/L while *R. toruloides* showed no growth inhibition up to 100 g/L. This shows that oleaginous yeasts have optimum concentration of substrates to grow on and the optimum concentration differs with species and strain.

The growth of *R. toruloides* in 100 g/L of pure glycerol was studied; the growth curve is shown in Figure 5.7.

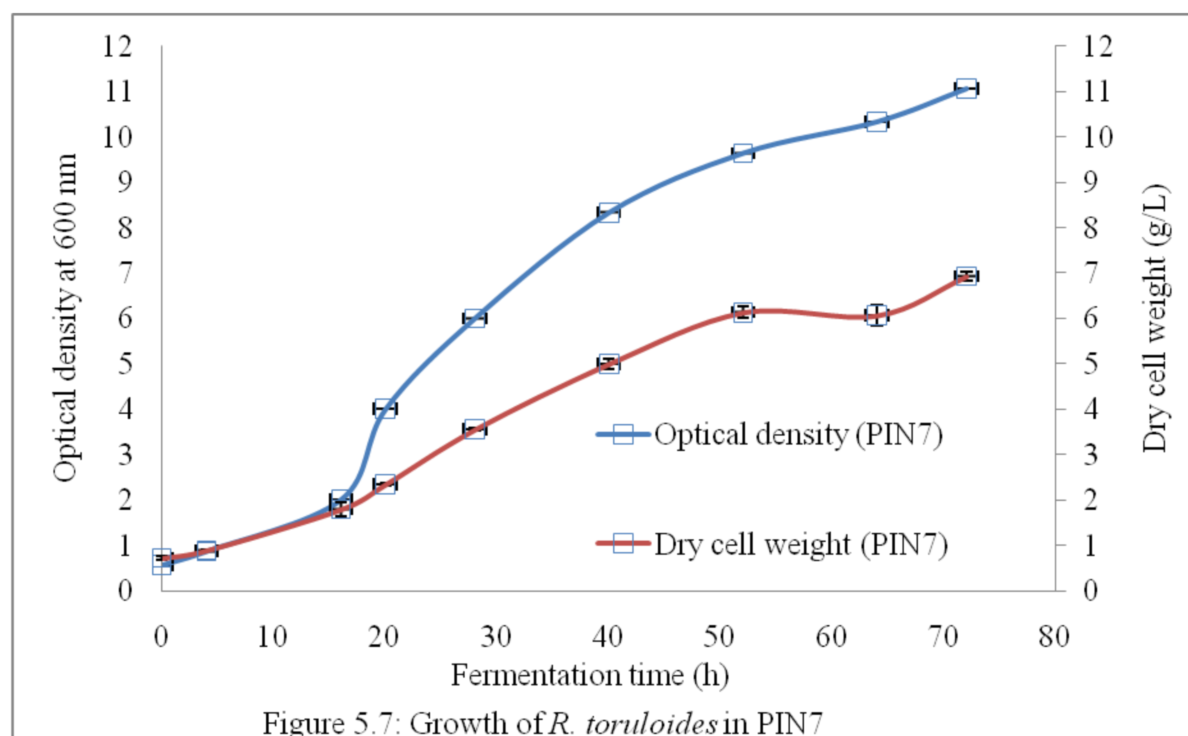


Figure 5.7: Growth of *R. toruloides* in PIN7

R. toruloides accumulated biomass of 6.93 ± 0.04 g/l.

5.3.2 Bioreactor studies on the growth of *Rhodosporidium toruloides* on pure glycerol.
The set-up of bioreactor for growing *R. toruloides* in a bioreactor using pure glycerol was the same with that of glucose. No foaming was observed, therefore anti-foam was not used.

The growth curve is shown in Figure 5.8 below, the lipid content and final biomass accumulated at the end of the fermentation period were 41 ± 0.10% and 13.09 ± 0.15 g/L respectively.

The accumulated biomass is higher than that of shake flask when grown in pure glycerol. Since the growth conditions in bioreactor and shake flasks are the same except the oxygen supply in the bioreactor, the high dry weight accumulated might be due to the high oxygen requirement of the yeast. This result is in support of El-Fadaly *et al*. (2009) that energy metabolism and synthesis of cellular components are dependent on dissolved oxygen.

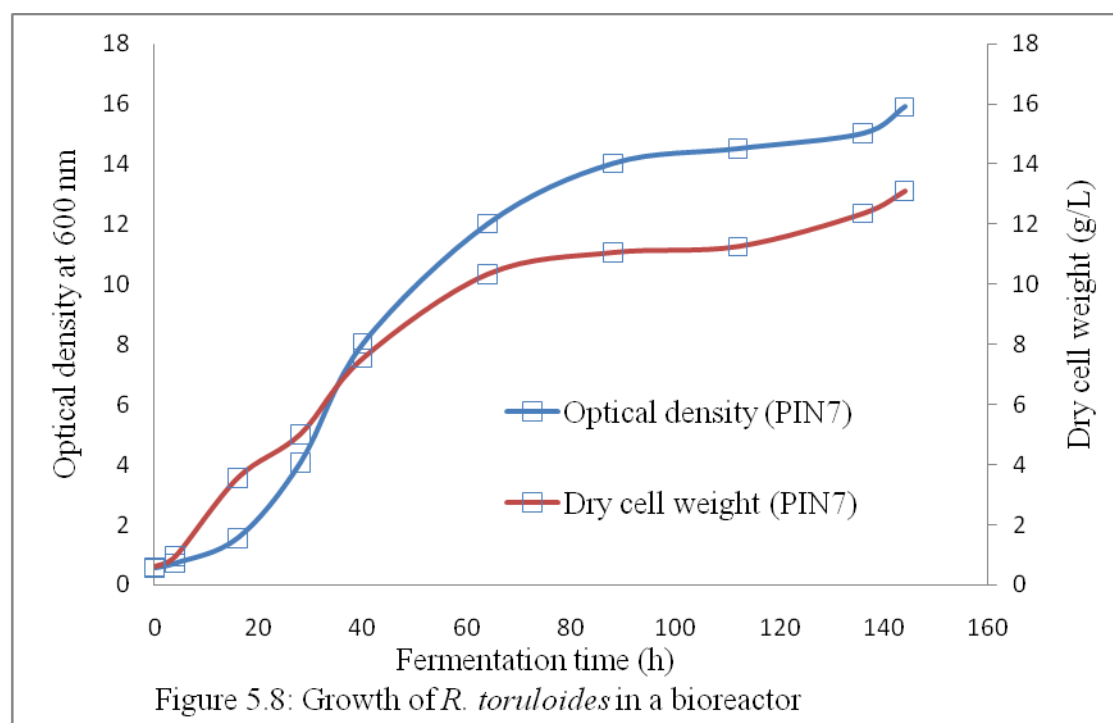


Figure 5.8: Growth of *R. toruloides* in a bioreactor

5.4 Utilization of crude glycerol as carbon source

This experiment was designed to ascertain whether *R. toruloides* could use crude glycerol as carbon source. Crude glycerol is a by product from biodiesel industries and would be economically if it could be used as carbon source by *R. toruloides*. Crude glycerol has been used to grow other oleaginous yeast such as *Cryptococcus curvatus* (Liang *et al*., 2010).but no literature has been written on the ability of *R. toruloides* to use crude glycerol as carbon source therefore it is interested to find out since *R. toruloides* is known for high accumulation of cell density and lipid (Li *et al*., 2007).

R. toruloides was grown on medium CIN1, CIN2, CIN3, CIN4, CIN5, CIN6, CIN7, CIN8, and CIN9 containing 3 g/L, 6 g/L, 8 g/L, 20 g/L, 40 g/L, 100 g/L, 130 g/L, and 160 g/L crude glycerol as carbon source for 72 hours after which the optical density and dry cell weight were measured. The experiment was carried out in triplicates.

Crude glycerol used in this research was bought from Double Green limited United Kingdom. The crude glycerol contains 60% glycerol and other impurities. From the results shown in Figure 5.9, there was an increase in dry cell weight when *R. toruloides* was grown in 3 g/L, 6 g/L, 8 g/L, and 20 g/L crude glycerol however, dry cell weight reduced drastically on further increase in concentration after 20 g/L.

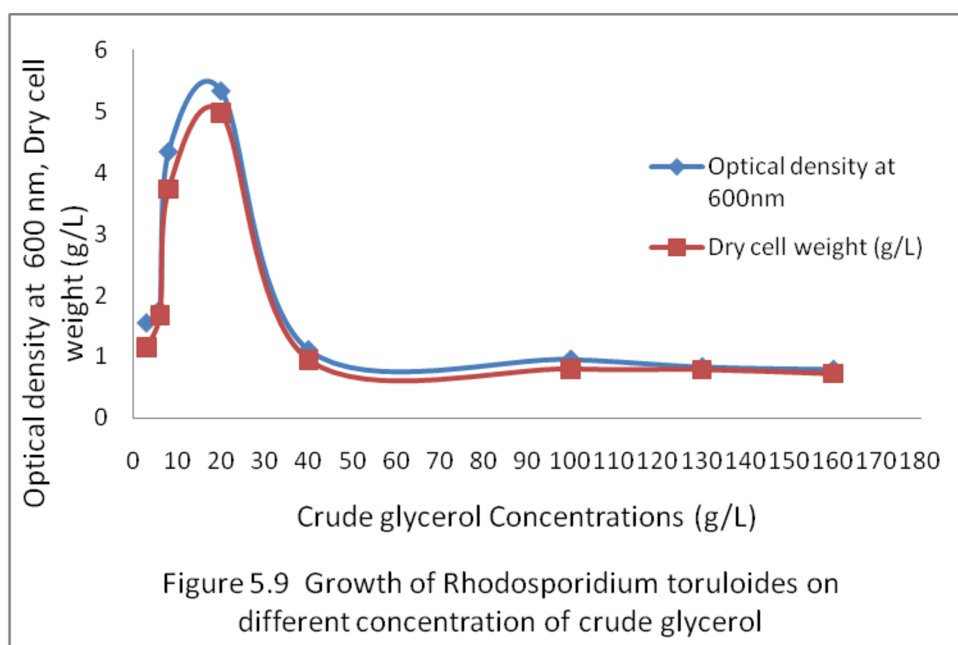


Figure 5.9 Growth of Rhodosporidium toruloides on different concentration of crude glycerol

From Figure 5.9, it is obvious that there was growth inhibition at crude glycerol concentration higher than 20 g/L which resulted in the decrease in biomass concentration. Pure glycerol is not toxic to *R. toruloides* cells since results in the previous section confirmed that *R. toruloides* can utilize pure glycerol as carbon source however what is toxic to the oleaginous yeast cell is the impurities in the crude glycerol such as methanol and soap.

5.5 Influence of nitrogen sources on cell density (biomass accumulation)

From the results on the growth of *R. toruloides* on different nitrogen sources, it is obvious that *R. toruloides* can utilize both inorganic and organic nitrogen as nitrogen source.

R. toruloides accumulated the highest cell density of 10.42 ± 0.80 g/L using peptone (GPEP) as nitrogen source while the lowest cell density of 6.52 ± 0.52 g/L was accumulated using corn steep liquor (GCSL) as nitrogen source as shown in Figure 5.10.

No remarkable difference in cell density was observed after 72 h and 144 h of fermentation using the four nitrogen sources.

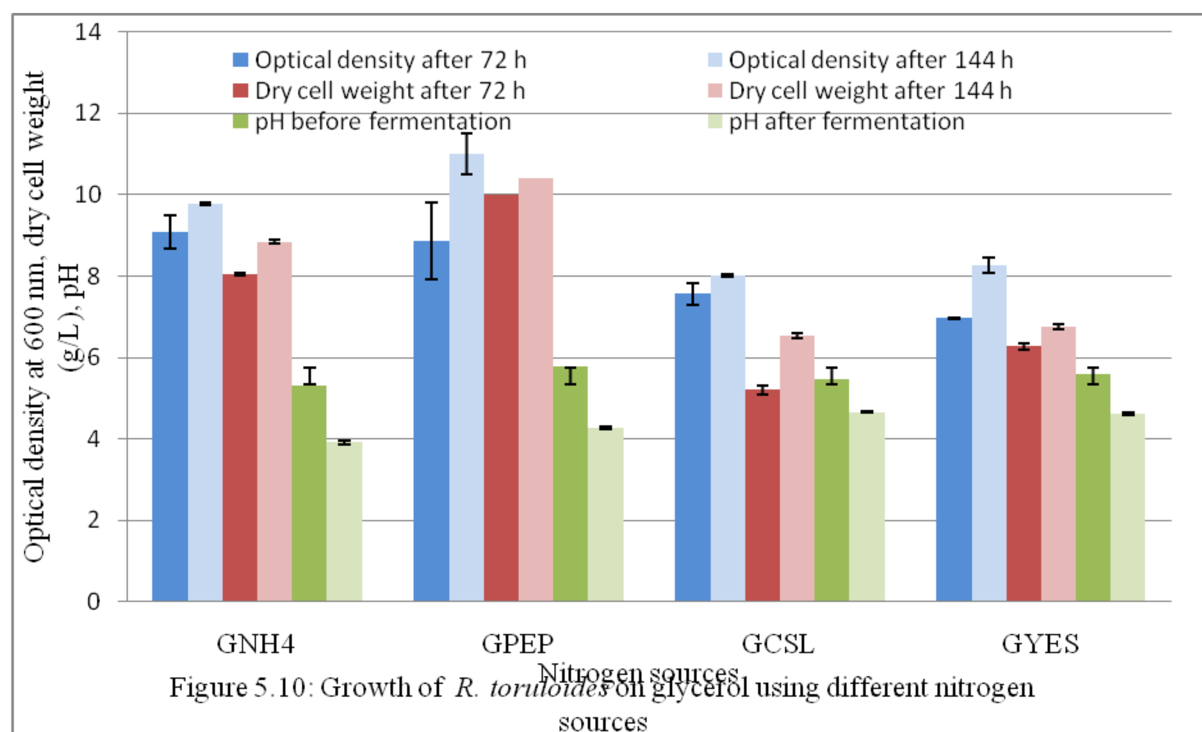


Figure 5.10: Growth of *R. toruloides* on glycerol using different nitrogen sources

When grown on pure glycerol using ammonium sulphate (PNH4), corn-steep liquor (PCSL), and yeast extract (PYES), it was observed that after 144 h, *R. toruloides* accumulated almost the amount of biomass which was higher than the amount of biomass accumulated when grown on peptone (PPEP) although there was significant difference in the accumulated biomass as shown in Figure 5.11. There was no remarkable difference in biomass accumulated after 72 h and 144 h when *R. toruloides* was grown in ammonium sulphate and peptone, however, there was much difference in accumulated biomass when yeast extract and corn-steep liquor were used as nitrogen source.

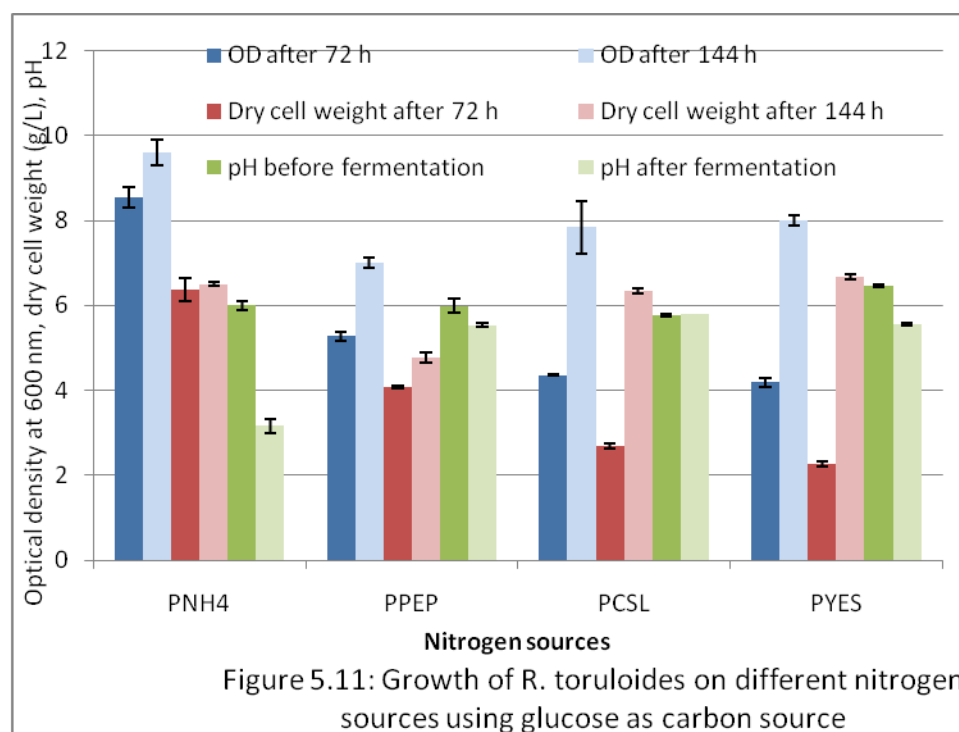


Figure 5.11: Growth of R. toruloides on different nitrogen sources using glucose as carbon source

An interesting observation is that *R. toruloides* accumulated almost the same cell density with corn steep liquor after 144 h. This indicates that corn-steep liquor which is cheap organic nitrogen can accumulate the same biomass as ammonium sulphate which is a very expensive nitrogen source and could be use to replace ammonium sulphate in order to reduce fermentation cost.

The lower cell densities obtained after 72 h with corn-steep liquor and yeast extract might be due to growth inhibition which might either be substrate or product inhibition. It might also be due to formation of complexes between corn-steep liquor and pure glycerol.

5.6 Summary

R. toruloides could grow at 100 g/L of glucose and pure glycerol without any noticeable inhibition. Highest cell density was achieved with pure glycerol than with glucose however, lipid content was higher in glucose. The results showed that crude glycerol can be utilized by

R toruloides as carbon source but a concentration above 20 g/L of crude glycerol is toxic to the yeast.

From the results, it was observed that oxygen supplied to *R. toruloides* increased the biomass accumulation. *R. toruloides* accumulated higher cell density in bioreactors were oxygen was supplied than in the shake flasks. The dry cell weights when grown in different carbon and nitrogen sources is summarised in Table 5.1

Nitrogen source is very important when growing *R. toruloides*. Higher cells density was got with peptone as nitrogen source when glucose was used as carbon source, however with glycerol as carbon source; the highest cell density was got when yeast extract was used as nitrogen source.

Table 5.1: Summary of results on growing *R. toruloides* on different medium

Mode of cultivation	Source of carbon	Dry cell weight (g/L)	Lipids (g/L)	Lipid yield	Lipid content (w/w%)
Batch (bioreactor)	glucose	10.45	5.00	0.48	48
Batch (Bioreactor)	Pure glycerol	13.09	5.37	0.41	41

CHAPTER SIX CONCLUSIONS AND FUTURE WORK

The ratio of carbon to nitrogen is very important in maximizing lipid production but the biomass accumulation is dependent on the source of carbon and nitrogen this is confirmed in the results discussed in the previous chapter.

R. toruloides could utilize crude glycerol as carbon source though there was growth inhibition after 20 g/L of crude glycerol, however, the growth conditions was not optimized Optimization of the growth condition might improve the biomass accumulation and lipid yield.

Future work is recommended in modelling the growth of different oleaginous using different carbon sources. Studies on optimizing the growth conditions of *R. toruloides* on pure glycerol is recommended since *R. toruloides* accumulated almost the cell density when grown on the two carbon source. More studies on the use of corn steep liquor as nitrogen source is recommended in order to optimize growth and lipid production of oleaginous yeasts since corn steep liquor is a cheap nitrogen source. From the results, corn steep liquor was used to accumulate almost the same cell density when ammonium sulphate was used.

In addition, more studies on the effects of oxygen supply on the growth of different species of oleaginous yeast is recommended since from this study, *R toruloides* accumulated higher cell density when oxygen is supplied in the bioreactor than in shake flasks .

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