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MICROBIAL LIPID PRODUCTION AND NUTRIENT REMOVAL FROM POTATO PROCESSING WASTEWATER USING OLEAGINOUS FUNGI

A dissertation submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

by

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TABLE OF CONTENTS

ABSTRACT…………………………………………………………………… i

ACKNOWLEDGEMENT…………………………………………………… iii

1.0 INTRODUCTION

1.1 BACKGROUND……………………………………………………………… 1

1.2 RESEARCH AIM AND OBJECTIVES………………………………………… 2

1.3 RESEARCH PROCEDURES………………………………………………… 3

1.4 STRUCTURE OF THE THESIS……………………………………………… 5

2.0 LITERATURE REVIEW

2.1 INTRODUCTION…………………………………………………………… 6

2.2 ENERGY SECURITY AND ENVIRONMENTAL CONCERNS…………... 6

2.3 BIODIESEL………………………………………………………………… 12

2.4 MICROBIAL LIPIDS……………………………………………………… 13

2.4.1 Oleaginous microorganims……………………………………………… 14

2.4.2 Brief history of microbial lipids…………………………………………… 15

2.4.3 Biochemistry of microbial lipid production ……………………………… 18

2.5 CULTIVATION MODES EMPLOYED FOR MICROBIAL LIPID PRODUCTION…………………………………………………………………… 22

2.5.1 Batch cultivation………………………………………………………… 22

2.5.2 Fed- batch cultivation…………………………………………………… 23

2.5.3 Continuous cultivation…………………………………………………… 23

2.6 REVIEW OF MICROBIAL LIPID PRODUCTION FROM RENEWABLE AND WASTE MATERIALS…… 24
2.6.1 Agricultural by products…… 24
2.6.2 Whey…… 25
2.6.3 Molasses…… 28
2.6.4 Glycerol…… 28
2.6.5 Sewage sludge…… 29
2.6.6 Monosodium glutamate wastewater…… 30
2.7 PROPERTIES OF SCO PRODUCED FROM WASTE MATERIALS…… 31
2.8 SUMMARY…… 31

3.0 ISOLATION, SCREENING AND IDENTIFICATION OF OLEAGINOUS FUNGI FROM IRISH SOILS

3.1 INTRODUCTION…… 32
3.2 MATERIALS AND METHODS…… 33
3.2.1 Soil sample collection…… 33
3.2.2 Isolation of oleaginous fungi…… 34
3.2.3 Screening for lipid production…… 34
3.2.4 Analytical methods…… 35
3.3 MOLECULAR CHARACTERIZATION OF FUNGAL ISOLATES…… 37
3.3.1 DNA extraction…… 37
3.3.2 PCR amplification…… 37
3.3.3 Nucleotide sequencing…… 38

3.4 RESULTS AND DISCUSSION…….. 38

3.4.1 Isolation and screening of oleaginous fungi…….. 38

3.4.2 Growth and microbial lipid production of isolates in the glucose containing medium……... 39

3.4.3 Growth and microbial lipid production of isolates in the starch containing medium……... 42

3.4.4 Identification of oleaginous fungi by using molecular techniques……..... 46

3.5 SUMMARY…….. 50

4.0 MICROBIAL LIPID PRODUCTION FROM POTATO PROCESSING WASTEWATER USING OLEAGINOUS FUNGI *ASPERGILLUS ORYZAE*

4.1 INTRODUCTION…….. 51

4.2 MATERIALS AND METHODS…….. 52

4.2.1 Characteristics of potato processing wastewater……................................ 52

4.2.2 Microorganisms and cultivation conditions……... 52

4.2.3 Dilution of potato processing wastewater…….. 53

4.2.4 Analysis…….. 54

4.2.5 Statistical analysis……... 55

4.3 RESULTS AND DISCUSSION…….. 55

4.3.1 Lipid production by *Aspergillus oryzae* ………………………………….. 55

4.3.2 Lipid composition ………………………………………………………. 60

4.3.3 Starch utilization and amylase secretion ……... 61

4.3.4 Removal of COD and nutrients from wastewater…….................................. 64

4.4 SUMMARY………………………………………………………… 66

5.0 UTILIZATION OF POTATO PROCESSING WASTEWATER FOR MICROBIAL LIPIDS AND GAMMA-LINOLENIC ACID PRODUCTION BY OLEAGINOUS FUNGI

5.1 INTRODUCTION…………………………………………………… 67

5.2 MATERIALS AND METHODS……………………………………… 67

5.2.1 Microorganisms………………………………………………… 67

5.2.2 Potato processing wastewater………………………………… 68

5.2.3 Cultivation conditions………………………………………… 68

5.2.4 Analytical methods……………………………………………… 69

5.3 RESULTS AND DISCUSSION…………………………………… 70

5.3.1 Growth and Lipid production of *A. flavus* I16-3 and *M.rouxii*………. 70

5.3.2 GLA production ……………………………………………….. 76

5.3.3 Nutrient removal from wastewater……………………………… 79

5.4 SUMMARY………………………………………………………… 84

6.0 CHARACTERIZATION OF MICROBIAL LIPID PRODUCTION BY *MUCOR ROUXII* WITH DIFFERENT CARBON SUBSTRATES

6.1 INTRODUCTION…………………………………………………… 85

6.2 MATERIALS AND METHODS……………………………………… 86

6.2.1 Microorganism and cultural conditions………………………… 86

6.2.2 Analytical methods……………………………………………… 87

6.3 RESULTS AND DISCUSSION…………………………………… 88

6.3.1 Biomass growth, carbon source consumption and lipid production…… 88

6.3.2 Substrate uptake rate and hydrolytic activity of *M.rouxii*……................. 93

6.3.3 Composition of microbial lipids under different carbon sources……… 95

6.4 SUMMARY……... 98

7.0 CONCLUSIONS AND RECOMMENDATIONS

7.1 OVERVIEW……... 99

7.2 CONCLUSIONS……... 99

7.2.1 Isolation of local oleaginous fungi from Irish soil……............................. 99

7.2.2 Microbial lipid production from potato processing wastewater by *Aspergillus oryzae*……... 100

7.2.3 Effects of nutrient addition on lipid and GLA production from potato processing wastewater by *Aspergillus flavus* I16-3 and *Mucor rouxii*……......... 100

7.2.4 Biochemical behaviors of oleaginous fungi cultivated with pure carbon sources…….. 101

7.2.5 Summary…….. 101

7.3 RECOMMENDATIONS FOR FUTURE RESEARCH………….. 102

BIBLIOGRAHY 104

APPENDICES

Appendix A………………….. 119

Appendix B …….. 122

teṣāḿ satata-yuktānāḿ
bhajatāḿ prīti-pūrvakam
dadāmi buddhi-yogaḿ taḿ
yena mām upayānti te
(Bhagavad Gita 10:10)

To those who are constantly devoted to serving Me with love, I give the understanding by which they can come to Me.

All the results of this thesis are a humble offering onto the lotus feet of The Supreme Personality of Godhead.

ABSTRACT

Microbial lipids can be used as a feedstock for biodiesel production, and use of biowastes as the substrate for microbial lipid production can reduce its production cost. The aims of this research include: (i) isolation and identification of local oleaginous species from Irish soils; (ii) feasibility of oleaginous fungi for microbial lipid production from potato processing wastewater, in addition with nutrient removals from wastewater; and (iii) microbial lipid production using pure carbon sources for comparison.

Local oleaginous fungi were isolated from Irish soils with the aim of producing microbial lipids with low cost starch substrates. 30 cultures were oleaginous with glucose as the carbon substrate; the maximum lipid content in biomass (44.3%) was observed in the isolate I16-3. When the isolates were grown on starch medium, 19 cultures among 30 cultures were able to accumulate lipids. The maximum lipid content (39%) was observed in I16-3. The isolate was identified as *Aspergillus flavus*. This study also explored the amylase secretion capability of isolated cultures and the maximum yield (34 IU/mL) was observed in the isolate I16-3. Identification of isolated fungi with molecular techniques revealed a number of non mucaraceous fungi including *Aspergillus* sp., *Penicillum* sp., *Tricoderma* sp., and *Aceremonium* sp. along with mucaraceous fungi *zygomycetes* sp., *Mortierella* sp. and *Umbliopsis* sp. These non mucaraceous oleaginous fungi would be of future interest in generation of the second generation biodiesel.

Potential use of potato processing wastewater for microbial lipid production was studied under various conditions. Using *Aspergillus oryzae,* diluting the wastewater by adding 25% of tap water achieved the maximum lipid yield of 3.5 g/L. It was observed that phosphate limitation may be the mechanism to stimulate microbial lipid production. At this dilution ratio, total soluble starch was utilized by 99% and the fungi secreted amylase of 53.5 IU/mL. Nutrient removal from potato processing wastewater was examined; at this dilution ratio, removals of chemical oxygen demand, total soluble nitrogen, total soluble phosphorus, ammonium nitrogen, orthophosphate and sulphate of 91%, 98%, 97%, 100%, 100% and 30% were achieved. Lipids of *A.oryzae* contained major fatty acids like palmitic acid (12.6%), palmitolic acid (4.3%), stearic acid (14.5%), oleic acid (22.5%), linolenic acid (5.5%), and linoleic acid (6.5%).

When external nutrients were added to raw potato processing wastewater for lipids and gamma -linolenic acid (GLA) production using two oleaginous fungi, *Aspergillus flavus* I16-3 and *Mucor rouxii*, the lipid and GLA yields were enhanced; 3.5 and 4.2 g/L of lipids, and 100 and 140 mg/L of GLA were produced by *Aspergillus flavus* I16-3 and *Mucor rouxii,* respectively. In addition, the wastewater was efficiently treated, when external nutrients were added to raw wastewater, with total soluble chemical oxygen demand removals of 60% and 90%, total soluble nitrogen removals of 100% and 98%, total soluble phosphorus removals of 92% and 81% by *Aspergillus oryzae* I16-3 and *Mucor rouxii*, respectively.

In the study with pure glucose, starch and cellulose as the carbon substrate, *Mucor rouxii* was cultured at an initial C/N ratio of 60. The highest lipids yield of 4.9 g/L was found with glucose as the carbon substrate. The lipid content in biomass for starch was less than for glucose. The maximum lipid yield was increased when the starch concentration was increased to 60 g/L and 5.8 g/L of lipids was produced. Cellulose supported *M.rouxii* biomass growth but did not support the lipid production. Significant quantities of α-Amylase (0.5 and 1.2 IU/mL) and cellulase (0.19 IU/mL) were produced by *M.rouxii* to hydrolyze the complex carbon sources. Microbial lipids comprised oleic, palmitic, stearic, linolenic and GLA fatty acids.

On the whole, this research has found non mucaraceous oleaginous fungi for low cost lipid production on starchy wastes. Isolated indigenous fungi have been proven to be efficient in microbial lipid production from potato processing wastewater. Our research also demonstrates the potential use of potato processing wastewater for microbial lipid production with simultaneous wastewater treatment.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

The use of fossil fuels is now widely recognized to be unsustainable due to the depletion of fossil fuel reserves and the emission of greenhouse gases into the environment. There are worldwide concerns on mitigation of greenhouse gas emission, which has already exceeded the "dangerously high" threshold of 450 ppm CO_2-e (Csavina et al., 2011). The transport sector is one of the major contributors to greenhouse gas emissions, because 27% of the global primary energy is used for transportation. Thus replacing fossil fuels used in this sector with sustainable biofuels will mitigate greenhouse gas emissions (Clarens et al., 2011). It is estimated that by 2050, biofuels could provide 27% of total transport fuels and in particular replace diesel, kerosene and jet fuel. The projected use of biofuels can avoid around 2.1 gigatonnes (Gt) of CO_2 emissions per year (IEA, 2010).

Biofuels are carbon neutral. Therefore, they are rapidly being developed. Among the biofuels, liquid fuels such as bioethanol and biodiesel are produced using sugar crops and vegetable oils (i.e., the first generation biofuels). However, their production unavoidably competes with the food manufacturing sector for land and water availability. Due to the first generation biofuels, the overall food price has risen by 15 - 25% (Sims et al., 2010). This effect has stimulated the interest in development of the second generation biofuels which are produced from non food biomass such as lignocellulosic feedstocks (cereal straw, sugarcane bagasse, forest residue, etc.) and biowastes.

Microbial lipids have recently been seen as a promising feedstock for the second generation biodiesel production because of their advantages over vegetable oils, such as no requirement for huge acres of land and a much shorter production time than energy crops. The microbial lipid production is also less affected by the seasonal change and the climate. Even though the microbial lipid production technology has been studied in the past 100 years, the number of oleaginous fungi currently in use is limited and generally belongs to the zygomycetes division. Most oleaginous zygomycetes are well known for production of lipids rich in poly unsaturated fatty acids (PUFA), especially gamma linolenic acid (GLA) which is of nutritional importance. On the other hand, if the microbial lipid contains too high GLA, for example more than 12%, its use for biodiesel production becomes unrealistic because of the strict European Union regulation

(EN14214 and 14213) (Feofilova et al., 2010). Hence, there is a need for isolation and identification of more oleaginous fungi for microbial lipids production, especially, oleaginous fungi capable of hydrolyzing various second generation feedstocks (Meng et al., 2009). Furthermore, isolation and screening of local species ensures protection of the local ecology which will be under threat while using imported species.

The major bottleneck in microbial lipid production is the high raw material cost, which accounts for 70% of the production cost (Azocar et al., 2010). Utilization of cheap raw materials for production of microbial lipids would reduce the production cost, consequently the cost of the second generation biodiesel (Huang et al., 2009; Li et al., 2011). Such raw materials include rice straw hydrolysate (Economou Ch et al., 2011), cheese whey (Vamvakaki et al., 2010), wheat bran (Peng and Chen, 2008), and sewage sludge (Angerbauer et al., 2008), etc.

Potato processing wastewater is one of the prospective low cost raw materials available in Ireland, since potato is grown over an area of 26,869 acres with a production of 43,6028.8 tonnes annually (Mbougueng et al., 2009). Processing of potato generates a large amount of wastewater (Gelinas and Barrette, 2007). In many cases potato processing wastewater is not properly treated to discharge but delivered to landfill. The main organic matter in potato processing wastewater is starch. Oleaginous fungi are able to accumulate lipids on different carbon sources especially polysaccharides such as starch (Papanikolaou et al., 2007).

Thus in this research potato processing wastewater was studied for low cost microbial lipid production with the aim to use the lipids as the feedstock for the second generation biodiesel. With the production of such high value products, the wastewater would be treated and its harm to the environment would be reduced.

1.2 RESEARCH AIM AND OBJECTIVES

The overall aim of this PhD research was to study the feasibility of microbial lipid production by oleaginous fungi with potato processing wastewater as the raw material. Specific objectives of this research were:

1. To isolate and screen indigenous oleaginous fungi from Irish soils and to identify isolated cultures;
2. To study the characteristics of microbial lipid production by oleaginous fungi from potato processing wastewater under various operational conditions;
3. To study biochemical behaviors of oleaginous fungi on various carbon sources.

Use of potato processing wastewater as the raw material for microbial lipid production has not yet been carried out. This is the novelty of this PhD research.

1.3 RESEARCH PROCEDURES

The entire research work was carried out in the Environmental Engineering Laboratory at NUI Galway. All fermentation experiments were carried out in aseptic conditions. Soil samples were collected from various locations in western Ireland and used for isolation study. Screening of isolated cultures for lipid production was carried out in the laboratory. DNA extraction, polymerase chain reaction (PCR), and sequencing techniques were used to identify the isolated cultures.

Batch fermentation studies were performed to evaluate the feasibility of use of potato processing wastewater for microbial lipid production by known and isolated oleaginous fungi. In addition, removals of contaminants, like chemical oxygen demand (COD), nitrogen and phosphorus, from the wastewater were examined.

During the batch fermentation study, dilution of raw potato processing wastewater with tap water was adopted to avoid the gelatinization of starch during sterilization. At various dilution ratios, the lipid yields of oleaginous fungi and nutrient removals from wastewater were studied. The profiles of long-chain fatty acids in microbial lipids were analyzed using gas chromatography.

In another fermentation experiment, external nutrients were added to raw potato processing wastewater to study microbial lipid and GLA production. Finally biochemical behaviors of oleaginous fungi on three different carbon sources - glucose, starch and cellulose - were studied. Similarities and difference in lipid accumulation, substrate uptake, enzymatic hydrolysis of sugars, and lipid composition were studied.

The procedures of the research work and structure of the PhD thesis are given in Fig. 1.1.

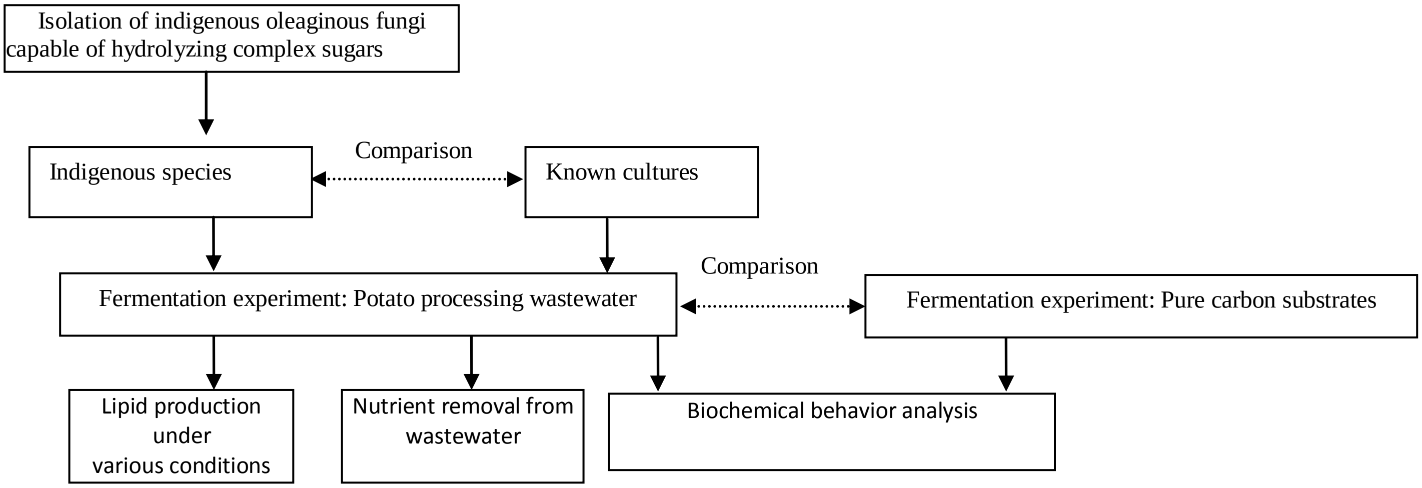

Isolation of indigenous oleaginous fungi capable of hydrolyzing complex sugars
Indigenous species
Comparison
Known cultures
Fermentation experiment: Potato processing wastewater
Comparison
Fermentation experiment: Pure carbon substrates
Lipid production under various conditions
Nutrient removal from wastewater
Biochemical behavior analysis

Figure 1.1 Diagram of research procedures and PhD thesis structure

Figure 1.1 describes the steps involved in this research. Firstly, indigenous oleaginous fungi were isolated from local Irish soils and screened for their capability in microbial lipid production. Then, fermentation experiment was carried out on the isolated fungal species and known fungal species with potato processing wastewater as the cultivation substrate. Both isolated and known cultures were compared for microbial lipid yields, nutrient removal efficiency and biochemical behaviors on potato processing wastewater. Finally, fermentation experiment was conducted with pure carbon sources including glucose, starch and cellulose as the cultivation substrates. The differences in microbial lipid production and biochemical behaviors with potato processing wastewater and pure carbon sources as cultivation substrates were compared.

1.4 STRUCTURE OF THE THESIS

The structure of the thesis is as follows:

Chapter 2 describes review of literatures concerning biodiesel production and biochemistry in association with microbial lipids. A review of waste materials used for microbial lipid production is also included in this chapter.

Chapter 3 describes the isolation and screening of oleaginous fungi from Irish soils, as well as the identification of isolated oleaginous species using molecular techniques.

Chapter 4 discusses the use of potato processing wastewater for microbial lipids production by ***Aspergillus oryzae***. The microbial lipid yield and nutrient removal efficiency were improved by dilution of the raw potato processing wastewater with tap water.

Chapter 5 studies production of microbial lipids and gamma linolenic acid by ***Aspergillus flavus I16-3*** and ***Mucor rouxii***.

In Chapter 6 pure carbon substrates, including glucose, starch and cellulose, were used to culture ***Mucor rouxii*.** The physiological responses of the fungus such as lipid production, substrate uptake, long-chain fatty acid composition in lipids and enzyme secretion are analyzed in this chapter.

Finally in Chapter 7 conclusions drawn from the present PhD research and recommendations for future research are presented.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Microbes have been exploited to produce different high value products such as enzymes, proteins, antibiotics, vitamins, etc. Use of microorganisms for production of lipids (commonly called as single cell oils) commenced during the 18th century in Germany. In the beginning, the intent was to use microbial lipids as an alternative to vegetable oils. Then, biochemical studies were conducted and key enzymes involved in microbial lipid production were explored. Special fatty acids such as gamma linolenic acid (GLA), arachidonic acid (AA) and docosahexaenoic acid (DHA) were identified and their benefits as neutraceuticals and food additives were popularized and are now being produced in a large scale. In the past decade microbial lipids have been considered as an alternative feedstock for biodiesel production due to the contemporary issues on climate change, renewable energy and food security. Recently, concerns have been given on the exploration of various cheap raw materials for economic microbial lipid production, which is considered as a solution to reduce biodiesel production cost and to achieve sustainable management of biowastes. In this chapter historical development of microbial lipids, biochemistry of lipid accumulation by oleaginous microorganisms, and lipid production from biowastes are reviewed and discussed.

2.2 ENERGY SECURITY AND ENVIRONMENTAL CONCERNS

Existing primary energy sources are mainly fossil fuels, such as crude oil, coal and natural gas. These energy sources are directly extracted or captured from natural sources and their consumption has almost doubled from 256 to 505 million gigajoule (GJ) since 1973 to 2007. Figure 2.1 shows the energy production in the world since 1971; the production of energy from different sources has risen gradually. Use of oil for energy production is higher than use of other sources. The balance reserves of coal, crude oil and natural gas in 2011 were 1,089 billion tons, 1,342 billion barrels and 6,300 trillion cubic feet, respectively (IEA, 2009). Decline of petroleum reserves is at a rate between 2% and 3% per year from 2010 (Campbell, 2006).

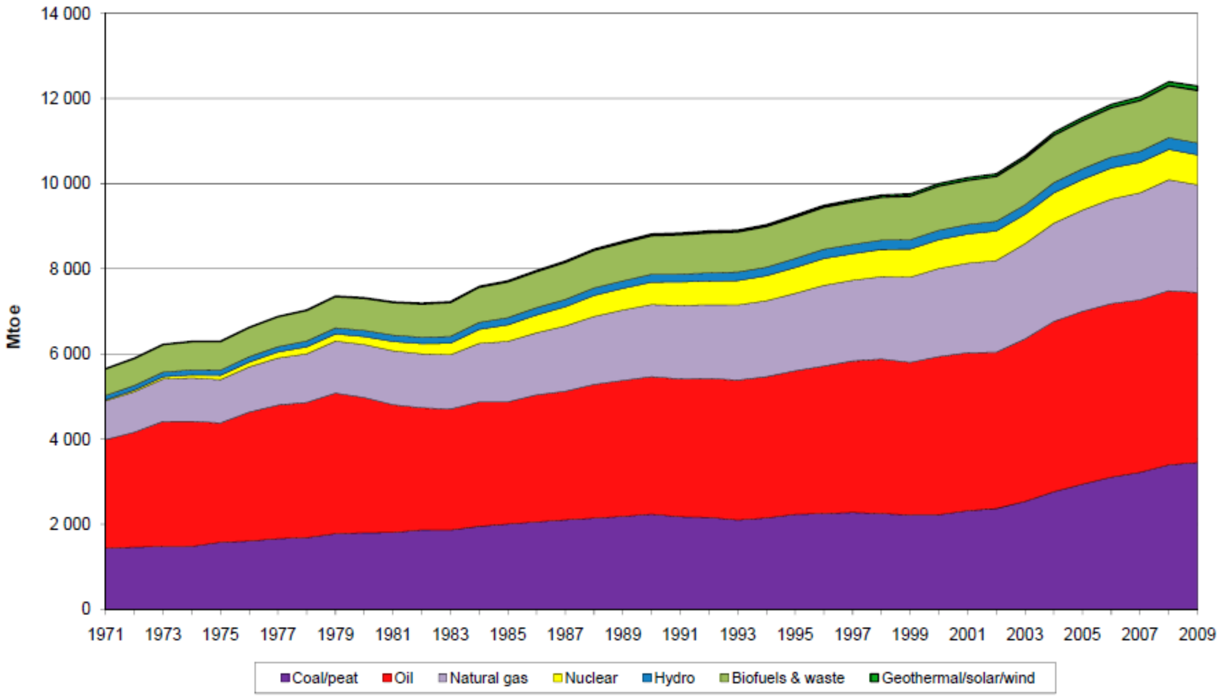


Figure 2.1 Worldwide energy production from 1971 to 2009 (Source: IEA, 2011)

Another concern about the usage of fossil fuels is the emission of greenhouse gases (GHG). When the primary energy sources are transformed to the secondary energy for commercial use the process releases GHG to the environment. For instance, burning of coal (primary energy) to generate electricity (secondary energy) released 7,000 Mt of CO_2 in 2009. Figure 2.2 shows CO_2 emission due to burning of different fuels is increased year by year. In 2008, among the total CO_2 emissions from burning fuels, 43%, 37% and 20% was released from burning of coal, oil and natural gas, respectively (IEA, 2009).

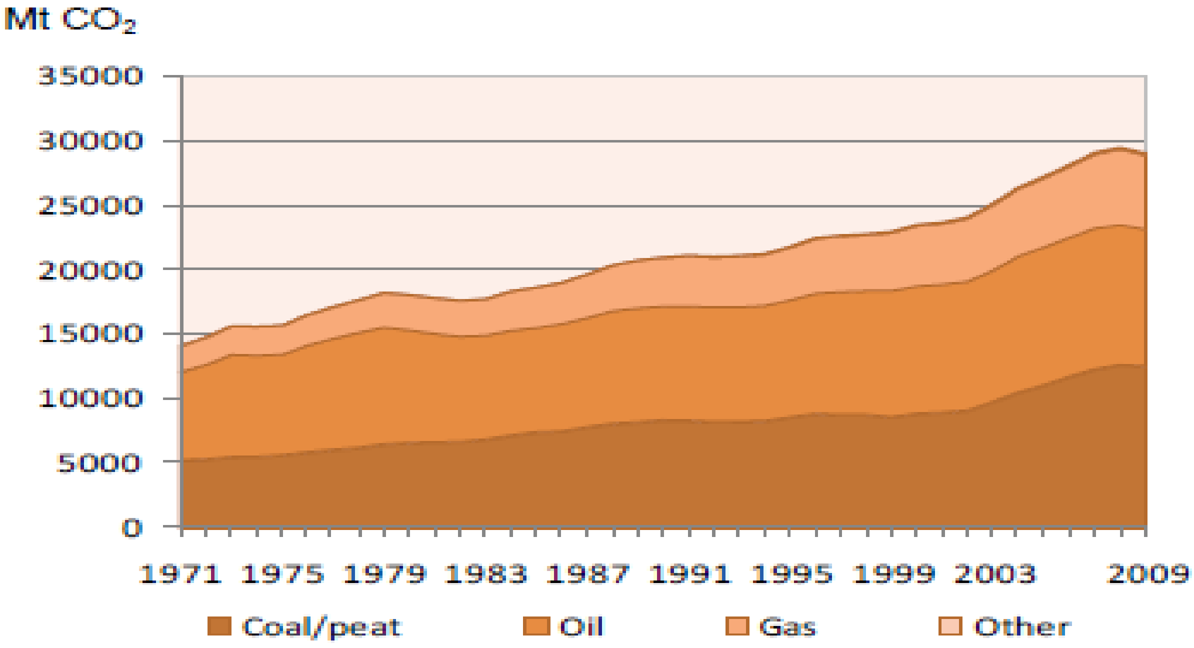


Figure 2.2 CO_2 emissions from burning of fuels (source: IEA, 2009)

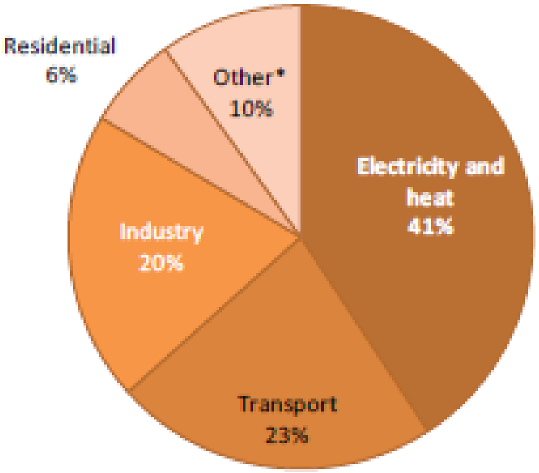


Figure 2.3 CO_2 emissions by the sector (source: IEA, 2009)

Among fossil energy sources 28% of total available energy is used in the transport sector worldwide (Antoni et al., 2007). A strong increase in global mobility is anticipated and the needs will triple by 2050. Today, 52% of total petroleum production is used for transportation. By 2030 this share is expected to increase to 57% (World Business Council for Sustainable Development, 2004). Burning of petroleum releases GHG to the environment, globally about 11,230.54 Mmt (million metric tonnes) of CO_2 was released due to burning of petroleum in 2009 (Batan et al., 2010) and 23% of the emission was from the transport sector (Fig. 2.3). On the whole, the GHG concentration in the atmosphere has exceeded the threshold of 450 ppm CO_2–equivalent (Schenk et al., 2008). Hence, the use of fossil fuels is now widely recognized as unsustainable due to depletion of resources and emissions of GHG into the environment. Reducing the use of fossil energy in transportation will play an important role in GHG emission mitigation.

Additionally, during the past two decades the risk and reality of environmental degradation has become more and more serious due to the environmental impact of human activities, with the sharp increase of world population, consumption and industrial activities (Achten et al., 2010). All these economic and geopolitical factors (high oil prices, environmental concerns and supply instability) have prompted policy makers and the public to put emphasis on renewable energy sources (Batan et al., 2010; Semwal et al., 2011).

Use of renewable energy is considered as the only option to reduce the emission of GHG. Renewable energy is the energy coming from natural sources like wind, light, air, tide and biomass (Antoni et al., 2007; Atsumi et al., 2008; Basha et al., 2009; Cheng, 2010). Among the renewable energy sources biofuels are one of the promising options which can replace the fossil fuels in the transport sector. It is estimated that by 2050, biofuels could provide 27% of total transport fuel and contribute in particular to the replacement of diesel, kerosene and jet fuel. This projected use of biofuels can avoid around 2.1 gigatonnes (Gt) of CO_2 emissions per year when produced sustainably (IEA, 2010). There are various types of biofuels available such as solid biomass, liquid fuels (bioethanol and biodiesel) and biogases (methane and hydrogen) (Fang et al., 2008; Fedosov et al., 2011). The major advantage of biofuels is environmental safety, since upon burning they release zero CO_2 (Pfromm et al., 2011). To reduce the CO_2 emission from the transport sector, the Office of the Biomass Programme, US Department of Energy, has developed a scenario for supplying 30 per cent of 2004 motor gasoline demand with biofuels by the year 2030 (Biswas et al., 2011). Similarly the European Union (EU) has developed a vision in which one fourth of the EU's transportation fuels will be derived from biofuels by 2030 (Achten et al., 2010).

Table 2.1 lists different types of biofuels presently used and they are either in the liquid form or gaseous form. Gaseous fuels such as methane and hydrogen have to be liquidised by cooling or compression to reduce their specific volume for storage. This process is energy intensive and would require development of additional infrastructure. In addition, these gases are difficult to transport. Whereas, liquid fuels such as biodiesel and bio hydrocarbons are easier to transport. Hence, transition from fossil fuels to selected liquid biofuels can be smooth and should not require any major engine modifications. While research on hydrocarbon production is in its preliminary stage, biodiesel production has grown significantly.

Based on the type of feedstock sources biofuels are classified as the first generation biofuels or the second generation biofuels. The first generation biofuels (biodiesel, bioethanol and biogas) are derived primarily from food crops such as cereals, sugar crops, animal fats and vegetable oils. The second generation biofuels are produced from non food crops such as crop by-products, biowastes, and lignocellulosic wastes.

The production process of the first generation biofuels is well known and commercial markets are in place. However, the sustainability of the first generation biofuels is under close scrutiny because their production is limited by:

1. Competition for land and water with the food and fibre production industry;
2. High production and processing costs; and
3. Uncertainty of the net greenhouse gas reductions once land use change is taken into account (Sims et al., 2010).

Therefore, more and more attention has been paid on the second generation biofuels.

Table 2.1 Types of biofuels with a focus on production with microorganisms (adopted from Antoni et al., 2007; Peralta-Yahya and Keasling, 2010; Rojo, 2008; Shi et al., 2011; Stephanopoulos, 2007; Wackett, 2011; Wackett, 2008)

Fuel	Chemical name	Sources	Process	Microorganisms involved if necessary
Biodiesel	fatty acid methyl esters (FAME)	vegetable oils, microbial oils, animal fats	transesterfication, heterotrophic fermentation, photosynthesis	microalgae, fungi, yeast, bacteria, engineered E.coli
Bioalcohols	ethanol	enzymatically hydrolyzed starch	homoacedogenic fermentation	yeasts such as *Saccharomyces cerevesiae*, *Pichia stiplis*
	n-butanol	Starch rich wastes	anaerobic fermentation	bacteria such as *Clostriduim acetobutylicum*, *Clostriduim thermococcus*
	methanol, methyl tret butyl ether (MTBE)	methanol, isobutane	acid catalysis	methanogenic bacteria
	propanol	starch rich wastes	anaerobic fermentation	E.coli
Bio hydrogen	hydrogen	Water	bio-photolysis	*Cynobacterium*
		organic substances	photo fermentation	*Anabena variablis*
		pectin, cellulose, xylan ethanol	heterotrophic fermentation	*Clostridium pytofermentas*, *Cladicellulosporium saccharolyticus*
Bio hydrocarbons	n-alkanes	sugars and organic acids	heterotrophic fermentation	*Vibrio furnassi*
	alkenes	branched chain fatty acids	head to head condensation	*Micrococcus* sp.
	isoprenoids	isopentyl- di – phosphate	enzymatic conversion	*Bacillus* sp.
Biogas	methane	cow manure, pig manure, slaughterhouse wastes	anaerobic digestion	methanogenic bacteria such as *Methanosaeta thermophila*

2.3 BIODIESEL

Biodiesel is a renewable liquid biofuel produced from vegetable oils, animal fats and waste cooking oil. In the transport sector, it can be effectively used after blended with fossil diesel or directly used. It has been demonstrated to have significant environmental benefits in terms of decreased global warming impacts**,** reduced emissions**,** greater energy independence and positive impacts on agriculture (Abdulla et al., 2011). It is reported that use of one kilogram of biodiesel reduces three kilograms of CO_2 equivalent emission (Zou and Atkinson, 2003). It reduces the emissions of particulates and other harmful contaminants, as it has an extremely low sulphur content, a high lubricity and fast biodegradability. A brief history of biodiesel is given in Table 2.2.

Table 2.2 History of biodiesel production

1900	Rudolph Diesel first ran engine on peanut oil
1900	Petroleum was cheap and became primary energy source
1930-1940	Vegetable oils were used for biodiesel
1947	Hydrocarbon from Tung oil in China was developed
1960	Large scale cultivation of *chlorella* in Japan started
1970	Global petroleum crisis
1970	Algae as a renewable energy source was considered due to the energy crisis
1970-1990	Usage of vegetable oils for energy was increased and search of other alternative sources began
1978- 1996	Aquatic Species Program (ASP) by National Renewable Energy Laboratory (NREL), USA
1990	Second global energy crisis, leading to consideration of microbial sources for alternative energy
2000	Open pond cultivation of algae was established.
2004	food vs fuel debate started
2005-2012	Development of the second generation biodiesel

The global biodiesel industry has grown significantly over the past decade. The global biodiesel market is estimated to reach 37 billion gallons by 2016, with an average annual growth rate of

42% (Shi et al., 2011). The EU is the leader in biodiesel production with Germany, France and Italy being the major producers. The total EU biodiesel production in 2010 was 9.5 million tonnes which was a 5.5 per cent increase over 2009 and the production has increased sharply with 22 million metric tonnes in 2011 (IEA, 2010). Biodiesel is also produced from Ireland; the production was 28,000 tonnes in 2010 and increased to 76,000 tonnes in 2011 (Kohler, 2011). Today, there are approximately 254 biodiesel plants in the EU. Biodiesel production in the United States has been increased dramatically in the past few years. The production in 2008 was 682,530,000 gallons, and has grown rapidly with the current production of 1 billion gallons in 2011 (IEA, 2010). According to the National Biodiesel Board, there are 147 plants in operation. A rapid expansion in biodiesel production is being observed not only in developed countries but also in developing countries such as China, Brazil, Argentina, Indonesia and Malaysia.

At present 95% of global biodiesel is made from vegetable oils like soybean oil, and rapeseed oil (Azocar et al., 2010). The land use for cultivation of biodiesel crops is increasing. In EU the amount of arable land used for biofuel production in 2006 was 17.5 million hectares in which 3 million hectares were used for growing vegetable oils to produce biodiesel. However, the demand for vegetable oils for human consumption is also rising. The world production of oilseed in 2010 is 453 Mmt, 10 percent more than 2009. Production of vegetable oils requires huge arable land, consumes time, threats food supply, and causes environmental problems such as defrostation, soil erosion and deterioration of the ecological status of water (Abdulla et al., 2011). Use of animal fats and waste cooking oils could not meet the whole demand for biodiesel and the production process needs to be standardized for those materials (Boey et al., 2009; Da Ros et al., 2010). Hence it is necessary to look for alternative oil sources which can be produced in a shorter time with less arable land requirement. Microbial oils are one of the promising options and offer many advantages over vegetable oils. They are considered as a feedstock for the second generation biodiesel production.

2.4 MICROBIAL LIPIDS

Microbial lipids, namely single cell oils (SCOs), are produced by oleaginous microorganisms such as algae, fungi and yeast, and are considered to be promising candidates for biodiesel production as their oil properties are similar to vegetable oils (Kumar et al., 2011). Moreover they do not require huge space and can be produced in a much shorter time than vegetable oils. Also they are less affected by seasons and the climate (Du et al., 2008). The major bottleneck in microbial lipids production is their raw material cost which accounts for 70 per cent of the total production cost. Recent research has been focused on various waste materials for economic lipids production. Several waste materials such as rice straw hydrolysate (Economou Ch et al., 2011), cheese whey (Vamvakaki et al., 2010), wheat bran (Peng and Chen, 2008), sewage sludge (Angerbauer et al., 2008), etc., have been examined for lipid production.

2.4.1 Oleaginous microorganims

Lipids (oils), proteins and carbohydrates are the three macromolecules occurring naturally in microbial systems. Lipids are an important structural component of the microbial cell membrane. Certain microorganisms (algae, yeast, fungi and bacteria) can accumulate more than 20 percent of lipids inside their cells as triacylglycerol (TAG) (Fig 2.4). Those microorganisms are called as oleaginous microorganisms. The oils thus produced are popularly called single cell oils (SCOs) (Ratledge, 2004). Known oleaginous microbes and their lipid contents are given in Table 2.3.

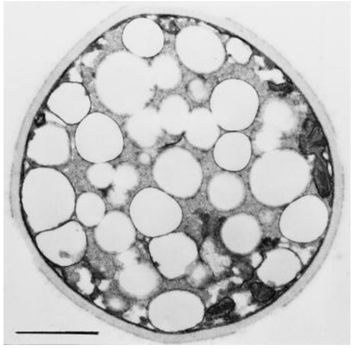


Figure 2.4 Electron micrograph of yeast *Apotrichum curvatum* grown in nitrogen limited medium (2 days) showing lipid droplets (Holdsworth et al., 1988).

Table 2.3 Oleaginous microorganisms available for lipid production (Meng et al., 2009)

Microorganisms	Oil content (% dry weight)
Microalgae	
Botryococcus braunii	25–75
Cylindrotheca sp.	16–37
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23
Bacteria	
Arthrobacter sp.	>40
Acinetobacter calcoaceticus	27–38
Rhodococcus opacus	24–25
Bacillus alcalophilus	18–24
Yeast	
Botryococcus braunii	25–75
Cylindrotheca sp.	16–37
Chlorella sp.	28–32
Rhodotorula glutinis	72
Fungi	
Aspergillus oryzae	57
Mortierella isabellina	86
Humicola lanuginose	75
Mortierella vinacea	66

2.4.2 Brief history of microbial lipids

Attempts to identify microbes for oil production as an alternative to vegetable oils were made in 1875 (Ratledge, 1989; Ratledge, 1992). The first initiative on biodiesel was started when Rudolph Diesel in 1900 used peanut oil to run an engine for several hours; he stated that vegetable oils in future would be one of sources of biodiesel (Griffin, 1993; Ma and Hanna, 1999). Agriculture had a great improvement in 1940s, resulting in identification and development of high yield plants. Consequently, microbial oils had been considered as an uneconomic option and its research was virtually ceased.

Microbial lipids research was developed in UK and US during 1950s and their commercial exploitation began. In the early 1960s, single cell proteins (SCPs) were produced with cheap n-alkanes as raw materials. SCP can be served as animal feed and also sometimes used for human consumption. Industries started commercial SCP production using different feedstocks (Tusé and Miller, 1984). The first open cultivation of *chlorella* algae for microbial lipid production took place in Japan in 1960. Since SCP production from cheap sources was a commercial success, it was thought that microbial lipids would also be an attractive option for commercialization (Ratledge, 1976; Ratledge and Ewing, 1978).

Nevertheless, further historical events overtook both the processes. During mid of the 20th century there was a second boom in the agriculture industry. Varieties of plant oils such as soybean, sunflower, rapeseed, etc., have been planted. By-products of those seed oils leave inexpensive animal feeds making SCP uneconomical. In addition, there is a significant decline in raw materials of the SCP process, due to the increase of oil prices. SCO production had a different development route; although SCO production was expensive there was a great demand for oils with special fatty acids. If microbes capable of accumulating nutritional fatty acids in their lipids could be identified, it was thought that SCO production would be commercially possible. Hence, the SCO production research was focused (and is currently being studied) on oils rich in polyunsaturated fatty acids (PUFA). Studies on biochemical mechanisms for lipid accumulation by oleaginous microbes started simultaneously in this period.

The global petroleum crisis happening in 1970 encouraged the research community to seek alternative oil sources for energy production. Coincidently, the agricultural industry in that period answered many of the requests in the energy sector: vegetable oils were used for biodiesel production; new strategies were developed; and edible and non edible plant oils were tested for biodiesel production. In the case of microbial lipids, algae were considered as potential producers due to their microscopic and photosynthetic nature (raw materials are sunlight and water) and attracted many researchers.

Table 2.4 History of microbial lipid production (adopted from Ratledge, 2004)

1873 - 1878	Consideration of microbes for commodity oils and fats
1920-1945	Identification of lipid producing microorganisms
1945	Boom in the agriculture
1950s	Interest in microbial lipids in US and UK
1960	Single cell protein (SCP) production from n- alkanes
1976-78	Single cell oil (SCO) production was initiated
1978-79	Development of new vegetable oil crops with greater yields
1980-1989	Priority of SCO research was given to oils rich in high value poly unsaturated fatty acids (PUFA)
1992	Production of Cocoa Butter Equivalents (CBE)
1999-2001	Production of microbial PUFAs and biochemical studies on microbial lipids
2001- 2005	Microbial lipids and PUFAs production using renewable and low cost materials
2005 onwards	Consideration of microbial lipids for the second generation biodiesel production; algal SCO production

Several research groups started exploiting native algal species for oil production. One among the major groups was the National Renewable Energy Laboratory (NREL) in USA which started the Aquatic Species Program (ASP) in 1978 to isolate and screen potential algae for microbial oil production. Thus among the microbes algae were considered primarily and studies on screening of algal strains, their biochemistry and physiology of oil accumulation, cultivation in outdoor facilities and genetic improvement of algae for higher oil yields were initiated.

The second petroleum crisis happening during 1990s had created a much serious concern than before. Production of renewable fuels and their significance in environmental protection has been repeatedly emphasised and as a result several renewable fuels are being developed and used. However, over the past decade use of arable land for cultivation of biodiesel crops has resulted in hiking food prices, degradation of soil quality and lack of land for food crops cultivation (Acquaye et al., 2011; Barker, 2008; Dietz et al., 2007). Therefore, SCO offers a positive replacement to vegetable oils. Hence, it has now being considered as a potential candidate for biodiesel feedstock production (Antoni et al., 2007; Nawabi et al., 2011; Shi et al., 2011; Wackett, 2008).

Currently intense genetic, metabolic and transcriptomic studies are being carried out to understand microbial lipid production and to find the means to improve the production capability of oleaginous microorganisms (Courchesne et al., 2009; Nawabi et al., 2011; Peralta-Yahya and Keasling, 2010; Rojo, 2008; Stephanopoulos, 2007; Wackett, 2008). The history of development of microbial lipids is summarized in Table 2.4.

2.4.3 Biochemistry of microbial lipid production

Microbes can naturally synthesize lipids for maintenance of cell membranes, storage of energy and communication. However, only a certain group of microorganisms can accumulate lipids more than 20% of their biomass and store them as triacylglycerol molecules.

Biochemistry of lipid production can be divided into four aspects: (1) nutrient deprivation; (2) cytosol synthesis of intermediates; (3) exchange of molecules between the cytosol and the mitochondria; and (4) lipid synthesis. Figure 2.5 illustrates the lipid production process and involvement of different enzymes.

2.4.3.1 *Nutrient deprivation*

Lipid production in oleaginous microorganisms starts when one of the growth nutrients (usually nitrogen) in the medium runs out and the carbon source is in excess. The cell accumulates excess carbon and converts it into lipids in the form of triacylglycerol. The high C: N ratio in the culturing medium is required for oleaginous microorganisms to accumulate lipids (Holdsworth and Ratledge, 1991). Generally, ammonium ion (ammonium sulphate or ammonium chloride) is served as the deficit nutrient. It should be noted that nitrogen is not the only limiting nutrient for lipid synthesis; if nitrogen is present in excess, limitation of other nutrients can also induce lipid accumulation. Beopoulos et al. (2009) observed that oleaginous yeast started microbial lipids accumulation when zinc, iron or magnesium was depleted. In recent studies it has been demonstrated that phosphate (Wu et al., 2010b) and sulphate (Wu et al., 2011) limitation can also induce lipids accumulation in oleaginous yeast *Rhodotorula glutinis* with the presence of excess nitrogen. This strategy is useful when wastewater is used as a raw material for lipid production, where many nutrients are excessive.

2.4.3.2 *Cytosol synthesis of intermediates*

Cytosol synthesis of intermediates can be classified into a few important groups based on the key enzymes involved as shown in Fig. 2.5.

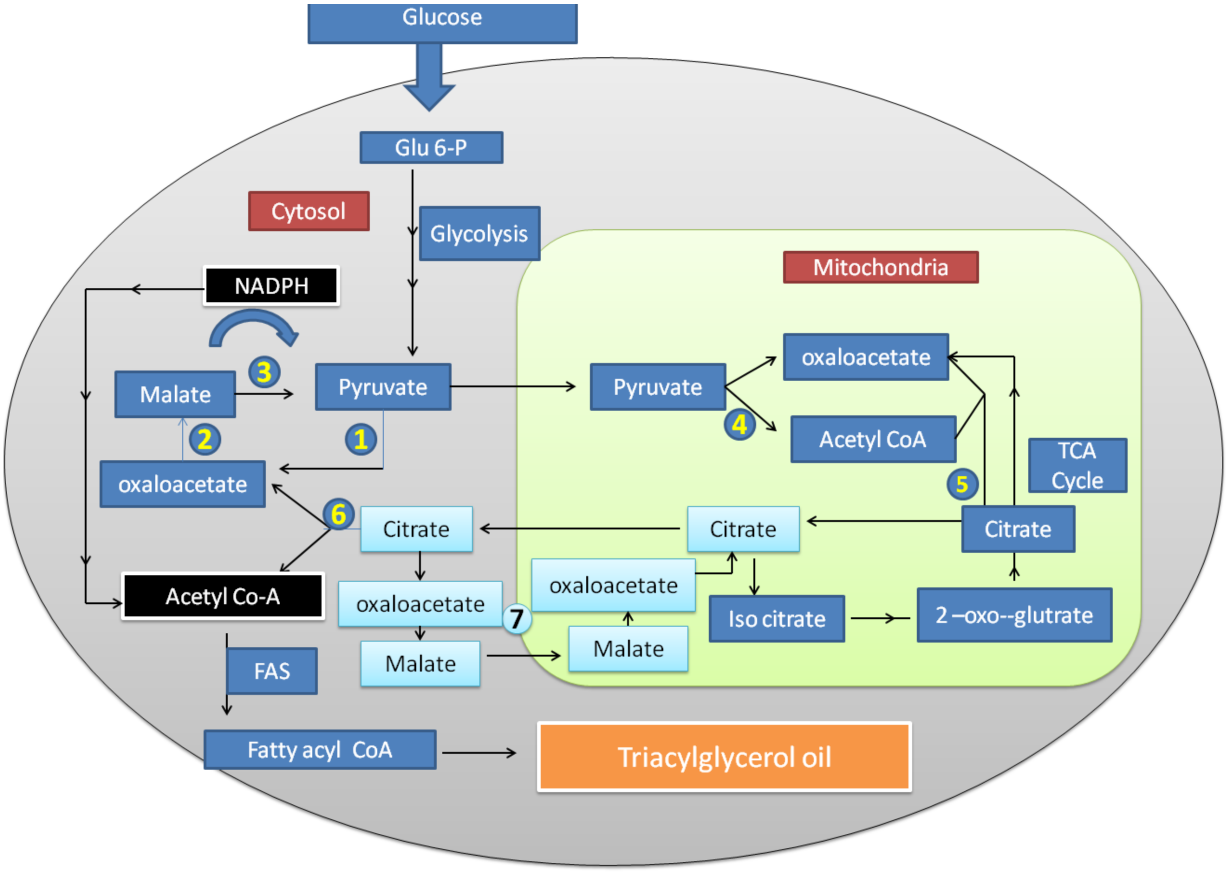

Glucose
Glu 6-P
Cytosol
Glycolysis
Mitochondria
NADPH
Malate
3
Pyruvate
Pyruvate
oxaloacetate
4
Acetyl CoA
2
1
oxaloacetate
5
TCA Cycle
6
Citrate
Citrate
Citrate
Acetyl Co-A
oxaloacetate
oxaloacetate
7
Iso citrate
2 –oxo--glutrate
Malate
Malate
FAS
Fatty acyl CoA
Triacylglycerol oil

Figure 2.5 Lipid synthesis in oleaginous microorganisms. 1. pyruvate decarboxylase; 2. malate dehydrogenase; 3. malic enzyme; 4. pyruvate dehydrogenase; 5. citrate synthase; 6. ATP citrate synthase; 7. citrate/ malate translocase (Adapted from publications of Ratledge, 2004; Ratledge, 2002; Ratledge and Wynn, 2002; Wynn et al., 2001).

Once the key nutrient in the medium runs out, the excess carbon source, for instance glucose, the most common simple sugar, is taken up by the cells and is subjected to catabolism through glycolysis. As continuation of glycolysis glucose undergoes changes in its structure and is cleaved. Phosphofructo kinase (PFK) is the enzyme involved in cleaving glucose into dihydroxyacetone phosphate (DHAP) and glyceraldehydes -3 - phosphate. This PFK enzyme is important in the lipid synthesis pathway. While the nitrogen source NH_4^+-N is taken up by cells, it forms a bond with enzyme PFK before the entire source of nitrogen in the medium is exhausted. This bond is necessary for PFK to be stable since the molecule is suppressed by citrate which is accumulated in the later stages of lipid synthesis. However, when the nitrogen level is high in the medium the regulation between NH_4^+ ions and PFK is not significant. This is confirmed when the yeast cells were grown in glutamate which had more NH_4^+ ions in comparison with NH_4Cl (Evans et al., 1983a; Evans et al., 1983b).

After cleavage glucose undergoes further changes and the end product pyruvate is formed. Pyruvate passes through the mitochondrion membrane to the mitochondrion matrix where it is cleaved into acetyl-CoA and oxaloacetate; this reaction is catalyzed by the pyruvate dehydrogenase enzyme (Fig. 2.5.). Then acetyl- CoA and oxaloacetate combine to form citric acid in the cytosol followed by entrance of acetyl-CoA into the tricarboxilic acid cycle (TCA cycle) (Ratledge, 2004; Ratledge, 2002).

2.4.3.3 *Exchange of molecules between the cytosol and the mitochondria*

The citric acid (citrate) accumulated must re-enter the cytosol, where it forms the precursor acetyl-CoA for fatty acid synthesis. Activation of fatty acid synthesis is conducted by exchange of citrate with malate. This exchange is commonly known as the citrate malate cycle. Initially, citrate should come out of the mitochondria and malate in the cytosol should enter the mitochondria; in the cytosol malate is converted into oxaloacetate by malate dehydrogenase enzyme. Then malate enters the mitochondria and citrate efflux happens. The reaction is tightly regulated. Firstly malate is bound with citrate/malate translocase protein inside the mitochondrion membrane and then citrate effluxes to the cytosol (Evans et al., 1983a; Evans et al., 1983c).

2.4.3.4 *Synthesis of lipids*

There are three key enzymes involved in lipid accumulation. Of course all the other enzymes are important during formation of lipids since glucose uptake. These three enzymes are ATP citrate lyase, malic enzyme and Acetyl-CoA carboxylase (Ratledge, 2004).

ATP citrate lyase

ATP citrate lyase is a key enzyme in lipid accumulation and it cleaves citric acid effluxing from the mitochondrion to acetyl-CoA and oxaloacetate. The produced acetyl-CoA further serves as a precursor for lipid synthesis. Thus acetyl-CoA is important in lipid accumulation. ATP citrate lyase is commonly found in oleaginous microorganisms. If ATP citrate lyase doesn't exist in the microbial system there will be no lipid production (Adams et al., 1997; Adams et al., 2002).

Malic enzyme

The role of malic enzyme is to generate the pool of nicotinamide adenine dinucleotide phosphate (NADPH) which is necessary for lipid synthesis. Produced NADPH joins with acetyl-CoA, the cleaved product of citrate, and initiates the lipid biosynthesis (Kendrick and Ratledge, 1992; Song et al., 2001). Malic enzyme is also found in association with the cell membrane where it helps in the passage of NADPH to the cell membrane for electron transport chains (Holdsworth et al., 1988). Malic enzyme activity seems to be suppressed when the medium is supplemented with sesamol which inhibits this enzyme (Wynn et al., 1997). Over expression of malic enzyme can lead to enhanced supply of NADPH pool and an increase in lipids by 2-3 folds in oleaginous fungi (Zhang et al., 2007). Similarly, when ATP citrate lyase is over expressed there is an increase in fatty acids accumulation by 16 folds but little difference in the composition of fatty acids is observed (Li et al., 2005; Zhang and Ratledge, 2008).

Acetyl-CoA carboxylase (ACC)

Acetyl-CoA carboxylase (ACC) is responsible for control of the metabolic efflux. This enzyme is integrated with endoplasmic reticulum and helps in the formation of malonyl-CoA which in turn initiates fatty acid synthesis. The study carried out by Davis et al. (2000) showed that overproduction of ACC in *E. coli* resulted in increased lipid production. Acetyl-CoA synthase gene is responsible for conversion of acetate into acetyl-CoA; when it is over expressed in E.coli a significant increase in uptake of acetate has been observed.

Synthesis of fatty acids

Once malonyl-CoA is synthesized, it is transferred by malonyl-CoA:ACP transacetylase to acyl-carrier protein (ACP) of the fatty acid synthase (FAS), a multi-enzymatic complex with Type I to Type IV (Subrahmanyam and Cronan, 1998). Type I FAS is found in yeast and vertebrates, which is a multifunctional protein (Verwoert et al., 1995).

FAS catalyzes fatty acid elongation by condensing malonyl-CoA molecules and acetyl-CoA. ACP, one of the FAS subunits, contains a thiol group that can form malonyl-ACP via forming thioesters with malonyl-CoA, and afterwards with the growing acyl chain in order to assure its transport (Subrahmanyam and Cronan, 1998). This resultant compound is first transformed via three successive reactions, i.e., reduction, dehydration and reduction, and then condensed with another malonyl-CoA. This cycle is repeated until the saturated chain of palmitic acid (16:0) or stearic acid (18:0) is formed (Subrahmanyam and Cronan, 1998). At last, ACP-thioesterase cleaves the acyl chain and liberates fatty acids (Courchesne et al., 2009).

2.4.3.5 *Lipid turnover*

Lipid turnover is a mechanism by which the accumulated lipids inside the cell are utilized for synthesis of lipid free biomass when the excess carbon source in the medium is completely exhausted (Botham and Ratledge, 1979; Kock and Ratledge, 1993). This mechanism, however, was observed very little before, but recently it has got a great attention due to the importance in industrial production of poly unsaturated fatty acids (Papanikolaou et al., 2002). This phenomenon is regularly observed in *Mucaraceous* fungi and oleaginous yeasts. It is very important to curb lipid turnover if the industry needs to achieve high yields of lipids or nutritional fatty acids. When the medium is supplemented with multiple limited nutrients this mechanism can be avoided or repressed (Papanikolaou et al., 2004).

2.5 CULTIVATION MODES EMPLOYED FOR MICROBIAL LIPID PRODUCTION

Batch, continuous and fed - batch modes have been employed in cultivation of microorganisms for lipid production. The type of microorganisms and nature of raw materials decide the cultivation mode.

2.5.1 Batch cultivation

Batch cultivation refers to culturing cells in a fixed volume of the medium under specific environmental conditions. Most of the studies in literature have been carried out using this cultivation mode which allows for determining optimal conditions for lipids accumulation. Therefore batch cultivation of oleaginous microorganisms is performed with a high C/N ratio to channelize the extra carbon into cells for lipids accumulation after the nitrogen limiting conditions are reached. As long as the nitrogen is not limiting, the culture remains in the exponential growth phase and biomass keeps growing. After the exhaustion of nitrogen, the exponential phase stops and the culture enters the lipid accumulation phase. It is employed when the hydrolysate or extracts of different substrates are used as raw materials (Chen and Walker, 2011; Li et al., 2007; Liu et al., 2010), such as cornstover hydrolysate, beet molasses, sweet sorghum juice, and hydrolysate of plant materials. When micro algae *Chlorella protothecoides* is grown on sweet sorghum juice in batch cultivation, the lipid yield was up to 5.1 g lipids/L medium (Gao et al., 2010).

2.5.2 Fed- batch cultivation

In fed-batch cultivation the batch is prolonged by intermittent or continuous feeding of nutrients into the bioreactor. This gives some control over the concentration of key nutrients and therefore fed-batch cultivation is usually used to overcome substrate inhibition or catabolite repression. It can improve the fermentation productivity by regulating the environmental variables to maximize the stability of the metabolic state. Mostly oleaginous yeasts are cultivated using the fed-batch method; this method has been proven to increase cell concentrations and lipid contents in yeasts (Amaretti et al., 2010; Beopoulos et al., 2009; Karatay and Donmez, 2010a). When the marine derived yeast *Rhodotorula muciloginosa* was cultured with a batch cultivation mode the lipid content was 49% but it rose to 52.9% with the fed-batch mode (Li et al., 2010; Ma et al., 2009; Wei et al., 2009). *Rhodotorula muciloginosa* grown on Jerusalem artichoke hydrolysate also yielded a high lipid content of 52.2% when the fed-batch cultivation mode was employed (Zhao et al., 2010).

2.5.3 Continuous cultivation

In continuous cultivation, the fresh culturing medium is continuously supplied to a well-stirred bioreactor and products and cells are simultaneously withdrawn. Continuous operation can be used when the raw materials are liquid. *Scenedesmus obliquous* a microalgae was employed in combined treatment of poultry litter, fish pond waste, and municipal secondary settling tank sludge in continuous cultivation and 10.9 g/L lipid yield was achieved (Mandal and Mallick, 2009). Municipal wastewater and dairy wastewater were treated with multi algae cultures and the maximum lipid content of 29% was achieved.

2.6 REVIEW OF MICROBIAL LIPID PRODUCTION FROM RENEWABLE AND WASTE MATERIALS

A variety of waste materials are rich in organic carbon in the form of lignocellulose, starch, sucrose, xylose, glycerol, etc. Production of microbial lipids using these low cost raw materials would significantly reduce the lipid production cost. The produced lipids have characteristics similar to vegetable oils and can be used as biodiesel feedstock (Shi et al., 2011). Various renewable and waste materials have been used for economic lipid production. In this review they are grouped under different categories: agricultural by products, industrial by products, glycerol and municipal wastes. These waste materials and their lipid production potentials are summarized in Table 2.5.

2.6.1 Agricultural by-products

When processing agricultural crops like rice, wheat, malt, barley, potato and sugar cane, enormous amounts of residues which are rich in carbohydrates are generated. These residues have already been proved to be the best source for bio ethanol production through yeast fermentation (Antoni et al., 2007). Microbial lipid production using agricultural by-products is a new concept. Depending on the nature of the by-products either solid state fermentation (sweet potato waste, rice bran, rice husk) or submerged fermentation (hydrolysate of the waste materials) can be carried out. Fungi can grow well under solid state conditions because of their hypal devilment and penetration into solid substrates (Pandey et al., 2000). Solid state cultivation on sweet potato waste using *A. niger, A. oryzae,* and *P.ostreatus* produced a lipid content of 8.71% of dry mass in addition with protein accumulation (Abu et al., 2000). Semisolid state cultivation of sweet sorghum using oleaginous fungi *Mortieriella isabelliana* yielded an oil content of 11% of dry substrate (Economou Ch et al., 2010). Pear pomace, an agro industrial by-product, yielded 12% (w/w) of dry fermented mass when solid cultivation using *Mortierella isabelliana* (Fakas et al., 2009a).

Solid state fermentation of rice, barley and finger millet as substrates for lipid production using *Pythium utimum* was able to accumulate lipids up to 14.6, 19.1 and 16.9% of dry mass, respectively (Stredansky et al., 2000). The oleaginous yeast *T. fermentans* grown on sulphuric acid treated rice straw hydrolysate and poor results were obtained due to the acidic condition and other toxic substances present in the hydrolysate. When different techniques were applied over the hydrolysate viz., liming and concentration, the levels of toxic substances were reduced and the oil yield was increased to up to 40% of dry biomass (Huang et al., 2009). Recent investigation on use of a mixture of rice straw and wheat bran for lipid production with two endophytic fungi has showed lipid yields of 60.32 - 84.30 mg/g of dry substrate alongside secretion of cellulase enzyme 1.21 - 2.51 FPU/g of dry substrate (Dey et al., 2011). The kinetic experiment on rice hull hydrolysate using *Mortierella isabelliana* yielded a lipid content of 64.3% at the C:N ratio of 57(Economou Ch et al., 2011). Solid state fermentation of wheat straw mixed with wheat bran by entophytic fungus *Microsphaerosis* sp. produced 42 mg/g dry mass of oils; the oil content rose to 74 mg/g dry mass with addition of cellulase enzyme (Peng and Chen, 2008).

2.6.2 Whey

Whey is a by-product of the cheese manufacturing industry. Whey contains many nutrients and is a good source of lactose; this makes it a suitable raw material for microbial lipid production. The approximate composition of whey is (%): water, 94; protein, 0.8; lactose, 4.30; ash, 0.55; and fat, 0.1. It is reported that with 100 kg of milk, 10 - 20 kg of cheese can be produced with generation of 80 - 90 kg of liquid whey. The low solid content and unfavourable lactose protein ratio make whey hard to be used. Lactose has been known to support biomass growth of many oleaginous fungi and lipid production (Evans and Ratledge, 1983a). Recent investigation on cheese whey as a renewable material for lipid production using oleaginous fungi *Mortierella isabellia, Thamidium elegans* and *Mucor* sp. has showed it is a notable raw material for low cost lipid production; 4 g/L of lipid was produced by *Mortierella isabellia* and cheese whey promoted biomass growth of *Thamidium elegans* and *Mucor* sp. (Vamvakaki et al., 2010).

Table 2.5 Utilization of different wastes for microbial lipid production

Type of waste materials [a]Carbon source/ [b] Nitrogen source	Microorganisms involved	Operational parameters	Mode of cultivation	Lipid yield (g/L) Lipid content (%)	Reference
Corn stover hydrolysate	*Trichosporan cutaneum*	30 °C,180 rpm, pH 5.8-6.0	Batch	23.5%	Huang et al., 2011
Volatile fatty acids (VFA) effluents from hydrogen production reactors	*Cryptococcuc curvatus*	25 °C,170 rpm, pH 7.3	Batch	75%	Chi et al., 2011
VFA(acetic :proponic:butyric)	*Cryptococcus albidus*	25 °C ,180 rpm, pH 6.0	Batch	27.8%	Fei et al., 2011
Switchgrass hydrolysate	*Rhodotorula glutinis*	30 °C , 180 rpm, pH 6.0	Batch	39%	Zhang et al., 2010
Cane molasses	*Trichosporan capitatum*	28 °C , 160 rpm	Batch	30.2%, 5.1 g/L	Wu et al., 2010
Biodiesel derived crude glycerol	*Schizochytrium limacium*	25 °C , pH 7.5-8	Continuous	90 g/L DHA	Ethier et al., 2011
Carpet industry effluent wastewater	*Microalgal consortium* *Chlamydomonas globosa* *Chlorella miniutissima* *Scenedesmus bijuga*	25 °C, 80 μmol/m^2/s 12:12 Day /night	Batch	5.3%	Chinnasamy et al., 2010
Beet molasses [a] Corn gluten[b]	*Cryptococcus curvatus*	28 °C , 72 h, pH 5.5, 200 rpm	Batch	1.2 g/L[a] 1.6 g/L[b]	Falady et al .,2009
Sweet sorghum juice	*Chlorella protothecoides*	28 °C , 220 rpm	Batch	5.1 g/L,52.5%	Gao et al., 2010
Cassava hydrolysate	*Chlorella protothecoides*	28 °C, 220 rpm	Batch	53%	Lu et al., 2010
Biodiesel glycerol	*Chlorella protothecoides*	28 °C , 250 rpm	Batch	0.098 g/L/h	O'Grady and Morgan, 2011
Poultry litter, fishpond waste, municipal secondary settling tank sludge	*Scenedesmus obliquous*	25 °C, 75 μmol/m^2/s, 14:10 Day /night	Continuous	9.47-10.49 g/L	Mandal and Mallick, 2009, 2010, 2011
Waste molasses	*Chlorella protothecoides*	28 °C, 220 rpm	Batch	40.8 g/L, 57.6%	Yan et al., 2011
Cassava starch	*Chlorella protothecoides*	28 °C and 35 °C, 220 rpm	Batch	49.34 g/L, 54.50%	Lu et al.

Sugarcane molasses	*Gordonia* sp.	30 °C and 120 rpm	Batch	50%	Gouda et al., 2008
Molasses	*Candida lipolytica* *Candida tropicalis* *Rhodotorula mucilaginosa*	30 °C and 100 rpm, pH 5, 4 days	Batch	up to 69.5%	Karatay and Donmez, 2010
Artificial wastewater	*Chlamydomonas reinhardtii*	25 °C, manual mixing 4 times daily	Batch	25.25%	Kong et al., 2010
Municipal wastewater, dairy wastewater	*Poly culture of algae* *Poly culture of algae*	18 days, CO_2 addition, 15 days, 23-25 °C	Semi continuous, continuous, batch	14-29%	Woertz et al., 2009
Biodiesel derived glycerol	*Lentinula edodes* *Aspergillus niger*	28 °C, 180 rpm, pH6	Batch	0.1 g/L 3.1-3.5 g/L	André et al., 2010
Sweet potato root meal	*Aspergillus niger*	28 °C, 180 rpm, pH6	Bath	3.17%	Abu et al., 2000
Industrial derivative of animal Sterain	*Yarrowia lipolytica*	28 °C, 180 rpm, pH6	Batch	0.44-0.54 g	Papanikolaou et al., 2002
Barley	*Cunningamella elegans*	28 °C, 180 rpm, pH6	Batch	15.8 g/g of biomass	Conti et al., 2001
Sweet sorghum juice	*Schizochytridium limacium*	Room temperature, 170 rpm	Batch	9.4 g/L,73.48 g/L	Liang et al., 2010
Sewage sludge	*Lipomyces starkeyi*	30 °C, 120 rpm	Batch	68%, 1 g/L	Angerbauer et al., 2008
Gylcerol, xylose and glycerol	*Rhodotorula glutinis*	112 rpm, 48 h	Batch	25% 34%	Easterling et al., 2009
Cassavastarch hydrolysate	*Rhodotorula muciloginosa*	160 rpm, pH 6	Batch, fed-batch	47.9% 52.9%	Li et al., 2010
Sweet sorghum	*Mortierella isabelliana*	28 °C, pH6	11 g/100 mg of biomas		Economou Ch et al., 2010
Starch wastewater	*Rhodotorula glutinis*	30 °C, 180 rpm, pH 5.5	Batch	35%	Xue et al., 2010
Jerusalem artichoke hydrolysate	*Rhodospirillum muciloginosa*	160 rpm, 25 °C	Batch, fed-batch	48% 52.2%	Zhao et al., 2010
Inulin extract from Jerusalem artichoke	*Rhodotorula muciloginosa TYJ15a coculturing Pichia guillier*	28 °C, pH 6, 170 rpm	Batch	56.6%	Zhao et al., 2011
Rice straw hydrolysate	*Trichosporan fermentas*	25 °C, pH 6, 160 rpm	Batch	40.1%	Huang et al., 2009
Wheat straw	*Cryptococcus curvatus*	28 °C, pH 6, 200 rpm	Batch	72%	Yu et al., 2011
Rice hull hydrolysate	*Morteriella isabelliana*	28 °C, pH 6-6.4, 180 rpm	Batch	64.3%	Economou Ch et al., 2011

2.6.3 Molasses

Molasses is a by product of the sugar manufacturing industry after boiling sugar syrup from sugar rich crops like sugar cane and sugar beet. It is rich in sugar, calcium, magnesium, potassium and iron and its price is comparatively lower than other crop residues. Its average composition in 75 percent of dry matter is (%): total sugars, 48-56; non-sugar organic matter, 9-12; protein, 2.5; potassium, 1.5-5.0; calcium, 0.4-0.8; magnesium, 0.06; phosphorus, 0.06-2.0; biotin, 1-3.0; and other minerals (Tewari et al., 2007).

Molasses are an attractive raw material for microbial lipid production. *Trichosporan fermentas* an oleaginous yeast when grown on pre-treated molasses yielded a lipid of 12.8 g/L and the lipid yield was increased as increasing concentrations of molasses (Zhu et al., 2008). A mixture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* was cultivated on waste from a seafood processing plant and a sugar cane plant. The highest biomass yield of 4.63 g/L and lipid yield of 2.88 g/L were obtained after five days of cultivation (Cheirsilp et al., 2011). The lipid production potential of *Candida lipolytica*, *Candida tropicalis* and *Rhodtorula mucilagionousa* on different concentrations of molasses was studied and 8% molasses solution gave the highest lipid contents of 59.9%, 46.8%and 69.5%, respectively, on the 5th day of cultivation (Karatay and Donmez, 2010b).

2.6.4 Glycerol

Glycerol (1, 2, 3-propanetriol) was first produced from saponification of olive oil in 1779. Presently, glycerol has a range of applications in food, beverage, pharmaceutical and cosmetic industries (Amaral et al., 2009). Two forms of glycerol are currently available: synthetic glycerol (10% of the market share) produced from chemical conversion of propylene and natural glycerol (90% of the market share) produced in the biodiesel industry (Papanikolaou and Aggelis, 2009). It is estimated that with the production of 10 kg of biodiesel by esterification of rapeseed oil, 1 kg of glycerol is generated (Meesters et al., 1996). As the biodiesel market has a sharp expansion, it is expected that a large amount of glycerol would be generated. For example 71 thousand tonnes of crude glycerol is estimated to be generated in US alone every year (Thompson and He, 2006). Glycerol can be used to manufacture citric acid (Papanikolaou and Aggelis, 2009), pigments (Andre et al., 2010) and other products (Amaral et al., 2009).

Utilization of glycerol for single cell oil production is a relatively new concept. Many filamentous fungi and yeasts with high cellular lipid contents have been successfully cultured using crude glycerol (Table 2.5). For example, a lipid content above 70% (w/w) of the cell dry weight was reported when batch cultivation of the fungus *Thamnidium elegans* (Chatzifragkou et al., 2011). The yeast *Rhodotorula glutinis can* also accumulate 60.7% (w/w) lipids in a fed-batch cultivation system (Saenge et al., 2011). Though high lipid contents have been observed in biomass, the overall amount of lipid produced per litre of the culturing medium remains relatively low, with the *Thamnidium elegans and Rhodotorula glutinis* producing total lipids of only 11.6 g/L and 6.1 g/L from the medium containing 90 and 95 g/L crude glycerol, respectively (Table 2.5). In addition, the overall lipid yield per gram of glycerol consumed is low. Ratledge (2002) reported a theoretical maximum lipid yield from glycerol of 0.3 g lipids per g glycerol consumed. However, much lower values are often observed. For example, a maximum lipid yield of only 0.08 g lipids/g glycerol consumed for *Y. lipolytica* using 50.5 - 135.1 g/L crude glycerol (Papanikolaou and Aggelis, 2009) and a slight increase yield of 0.10-0.15 g lipids/g glycerol consumed was reported with *Schizochytrium limacinum* (Ethier et al., 2011). Batch cultivation has been widely used to investigate lipid production from glycerol, while fed-batch systems are also adopted as they enable an increase in the cell density and the lipid content (Beopoulos et al., 2009). This is largely due to a better control of substrate levels preventing substrate inhibition at high glycerol concentrations (Saenge et al., 2011).

Batch cultivation of *Cryptococcus curvatus* was negatively impacted by the impurities found in crude glycerol compared to pure glycerol (Liang et al., 2010). Growth was reduced by 2.5 times when the crude glycerol level was increased from 20 g/L to 40 g/L and almost no growth was observed at 60 g/L. Switching the batch cultivation to a fed-batch setup allowed *C. curvatus* to reach cell densities comparable to those observed in pure glycerol batch cultivation systems. (Table 2.5).

2.6.5 Sewage sludge

Dufreche et al. (2007) attempted to extract lipids from sewage sludge of municipal wastewater treatment plants. Annual production of dried sewage sludge is 6.2×10^6 t in USA and it is mainly used as a fertilizer for agricultural lands or is delivered to landfill. Recent restrictions on the landfill and land use of sewage sludge require alternative sludge disposal approaches. Sewage sludge contains approximately 20% of ether soluble grease and fats (Konar et al., 1994); this could be converted into fatty acid methyl esters (FAMEs). Additionally, the cell membrane of microorganisms in the sewage sludge is composed of phosphorus lipids which can be converted into fatty acid alkyl esters via acid and base catalysed transesterification. Lipids can be extracted from sewage sludge using hexane, methanol, acetone and super critical CO_2. Extraction of lipids with a mixture of n-hexane, methanol, and acetone gave the largest conversion into biodiesel, up to 4.41 per cent based on total dry weight of sludge, compared with the other solvents. In situ transesterfication of dried sludge resulted in a yield of 6.23 per cent. If fifty percent of municipal wastewater plants would adopt lipid extraction and transesterification, enough biodiesel could be produced to replace 0.5 per cent of the national petroleum diesel demand in US. Angerbauer et al. (2008) studied the conversion of sewage sludge into microbial oils using *Lipomyces starkeyi* and assessed potential accumulation of lipids on a synthetic medium containing 50 g of raw sewage sludge, glucose and ammonium nitrogen. Lipids accumulation strongly depended on the C/N ratio. The highest content of lipids in biomass (68%) was measured at a C/N ratio of 150; at a C/N ratio of 60 the lipid content was 40%. Raw sewage sludge had no inhibitory effects on biomass growth or lipid accumulation. Pre-treatment (acid hydrolysis and ultra sonic treatment) led to accumulation of lipids by *L. starkeyi* with the highest lipid yield of 1 g/L sludge.

2.6.6 Monosodium glutamate wastewater

Since 1960s monosodium glutamate production has been increased annually in China and the wastewater from this industry is also an attractable fermentable medium (Jia et al., 2006). It has high concentrations of COD (10,000 - 40,000 mg/L), ammonium nitrogen (15,000 - 25,000 mg/L), sulphate (15,000 - 30,000 mg/L) and very low pH (about 2.0). The treatment of such wastewater requires a large amount of energy. Hence Xue et al. (2006) studied its potential use as a fermentable medium for lipids production using *Rhodotorula glutinis*. Since the wastewater contained nitrogen, favouring biomass growth but not lipids accumulation, glucose was added at various levels using three methods, namely initial addition, fed batch addition and feedback addition. Of the three methods, the feedback addition yielded the highest biomass growth (25 g/L), lipid content in the biomass (20%) and COD degradation (45% removal).

2.7 PROPERTIES OF SCO PRODUCED FROM WASTE MATERIALS

The similarity between microbial oils and vegetable oils is the main criteria for using SCO as a potential feedstock for biodiesel production. Microbial oils produced from different wastes must display lipid properties similar to vegetable oils, and be in accordance with national and international standards. Among the properties SCO should have major fatty acids of palmitic, stearic, linonelic and linolenic acids and the linolenic acid content should not be more that 12% (Baber et al., 2005). The single cell oil produced by *Mortierella* sp. had many similar physio chemical properties (Kumar et al., 2011): the oil contained mysteric acid (0.45%), palmitic acid (19.6%), palmitoleic acid (0.3%), stearic acid (11.5%), oleic acid (38.2%), linoleic acid (4.8%), arachidic acid (3.3%), gamma linolenic acid (8.1%) and behenic acid (0.2%). In addition, the fungal oil had similar kinetic viscosity (54.8 mm^2/s at 40 °C), water content (3.9%), flash point (218 ^oC), pour point (7.0 ^oC), ash content (0.6%), carbon residue (0.08%), acid value (28.2 mg KOH/g of oil) and fire point (230 ^oC).

Scenedesmus obliquous when cultivated on a mixture of poultry litter, fish pond waste and municipal secondary settling tank sludge displayed a high amount of palmitic acid (24.5%), a slightly high linolenic acid (12.5%), and a positive oxidation stability (Mandal and Mallick, 2010). *Cholorella prothecoides* cultivated on sweet sorghum juice had high oleic acid (32.5%) and low linolenic acid (6.2%) levels in its SCOs, which was suitable for a biodiesel feedstock (Gao et al., 2010). Similarly *Trichosporan capitatum* grown on cane molasses yielded microbial lipids with a high oleic acid content of 80% which was a good feedstock for biodiesel (Wu et al., 2010a). Algae cultivated on starchy materials yielded lipids with a less GLA content in lipids (5.7%) which could be used for biodiesel producer (Lu et al., 2010). All the research mentioned above shows a positive position of microbial lipids as a potential feedstock for biodiesel production.

2.8 SUMMARY

In this chapter energy security, environmental concerns, and usage of microbial lipids for biodiesel production was discussed. Current research on use of different waste materials for low-cost microbial lipid production was reviewed, in addition with the microbial lipid properties for biodiesel production.

CHAPTER THREE

ISOLATION, SCREENING AND IDENTIFICATION OF OLEAGINOUS FUNGI FROM IRISH SOILS

Chapter Three – Isolation, screening and Identification of oleaginous fungi

3.1 INTRODUCTION

Oleaginous microorganims can accumulate lipids more than 20% inside their cells. Microbial lipids will be one of the major feedstocks for production of the second generation biodiesel (Li et al., 2008; Liu and Zhao, 2007; Lu et al., 2008; Meng et al., 2009b; Papanikolaou et al., 2010). The number of oleaginous microorganims currently in use for energy production is limited; for example, of the 600 yeasts only 25% are known to produce lipids and among 60,000 fungal species less than 50% are known to accumulate lipids (Ratledge, 1989). Although the numbers seem large, the number of oleaginous microorganims in use is limited (Subramaniam et al., 2010), since the microbial lipid production research and application until now is focused on production of special lipids rich in poly unsaturated fatty acids (such as gamma linolenic acid and arachidonic acid). Oleaginous fungi used for these special lipids production are mainly cultures belonging to the Mucorales order. Use of these already established mucaraceous oleaginous fungi for biodiesel production has a problem: their microbial oils would contain a high linolenic acid content, which does not satisfy the European standards (EN14214 and 14213). Feedstocks for the second generation biodiesel include biomass, agricultural and forestry residues, and biowastes. In order to utilize these residues for lipid production oleaginous microorganims should have respective enzymes to breakdown complex organic compounds, like cellulose, starch, etc. Otherwise external enzymes will be added, thus increasing the lipid production cost. It is reported that when oleaginous fungi *M. isabelliana ATHUM* 2935 and *C. echinulata* ATHUM 4411 were grown on renewable carbon sources (starch, pectin and lactose) their lipid yields were greatly influenced by enzymes (amylase and polygaluctronase) secreted (Papanikolaou et al., 2007). Hence, it is of particular interest to isolate more oleaginous microorganims for the second generation biodiesel production and such isolates should have enzyme secretion capability (Meng et al., 2009a). Pan et al. (2009) tried to isolate oleaginous yeasts with xylose assimilating capability for utilizing lingo-cellulosic wastes for the second generation biodiesel production. Furthermore, the oleaginous microorganisms must be safe to human beings, local ecology and local environment. Thus, it is necessary to find indigenous species, study their oil production capability and utilize them for microbial oil production.

Hence in this study Irish soils were explored for isolation and screening of indigenous oleaginous fungi with amylase secreting capability.

3.2 MATERIALS AND METHODS

3.2.1 Soil sample collection

Fifty undisturbed soil samples were collected from various locations in western Ireland and used for isolation of oleaginous fungi. Upper layer (5 -15 cm) undisturbed soils were collected with sterile polyethylene bags, brought to the laboratory and used immediately for isolation studies. The sampling locations are given in Fig.3.1.

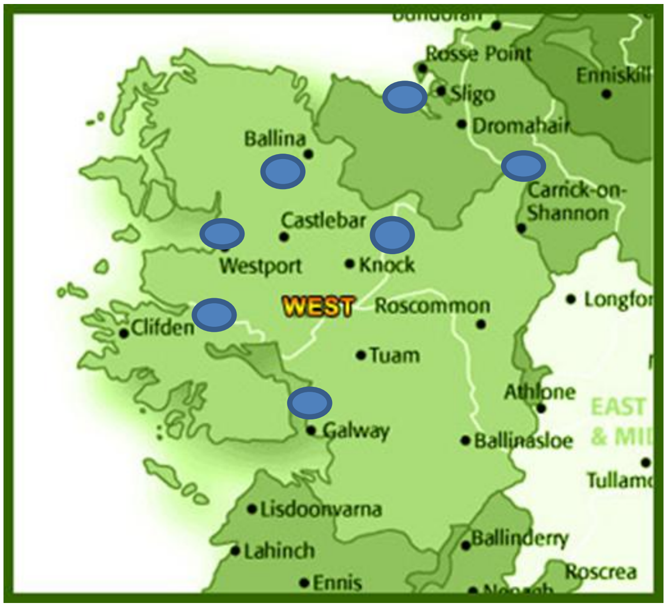

Rosse Point
Sligo
Dromahair
Enniskill
Ballina
Carrick-on-Shannon
Castlebar
Westport
Knock
WEST
Roscommon
Longfor
Clifden
Tuam
Athlone
EAST
& MI
Galway
Ballinasloe
Tullam
Lisdoonvarna
Lahinch
Ballinderry
Roscrea
Ennis

Figure 3.1 Soil sampling sites in western Ireland (Blue dots indicate the sites)

3.2.2 Isolation of oleaginous fungi

Fungi were isolated from soil samples by means of the serial dilution and plating method. Ten gram of each soil sample was suspended in 90 mL of sterile distilled water added in 250 mL conical flasks; the flasks were shaken vigorously for five minutes. The suspensions were serially diluted up to one thousand folds. One mL of the one thousand dilution aliquot was transferred aseptically in a Class II biosafety cabinet (Fisher Scientific, Ireland) onto sterile Petri dishes and then 10 - 15 mL of molten PDA medium was poured into the Petri dishes. Filter sterilized Kanamycin (35 ppm) was added into the sterilized PDA medium before plating. The plates were incubated at 30 °C for 5 days. Single fungal colonies were isolated and transferred repeatedly to new plates until pure cultures were obtained, which were maintained in PDA slants and stored at 4 °C.

3.2.3 Screening for lipid production

Cultures with a good mycelial morphology were selected for screening. Mycelium was taken from disks using a cork borer from the actively growing area and was pre-cultured in the Yeast Extract Malt Extract (YEME) broth for 2 days. The composition of the medium was (g/L): glucose, 10; peptone, 5; yeast extract, 3; and malt extract, 3. After two days, the mycelium was harvested, and then mycelium with a wet weight less than 10 g was transferred to 10 mL sterile plastic tubes (Fisher scientific, Ireland). 5 - 8 sterile glass beads (5 mm diameter) were added and the mycelium was homogenised using vortex for two min. 0.8 mL of the homogenised mycelium was then transferred into 250 mL conical flasks containing 50 mL of the lipid producing medium, which consisted of (g/L): glucose, 30; yeast extract, 1.5; KH_2PO_4, 7; Na_2HPO_4, 5; $MgSO_4$, 1.5; $FeCl_3$, 0.08; $ZnSO_4$, 0.01; $CaCl_2$, 0.1; $MnSO_4$, 0.1; $CuSO_4$, 0.1; and $CO\ (NH_3)_2$, 0.1. The medium had a pH value of 5.5. Glucose in this medium was replaced with 30 g/L of starch while studying the capability of isolated oleaginous fungi in lipid production with starch as the carbon source.

Then, the cultures were incubated in an orbital shaker incubator (Fisher Scientific, Ireland) at a shaking speed of 160 rpm and an incubation temperature of 30±1 ^{o}C. Flasks were periodically removed from the shaker incubator and the samples were filtered using 0.09 mm stainless steel sieve (Starsdet, Germany). Filtrates were centrifuged again at 5000 rpm for 5 min and used to analyze glucose, starch and amylase. The biomass was used for extraction of lipids.

3.2.4 Analytical methods

3.2.4.1 *Residual glucose analysis*

Remaining glucose in the culturing supernatant was analyzed using the DNS method (Miller, 1959). 1 mL of the supernatant samples was added into 15 mL glass tubes and the liquid volume in the tubes was equalized to 3 mL by adding distilled water. The tubes were then added with 3 mL of DNS reagent and were placed in a water bath at 90 ^{o}C for 5 min. 1 mL of 40% Rochelle salt solution (potassium sodium tartrate) was added to the tubes when the contents were still warm. After the tubes were cooled to the ambient temperature, the intensity of a dark red color was read at the wavelength of 510 nm using a spectrophotometer (Hach Lange, Ireland). 1 mL of distilled water was added in a 15 mL glass tube and used as the blank sample; the procedure mentioned above was repeated. A series of glucose solutions with known glucose mass (0 – 500 µg) were run in parallel for the purpose of calibration.

3.2.4.2 *Residual starch analysis*

Residual starch in the culturing supernatant was analyzed according to the phenol sulphuric acid method (Dubois et al., 1956). 0.1 mL of the supernatant sample was added in 15 mL glass tubes and the liquid volume was made up to 1 mL with distilled water. 1 mL of phenol and 5 mL of 96% sulphuric acid solution were added into the tubes. The contents were well mixed by inverting the tubes several times and left undisturbed for 10 min. Then, the tubes were shaken and placed in a water bath at 25-30 ^{o} C for 20 min. The contents were cooled at ambient temperature and the colour intensity was read at the wavelength of 490 nm using the spectrometer. The blank sample was 1 mL of distilled water with the procedure mentioned above repeated. The same procedure was followed to standard glucose solutions containing 0 - 500 µg glucose for the purpose of calibration.

3.2.4.3 *Screening for amylase secretion*

Preliminary screening of fungi with amylase secretion was conducted on 2% starch agar medium in plates. A disk of actively growing mycelium was placed in the centre of the plates, incubated at 30 °C and examined after 24 - 48 h. α-amylase producing cultures were selected by the formation of a clearance zone when the plates were flooded with iodine solution (1% iodine in 2% potassium iodide, w/v). Amylase secretion of positive cultures was quantified after incubating these species with the lipid producing medium containing 3% starch for five days. The amylase activity was measured by mixing 1 mL of starch solution with 1 mL of properly diluted culturing supernatant in a tube, followed by incubation at 27 ^{o}C for 15 min. Then 2 mL of DNS solution was added and the mixture was heated in a boiling water bath for 5 min; when the tubes were still warm, 1 mL of potassium sodium tartrate solution was added and the tubes were cooled to ambient temperature. The liquid volume was then made up to 10 mL with distilled water and the colour intensity was read at the wavelength of 560 nm using the spectrophotometer. One unit of amylase activity (IU/mL) was defined as the number of mg of maltose liberated by 1 mL of 1% enzyme solution per 5 min (Bernfeld, 1955).

3.2.4.4 *Biomass analysis*

Fungal biomass was analyzed by filtering the contents in conical flasks using 0.09 mm stainless steel sieve (Starsdet, Germany). Filtered biomass was washed twice with distilled water and then dried at 100 ^{o}C until a constant weight.

3.2.4.5 *Lipid extraction*

Lipid extraction from dried biomass was performed according to the Bligh and Dyer method. 100 mg of dry biomass was weighed accurately and added into a *reacti* vial (Fisher Scientific, Ireland). The lipids were extracted from the biomass using 3 mL of chloroform and methanol mixture (2:1; v/v) overnight by shaking the tubes gently on a mini shaker (Starsdet, Ireland). Then lipids were separated by adding 1 mL of water followed by centrifugation (Sigma Aldrich, Ireland) at 3000 rpm for 10 minutes. The lower liquid phase containing lipids was then transferred using pasteur pipette; the solvents in the extracts were evaporated using dry nitrogen gas and the lipids were measured gravimetrically (Bligh and Dyer, 1959).

3.2.4.6 *Equations*

The lipid content in biomass, $Y_{L/X}$, was calculated using Equation 3.1:

$$Y_{L/X} = \frac{L_{max}}{X} \quad \text{[Eq. 3.1]}$$

where,

L_{max}, maximum lipid yield, g/L; and

X, biomass yield corresponding to the maximum lipid yield, g/L.

The amount of biomass yield with respect to the substrate consumption, $Y_{X/C}$, is calculated using Equation. 3.2 :

$$Y_{X/C} = \frac{X}{(C_i - C_f)} \quad \text{[Eq. 3.2]}$$

$Y_{X/C}$, biomass yield with respect to carbon substrate consumed, g biomass/g substrate;

X , fungal biomass yield at time t, g/L;

$C_{i,}$ initial carbon substrate concentration, g/L; and

$C_{f,}$ carbon substrate concentration at time t, g/L.

The lipid yield with respect to carbon substrate consumption, $Y_{L/C}$, was calculated using Equation 3.3:

$$Y_{L/C} = \frac{L_{max}}{(C_i - C_f)} \qquad \text{[Eq. 3.3]}$$

Where,

$C_{f,}$ carbon substrate concentration at time t corresponding to the maximum lipid yield, g/L.

3.3 MOLECULAR IDENTIFICATION OF FUNGAL ISOLATES

3.3.1 DNA extraction

Three loops of oleaginous fungal cultures were scrapped from PDA plates and inoculated in YEME broth (Section 3.2.3) and incubated for 24 – 48 hrs in a shaker incubator (Fisher Scientific, Ireland) at a shaking speed of 160 rpm at 30±1 ^{o}C. Genomic DNA of the cultures was extracted using Cambio Microbial DNA Isolation Kit (CAMBIO, UK) according to the manufacturer’s protocol.

3.3.2 PCR amplification

Polymerase chain reaction (PCR) amplification of 18s rDNA gene was carried out using a master cycler gradient thermo cycler (Eppendorf, Germany). PCR reactions were performed in 25-µl volumes containing 1 µl of DNA, 12 µl of PCR master Mix (Applied Biosystems, UK), 2 µl each forward and reverse primers and 8.0 µl of PCR water (Sigma, Ireland). The forward primer was EF4 (5’-GGAAGGGRTGTATTTATTAG-3’) and the reverse primer was EF3 (5’-

TCCTCTAAATGACCAAGTTTG-3’). The thermo cycle patterns were as follows: 94 ^{o}C for 3 min (one cycle); 94 ^{o}C for 1 min, 50 ^{o}C for 1 min, 72 ^{o}C for 2 min (40 cycles); and 72 ^{o}C for 10 min (one cycle). PCR products with a correct size were confirmed by 1% agarose gel electrophoresis.

3.3.3 Nucleotide sequencing

The sequencing of purified PCR products containing 1.5 - 1.6 kb of 18s rDNA was carried out by means of automated sequencing **(**Euro Fins Biotech Pvt. Ltd., Germany**).** The sequence data generated were subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/Blast). New gene sequences of isolates were submitted to the NCBI.

3.4 RESULTS AND DISCUSSION

3.4.1 Isolation and screening of oleaginous fungi

A total of 247 fungi cultures were isolated from soil samples. Fifty isolates showed good morphological growth on PDA plates and were screened for lipid production on the medium containing 3% glucose or 3% starch, respectively (Fig. 3.2). Next to lignocellulosic materials starchy wastes are abundant and can be a major alternative substrate for oleaginous fungi. Therefore isolating oleaginous fungi with particular reference to starch utilization is a worthy study.

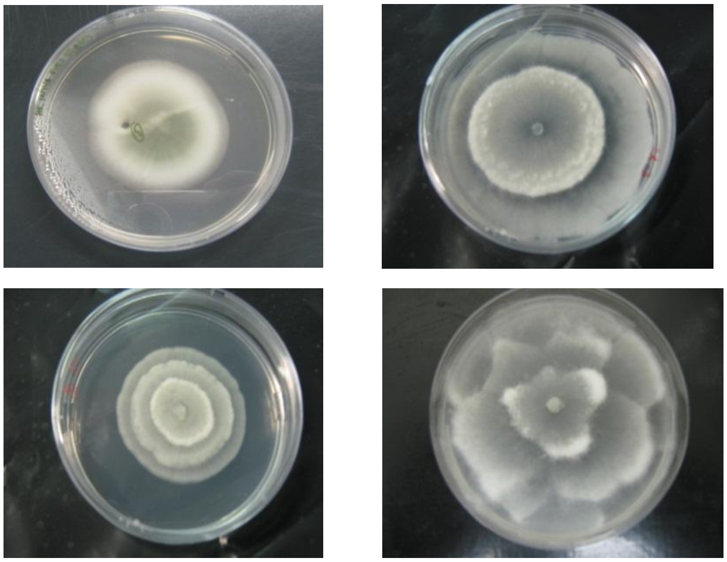


Figure3.2 Examples of morphological variations of fungi isolated from Irish soils (top left, Isolate I17-1; top right, Isolate 2A31; bottom left, Isolate I19-2; bottom right, Isolate I5-10).

3.4.2 Growth and microbial lipid production of isolates in the glucose containing medium

Among the fifty fungal isolates screened for lipid production, 30 cultures were able to convert glucose into lipids with the lipid contents in biomass ranging from 20% to 44% within 7 days of cultivation. Therefore, these 30 cultures were oleaginous. Glucose supported biomass growth (4.4 to 11.8 g biomass yield/ L medium) and was consumed by all the 30 cultures (11.0 to 29.3 g glucose consumed/L medium) (Table 3.1). The maximum lipid content in biomass was observed in the isolate I16-3, up to 44.3%. The lipid yield with respect to glucose consumed, $Y_{L/C}$ (g lipids/g glucose consumed), is calculated using Equation 3.3. $Y_{L/C}$ values were in the range of 4.7 - 14.0 g lipids/100 g of glucose consumed. The higher $Y_{L/C}$ values demonstrate the higher the substrate conversion efficiency of the isolated oleaginous fungi. The isolate I16-3 had the maximum $Y_{L/C}$ of 14.0 g of lipids yield per 100 g of glucose consumed. 8 cultures (I1A1, I1A3, I1A5, I5-8, I8-1, I14-5, I16-3 and I18-1) had $Y_{L/C}$ higher than 10 g of lipids yield per 100 g of glucose consumed. In our study, glucose was not completely utilized by any of the selected fungi; hence occurrence of lipid turnover was not observed. With the glucose concentration of 30 g/L the maximum $Y_{L/C}$ values obtained for *Mortierella isabelliana* and *Cunninghiamella echinulata* were 10.6 and 11.3 g lipids/100 g of glucose consumed, respectively (Papanikolaou et al., 2007). Our present study identified oleaginous fungi with higher $Y_{L/C}$ values. The literature also reports much lower $Y_{L/C}$ values. For example, *Z.moelleri* MUCLL1430 had $Y_{L/C}$ of 4.23 g lipids/100 g of glucose consumed, *M.ramannia* ATHUM2922 had 5.0 g lipids/100 g of glucose consumed, *R.stolonifer* LGAM (9)1 had 4.5 g lipids/100 g of glucose consumed, *R.stolonifer* BPIL 1676 had 2.8 g lipids/100 g of glucose consumed, and *Mucor rouxianus* CBS120-08 had 1.2 g lipids/100 g of glucose consumed when cultivated on glucose (Kavadia et al., 2001).

Table 3.1 Characterization of oleaginous fungi grown on the glucose containing medium (cultivation for 7 days)

Isolate name	X (g/L)	L_{max} (g/L)	Lipid content (%)	Consumed Glucose (g/L)	$Y_{L/C}$ (g lipid/100 g glucose)
I1-1	9.8±0.2	2.0±0.3	20.2	25.5±3.1	7.8
I1-2	7.6±0.4	1.5±0.4	20.1	26.0±0.4	5.9
I1-3	5.8±0.3	1.7±0.2	29.2	25.8±2.2	6.6
I1A1	5.2±0.5	1.2±1.0	23.2	11.0±1.0	11.0
I1A3	9.5±0.5	2.4±0.5	25.5	21.9±1.7	11.0
I1A10	9.7±0.1	2.5±1.0	25.3	25.8±2.5	9.5
I1A5	7.5±0.4	2.0±0.6	26.2	15.7±3.1	12.5
I2A2	4.4±0.2	1.5±0.8	34.0	21.1±0.8	7.1
I2A3	4.6±0.1	1.5±0.2	33.3	28.9±3.4	5.3
I2A31	5.7±0.1	1.6±1.0	28.9	27.3±4.1	6.0
I5-3	4.1±0.7	1.2±0.2	30.2	26.4±3.4	4.7
I5-5	4.8±1.0	1.4±0.8	29.7	23.7±1.9	6.0
I5-6	4.9±0.3	1.4±0.8	29.4	29.0±0.7	5.0
I5-7	7.2±0.4	1.9±0.8	27.1	24.1±0.4	8.1
I5-8	9.4±0.8	3.5±0.2	36.9	27.5±0.7	12.6
I5-10	5.9±0.3	1.8±0.4	30.3	25.4±1.9	7.1
I8-1	11.8±0.4	2.6±0.6	22.3	26.0±4.2	10.2
I10-1	10.2±0.2	2.4±0.3	23.5	26.7±3.2	9.0
I10-2	6.2±0.4	1.5±0.2	24.7	28.6±0.7	5.4
I13-1	5.8±0.3	1.3±0.1	22.0	26.9±1.2	4.7
I14-2	6.5±0.2	1.5±0.1	22.9	27.5±3.6	5.4
I14-4	8.7±0.7	1.8±0.4	20.7	21.1±2.7	8.5
I14-5	9.3±0.5	2.5±0.7	27.2	24.3±2.8	10.4
I15-1	6.3±0.9	1.4±0.3	22.3	14.6±0.6	9.6
I16-3	9.3±0.3	4.1±0.5	44.7	29.3±2.4	14.0
I16-4	7.3±0.1	1.9±0.2	25.4	24.8±0.2	7.5
I17-1	5.7±0.4	2.4±0.3	42.6	28.7±0.3	8.5
I17-2	7.3±0.5	1.8±0.2	24.5	27.9±1.2	6.4
I18-1	7.2±0.9	2.9±0.2	39.6	22.3±3.4	12.8
I19-2	6.5±0.5	1.3±0.5	20.2	16.4±2.1	8.0

3.4.3 Growth and microbial lipid production of isolates in the starch containing medium

The same fifty isolates were grown on the medium containing 3% starch to test their capability in lipid accumulation with starch as the carbon substrate. After 7days of incubation 19 cultures were able to accumulate lipids; interestingly all the 19 cultures were among the 30 cultures which were able to accumulate lipids in the medium containing 3% glucose (Table 3.2). Other 11 cultures did not accumulate lipids in the starch containing medium; this might be because glucose is a simple carbon source. A noticeable amount of biomass was produced (4.8 - 15.0 g/L) and the maximum biomass value obtained (among the 19 cultures) in the starch containing medium was higher than in the glucose containing medium (15.0 against 11.8 g/L), confirming that starch is a good biomass source for oleaginous fungi (Ahmed et al., 2006; Papanikolaou et al., 2007). All the 19 cultures utilized starch above 15 g/L (Table 3.2). The lipid content in biomass ranged from 19% to 39%; the maximum lipid content (39%) was observed in the same isolate I16-3. Interestingly, the isolate had a slightly higher lipid yield (4.3 g/L) when grown on 3% starch medium than 3% glucose medium (4.1 g/L). Moreover, the maximum lipid yield obtained by Isolate I16-3 (4.3 g/L) is comparable with other researchers' data. For instance, 3.7 and 3.8 g/L of lipids were produced by *Mortierella isabelliana* and *Cunninghiamella echinulata* when grown on 3% starch (Papanikolaou et al., 2007). 3.4 g/L microbial lipids were produced when *Aspergillus oryzae* was cultured on 4% starch. Our isolate possessed a higher lipid yield than reported species.

In this study seven isolates (I1-1, I1-3, I13-1, I15-1, I16-3, I17-1 and I19-2) had $Y_{L/C}$ values above 10 g lipids/100 g of glucose consumed and a maximum value of 19.3 g lipids/100 g of glucose consumed was observed in the isolate I16-3 (Table 3.2). $Y_{L/C}$ values of 14.9 and 15.2 g lipids/100 g of glucose consumed were obtained when *Mortierella isabelliana* and *Cunninghiamella echinulata* were cultured on the medium containing 3% starch. Much lower $Y_{L/C}$ of 3.5 g lipids/100 g of starch consumed was obtained when *Aspergillus oryzae* was grown on 4% starch. These results suggest that Isolate I16-3 is a potential starch utilizing culture; this is probably because of its high amylase activity.

Amylase is the primary enzyme responsible for starch hydrolysis. It is reported that, when complex carbon sources are used for microbial lipid production, the capability of oleaginous fungi in secretion of amylase is critical to obtain high lipid yields (Papanikolaou et al., 2007). Hence, in the present study, all 50 isolates were screened for amylase secretion on plates and the positive cultures were tested with the lipid producing medium containing 3% starch. The results showed that, among the 50 isolates screened, 19 isolates were positive (+ve) for amylase secretion with the width of the clearing zones ranging from 2 to 20 mm and the width of the biomass ranging from 3 to 40 mm (Table 3.3). The result shows that the isolates which were positive were all oleaginous. The isolate I16-3 achieving the highest lipid yield possessed the highest amylase activity with a 20 mm clearing zone in plates and 34 IU/mL of amylase was secreted in the liquid medium (Tables 3.2 and 3.3).

Table 3.2 Characterization of isolated oleaginous fungi cultured in the starch containing medium (cultivation for 7 days)

Isolate name	X (g/L)	L_{max} (g/L)	Lipid content (%)	Consumed Starch (g/L)	$Y_{L/C}$ (g lipid/100 g starch)	Amylase secretion (IU/mL)
I1-1	10.5±0.4	3.1±0.8	29.5	26.9±1.4	11.5	24.6±0.3
I1-3	15.0±1.0	3.5±0.6	23	27.5±1.7	12.6	22.1±0.4
I2A3	5.8±0.9	1.7±0.4	29.1	23.6±0.4	7.2	20.2±1.6
I5-3	7.8±0.9	2.0±0.3	25.1	20.9±1.0	9.4	22.2±0.1
I5-5	5.4±0.8	1.3±0.2	23.7	22.9±0.7	5.6	24.3±1.9
I5-8	5.2±0.4	1.8±0.4	34.4	25.5±0.5	7.0	25.7±1.2
I5-10	8.9±0.4	2.3±0.6	25.3	25.8±1.4	8.7	27.9±1.4
I13-1	11.7±0.6	2.6±0.2	22.6	15.7±0.6	16.8	28.4±1.6
I14-2	7.0±0.2	2.3±0.8	33.5	27.6±0.7	8.5	24.5±1.4
I14-4	5.6±0.8	2.0±0.4	35.3	26.6±0.2	7.4	27.5±1.2
I14-5	6.9±0.5	2.2±0.1	32.5	28.6±0.6	7.8	28.4±1.6
I15-1	10.9±0.6	2.5±0.8	22.5	21.1±0.6	11.7	30.2±2.0
I10-1	10.5±0.9	2.2±0.5	20.5	24.3±2.4	8.9	32.3±1.3
I16-3	11.0±1.1	4.3±0.7	39.4	22.5±0.7	19.3	34.6±2.3
I16-4	9.5±0.8	2.0±0.8	20.8	27.9±0.2	7.1	27.5±0.4
I17-2	8.4±0.3	2.3±0.6	27.6	25.5±0.5	9.1	26.9±0.3
I17-1	12.4±0.6	3.7±0.5	29.6	24.8±2.9	14.8	30.8±0.6
I18-1	7.8±0.5	2.1±0.3	27.2	24.8±1.8	8.6	24.3±0.8
I19-2	8.9±0.3	2.2±0.4	24.6	21.1±2.2	10.4	22.9±0.3

In order to use low-cost starchy substrates as raw materials for microbial lipid production, it is important to use oleaginous fungi with amylase secretion capability so as to reduce the lipid production cost. However, in the literature very few oleaginous microbes with amylase secretion capability for utilization of starchy wastes have been reported; they were *Aspergillus oryzae, M. isabelliana ATHUM 2935* (0.12 IU/mL) and *C. echinulata* ATHUM 4411 (0.19 IU/mL) (Papanikolaou et al., 2007). Other studies on use of starch based wastes for microbial lipid production either utilize commercial amylase or use amylase positive cultures to hydrolyse starch before using the hydrolysate for microbial lipids production. For instance, enzymes alpha amylase and gluco amylase were added to hydrolyze cassava starch for lipid production by micro algae *Chlorella protothecoide* (Wei et al., 2009; Lu et al., 2011; Lu et al., 2010). Li et al. (2010) used *S. fibuligera* amylase secreting culture to produce crude amylase, which was then added to cassava starch hydrolysate for lipid production by *Rhodotorula mucilaginosa* TJY15a.

In the present study, 19 oleaginous fungi were observed to have amylase secretion capability. This result would be useful for low-cost lipid production by using starchy wastes.

Table 3.3 Screening of oleaginous fungi with amylase secretion

Isolate name	Width of the clearing zone (mm)	Width of the biomass (mm)
I1-1	10±2	10±3
I1-3	5±2	8±1
I2A3	3±1	10±3
I5-3	3±1	5±4
I5-5	2±2	5±5
I5-8	9±1	5±2
I5-10	5±2	5±1
I13-1	7±1	4±3
I14-2	4±3	7±2
I14-4	5±2	10±1
I14-5	2±3	7±3
I15-1	12±1	10±2
I15-4	3±2	2±1
I16-3	20±2	10±3
I16-4	3±3	10±3
I16-5	6±2	4±4
I17-1	9±3	1±5
I18-1	7±2	8±2
I19-2	3±1	3±1

3.4.4 Identification of oleaginous fungi using molecular techniques

Sequencing of 18s rDNA of the 30 oleaginous isolates shows that they belonged to seven different genus: *Trichoderma* sp., *Penicillium* sp., *Aspergillus* sp., *Mortierella* sp., *Zygomycetes* sp., *Acremonium* sp. and *Umbiliopsis* sp. (Fig. 3.4). Among the seven genus 13 isolates showed 99% similarity with already reported sequences of reference strains identified by BLAST analysis and the sequences were submitted to the NCBI gene bank (Acc. No: **JF895924**, **JF895925**, **JF895926, JF895927, JF895928, JF895929, JF895930**, **JF912414, JF912415, JF912416, JF912417, JF912418 and JF912419**). Out of the 30 isolates, seven, ten, seven and three isolates were identified as *Trichoderma* sp., *Penicillium* sp., *Aspergillus* sp. and *Zygomycetes* sp., respectively; one each of *Mortierella* sp., *Acremonium* sp. and *Umbliopsis* sp. was identified (Table 3.4). Most of the studies in the literature related to oleaginous fungi used the Mucorales order; very few, to our best knowledge, only one genus *Aspergillus* which is non mucaraceous fungi have been used for lipid production. In addition, oleaginous fungi belonging to the Mucorales order are always used for production of lipids with special long- chain poly unsaturated fatty Acids (PUFA), such as gamma linolenic acid (GLA) and arachidonic acid (AA). These fatty acids have nutritional importance. The present study explored fungi which were capable of producing lipids and did not belong to the Mucorales order. *Aspergillus* a known oleaginous fungus belongs to the Deutromycetes order, *Tricoderma* belongs to the Hypocreales order, and *Penicillum* belongs to the Eurotiales order. These fungi could be attractive alternative fungi for microbial lipid production after further research. Furthermore, their amylase secretion capability could be an additional advantage when using starch wastes for low-cost lipid production. *Acremonium* sp. is well known for hydrolysis of lignocellulosic materials (Fugi et al., 2009), while in this study, it is found that *Aceremonium* sp. (I18-1) possessed starch utilizing capability and oleaginous nature. This is reported in the first time to the best of our knowledge. Therefore, this culture might be used for simultaneous saccharification and fermentation (SSF) of lignocellulosic materials for lipid production. This could be an attractive option for producing the second generation biofuels. When the microbial oil is used for biodiesel applications, European standards (EN14214 and 14213) regulate that the PUFA content in the microbial oil should not be more than 12%. Our study has explored non mucaraceous oleaginous fungi whose PUFA contents presumably would not exceed the limit.

Table 3.4 Blast sequence analysis of isolated oleaginous fungi

Isolate Name	Closest match in NCBI database	NCBI submission		
		Isolate name	Gene bank accession No.	Sequence similarity (%)
I17-1, I17-2, I13-1, I10-1, I16-3, I16-4, I10-2	*Aspergillus* sp.	I16-3,I17-1	JF 895924, JF912418	99
I14-2, I14-4, I5-7, I5-5, I14-5, I8-1, IA10, IA3, IA1	*Penicillum* sp.	I1A1,I5-5,I8-1,I14-4,I19-2	JF 895926, JF895928, JF895930, JF912416, JF912419	99
I19-2, I15-1, I1-2, I1-1, I5-8, I1-3, I5-6	*Trichoderma* sp.	I1-1,I1-3,	JF895925, JF912414	99
I2A31, I2A2, I2A3	*Zygomycetes* sp.	I2A3	JF912415	99
I1A5	*Umbleopsis* sp.	I1A5	JF895927	99
I5-10	*Mortierella* sp.	I5-10	JF895929	99
I18-1	*Acremoium* sp.	I18-1	JF912418	99

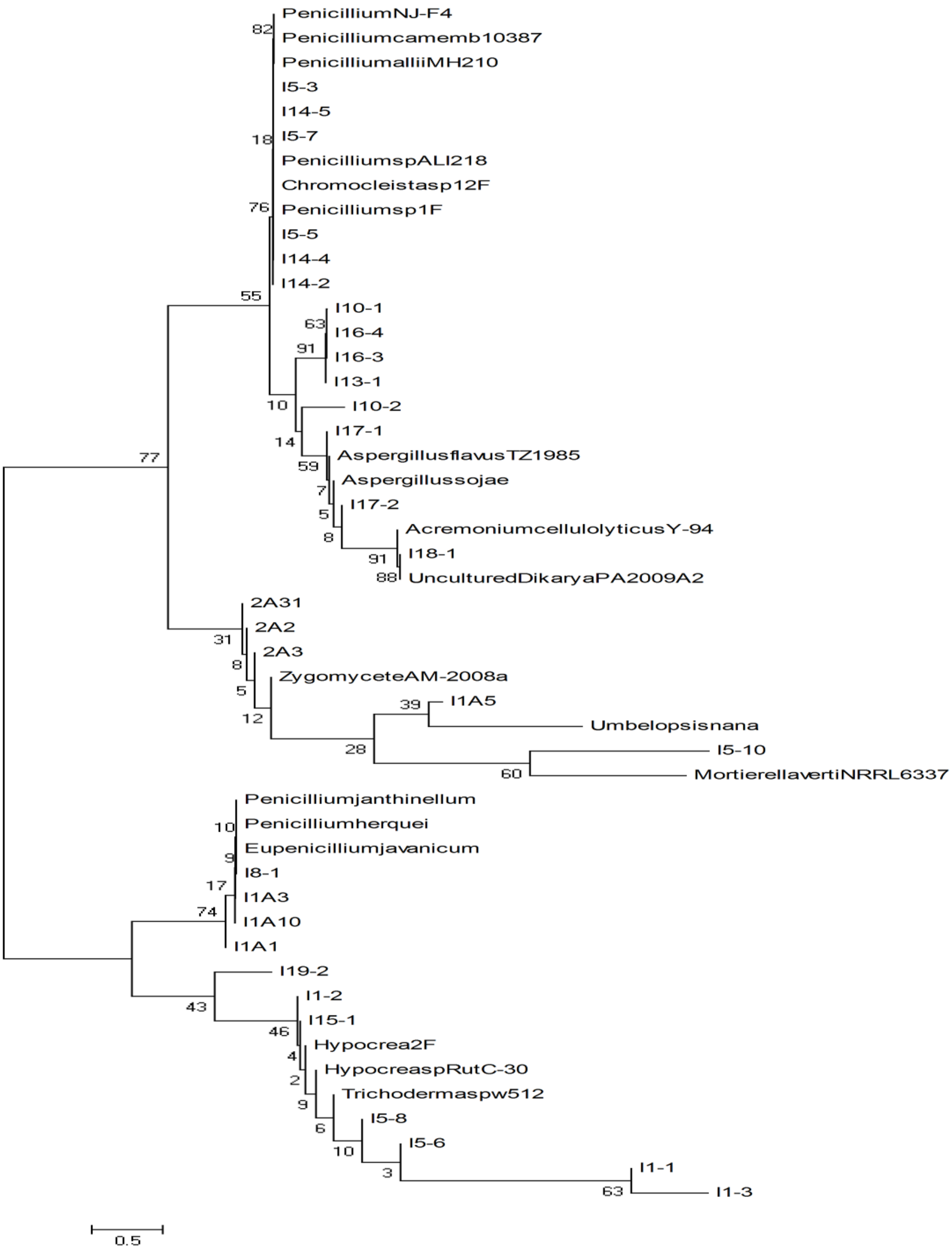

PenicilliumNJ-F4
82
Penicilliumcamemb10387
PenicilliumalliiMH210
I5-3
I14-5
18
I5-7
PenicilliumspALI218
Chromocleistasp12F
76
Penicilliumsp1F
I5-5
I14-4
I14-2
55
I10-1
63
I16-4
91
I16-3
I13-1
10
I10-2
I17-1
14
77
AspergillusflavusTZ1985
59
Aspergillussojae
7
I17-2
5
AcremoniumcellulolyticusY-94
8
I18-1
91
UnculturedDikaryaPA2009A2
88
2A31
2A2
31
2A3
8
ZygomyceteAM-2008a
5
I1A5
39
12
Umbelopsisnana
28
I5-10
60
MortierellavertiNRRL6337
Penicilliumjanthinellum
Penicilliumherquei
10
Eupenicilliumjavanicum
9
I8-1
17
I1A3
74
I1A10
I1A1
I19-2
I1-2
43
I15-1
46
Hypocrea2F
4
HypocreaspRutC-30
2
Trichodermaspw512
9
I5-8
6
I5-6
10
3
I1-1
63
I1-3
0.5

Figure 3.3 Phylogentic analysis of 18s rDNA of isolated oleaginous fungi

3.5 SUMMARY

In this chapter isolation of indigenous oleaginous fungi from Irish soils was carried out and the capability of those fungi in lipid production with the media containing glucose and starch was studied. 50 out of 247 isolates were screened with the medium containing 3% glucose or 3% starch. Best performing cultures were sequenced. Many non mucaraceous oleaginous fungi with amylase secretion capability were reported in this study, which could be used for low cost lipid production using starchy waste materials as carbon substrates.

CHAPTER FOUR

MICROBIAL LIPID PRODUCTION FROM POTATO PROCESSING WASTEWATER USING OLEAGINOUS FUNGI *ASPERGILLUS ORYZAE*

Chapter Four - Microbial lipid production from potato processing wastewater by *A. oryzae*

4.1 INTRODUCTION

Currently, the major source of biodiesel is vegetable oils, with 95% of biodiesel produced from edible plant oils (Gui et al., 2008). This would compete with the food sector, although currently a variety of non edible oils are being used (Abdulla et al., 2011).

As discussed in Chapter 2, microbial oils can replace vegetable oils for energy production due to their advantages over vegetable oils and animal fats, like short life cycles, less labor requirement, less land requirement, less influence by the season and climate change, and easy scale - up (Li et al., 2008). The major bottleneck in using microbes for oil production is the high cost of raw materials. Thus, utilization of cheap materials needs to be explored.

In this study, potato processing wastewater was studied for microbial lipid production using *Aspergillus oryzae*. Potato is a main carbohydrate source in Irish diet, and it was grown over an area of 23,403 acres in 2011 (Farrell and Molloy, 2011). The potato processing industries producing chips, peeled potatoes and wedges, etc., generate a large amount of starchy wastewater which contains high concentrations of chemical oxygen demand (COD), 5-day biochemical oxygen demand (BOD_5) and suspended solids (SS). Commonly, the potato processing wastewater is directed to landfill without treatment, which causes serious environmental damage (Kosseva, 2009). Different methods, such as aerobic biotechnologies, lagoons, land application and electro coagulation, have been used or developed to treat the potato processing wastewater (Abu et al., 2000; Huang et al., 2005; Mishra et al., 2004). Production of high value products from this wastewater has not been studied much. Very limited studies have been conducted using potato processing wastewater for microbial lipids production. To our best knowledge, only Xue et al. (2010) used oleaginous yeast *Rhodotorula glutinis* for lipid production from corn starch wastewater, a type of starchy wastewater. In the present study, we attempted to produce microbial lipids from potato processing wastewater using *Aspergillus oryzae*, and to study nutrient removal efficiencies.

4.2 MATERIALS AND METHODS

4.2.1 Characteristics of potato processing wastewater

Potato processing wastewater was collected from a local vegetable processing industry Glynn Fruit and Vegetables Ltd, Galway, Ireland. Characteristics of the potato processing wastewater are given in Table 4.1. The wastewater had very high concentrations of COD, total solids, starch and other nutrients.

Table 4.1 Characteristics of potato processing wastewater (pH = 4.5)

Characteristics	Value (g/L)
Total solids	26-42
Total suspended solids	2.5-3.8
Volatile suspended solids	2.2-3.5
Total soluble starch	30-36.2
Total Kjeldahl nitrogen (TKN)	0.4-0.62
Total soluble phosphorus (TSP)	0.2-0.56
Ammonium nitrogen (NH_4^+-N)	190 (mg/L)
Orthophosphate (PO_4^{3-}-P)	103 (mg/L)
Sulphate (SO_4^{2-})	102 (mg/L)
Total soluble COD	35-40

4.2.2 Microorganisms and cultivation conditions

Four different strains of *Aspergillus oryzae* were obtained from the German Culture Collection Centre (DSMZ, Germany), DSM 1861, DSM 1862, DSM 1147 and DSM 63303, and were stored in the laboratory on potato dextrose agar plates at 4 °C. In order to find out the best fungus among the four cultures, they all were cultivated on potato processing wastewater with the procedure briefed as follows: (1) 50 mL of properly mixed potato processing wastewater was added into 250 mL conical flasks and was autoclaved at 121 °C for 20 min; (2) two days old fungi cultures grown on YEME broth was homogenized and 0.8 mL of the mycelium suspension was added into each flask as described in Section 3.2.3; and (3) the flasks were placed in an orbital shaker incubator (Fisher Scientific, Ireland) at a shaking speed of 160 rpm and an incubation temperature of 30±1 °C. The fermentation lasted for seven days. The results are given in Table 4.2.; the best performing culture was selected for further studies.

Table 4.2 Selection of *Aspergillus oryzae* strain for lipid production

Strain	**Lipid yields, L_{max} (g/L)**	**Maximum amylase secreted (IU/mL)**	**Starch utilization (%)**
DSM 1861	2.4±0.3	31±1.2	68.0±2.4
DSM 1862	2.8±0.1	35±2.3	72.3±3.4
DSM 63303	1.7±0.2	27±4.3	56.2±5.7
DSM 1147	1.5±0.4	24±5.2	63.3±4.3

Among the four strains tested, DSM 1862 was the best ($p<0.05$) for lipid production, starch utilization and amylase secretion (Table 4.2). Hence, *Aspergillus oryzae* strain DSM 1862 was chosen for the further study.

4.2.3 Dilution of potato processing wastewater

Some researchers did not sterilize starchy wastewater for fermentation. For example, Haung et al. (2003) used potato processing wastewater to produce lactic acid from *Rhizopus arrhius* without sterilization in order to avoid additional costs of the sterilization plant. Recently, Xue et al. (2010) constructed a pilot-scale plant, producing microbial oil from corn starch wastewater using oleaginous yeast. They did not sterilize the wastewater. However, in our research, one of the aims was to study the potential of pure *Aspergillus oryzae* in microbial lipid production, and its potency of nutrient and COD removal. Since non aseptic fermentation cannot ensure growth of a single pure culture, aseptic fermentation was carried out in this study. As a result of sterilization, solidification of starch occurred in the flasks, causing the fungi difficult to branch its filaments. To overcome this problem simple dilution of the raw wastewater with tap water was performed and then the diluted potato wastewater was sterilized. Different dilution ratios were carried out: 25% (75 potato processing wastewater: 25 tap water; v/v), 50% (50 potato processing wastewater: 50 tap water; v/v) and 25% (25 potato processing wastewater: 75 tap water; v/v). In all the trials raw potato wastewater (no dilution) was used for comparison. Nutrient levels in the wastewater at the three dilutions were reduced but there was no change in the C: N ratios.

The cultivation of DSM1862 in the four types of wastewater was conducted using the procedure described in Section 4.2.2.

4.2.4 Analysis

Flasks were removed from the incubator at designed time intervals and subjected to analysis. Samples taken from the flasks were centrifuged at 5000 rpm for 10 min. The supernatant was used for analysis of pH, COD, total soluble starch, amylase activity, total soluble nitrogen (TSN) and total soluble phosphorous (TSP). Ammonium nitrogen and orthophosphate were measured using a Konelab 20 Analyzer (Thermo Clinical Lab Systems, Vantaa, Finland). Biomass samples were dried at 100 ^{o}C until a constant weight and were used for lipids extraction using 2:1 (v:v) chloroform and methanol mixture (Bligh and Dyer, 1959); the detailed procedure was described in Section 3.2.4.5. COD was measured according to the standard APHA methods (American Public Health Association et al., 1999). Total soluble starch was measured using the phenol sulphuric acid method (Dubois et al., 1956) and the method was detailed in Section 3.2.4.2. The amylase activity was measured using the DNS method (Bernfeld, 1955) (Section 3.2.4.3). TSN and TSP were measured by the total nitrogen and total phosphorus kits (Hach Lange, Ireland) according to the supplier's protocol.

The composition of lipids was measured after direct transesterfication (Lewis et al., 2000). A known amount of biomass was added in ***reacti*** vials (Fisher Scientific, Ireland) with a fresh solvent mixture of methanol: hydrochloric acid: chloroform (10:1:1) (v/v). One mg of heptadeconoic acid (17:0) was added as the internal standard. The suspension was vortexed for 2 min and kept at 90 ^{o}C for 30 mins for the transesterfication reaction to take place. Then the samples were taken out and cooled down at ambient temperature. 1 mL of distilled water was added and the suspension was centrifuged at 1500 rpm for 5 min. The lower aqueous phase containing fatty acid methyl esters (FAME) formed was then transferred into new clean tubes for gas chromatography (GC) analysis. Concentrations of fatty acids were measured using gas chromatography (7890A, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and a capillary column DB225 (30 m × 0.25 m × 0.25 µm film thickness). The injection temperature was 220 ^{o}C; the initial column temperature was 160 ^{o}C and rose to the final temperature of 190 ^{o}C at a rate of 3 ^{o}C per min; the detector temperature was 270 ^{o}C. The carrier gas was helium at a flow rate of 1.3 mL/min. The FAME composition of lipids was determined by comparing the retention time and peak area of samples with the internal standard and the FAME mix (Sigma, Ireland).

4.2.5 Statistical analysis

Statistical analysis was performed by paired t-test for two sample means using statistical software MINITAB (version 16). p value < 0.05 was set as cut off to estimate statistical significance.

4.3 RESULTS AND DISCUSSION

4.3.1 Lipid production by *Aspergillus oryzae*

Lipid production by *Aspergillus oryzae* in different dilution potato processing wastewaters was monitored, in addition with principal nutrients, namely $NH_4{}^{+}$ - N and PO_4^{3-} -P ions. After commencement of the fermentation, concentrations of NH_4^{+} - N and PO_4^{3-} -P decreased (Fig. 4.1). Lipid accumulation was observed after the 3rd day, when the complete depletion of PO_4^{3-} -P occurred and there were low levels of NH_4^{+}-N ions remaining in the wastewater (Fig. 4.1a, 4.1b, 4.1c, 4.1d, and Table 4.3). Hence, it can be inferred that PO_4^{3-}-P limitation might have mediated lipid accumulation in *Aspergillus oryzae* even though the wastewater had higher initial concentrations of NH_4^+-N (190, 143, 95 and 40 mg/L in the raw wastewater, wastewater at 25%, 50% and 75% dilution ratios, respectively).

Table 4.3 Nutrient concentrations in potato processing wastewater during fermentation

Wastewater	Initial NH_4^+-N (mg/L)	Initial PO_4^{3-}-P (mg/L)	NH_4^+-N on the 3rd day (mg/L)	PO_4^{3-}-P on the 3rd day (mg/L)
Raw	190±0	100±0	9.2±4.3	0.1±0.1
25%	143±0	73.5±2.1	7.6±0.8	0.0±0.0
50%	95±0.8	50.0±0	6.1±0.4	0.1±0.1
75%	40±4.4	24.5±0.7	6.8±1.4	0.0±0.0

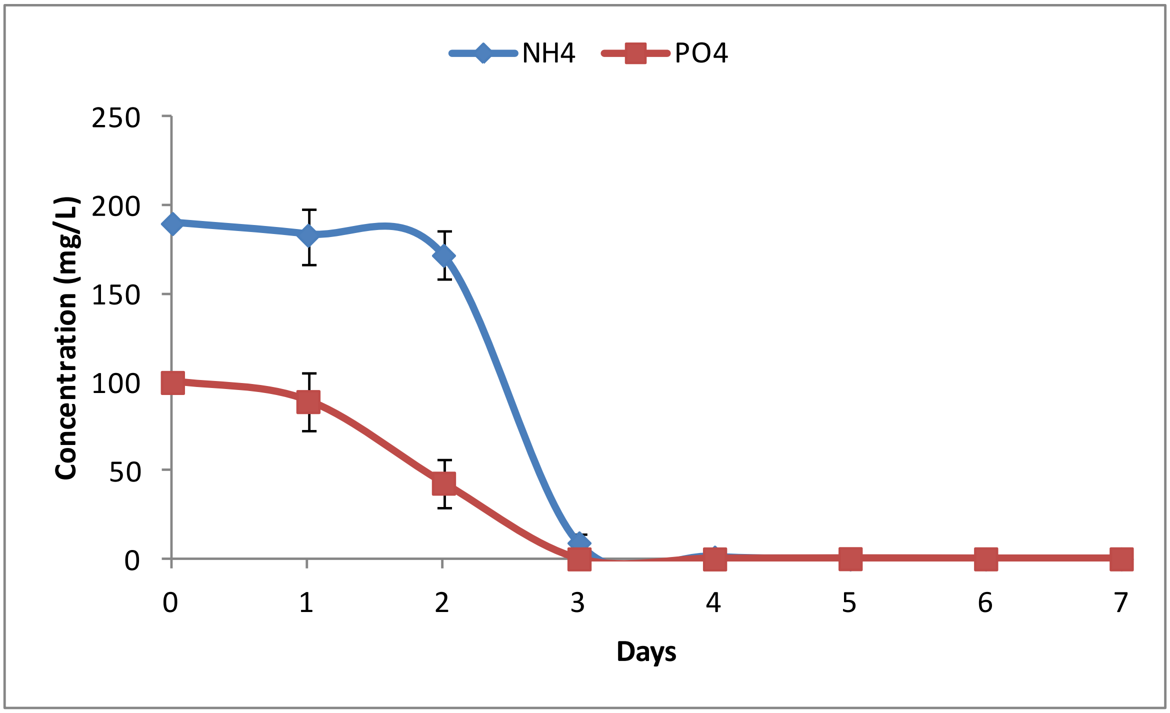


Figure 4.1a. Depletion of NH_4^+-N and PO_4^{3-}-P ions in raw potato processing wastewater

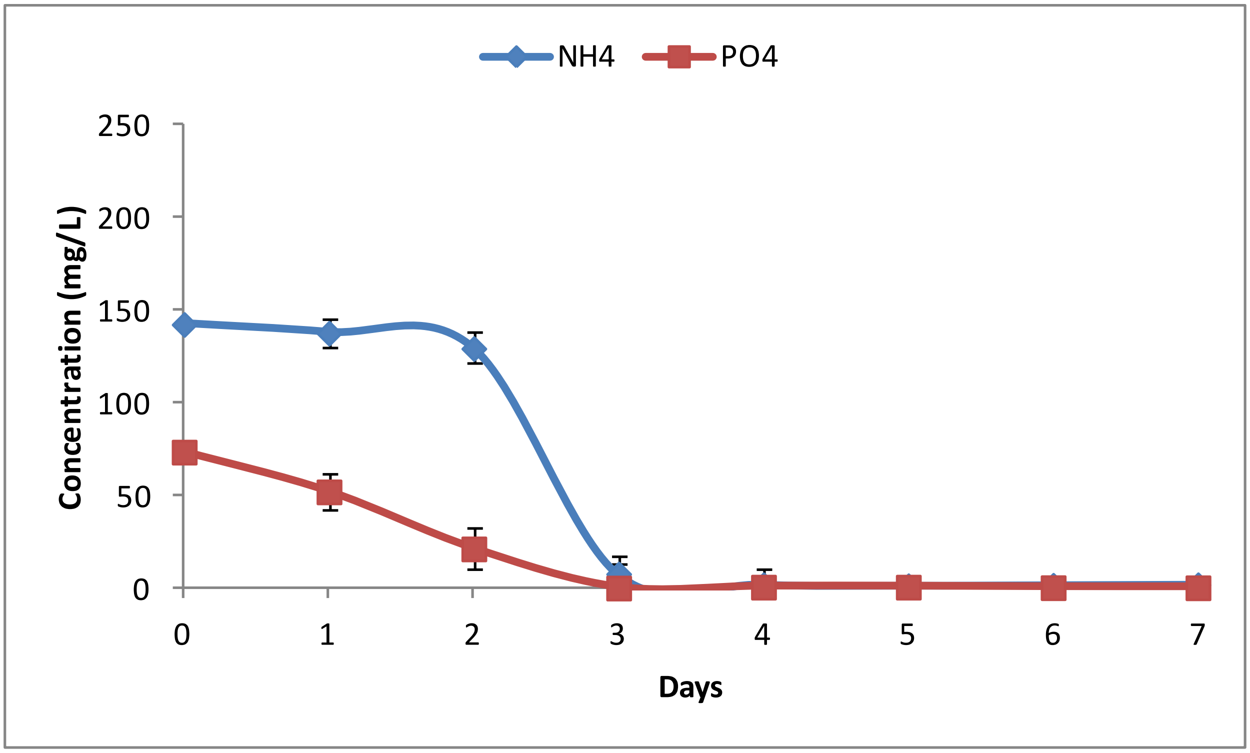


Figure 4.1b. Depletion of NH_4^+-N and PO_4^{3-}-P in the wastewater at 25% dilution

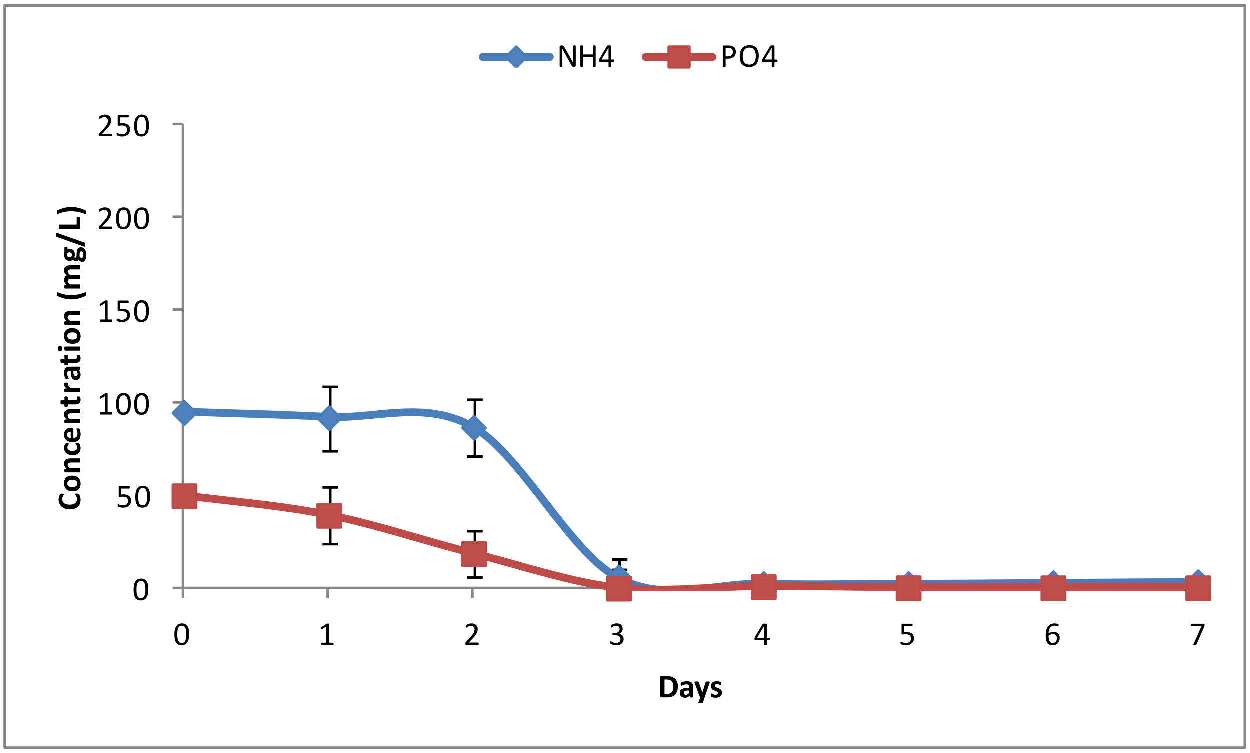


Figure 4.1c. Depletion of NH_4^+-N and PO_4^{3-}-P in the wastewater at 50% dilution

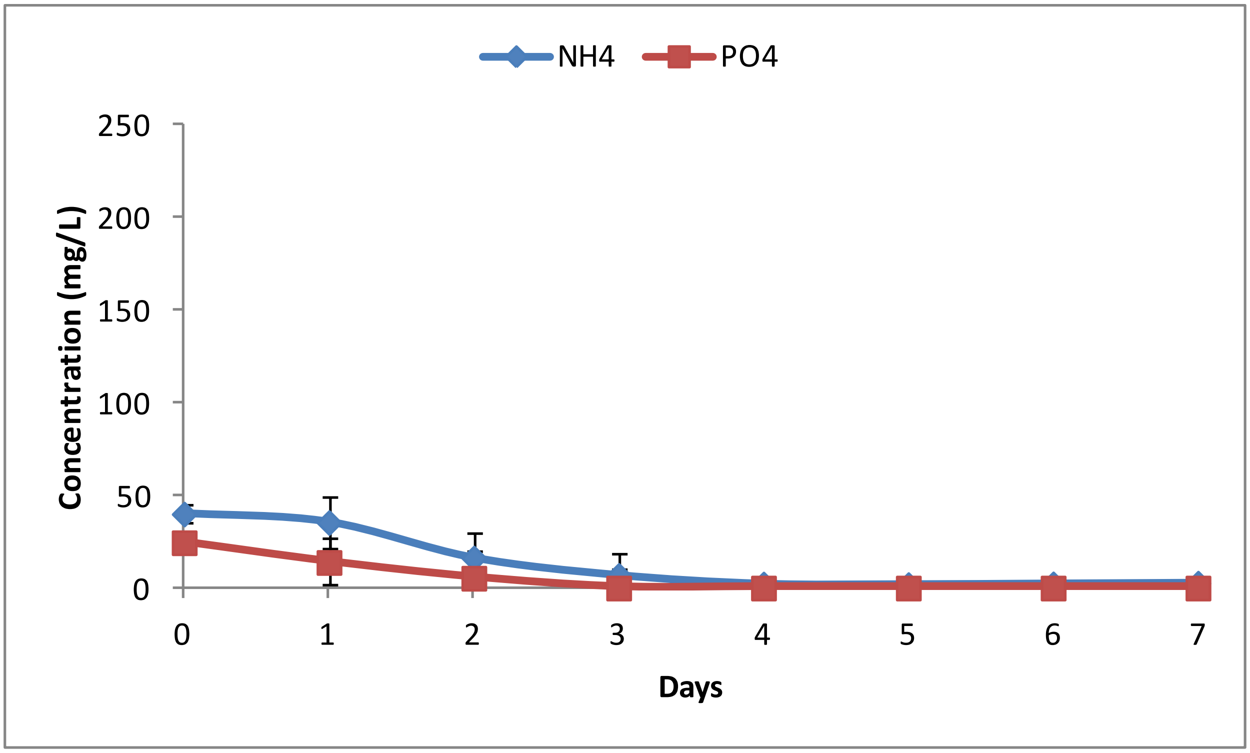


Figure 4.1d. Depletion of NH_4^+-N and PO_4^{3-}-P in the wastewater at 75% dilution

Microbial lipid production usually requires a preliminary step of nitrogen source deficit in order for the cells to channelize excess carbon into cells for lipid synthesis (Ratledge, 2004). While, Papanikolaou et al. (2002) found that when the carbon source was fatty acids and their derivatives, lipid accumulation by oleaginous organisms didn't require nutrient deficit, and lipid accumulation and nutrient reduction happened simulatneously. Deficit of nitrogen in the form of ammonium nitrogen is widely studied. Apart from nitrogen source deficit, phosphorous, magnesium, zinc and iron deficit was also able to induce microbial lipid accumulation by oleaginous yeasts *Yarrowia lipolytica Candida* sp. and *Rhodotorula glutinis*; the lipid yield with respect to the substrate consumption, $Y_{L/C}$, was up to 0.11 - 0.22 g lipids / g of glucose consumed (Beopoulos et al., 2009). Recently, lipid accumulation mediated by phosphate deficit (Wu et al., 2010) and sulphate deficit (Wu et al., 2011) by oleaginous yeast *Rhodosporidium toruloides* has been reported.

To the best of our knowledge no studies have been conducted to study the depletion of nutrients for lipid production in wastewater conditions, with specific reference to *Aspergillus oryzae*. The present study has observed for the first time that in potato processing wastewater PO_4^{3-} limitation would induce lipid accumulation. Wu et al. (2010) also suggest that phosphate limitation could lead to higher lipid contents in oleaginous microbes although nitrogen is present in high concentrations.

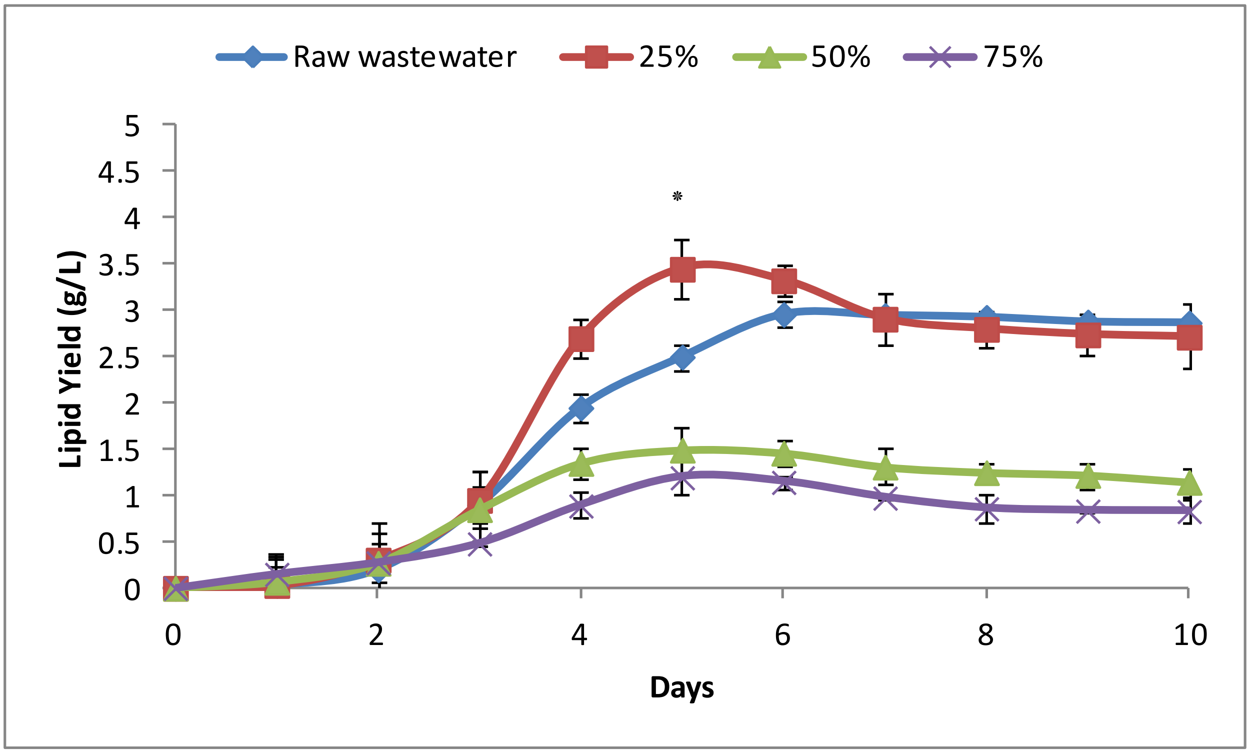


Figure 4.2 Lipid productions by *Aspergillus oryzae* in potato processing wastewater at different dilution ratios

Lipid accumulation gradually increased after the fermentation commenced, and the highest lipid accumulation was observed on the 5th day in the diluted wastewater and on the 6th day in the raw wastewater. The highest lipid yield (3.5 g/L) was achieved at the dilution ratio of 25% ($P < 0.05$), followed by 3.0 g/L in the raw wastewater, 1.5 g/L at the dilution ratio of 50% and 1.2 g/L at the dilution ratio of 75% (Fig.4.2.). Filamentous fungi have a great potential in producing value added products. Fungal genus *Aspergillus* sp. has been examined to treat various wastewaters and simultaneously recover high value products like fungal protein, lactic acid, and amylase (Sankaran et al., 2010). However, its potential on single cell oil production has not been studied before.

This study shows *Aspergillus oryzae* accumulated 3.5 g/L of lipids within 5 days of fermentation, indicating that potato processing wastewater can be utilized to produce single cell oils quickly. In the present study the lipids yield with respect to soluble starch consumption, $Y_{L/C}$, was in the range of 0.11 – 0.16 g lipids /g soluble starch. A similar lipid yield around 0.15 g lipids/g glycerol was obtained when microalgae *Schizochytrium limacinum* was grown on biodiesel derived glycerol (Chi et al., 2007). Oleaginous fungi *C. echinulata* ATHUM 4411 grown on pure starch had a conversion yield of 0.15 g lipids/g starch (Papanikolaou et al., 2007). The results obtained in the present study, 0.11 - 0.16 g lipids/g soluble starch, with potato processing wastewater as the cultivation substrate were comparable and this is encouraging. Under ideal conditions for single cell oil production the maximum lipid yield on glucose consumption can rarely be higher than 0.22 g lipids/g glucose (Ratledge and Cohen, 2008). Interestingly, Papanikolaou and Aggelis (2011) have recently found that the lipids conversion yields from glycerol can be much higher than that from pure glucose, up to 0.30 g lipids/g glycerol. The yield obtained in our study gives a positive solution for economic production of lipids using potato processing wastewater and there is an ample scope for improving the conversion yield by optimizing nutritional and growth conditions. The lipid content in the fungal biomass was estimated as high as ˜40% in *Aspergillus oryzae*, which is comparable with other studies. Xue et al. (2010) obtained a lipid content of 35% in oleaginous yeast *Rhodotorula glutinis* cultivated on corn starch wastewater supplemented with waste syrup. Our study showed higher lipid contents without supplementation of external nutrients.

When cultivated on C6 compounds, after complete exhaustion of carbon sources, oleaginous microbes utilize accumulated lipids to produce biomass. This phenomenon of lipid turnover has been routinely observed in many oleaginous microbes belonging to the Mucorales order. In the present experiment lipid turnover was observed at the dilution ratios of 25%, 50% and 75% but not in the raw wastewater (Fig.4.2.). Therefore, the fermentation time should be controlled at 5-6 days for lipid production.

4.3.2 Lipid composition

The long-chain fatty acid profiles (C14:0 - 18:0) in the lipids are given in Table 4.4. A variety of long-chain fatty acids were found in the microbial lipids and the major fatty acids were oleic acid

(22.5%), stearic acid (14.5%) and palmitic acid (12.6%). The linolenic acid content was only 5.5% and was less than 12%. European regulation (EN 14213 and EN14214) stipulates that, if microbial oil is used for biodiesel feedstock the linolenic acid content in microbial oil should not be above 12%. Therefore, the lipid produced in this study is a suitable feedstock for biodiesel production.

Table 4.4 Fatty acid profiles of the microbial lipids obtained from *A.oryzae*. The analysis of fattyacids was made with maximum lipid content obtained on 5th day of fermentation. The data represented are the mean value obtained from three replicates.

Fatty acid		Wt (%)
C 14:0	Mystric acid	4.0
C 16:0	Palmitic acid	11.6
C 16:1	Palmitolic acid	15.6
C 18:0	Stearic acid	19.3
C 18:1	Oleic acid	30.3
C 18:2	Linoleic acid	6.5
C 18:3	Linolenic acid	5.5
C20:0	Arachidic acid	2.0
C22:0	Bechinic acid	2.3

4.3.3 Starch utilization and amylase secretion

Utilization of soluble starch by *Aspergillus oryzae* at different dilution ratios was also studied. Almost complete utilization of soluble starch was observed at dilution ratios of 25%, 50% and 75% with the utilization rates of 99.9%, 99.5% and 96.0%, respectively (Fig.4.3). The soluble starch utilization rate was only 70% in the raw wastewater, which could be due to the much higher initial soluble starch concentration (Fig.4.3).

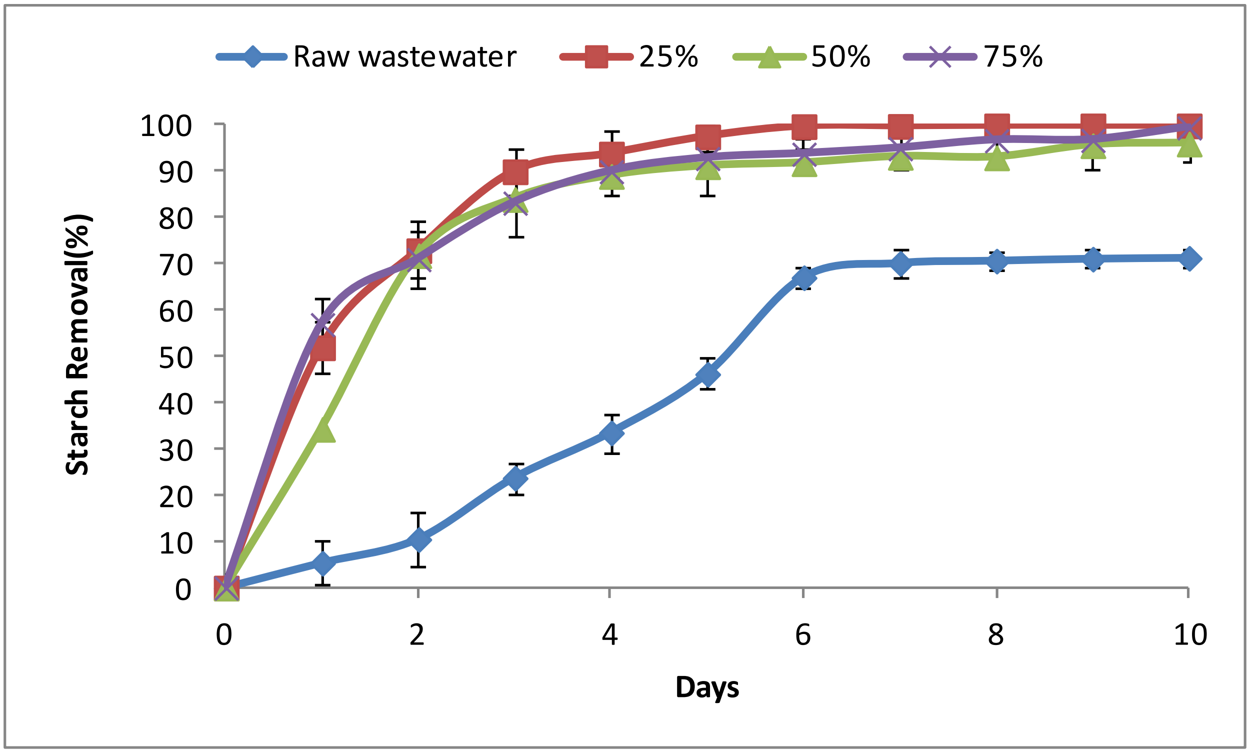


Figure 4.3 Starch utilization by *Aspergillus oryzae*

It is known that starch utilization is greatly influenced by secretion of amylolytic enzymes. A significant amount ($p<0.05$) of amylase secretion (the maximum amylase activity was 53.5 IU/mL) was observed in the potato processing wastewater at the dilution ratio of 25%, followed by at 50% dilution ratio (39.5 IU/mL) and in raw wastewater (31 IU/mL). The least amylase secretion was observed at the dilution ratio of 75% with an amylase activity of 26.5 IU/mL (Fig.4.4).

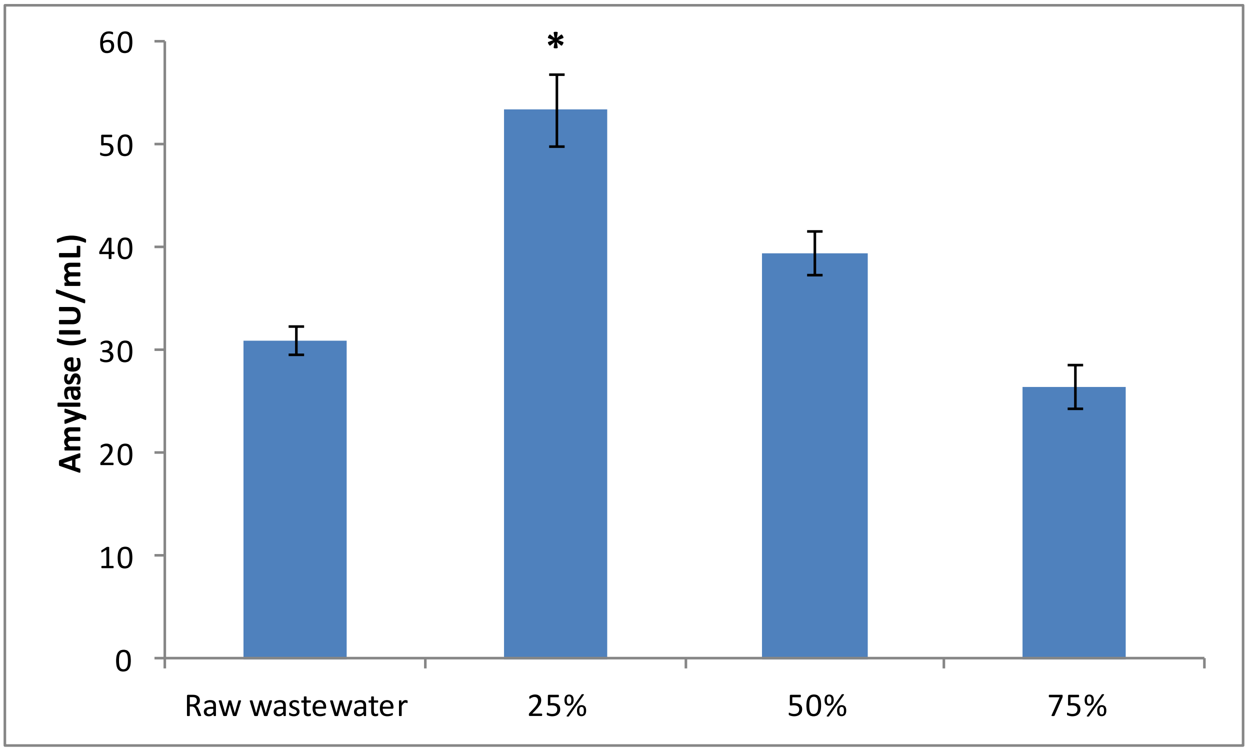


Figure 4.4 Amylase secretion by *Aspergillus oryzae* in potato processing wastewater at different dilutions (* symbol indicates the value is significantly higher than at other dilution ratios; $p < 0.05$)

The result indicates that the starch concentration should be kept below a certain level for optimal lipid production. For instance, when cassava starch hydrolysate was used for microbial lipid production by oleaginous yeast, 2% of cassava starch was found to be optimum (Li et al., 2010). Similarly, when wheat bran, a rich source of cellulose, was utilized for lipid production, 2% of cellulose was found to be optimum. The reason may be the toxicity caused by high concentrations of complex sugars to cells (Hui et al., 2010). The present study also suggests that, when starchy waste material is used for microbial lipid production there will be no need to add external amylase to hydrolyze starch, since *Aspergillus oryzae* can secrete amylase and accumulate lipids simultaneously. Some other researchers added external amylase to hydrolyze the starchy waste for lipid production. For example, *S. fibuligera* was used to produce crude amylase which was then added to cassava starch hydrolysate for lipid production by *Rhodotorula mucilaginosa* TJY15a (Li et al., 2010). Wei et al. (2009) added commercial enzyme alpha amylase and gluco amylase to hydrolyze cassava starch for lipid production by micro algae

Chlorella protothecoide. Since external addition of amylase is not necessary for lipid production by *Aspergillus oryzae*, the lipid production cost can be reduced.

4.3.4 Removal of COD and nutrients from wastewater

In addition with lipid production and amylase secretion, the potential of *Aspergillus oryzae* to remove COD, TSN, TSP, NH_4^+-N, PO_4^{3-} -P and SO_4^{2-} from potato processing wastewater was studied. The removal percentages of these contaminants are given in Table 4.5. The highest removals of COD, TSN and TSP were achieved at the dilution ratio of 25% ($P<0.01$).

COD removal of *Aspergillus oryzae* is comparable with other studies. Xue et al. (2010) studied lipid production from corn starch wastewater using oleaginous yeast *Rhodotorula glutinis* by means of non aspectic fermentation and achieved only 80% of COD reduction. Our study suggests that oleaginous fungi could be a better candidate for nutrient removal from starchy wastewater. Mishra et al. (2004) used potato chips industry wastewater for microbial protein production by mixed cultures of *Aspergillus foetidus* and *Aspergillus niger* and achieved COD reduction of 90%. In the present study, by using one pure culture *Aspergillus oryzae* COD reduction of 91% was achieved. In addition, at the end of the fermentation, complete removals of NH_4^+ and PO_4^{3-} were observed, in addition with 30% of SO_4^{2-} removal. This indicates that all these nutrients were used by the fungal species in the lipid accumulation process.

Table 4.5 Removals of COD, total soluble nitrogen (TSN) and total soluble phosphorus (TSP) by *Aspergillus oryzae* from potato processing wastewater at different dilutions (%)

Nutrients	Raw wastewater	25%	50%	75%
COD	74.2±1.9	91.3±1.3	85.7±2.7	66.6±3.1
TSN	77.6±2.1	97.7±1.1	86.5±0.9	94.6±3.2
TSP	31.5±1.4	97.1±0.2	90.8±0.4	94.7±0.2

Figure 4.5 show that COD removals achieved, *r*, were able to correlate with the maximum amylase activity, *E*, as follows:

$r = 0.89\ E + 46\ (R^2 = 0.90)$

This indicates the reason for efficient COD removals would be the capability of *A. oryzae* in amylase secretion, which helped to hydrolyze starch.

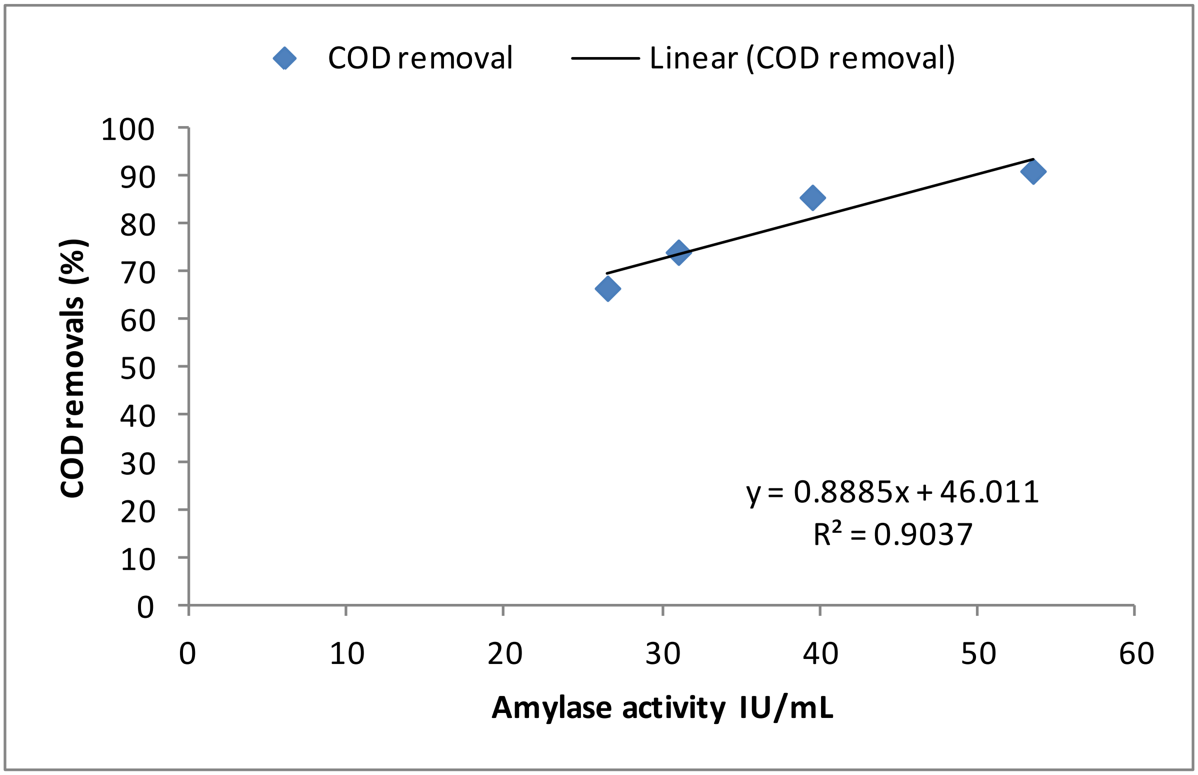


Figure 4.5. Correlation between COD removals and the maximum amylase activity.

4.4 SUMMARY

In this chapter potato processing wastewater was tested for low cost lipid production by Aspergillus *oryzae*. The results show that it could be a potential raw material for microbial lipid production using oleaginous fungi. The lipids produced had a long-chain fatty acid composition suitable for biodiesel production. In addition, this technology would offer a method to efficiently remove nutrients from wastewater.

CHAPTER FIVE

UTILIZATION OF POTATO PROCESSING WASTEWATER FOR MICROBIAL LIPIDS AND GAMMA-LINOLENIC ACID PRODUCTION BY OLEAGINOUS FUNGI

Chapter Five -Production of microbial Lipids and GLA using oleaginous fungi

5.1 INTRODUCTION

Value-added products such as biogas, lactic acid, and microbial proteins have been produced using starch rich wastewater (Abu et al., 2000; Gelinas and Barrette, 2007; Mishra et al., 2004). However, use of potato processing wastewater to produce high value products has not been studied in Ireland (Wijngaard et al., 2012). In the past decade, production of microbial lipids and polyunsaturated fatty acids has gained lots of attention because of their use as biodiesel feedstock, neutraceuticals and food additives (Boswell et al., 1996; Horrobin, 1992; Ratledge, 2004). Utilization of various low cost substrates to produce microbial lipids and polyunsaturated fatty acids is among current research topics in order to make the process economically feasible (Economou Ch et al., 2010; Fakas et al., 2009; Shene et al., 2010). To date, very few investigations have been carried out on use of potato processing wastewater for microbial lipids and gamma- linolenic acid (GLA) production. In this chapter, two oleaginous fungi, *Aspergillus flavus* I16-3 and *Mucor rouxii*, were used to produce microbial lipids ad GLA from potato processing wastewater. This study also investigated simultaneous removal of nutrients (COD, nitrogen and phosphorus) from wastewater.

5.2 MATERIALS AND METHODS

5.2.1Microorganisms

Aspergillus flavus I16-3 (Gene Bank ID: JF895924), an oleaginous, amylolytic and neutrophilic fungus, was isolated from Irish soils (Chapter 3). Mucaraceous fungi *Mucor rouxii* DSM1191, a known lipid and GLA producer, was provided by German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Both the cultures were stored in the laboratory on potato dextrose agar slants at 4 ^{o}C.

5.2.2 Potato processing wastewater

The potato processing wastewater was collected from the Glynn fruit and vegetable processing plant, Galway, Ireland, and was stored in the laboratory at 4 ^{o}C. The wastewater contained peeled potato skin, soil particles, and potato solids. Characteristics of wastewater are described in Table 5.1. It had high starch and low nitrogen contents with the total soluble starch to total soluble nitrogen ratio of 72. pH of the potato processing wastewater was 4.5 - 5.5, which was slightly lower than the optimum value of 5.5 - 7.0 for lipid accumulation by oleaginous fungi (Wu et al., 2011).

Table 5.1 Characteristics of potato processing waste water (pH = 4.5 – 5).

Characteristics	Value (g/L)
Total solids	26-42
Total suspended solids	2.5-3.8
Volatile suspended solids	2.2-3.5
Total soluble starch	30-36.2
Total Kjeldahl nitrogen (TKN)	0.4-0.62
Total soluble phosphorus (TSP)	0.2-0.56
Ammonium nitrogen (NH_4^+ -N)	190 (mg/L)
Orthophosphate (PO_4^{3-} -P)	103 (mg/L)
Sulphate (SO_4^{2-})	102 (mg/L)
Total soluble COD	35-40

5.2.3 Cultivation conditions

Cultivation of *A. flavus* I16-3 and *M. rouxii* in potato processing wastewater for lipid production was studied under two conditions - raw wastewater and synthetic wastewater. The synthetic wastewater was made by adding 10 mL of extra nutrient solutions to every 40 mL of the raw wastewater; the nutrient solution was made from commonly used lipid producing medium which contained (in g/L): $CaCl_2$, 0.1; KH_2PO_4, 2.5; $FeSO_4$, 0.02; NH_4Cl, 0.01; $MgSO_4$, 0.5; $MnSO_4$, 0.003; and CuSO4, 0.002 (Papanikolaou et al., 2004). In comparison with the raw wastewater, the change of the C:N ratio due to the addition of nutrients was ignorable.

During the experiment, properly mixed potato processing wastewater (raw and synthetic) was added into 250 mL conical flasks, 50 mL each, and autoclaved at 121 ^{o}C for 20 min. The pH value of the wastewater was adjusted to 6.0±0.5 using 1 N NaOH before sterilization and then confirmed after sterilization. The sterilization changed characteristics of the potato processing wastewater to a certain extent, for example, occurrence of starch solidification; effects of starch solidification on lipid production are described in Chapter 4. 0.8 mL homogenized mycelial suspension of the two oleaginous fungi (the detailed procedure for preparation of mycelial suspension is described in Section 3.2.3) was inoculated in each flask. Then the flasks were incubated in a shaker incubator; the incubation conditions are described in Section 3.2.3.

5.2.4 Analytical methods

Flasks were removed from the incubator at designed time intervals and subjected to analysis. Samples taken from the flasks were centrifuged at 5000 rpm for 10 min. The supernatant was used for analyzing pH, COD, total soluble starch, amylases activity, total soluble nitrogen (TSN) and total soluble phosphorous (TSP). Total soluble starch was measured using the phenol sulphuric acid method (Dubois et al., 1956) and is detailed in Section 3.2.4.2. Amylase activity was measured using the DNS method (Bernfeld, 1955) as described in Section 3.2.4.3. COD was measured according to standard APHA methods (American Public Health Association et al., 1999). TSN and TSP were measured by the total nitrogen and total phosphorus kits (Hach Lange, Ireland) according to the supplier's protocol. Biomass samples were dried at 80 ^{o}C until a constant weight and were used for lipids extraction using 2:1 (v: v) chloroform and methanol mixture (Bligh and Dyer, 1959); the detailed procedure is described in Section 3.2.4. Measurement of GLA was performed by direct transesterfication of biomass (Lewis et al., 2000). FAME was prepared as described in Section 4.2.5. The content of GLA in the FAME was measured by means of gas chromatography with an FID detector using conditions as given by manufacturer as Follows: the injector temperature was 250 ^{o}C, the oven temperature was programmed at 210 ^{o}C isothermal, and the detector FID temperature was 250 ^{o}C; a split ratio of

100 mL/min; and the carrier gas was helium gas. After the chromotogram was obtained, the GLA content was calculated using the following formula:

$$GLA = \frac{AL}{\sum A - AEI} 100\% \quad \text{[Eq. 5.1]}$$

where, GLA, content of GLA in microbial lipids (%);
AL, the peak area due to GLA;
ΣA, the total peak area due to the FAME (C14:0 to C24:1); and
AEI, peak area of methyl heptadecanoate (internal standard).

5.3 RESULTS AND DISCUSSION

5.3.1Growth and lipid production of *A. flavus* I16-3 and *M.rouxii*

Kinetic studies on biomass growth and lipid production of both fungi were studied under the two wastewater conditions - raw and synthetic wastewater. Potato processing wastewater did not show any inhibitory effects on biomass production (Fig. 5.1).

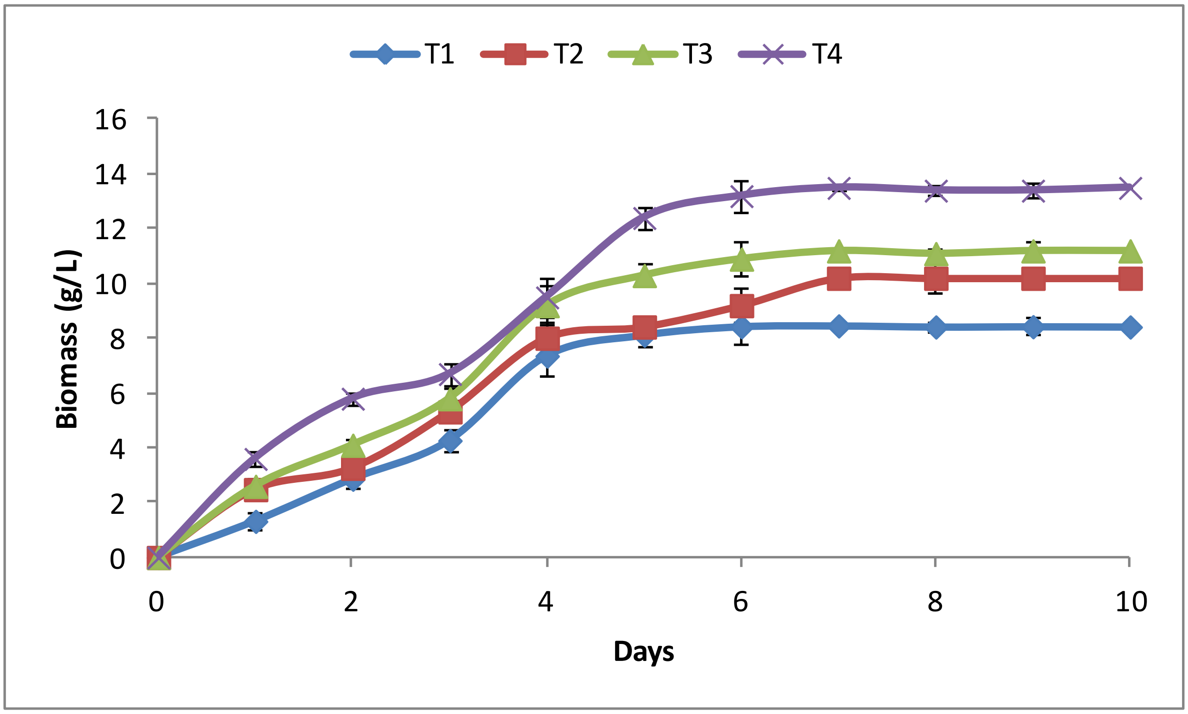


Figure 5.1 Biomass production of *Aspergillus flavus* I16-3 (T1: raw wastewater; T2: synthetic wastewater) and of *Mucor rouxii* (T3, raw wastewater; T4, synthetic wastewater)

Figure 5.1 shows that production of *M.rouxii* biomass was higher than that of *Aspergillus flavus* I16-3. In all the trials synthetic wastewater supported higher biomass growth for both fungi than the raw wastewater (Table 5.1 and Fig 5.1). Biomass yields of both fungi with respect to total soluble starch consumed ($Y_{X/C}$, Chapter 3) were up to 0.3 - 0.48 g biomass/g starch consumed. In general oleaginous fungi grown on renewable sugars such as starch, pectin and lactose have a maximum biomass yields of 0.19 - 0.54 g biomass/g carbon source consumed (Papanikolaou et al., 2007), and starch has been proven to be an excellent substrate for biomass production by oleaginous fungi (Kavadia et al., 2001; Papanikolaou et al., 2007). Our results present higher $Y_{X/C}$ values (0.48 g biomass/g starch) for *M.rouxii* grown in raw wastewater than those reported in the literature in which *Morterialla isabellina* ATHUM 2935 grown on pure starch produced 0.42 g biomass/g starch consumed.

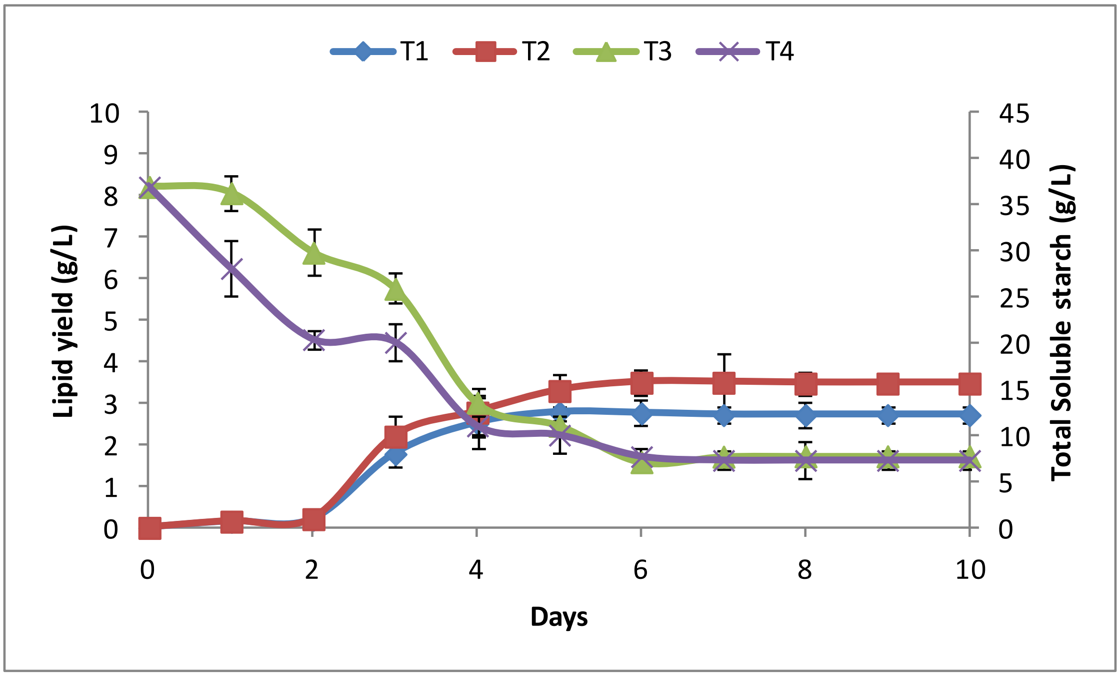
Taking into account that the substrate in our investigation was potato processing wastewater our results on lower yields can be easily justified. The kinetics of lipid accumulation and sugar consumption are shown in Fig.5.2.

Table 5.2 Growth of *A.flavus* and *M.rouxii* in potato processing wastewater

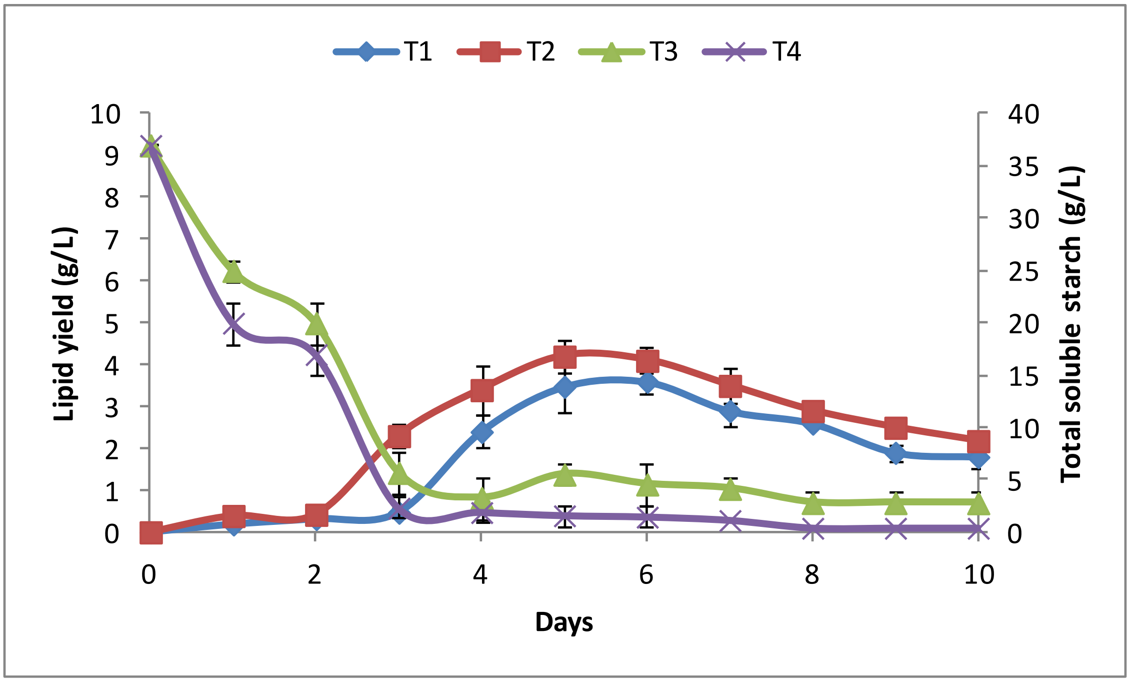
Culture	Conditions	X(g/L)	L_{max} (g/L)	TSS_f(g/L)	$Y_{X/C}$(g/g)	Lipid content (g/g)	$Y_{L/C}$ (g/g)
A.flavusI16-3	Raw	8.5±1.2	2.8±0.23	7.7±2.3	0.30	0.32	0.10
	Synthetic	10.2±1.4	3.5±0.34	7.3±2.1	0.35	0.34	0.12
M.rouxii	Raw	11.2±1.3	3.6±0.24	2.79±0.5	0.48	0.32	0.15
	Synthetic	13.5±1.4	4.2±0.29	0.39±0.1	0.37	0.31	0.11

TSS: total soluble starch; TSS_f: final soluble starch concentration in the media

Calculation of parameters such as $Y_{X/C,}$ lipid content, $Y_{L/C}$ are given in Section 3.2.4.6, Chapter 3 ,



(a)



(b)Figure 5.2. Lipid production and total soluble starch utilization by *Aspergillus flavus* I16-3 (a) and *Mucor rouxii* (b) (T1: lipid yield in the raw wastewater; T2: lipid yield in the synthetic wastewater; T3: total soluble starch in the raw wastewater; T4: total soluble starch in the synthetic wastewater).

Accumulation of lipids in *A. flavus* started two days after the fermentation commenced and the lipid yield reached the maximum value (L_{max}) of 2.8 g/L in the raw wastewater and 3.5 g/L in the synthetic wastewater on Day 5 (Fig. 5.2a). The lipid yields of *A. flavus* are comparable with those of other widely studied SCO producers such as *Morterialla isabellina* ATHUM 2935 and *Cunninghamella echinulata* ATHUM 2935, which produced L_{max} of 3.7 and 3.8 g/L, respectively when they were cultivated on starch. Hence, the isolated indigenous species *A. flavus* could be considered as a potential lipid producer while growing on starch or related wastewater sources.

M. rouxii presented a higher lipid accumulation capacity ($p<0.01$) under both conditions than *A. flavus* and started to accumulate lipids three days after the fermentation commenced. On Day 5 the lipid yield reached the peak values of 3.6 g/L in raw wastewater and 4.2 g/L in the synthetic wastewater (Fig. 5.2b). Microbes belonging to the Mucorales Order are better candidates for high lipid production. Similar research conducted by Ahmed et al. (2006) obtained higher lipids yields by *Mucor sp.* RRL 001 (L_{max} = 5.8 g/L) when cultivated on tapioca starch. Lipids contents in biomass ($Y_{L/X}$) ranged 0.31-0.34 g/g for both the fungi; similar results, 0.36 g/g, were observed when *Morterialla isabellina* ATHUM 2935 was grown in pure starch (Papanikolaou et al., 2007). The lipid yield with respect to soluble starch consumed ($Y_{L/C}$) value was higher ($p<0.05$) for *M.rouxii* in the raw wastewater than in the synthetic wastewater (Table 5.1). In our present investigation the Ls values of both fungi, when grown on potato processing wastewater, ranged from 0.1 to 0.15 g lipids per g of total soluble starch consumed. Similar values 0.15 g lipids per g of pure starch consumed were observed in oleaginous *zygomycetes* (Papanikolaou et al., 2007). Generally, when various sugars and polysaccharides are utilized as the carbon sources, $Y_{L/C}$ values of 0.18 - 0.24 g lipids per g of substrate consumed are considered optimal (Ratledge, 1993). The reason for lower $Y_{L/C}$ values in this study could be that the carbon substrate used in those studies were pure and the microbes can demonstrate much more efficient conversion of carbon than in potato processing wastewater.

Occurrence of reserve lipid turnover was not observed in *Aspergillus flavus* I16-3 but was observed in *M.rouxii*. The soluble starch consumption by *M.rouxii* was higher and it consumed most of available soluble starch under raw and synthetic wastewater conditions. In contrast soluble starch concentrations decreased from 36.2 g/L initially to 7.7 g/L on Day 6 under both conditions for *Aspergillus flavus* I16-3 (Fig. 5.2a and b). After the fermentation time was extended to 10 days, a significant amount ($p<0.01$) of soluble starch still remained in the medium. The reason that no lipid turnover occurred in *Aspergillus flavus* I16-3 was due to the availability of surplus carbohydrates in the medium.

Table 5.3 Specific uptake rates of total soluble starch in raw and synthetic wastewater by *A. flavus* and *M. rouxii*

		Total soluble starch specific uptake rate TSS_U (g/g.h)	
Cultures	Fermentation time	Raw	Synthetic
A.flavus I16-3	24	0.145	0.148
	48	0.035	0.098
	72	0.028	0.002
M.rouxii	24	0.192	0.196 *
	48	0.051 $	0.022
	72	0.010	0.009

Symbol (* and $) indicates there was statistically significant difference ($p<0.05$) for the specific uptake rates of the two oleaginous fungi.

The specific uptake rate for total soluble starch, TSS_U (g soluble starch/g biomass.h), can be calculated using the formula as follows:

$TSS_U = -\frac{dtss}{dt}\frac{1}{X}$ [Eq. 5.2]

where, TSS, substrate concentration (g/L); and X, biomass concentration at time t of consideration, g/L.

Calculation of TSS_U is given in Table 5.3. Table 5.3 shows that *M. rouxii* generally up took more total soluble starch than *A. flavus* I16-3. This can be confirmed by the kinetics of total soluble starch utilization pattern (Fig. 5.2). Consumption of total soluble starch was higher ($p<0.01$) in the synthetic wastewater than in the raw wastewater for both fungi (Fig. 5.2 a and b). The reason might be that addition of nutrients into potato processing wastewater enhanced the uptake of soluble starch by both fungi. The higher TSS_U values were obtained within 48 hrs (early stage) of cultivation (Table 5.3), which indicates that the carbon flow was channeled into cells for lipid production, followed by achievement of L_{max} values on 5-6 days of cultivation (Fig. 5.2 a and b). Addition of nutrients resulted in higher L_{max} values because both fungi demonstrated higher TSS_U values in synthetic wastewater conditions.

The potato processing wastewater was revealed as a good low cost substrate. This study shows that it is feasible to use it for microbial lipid production and to reduce total production cost since it is available free of cost.

5.3.2. GLA production

A comparison of GLA production in the raw and synthetic wastewater conditions by the two oleaginous fungi is given in Table 5.4. GLA production rose with the fermentation time. Non-negligible GLA yields (60 and 100 mg/L in the raw wastewater and synthetic wastewater) by *Aspergillus flavus* I16-3 were observed during 192 hours of fermentation. *Mucor rouxii* yielded 100 mg/L of GLA in the raw wastewater and 140 mg/L in the synthetic wastewater. GLA percentages in lipids were 2.2% and 2.9% for *Aspergillus flavus* I16-3 in the raw wastewater and the synthetic wastewater, respectively (Table 5.4). Lipids of *Mucor rouxii* contained higher percentages of GLA (5.2% and 5.7%, respectively) under the raw wastewater and the synthetic wastewater conditions. The maximum yield of GLA per g of total soluble starch consumption was 2.1 and 3.0 mg/g soluble starch consumed for *Aspergillus flavus* I16-3, and 3.0 and 3.8 mg/g soluble starch consumed for *Mucor rouxii* in the raw and synthetic wastewater, respectively (Table 5.4).

Generally, oleaginous fungi belonging to the *zygomycetes* division are known for GLA production (Conti et al., 2001; Kendrick and Ratledge, 1992). *Aspergillus* sp., which belongs to the *ascomycetes* division, has been mentioned in the literature as a potential oleaginous fungus (Subramaniam et al., 2010). Nevertheless, its ability in GLA accumulation has not been reported yet. In this study 60 and 100 mg/L of GLA was accumulated by *Aspergillus flavus* I16-3 in the raw and synthetic wastewater, respectively. To the best of our knowledge this is the first report on GLA production using *Aspergillus* sp. and from potato processing wastewater. GLA contents in lipids were low, and they were not

Table 5.4 GLA production by *Aspergillus flavus* I16-3 and *Mucor rouxii*

		Raw wastewater			Synthetic wastewater		
	Cultivation Time (h)	GLA (mg/L)	GLA/lipids (%)	GLA/TSS (mg/g)	GLA (mg/L)	GLA/lipids (%)	GLA/TSS (mg/g)
Aspergillus flavus I16-3	96	20±1.2	0.7±0.1	0.9	23±1.4	0.8±0	0.9
	144	40±1.5	1.5±0.3	1.4	62±2.3	1.7±0.2	2.4
	192	60±1.2	2.2±0.1	2.1	100±3.1	2.9±0.1	3.4
	216	60±2.1	2.2±0.2	2.1	90±2.1	2.6±0.1	2.4
Mucor rouxii	96	40±1.3	1.6±0	1.2	42±2.2	1.2±0	1.2
	144	60±0.4	1.6±0.1	1.9	73±1.3	1.7±0	2.1
	192	80±2.5	3.1±0.2	2.3	95±1.4	3.2±0	2.6
	216	100±1.2	5.2±0.3	3.0	140±1.7	5.7±0	3.8

TSS: total soluble starch consumed

greatly increased after prolonging the fermentation time (after Day 7) because there was no lipid turnover occurring for *Aspergillus flavus* I16-3.

On the other hand *Mucor rouxii* produced much higher yields of GLA, with the yields of 100 and 140 mg/L in the raw wastewater and the synthetic wastewater, respectively, confirming that lower fungi belonging to the Mucorales order are efficient GLA producers. The yields of GLA produced by *Mucor rouxii* are comparable to other strains in the literature. *Mucor rouxianus* CBS120-08 cultivated on glucose produced 80 mg GLA/L of medium (Kavadia et al., 2001) . The comparison shows that potato processing wastewater can replace glucose as a culturing medium for GLA production. The GLA percentage in lipids was low in the present study (5.2% and 5.7% in the raw and synthetic wastewater), in comparison with other Mucorales cultures, due to the high lipid yields. The GLA content in lipids was increased as the fermentation time was elongated, when the lipid turnover took place. This was due to its ability to use the accumulated lipids after exhaustion of soluble carbon sources, thereby increasing the GLA contents in lipids. This phenomenon has also been observed in other mucaraceous fungi (Kock and Ratledge, 1993).

Hence this current method provides an alternative way for low cost lipids and GLA production. The yields of lipids and GLA can be significantly improved by optimizing nutritional and growth parameters for both the fungi in future studies.

5.3.3 Nutrient removal from wastewater

The two fungi significantly reduced COD and nutrients from the potato processing wastewater, which would be beneficial for environmental protection. *Aspergillus flavus I16-3* reduced the soluble COD concentration in the raw wastewater from 40 to 14 g/L as a result of 7 days' fermentation. Addition of extra nutrients into the raw wastewater did not enhance COD removal with the final soluble COD of 16 g/L. *M.rouxii* was able to reduce COD to 12 g/L from the raw wastewater and its efficiency was increased when treating the synthetic wastewater, with the final soluble COD concentration of 4 g/L (Fig. 5.3).

Aspergillus flavus I16-3 reduced TSN by 98% from the raw wastewater, and removed it completely when treating the synthetic wastewater. The TSN removal efficiency of *Mucor rouxii* was 95% from the raw wastewater and 98% from the synthetic wastewater (Fig. 5.4). The TSP removal efficiency achieved by *Aspergillus flavus* I16-3 was 84% and 92% from the raw wastewater and the synthetic wastewater, respectively. *Mucor rouxii* removed 85% phosphorus from raw wastewater and 81% from the synthetic wastewater (Fig.5.5).

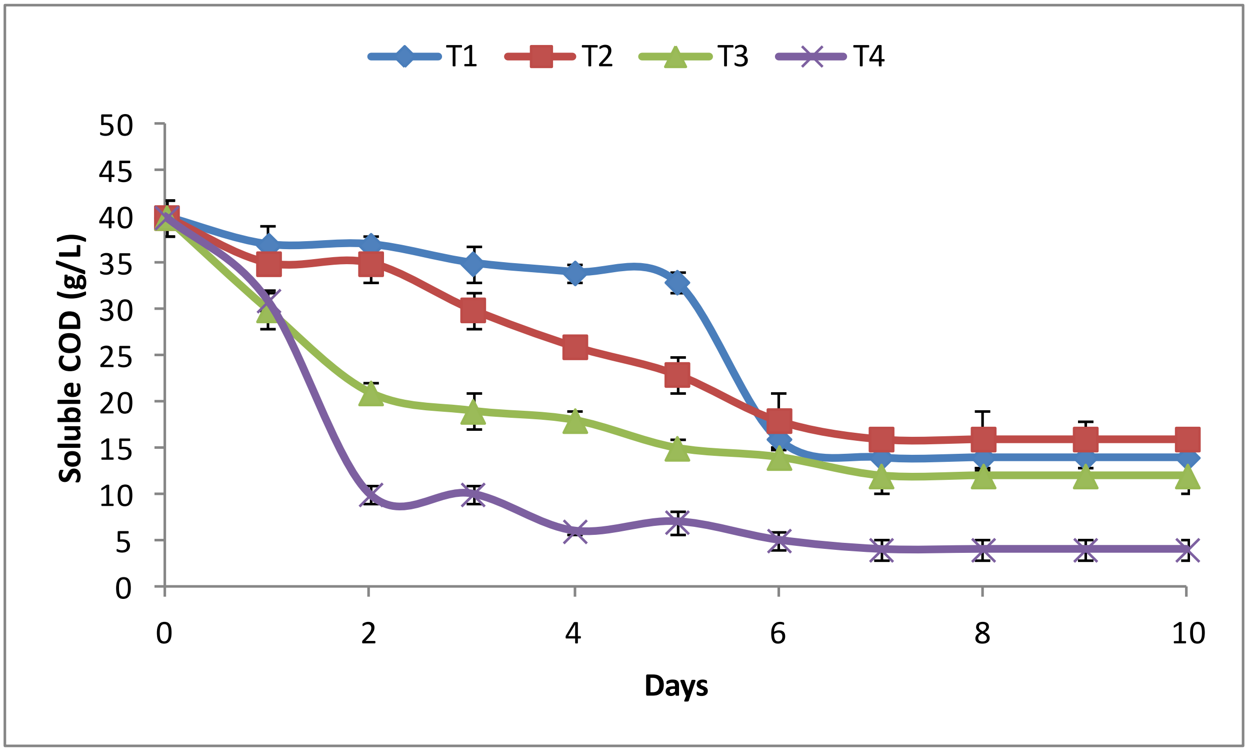


Figure 5.3 Total soluble COD reduction by *Aspergillus flavus* I16-3 (T1, T2) and *Mucor rouxii* (T3, T4) (T1 and T3: raw wastewater; T2 and T4: synthetic wastewater)

In the primary anabolic process of lipid accumulation, ammonium ions from the medium are utilized by oleaginous fungi. As a consequence, the fungi grow slowly and initiate the excess carbon source into lipids. Hence, it could be assumed that the TSN reduction in potato processing wastewater might have supported lipid production. Since the wastewater contained essential nutrients especially nitrogen and phosphorus, both oleaginous fungi didn’t require addition of any other external nutrients (except micro nutrients); this can further reduce the cost of lipid production.

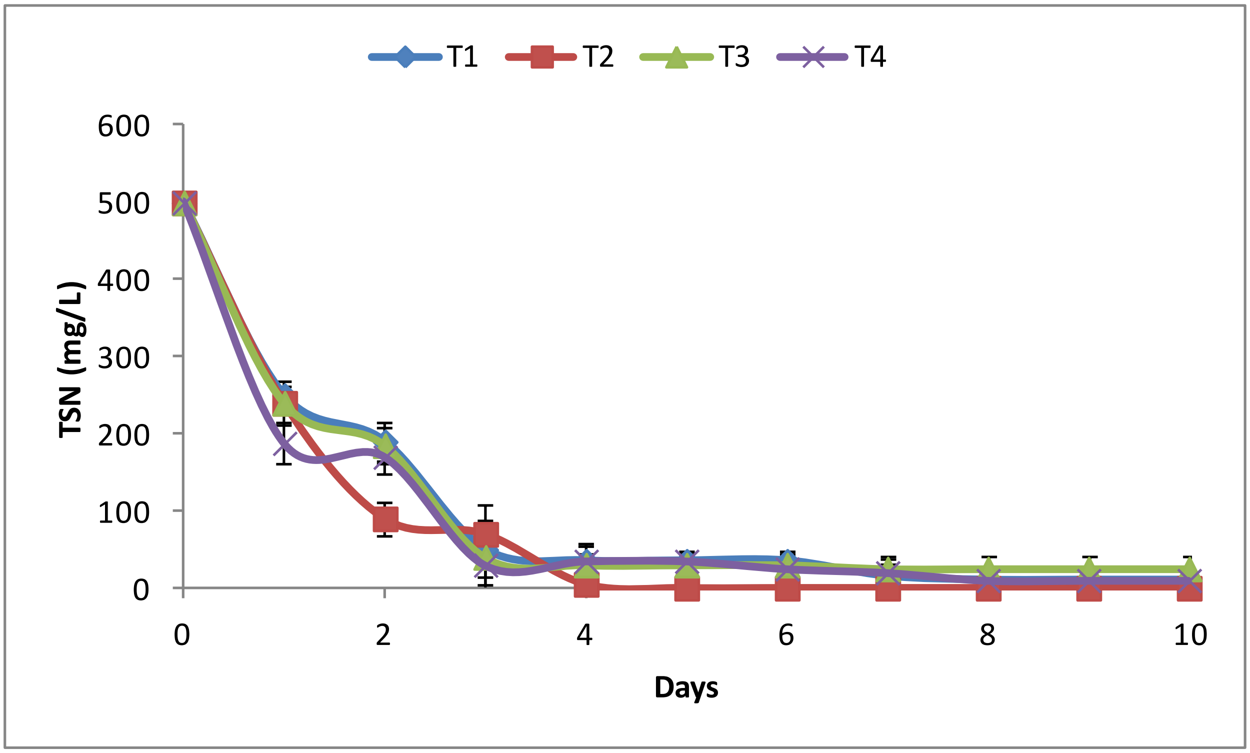


Figure 5.4 Removal of total soluble nitrogen by *Aspergillus flavus* I16-3 (T1, T2) and *Mucor rouxii* (T3, T4) (T1 and T3: raw wastewater; T2 and T4: synthetic wastewater)

Changes in pH values during the whole incubation period were monitored. For *Aspergillus flavus* I16-3, pH slightly rose in the first two days and then gradually decreased to final values of 5.3 and 4 at the end of the experiment in the raw and synthetic wastewater, respectively (Fig. 5.6). Reduction in pH during fermentation was due to secretion of organic acids such as citric acid (a fatty acid synthesis intermediate) and volatile fatty acids by the fungi (Evans and Ratledge, 1983; Papanikolaou and Aggelis, 2009; Ratledge, 2000).

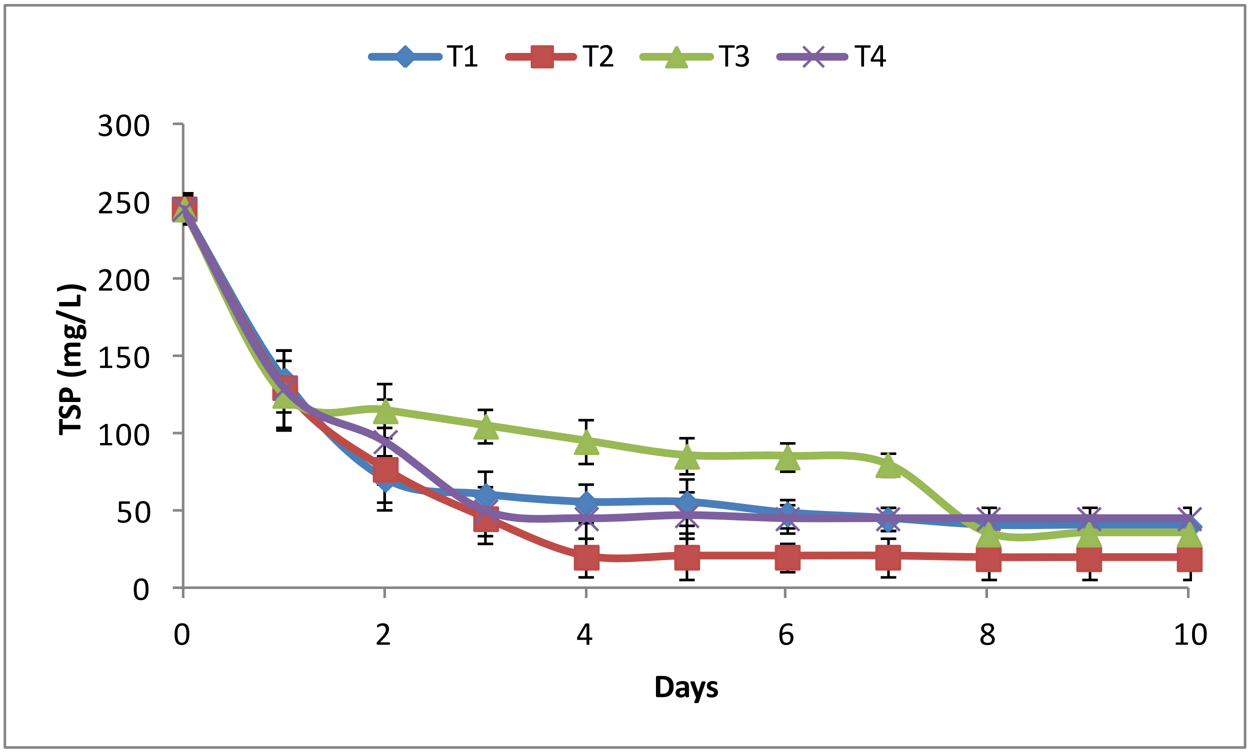


Figure 5.5 Removal of total soluble phosphorus by *Aspergillus flavus I16-3* (T1, T2) and *Mucor rouxii* (T3, T4) (T1 and T3: raw wastewater; T2 and T4: synthetic wastewater)

On the contrary, for *Mucor rouxii*, pH gradually rose to about 8 until Day 10. A similar phenomenon was reported when oleaginous fungi were grown on orange peel: the pH of the medium was stable for a few days and then rose until the end of the fermentation (Gema et al., 2002). However, the reason for pH increase has not been known and further research should be carried out.

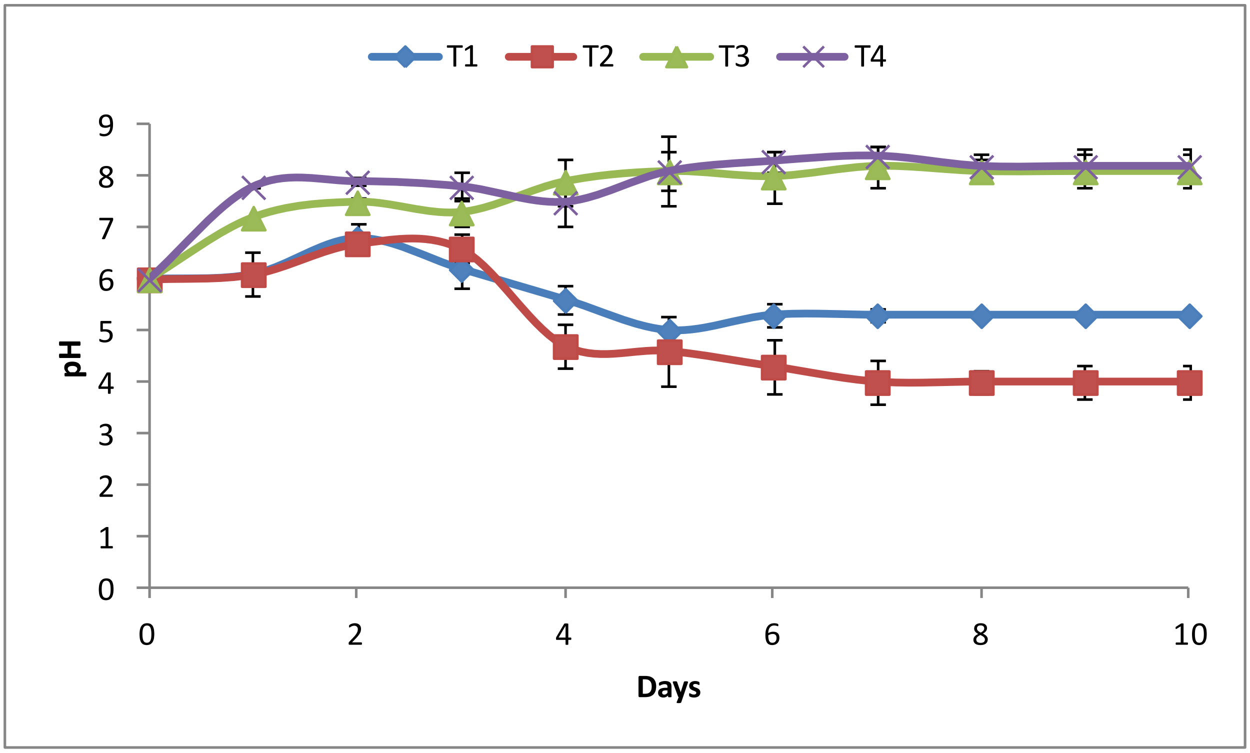


Figure 5.6. pH profiles during the experiment for *Aspergillus flavus* I16-3 (T1, T2) and *Mucor rouxii* (T3 and T4) (T1 and T3: raw wastewater; T2 and T4: synthetic wastewater)

5.4 SUMMARY

Potato processing wastewater can be used as an alternative feedstock for microbial lipids and GLA production by two oleaginous fungi (*Aspergillus flavus* I16-3 and *Mucor rouxii*). In addition, during the treatment, COD, nitrogen and phosphorus can be effectively removed. Hence, this study provides an environmentally sound method not only for potato processing wastewater treatment but also for recovering high value products.

CHAPTER SIX

CHARACTERIZATION OF MICROBIAL LIPID PRODUCTION BY *MUCOR ROUXII* WITH DIFFERENT CARBON SUBSTRATES

Chapter Six- Characterization of microbial lipid production with pure carbon substrates

6.1 INTRODUCTION

In the previous chapters, different types of oleaginous fungi including Mucoraceous (*Mucor rouxii*) and non Mucaraceous species (*Aspergillus oryzae* and *Aspergillus flavus* I16-3) were tested for their lipid accumulation capability with potato processing wastewater as the culturing medium under different conditions. In this chapter, *M. rouxii* was cultivated with three pure carbon sources (glucose, starch and cellulose) to compare its lipid production and physiological responses between potato processing wastewater and pure carbon sources.

It has been demonstrated in the literatures that, oleaginous microorganisms (yeasts and fungi) exhibit various characteristics in both lipid yields and lipids' fatty acid composition when cultivated with difference carbon substrates despite that these carbon substrates are biochemically similar (Ahmed et al., 2006; Papanikolaou et al., 2002; Papanikolaou et al., 2007). For instance, *Mortierella isabelliana* and *Cunninghamella echniulata*, two oleaginous fungi cultivated on glucose, starch, pectin and lactose based media, showed different biomass production. Glucose and starch was suitable for biomass growth of the two fungi; lactose favoured biomass production of *M.isabelliana* but did not support the growth of *C.echniulata.* Both fungi produced more lipids with glucose as the carbon substrate than with starch. Pectin was an inadequate substrate for biomass growth and lipid production for *C.echniulata*, but it supported the growth of *M.isabelliana* and lipid production.

Cellulose is the most abundant organic carbon source in the nature. However, there are very limited studies on direct fermentation of cellulose into microbial lipids by oleaginous microorganisms. Starch is another abundant carbon source. In this chapter, physiological responses of oleaginous fungi such as, biomass production, substrate uptake, secretion of hydrolytic enzymes, and lipid accumulation with cellulose or starch as the organic carbon substrate were studied. Glucose, one of the simplest sugars, was also studied as a comparison study. *M. rouxii*, a known lipid and GLA producer (Ahmed et al., 2006; Conti et al., 2001; Kavadia et al., 2001), was used in this study.

6.2 MATERIALS AND METHODS

6.2.1 Microorganism and cultural conditions

Mucaraceous fungi *Mucor rouxii* DSM1191 was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The culture was stored in the laboratory on potato dextrose agar slants at 4 ^{o}C. The lipid production medium consisted of three groups of components: i) mineral salts containing (g/L), $CaCl_2$, 0.1; KH_2PO_4, 2.5; $FeSO_4$, 0.02; NH_4Cl, 0.01; $MgSO_4$, 0.5; $MnSO_4$, 0.003; and $CuSO_4$, 0.002; ii) nitrogen source of 0.5 g/L $(NH_4)_2$ SO_4; and iii) carbon source. Three types of carbon sources were examined and they were glucose, starch and cellulose (Sigma Aldrich, Ireland): glucose and cellulose concentrations tested were 30 g/L and two starch concentrations were studied, 30 and 60 g/L.

Inoculation of the fungal culture was performed as follows: 1.0 g of mycelia were taken from potato dextrose agar plates and cultured in yeast extract malt extract agar (YM agar) broth containing 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, and 3 g/L malt extract (pH of the medium was 5.5) for 48 h in a shaker incubator at 30±1 ^{o}C at 180 rpm. After 48 h the mycelium was harvested and homogenized using sterile glass beads (0.5 mm in diameter) by vortexing for 2 min (Minivortex, Sigma, Ireland). 0.8 mL of the homogenized mycelium suspension was used as inoculum in the fermentation experiment.

All the fermentation experiments were performed in 250 mL conical flasks containing 50 mL of the lipid production medium which was sterilized at 121 ^{o}C for 20 min. pH of the medium was adjusted to 6.0±0.5 using 1 N NaOH before sterilization and then confirmed after sterilization using a pH probe (Hanna instruments, Ireland). Flasks were incubated at 30±1 ^{o}C in the shaker incubator at 180 rpm under aerobic conditions. All the trials were conducted in triplicates. Regardless of the carbon sources used, pH values of the medium did not change significantly (5.8 - 6.5) during the whole fermentation period. However, when the same fungi *M. rouxii* was cultivated on potato processing wastewater, pH varied and rose from 6 at the beginning to 8 at end of the fermentation (Section 5.3.2, Chapter 5). This clearly shows that different culturing media would significantly affect the physiological responses of fungi.

6.2.2 Analytical methods

Flasks were removed from the shaker incubator at designed time intervals and subjected to analysis. The detailed procedures for biomass analysis and lipid extraction are given in Section 3.2.4.4 and 3.2.4.5, respectively. Profiles of long-chain fatty acids in microbial lipids were analyzed after direct transesterfication and then FAMEs were analysed using gas chromatography; the detailed procedure is given in Section 4.2.4. Residual glucose was measured by the DNS method (Miller, 1959) (Section 3.2.4.1). Starch was measured by the phenol sulphuric acid method (Section 3.2.4.2). Cellulose was measured according to the method adopted by Updegraff (1969). Briefly, 2 mL of properly diluted supernatant (the procedure for obtaining supernatant is described in Section 3.2.4.1), which should contain approximately 100 µg of cellulose, was added in a 15 mL glass tube, 10 mL of 67% sulphuric acid was added and the tube was left undisturbed for 1 hr. After 1 hr, 1 mL solution was taken from the glass tube, added to a 250 mL conical flask, and diluted to 100 mL with distilled water. One mL of this diluted solution was transferred to a new 15 mL tube and 10 mL of anthrone reagent was added. After stirred, the tube was then heated in a water bath (90 ^{o}C) for 10 min. After cool down at the ambient temperature, the color intensity at 630 nm was measured using a spectrometer (Hach Lange, Ireland). Distilled water added with anthrone reagent was used as the blank for the spectrometry measurement with the procedure mentioned above. Cellulose with known mass (40 - 200 µg) was used to obtain the calibration curve for quantification of cellulose in the samples by repeating the procedure mentioned above. α – amylase activity was measured using the method adopted by Bernfeld (1955) (Section 3.2.4.3). Cellulase activity was measured using the protocol described by Denison and Koehn (1977): briefly, whatman No.1 filter paper was cut (7 mm diameter) using a paper punch and added into a 15 mL glass tube. Then, 0.5 mL of properly diluted sample supernatant was added to the tube. The mixture was placed in a water bath at 50 ^{o}C for 1 hr. Immediately after removing the mixture from the water bath, 0.5 mL of DNS reagent was added and the tube was heated again in a water bath at 90 ^{o}C for 5 min to terminate the enzyme activity. While the tube was warm 1 mL of Rochelle salt solution was added. After cooling to ambient temperature, the aqueous volume in the tube was made up to 5 mL by adding distilled water. The absorbance of the mixture was measured at 540 nm using the spectrometer. The calibration curve was prepared with pure glucose with mass in the range of 50 µg - 1000 µg.

One unit of enzyme activity (IU/mL) was expressed as mg of glucose released per min per mg of cellulose. Released glucose was measured by the DNS method (3.2.4.3).

6.3 RESULTS AND DISCUSSION

6.3.1 Biomass growth, carbon source consumption and lipid production

The results of biomass growth and lipid production of *M. rouxii* on different carbon sources show that a noticeable biomass yield (X) was obtained (Table 6.1). Glucose supported biomass growth of *M.rouxii*, and almost complete consumption of glucose was observed leaving only 0.23 g/L of glucose in the medium within 5 days of cultivation (Fig. 6.1). It is obvious that most of oleaginous fungi can utilize glucose more rapidly than starch and cellulose. Starch seemed to be the best for supporting biomass production among the carbon sources tested, producing a higher biomass yield than glucose (15.5 g/L against 13.2 g/L). Other researchers have found that starch is less efficient for biomass production for *Mucor* sp. (Ahmed et al., 2006; Hansson and Dostálek, 1988), which is opposite to our research results. Papanikolaou et al. (2007) observed similar results of increased biomass growth for cultures *C.echinulata* and *M.alpina* grown on starch over glucose. Uptake of starch was found to be significant when the initial starch concentration (C_i) was 30 g/L, and most of the soluble starch was utilized within 7 days of fermentation (Fig. 6.1), leaving only a small amount of starch in the medium (Table 6.1). Although more glucose was consumed than starch was, the biomass yield with respect to the consumption of the carbon source ($Y_{X/C}$) for glucose was less than for starch (Table 6.1). Since most of the starch was consumed when C_i was 30 g/L, in order to study the effect of increased starch concentrations on lipid accumulation (without altering the nitrogen concentration), C_i of 60 g/L was examined. In this case, almost 30% of starch was not consumed when the fermentation experiment was ended.

Table 6.1 Growth and lipid production of *M.rouxii* on different carbon sources

Carbon source	C_i (g/L)	Time (hr)	X (g/L)	L_{max} (g/L)	C_f (g/L)	$Y_{X/C}$ (g/L)	Lipid content (%,wt/wt)	$Y_{L/C}$ (g/g)
Glucose	30	144	12.3±0.91	4.9±0.4	0.23±0.01	0.44	39.8	0.16
Starch	30	144	14.4.±1.2	3.9±0.2	1.8±0.2	0.51	27.1	0.14
	60	144	13.4±1.1	5.8±0.5	20±0.98	0.34	43.3	0.15
Cellulose	30	350	7.4±0.3	0.05±0.001	20.5±1.2	0.78	0.7	0.01

1. Calculation of parameters such as $Y_{X/C,}$ lipid content, $Y_{L/C}$ are given in Section 3.2.4.6, Chapter 3

2. Ci: initial substrate concentration; C_f: substrate concentration at time t.

On the other hand, biomass production was not affected by the increase in the initial starch concentration; very slight reduction in biomass was observed when starch was increased from 30 g/L to 60 g/L (14.4 against 15.5 g/L). When the initial starch concentration was increased to 60 g/L, $Y_{X/C}$ values were also lower than those when the carbon substrates were glucose or 30 g/L starch (Table 6.1).

Cellulose supported the biomass growth of *M.rouxii*. The biomass yield was up to 7.4 g/L at a cellulose concentration of 30 g/L. Two thirds of cellulose was not consumed even though the fermentation time was extended to 350 hr. $Y_{X/C}$ values were much higher for cellulose than glucose and starch (Table 6.1). This is the first report in biomass growth of *Mucor rouxii* on cellulose, since lignocellulosic raw materials are abundant and cheap in the nature.

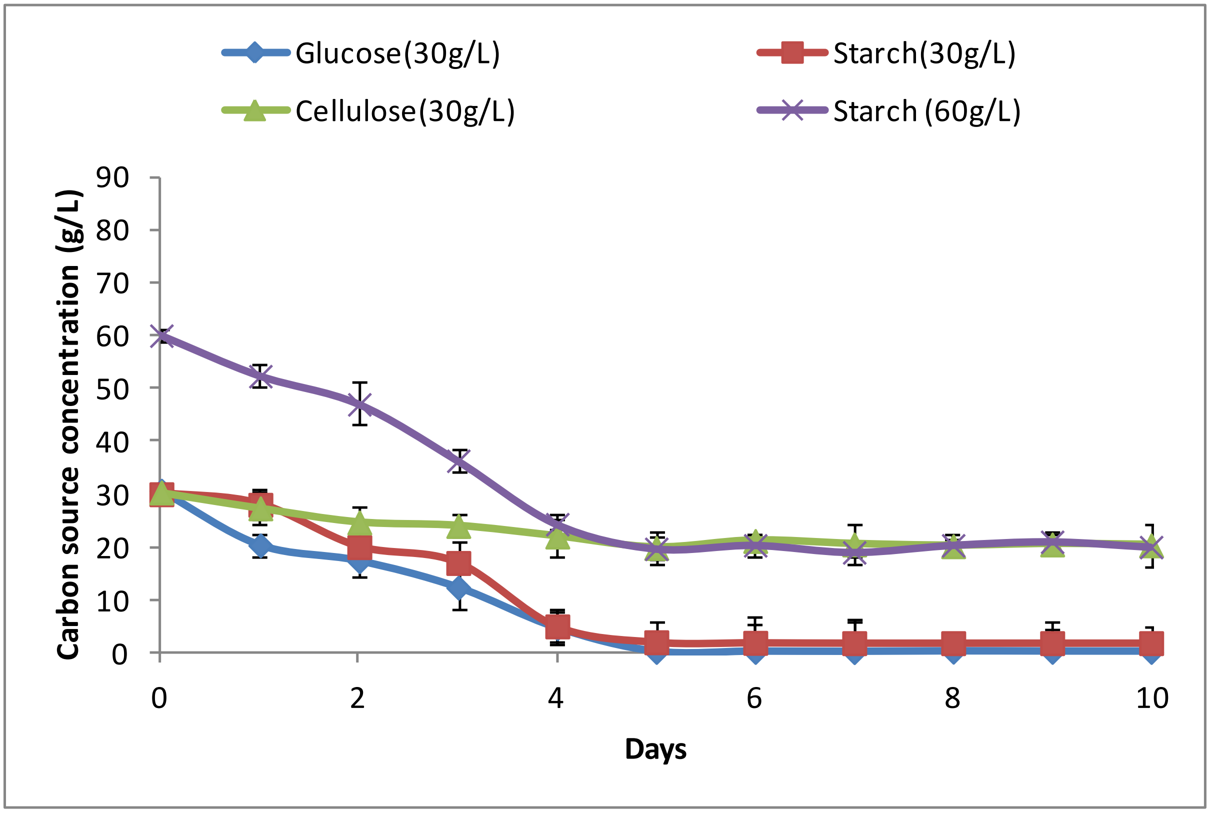


Figure 6.1 Utilization of different carbon sources by *M.rouxii*

Lipid accumulation commenced after complete exhaustion of ammonium ions in the medium. Regardless of the carbon sources used complete exhaustion of ammonium nitrogen was observed at 68±5hr and the depletion pattern was similar for all carbon sources (Fig. 6.2). The maximum lipid yield when glucose was the carbon substrate, 4.9 g/L, was higher than when starch was the carbon substrate, 3.9 g/L. The lipid contents in the dry biomass, $Y_{L/X,}$ were 39.8% and 27.1% when the carbon substrates were 30 g/L glucose and starch, respectively. Glucose, being a simple sugar, has supported the maximum lipid yield for most oleaginous microbes (Gema et al., 2002; Gill et al., 1977; Kavadia et al., 2001). However, higher L_{max} values for starch than for glucose were also observed when *C. echinulata* CCRS 3180 and *C. echinulata* ATHUM 4411 were grown on starch (Chen and Chang, 1996; Chen and Liu, 1997; Papanikolaou et al., 2007).

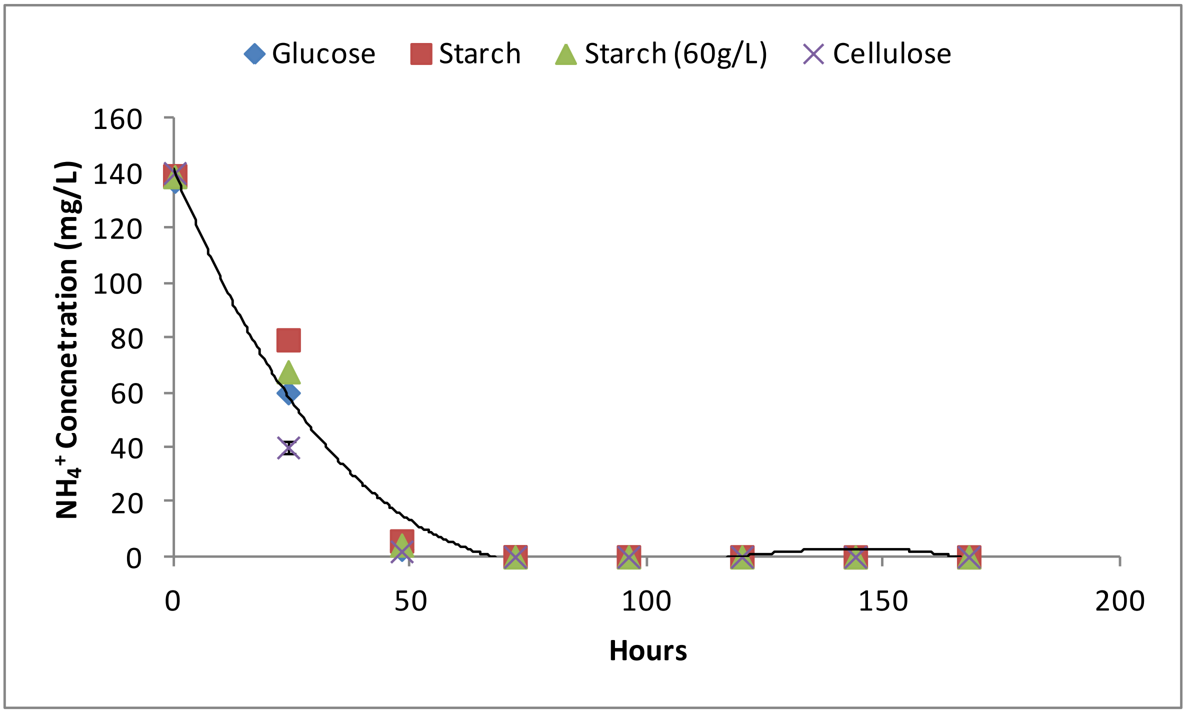


Figure 6.2 Depletion of ammonium nitrogen concentrations in different carbon sources

Papanikolaou et al. (2007) observed *C.echinulata* had higher $Y_{L/X}$ values (28%) than *M.rouxii* (27.1%). When the concentration of starch was 60 g/L the maximum lipid yield, L_{max}, was increased to 5.8 g/L (Table 6.1).

The research results show that cellulose did not support microbial lipid production. Although the biomass yield was up to 7.4 g/L, the lipid yield and the lipid content in biomass were much lower than for glucose and starch (Table 6.1).

The lipid yields with respect to carbon substrate consumption, $Y_{L/C}$, were almost similar for glucose and starch (both concentrations) as the carbon substrates (Table 6.1), but the value was very low for cellulose (0.01 g lipids/g cellulose consumed) than for other sources. Although the fungi produced a considerable amount of cellulase, it seemed that the reducing sugar produced was used for biomass production. Another reason could be the errors in biomass measurement caused by the unconsumed cellulose. The reason for the poor lipid yield on cellulose could be feedback inhibition by the substrate. Further studies should be conducted to optimize the cellulose concentration for obtaining high lipid yields.

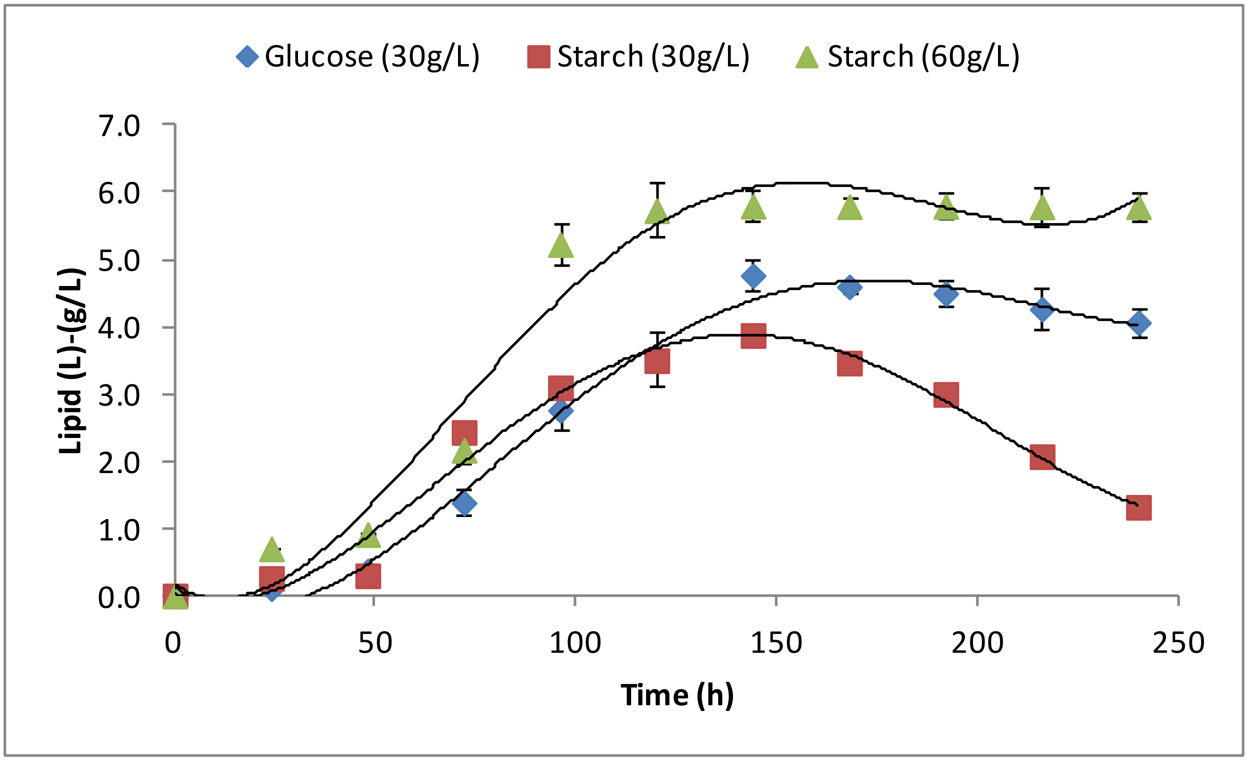


Figure 6.3 Lipid productions by *M.rouxii* with different carbon sources

In the experiment with 30 g/L glucose as the carbon source because a low amount of glucose was left in the medium, lipid turnover occurred. When the carbon source was 30 g/L starch, although starch was not utilized completely lipid turnover was observed. When 30 g/L cellulose and 60 g/L starch were used as the carbon substrates, lipid turnover did not take place probably due to the presence of excess organic carbon substrates in the medium (Fig. 6.3).

6.3.2 Substrate uptake rate and hydrolytic activity of *M.rouxii*

Specific uptake rates for different carbon sources by *M.rouxii* were calculated using Eq. 5.2 in which TSS was replaced with respective carbon substrate, and the results are presented in Table 6.2. At the initial carbon substrate concentration of 30 g/L, the specific substrate uptake rate of glucose was higher than those of starch and cellulose. In the first 24 hr, the specific substrate uptake rate for glucose was much higher and the rate was reduced after 48 hrs (Table 6.2) and the complete exhaustion of glucose was found within 5 days of fermentation (Fig. 6.1). This rapid uptake rate within 24 h indicates that glucose was channeled into cells for lipid synthesis. Specific uptake rates for starch at both levels and for cellulose were lower than that of glucose in the first 24 hrs. This suggests that complex carbon sources cannot be uptaken directly as glucose

Table 6.2 Specific substrate uptake rates of *M.rouxii* S_u (g substrate/g microorganism.h)

Carbon source	Fermentation time	Average substrate uptake rate S_u (g/g.h)
Glucose (30 g/L)	24	0.341
	48	0.109
	72	0.065
Starch (30 g/L)	24	0.076
	48	0.024
	72	0.048
Starch (60 g/L)	24	0.063
	48	0.082
	72	0.046
Cellulose (30 g/L)	24	0.053
	48	0.006
	72	0.01

by *M.rouxii*. It is reported that the uptake of complex carbon sources by oleaginous mucorales is greatly influenced by the secretion of hydrolytic enzymes (Papanikolaou et al., 2010; Papanikolaou et al., 2007). Enzyme secretion was observed in the experiment when cultivated on starch and cellulose (Fig. 6.4). When the carbon substrate was 30 g/L starch, amylase secretion started from the 1st day of fermentation with 0.3 IU/mL, the maximum amylase activity was 0.5 IU/mL on the 5th day and thereafter declined. A similar pattern of amylase secretion was observed with the maximum activity of 1.2 IU/mL when the starch concentration was 60 g/L (Fig. 6.4). The obtained amylase values (0.5 and 1.2 IU/mL) in this study were high, in comparison with that of the fungal culture *M. isabelliana ATHUM 2935*: when it was grown on starch, the maximum amylase secreted was 0.12 IU/mL within 24 hr of fermentation (Papanikolaou et al., 2007). This suggests that *M.rouxii* is better than *M. isabelliana ATHUM 2935* for starch hydrolysis. In contrast, the production of cellulase was different from amylase. The quantity of cellulase secretion was very low during the first day, increased from the 2nd day and reached the maximum activity on the 4th day (0.19 IU/mL).

The concentrations of reducing sugars in the medium containing 30 g/L starch were negligible (the maximum concentration was 0.12 g/L of glucose equivalents) after the 6th day, suggesting that most of the reducing sugars released were being utilized simultaneously. It is also worth mentioning the fact that there was no secretion of amylase or cellulase measured when glucose was the carbon source. Hence it is revealed that enzyme secretion of *M.rouxii* is substrate specific.

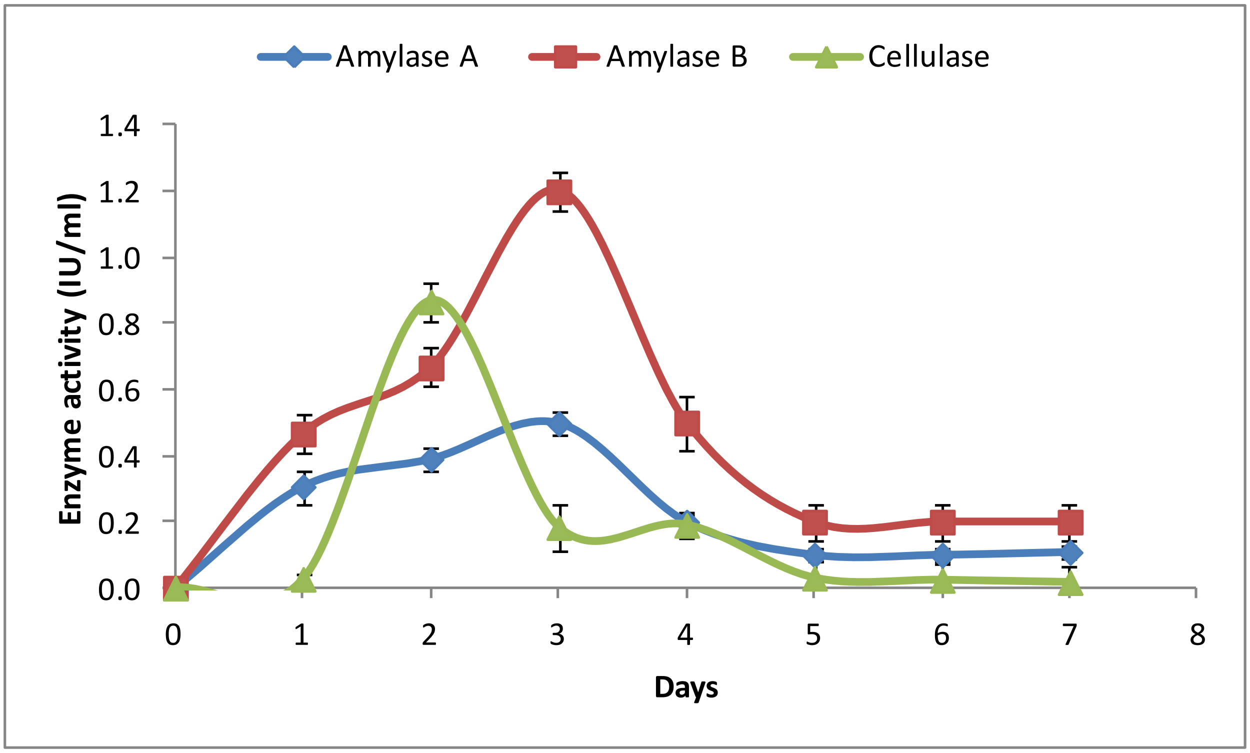


Figure 6.4. Secretion of amylase and cellulase by *M.rouxii* under different carbon sources (Amylase A and B indicate amylase secretion when starch was 30 g/L and 60 g/L, respectively).

6.3.3 Composition of microbial lipids produced under different carbon sources

The composition of long-chain fatty acids in microbial lipids is given in Table 6.3. With the three carbon sources tested, oleic acid (C18:1) was the predominant long-chain fatty acid present in microbial lipids. This phenomenon was also observed by other researchers (Ahmed et al., 2006; Papanikolaou et al., 2010; Somashekar et al., 2003): for oleaginous *Zygomycetes* cultured with renewable carbon sources oleic acid is the predominant long-chain fatty acid contained in microbial lipids. This may be due to an increased Δ^9 dehydrogenase activity in the oleaginous microorganims. Then palmitic acid (C16:0) shared second most part of the lipid produced by *M.rouxii*, followed by stearic acid (C18:0), linolenic acid and GLA. Higher contents of GLA were found in the microbial lipids with starch and cellulose as the carbon substrates than with glucose as the carbon source. However for the given glucose concentration contents of GLA were higher than other mucaraceous cultures. Starch had higher GLA than glucose and the concentration was 11.2 % when the concentration of starch was 60 g/L. Similar results of GLA content was found in *Mucor* sp. RRL001 when they were cultivated with tapioca starch (Ahmed et al., 2006). The comparison of GLA contents in microbial lipids of *Mucor rouxii* and *A. oryzae* which is non mucaraceous (Chapter 4) shows that much higher percentages of GLA was produced by mucaraceous fungi (*Mucor rouxii*) (Table 6.3) than non mucaraceous species, suggesting that mucaraceous fungi are best GLA producers.

Table 6.3 Lipid composition of *M.rouxii* (wt %) grown on different carbon sources under nitrogen limited conditions. Given that the lipid content was negligible the fatty acid composition was not given.

Carbon source	Fermentation time (hours)	PA (C16:0)	SA (C18:0)	OA (C18:1)	LA (C18:2)	GLA (C18:3)	ARA (C20:0)	BA (C22:0)
Glucose (30 g/L)	240	18.8	7.7	47.2	9.11	7.2	3.8	3.5
Starch (30 g/L)	238	20.6	6.2	46.3	8.3	9.2	3.9	2.5
Starch (60 g/L)	240	20.0	4.0	50.0	9.4	11.2	1.7	2.1

6.4. SUMMARY

In this chapter biochemical behaviors of *M.rouxii* were studied with three carbon substrates – glucose, starch and cellulose. All the carbon sources supported biomass growth; particularly, starch had a higher biomass growth than others. Interestingly cellulose supported good biomass growth suggesting further research should be conducted on this carbon source. Glucose had a higher lipids yield than starch did; cellulose did not support lipid production for *M.rouxii.* A high starch concentration supported a high GLA content in lipids; the maximum GLA content of 11.2% was found with 60 g/L of starch as the carbon substrate.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 OVERVIEW

In this research microbial oil production by oleaginous fungi using low cost raw materials was studied. The following research work has been conducted: (i) isolation and identification of local oleaginous fungi; (ii) potential of potato processing wastewater for low cost lipid production using oleaginous fungi, in addition with nutrient removal from wastewater; and (iii) comparison of biochemical behaviors of oleaginous fungi on pure carbon sources including glucose, cellulose and starch.

7.2 CONCLUSIONS

7.2.1 Isolation of local oleaginous fungi from Irish soils

In this study, indigenous oleaginous fungi were isolated from Irish soils and identified using molecular techniques. The following results were obtained:

1. Fifty undisturbed soil samples were collected from various locations in western Ireland and used for isolation. A total of 247 fungi cultures were isolated, and fifty isolates were screened for growth, lipid yields, and amylase secretion.
2. When cultivated with glucose as the carbon substrate, 30 cultures were able to convert glucose into lipids. The lipid contents were in the range of 20% - 44%. The maximum lipid content in biomass was observed in the isolate I16-3 (44.3%).
3. 19 out of 50 isolates were oleaginous and amylase positive when cultivated with the medium containing 3% starch as the carbon substrate. The lipid content in biomass ranged 20.9% - 39.0%. The isolate I16-3 yielded the maximum lipid content in biomass (39.0%).

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4. Sequencing of 18s rDNA of the 30 oleaginous isolates shows that they belonged to seven genus: *Trichoderma* sp., *Penicillum* sp., *Aspergillus* sp., *zygomycetes* sp., *Mortierella* sp., *Acremonium* sp. and *Umbliopsis* sp. Among the seven genus 13 isolates showed 99% homology with already reported sequences of reference strains identified by BLAST analysis and the sequences were submitted to the NCBI gene bank.

7.2.2 Microbial lipid production from potato processing wastewater by *Aspergillus oryzae*

Microbial lipid production from potato processing wastewater by oleaginous filamentous fungus *Aspergillus oryzae* in aseptic conditions was studied. The results from this study are as follows:

1. Sterilization of the potato processing wastewater resulted in a thick gelatinized starch medium, causing the fungi to grow slow. In order to overcome this problem, raw wastewater was diluted with tap water at three dilution ratios (25%, 50% and 75%) and used in the fermentation experiment.
2. Dilution of wastewater enhanced lipid production, starch utilization and amylase secretion. The dilution ratio of 25% was optimum for lipid production (3.5 g/L) and starch utilization (99.9%). The amylase activity was up to 53.5 IU/mL at 25% dilution.
3. Phosphate limitation may be the mechanism to stimulate lipid accumulation.
4. Removals of COD, TSN, TSP, ammonium nitrogen, orthophosphate and sulphate were up to 91%, 98%, 97%, 100%, 100% and 30% at the 25% dilution ratio, respectively.
5. Lipids contained palmitic (12.6%), palmitolic (4.3%), stearic (14.5%), oleic (22.5%), linolenic (5.5%) and linoleic acids (6.5%). The lipids could be used as a biodiesel production feedstock.

7.2.3 Microbial lipid and GLA production from potato processing wastewater by *Aspergillus flavus* I16-3 and *Mucor rouxii*

This study examined addition of nutrients to potato processing wastewater for lipids and GLA production by two oleaginous fungi, *Aspergillus flavus* I16-3 and *Mucor rouxii*. Conclusions drawn are as follows,

1. Without addition of any external nutrients, 2.8 and 3.6 g/L of lipids were produced by *Aspergillus flavus* I16-3 and *Mucor rouxii*, respectively, with corresponding maximum GLA yields of 60 and 100 mg/L.
2. Addition of nutrients to raw wastewater enhanced lipids and GLA yields; 3.5 and 4.2 g/L of lipids, and 100 and 140 mg/L of GLA were produced by *Aspergillus flavus* I16-3 and *Mucor rouxii*, respectively.

3. The wastewater was efficiently treated, with total soluble COD, TSN and TSP removals up to 60% and 90%, 100% and 98%, and 92% and 81% by *Aspergillus flavus* I16-3 and *Mucor rouxii*, respectively.

7.2.4 Biochemical behaviors of oleaginous fungi cultivated with pure carbon sources

Mucor rouxii was cultivated in the medium containing glucose, starch and cellulose with an initial C/N ratio of 60. The biochemical behaviors of *Mucor rouxii* were examined:

1. The highest lipid yield (4.9 g/L) was found with glucose as the carbon substrate. Starch was good for biomass production (15.5 g biomass/L medium). The Lipid content in biomass with starch as the carbon substrate was less than glucose (25%). The maximum lipid yield was increased (up to 5.8 g/L) with increasing the starch concentration to 60 g/L.
2. Cellulose did not support lipid production.
3. Significant quantities of α-amylase (0.5 and 1.2 IU/mL) and cellulase (0.19 IU/mL) were produced.

7.2.5 Summary

Overall, in this research production of microbial lipids by oleaginous fungi, isolated indigenous species and known species, was examined with potation processing wastewater and pure organic matter as cultivation substrates. The difference in microbial lipid production, nutrient removal and biochemical behaviors among different species and among various cultivation substrates was compared. The maximum lipid yield of *M.rouxii* with pure starch as the carbon source was 3.9 g/L (Chapter 6). When potato processing wastewater was used as the raw material, the culture *Aspergillus oryzae* accumulated a maximum lipid yield of 3.5 g/L. Another isolated culture *Aspergillus flavus I16-3* produced 3.5 g/L and 4.3 g/L of lipids when cultivated on potato processing wastewater (Chapter 5) and pure starch (Chapter 3), respectively. This suggests that, there are non mucaraceous oleaginous fungi able to accumulate a considerable amount of lipids when grown on renewable carbon sources such as starch and potato processing wastewater.

It is reported that the optimum lipid yield with respect to starch consumed is 0.15 g lipids/g starch consumed (Papanikolau, et al. 2007). In our present study the lipid yield of 0.1 g/g soluble starch (raw potato processing wastewater) and 0.12 g/g soluble starch (with addition of nutrients into potato processing wastewater) by *Aspergillus flavusI16-3,* 0.15 g/g soluble starch (raw potato processing wastewater), 0.11 g/g soluble starch (with addition of nutrients) and 0.15 g/g starch (pure starch) by *M.rouxii*, and 0.16 g/g soluble starch (potato processing wastewater) by *Aspergillus oryzae* was achieved. These results show that potato processing wastewater can be used a raw material. It also indicates that non mucaraceous oleaginous fungus *Aspergillus* sp. has a high potential for microbial lipid accumulation. It is worth using these fungi for lipid production with renewable carbon substrates in future. The lipid production potential of other species isolated and identified - *Trichoderma* sp, *Penicillum* sp., *Acremonium* sp. and *Umbliopsis* sp. has not been examined in this study. These fungi can secrete amylase to hydrolyze starch or starchy wastes. These four new genuses can be an addition to the existing oleaginous cultures for lipid production.

It is found that a maximum of 4.2 g of lipids can be produced from 1 liter of potato processing wastewater. This 4.3 g of lipids could approximately yield 4 mL of biodiesel via transesterfication. In the Glynn Fruit and Vegetables Ltd, Galway, 2 m^3 wastewater is generated per day when processing 1 ton (1000 kg) of potato. By using the research results obtained from this PhD research, 8 L of biodiesel can be produced from the wastewater generated produced per day. Currently, the company is spending around 5000 euros per month to dispose the potato processing wastewater. If the proposed technology would be adopted the company can produce biodiesel, which can offset the wastewater processing cost. In addition, nutrient concentrations in the wastewater would be greatly reduced by using this technology; this could reduce the wastewater treatment cost.

7.3 RECOMMENDATIONS FOR FUTURE RESEARCH

1. The lipid yields when using potato processing wastewater as the raw material should be optimized after examining various operational conditions.
2. The fed-batch and continuous fermentation of potato processing wastewater using laboratory-scale and pilot-scale fermentors was not studied in this PhD research and should be carried out in future.

3. Biodiesel properties should be tested in detail using standard methods after produced microbial lipid is used to synthesize biodiesel.

4. Biochemical behaviors of *Aspergillus oryzae I16-3*, the indigenous oleaginous fungi, should be carried out in future.

5. Detailed cost-benefit analysis using potato processing wastewater as the raw material for microbial lipids production should be carried out.

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APPENDIX A

Published gene sequences

Appendix A

GENE BANK ENTRIES

Iniya kumar,M., Xiao, L. and Zhan, X. 2011. *Aspergillus* sp. I16-3 18s rDNA partial sequence. NCBI- Acc.No: **JF895924**.

Iniya kumar,M., Xiao, L. and Zhan,X. 2011. *Trichoderma* sp.I1-1 18s 18s rDNA partial sequence. NCBI- Acc.No: **JF895925**.

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. *Penicillium* sp. I1A1 18s rDNA partial sequence.NCBI- Acc.No: **JF895926**.

Iniya kumar,M., Xiao, L. and Zhan,X. 2011. *Umbelopsis* sp. I1A5 18s rDNA partial sequence.NCBI- Acc.No: **JF895927**.

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. *Penicillium* sp. I5-5 18s rDNA partial sequence.NCBI- Acc.No: **JF895928**.

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. *Mortierella* sp. I5-10 18s rDNA partial sequence.NCBI- Acc.No: **JF895929**.

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. *Penicillium* sp. I8-1 18s rDNA partial sequence.NCBI- Acc.No: **JF895930**.

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. *Trichoderma* sp. I1-3 18s rDNA partial sequence.NCBI- Acc.No: . **JF912414**

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. Zygomycete sp. I2A3 18s rDNA partial sequence.NCBI- Acc.No: . **JF912415**

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. *Penicillium* sp. I14-4 18s rDNA partial sequence.NCBI- Acc.No: . **JF912416**

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. Acremonium sp. I18-1 18s rDNA partial sequence.NCBI- Acc.No: .**JF912417**

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. Aspergillus sp. I17-1 18s rDNA partial sequence.NCBI- Acc.No: . **JF912418**

Iniya kumar,M., Xiao,L. and Zhan,X. 2011. Penicillium sp. I19-2 18s rDNA partial sequence.NCBI- Acc.No: **. JF912419**

Gene bank entries published in NCBI website results associated with chapter 3

APPENDIX B

Laboratory study data

Appendix B

Depletion of nutrients in raw wastewater

Days	NH4	PO4
0	142.50±0.00	73.50±2.12
1	137.79±3.54	52.00±1.41
2	129.50±1.48	21.17±1.65
3	7.61±0.75	0.01±0.83
4	1.81±0.35	0.49±0.13
5	1.27±0.01	0.49±0.13
6	1.52±0.01	0.20±0.00
7	1.80±0.01	0.15±0.07

Depletion of nutrients in 25% dilution

Days	NH4	PO4
0	95.00±0.71	50.00±0.00
1	92.19±11.41	39.46±8.73
2	87.00±5.28	18.74±5.32
3	6.08±0.35	0.05±0.07
4	2.54±0.19	0.85±0.49
5	2.51±0.00	0.27±0.09
6	3.01±0.00	0.25±0.07
7	3.53±0.00	0.25±0.07

Depletion of nutrients in 50% dilution

Days	NH4	PO4
0	40.00±4.41	24.50±0.71
1	35.42±3.41	13.84±2.60
2	16.25±3.19	5.32±4.02
3	6.84±1.46	0.00±0.07
4	2.18±1.15	0.00±4.14
5	1.89±1.89	0.00±0.14
6	2.27±2.60	0.00±0.14
7	2.66±2.45	0.00±0.14

Depletion of nutrients in 75% dilution

Appendix B

Lipid production by *A.oryzae* under different dilutions of potato processing wastewater

Days	100%	25%	50%	75%
0	0	0	0	0
1	0.03±3.1	0.03±0.0	0.065±0.00	0.15±0.07
2	0.2±0.1	0.305±0.0	0.27±0.01	0.275±0.04
3	0.9±0.1	0.95±0.1	0.85±0.01	0.48±0.03
4	1.95±6.1	2.7±0.1	1.35±0.07	0.89±0.01
5	2.5±0.1	3.45±0.1	1.493±0.07	1.195±0.01
6	2.96±0.1	3.325±0.0	1.46±0.01	1.145±0.06
7	2.95±0.1	2.91±0.0	1.31±0.03	0.975±0.01
8	2.93±0.1	2.8±0.0	1.25±0.01	0.86±0.01
9	2.88±0.0	2.74±0.0	1.22±0.01	0.835±0.01
10	2.87±0.0	2.715±0.0	1.145±0.01	0.83±0.00

Starch utilization *by A.oryzae* under different dilutions of potato processing wastewate

Days	100%PWW	25%PWW	50%PWW	75%PWW
0	0	0	0	0
1	5.36±2.70	51.96±2.13	34.33±1.04	56.96±2.28
2	10.50±0.73	72.96±0.39	71.88±1.09	71.03±1.05
3	23.76±0.71	90.03±1.12	84.04±3.34	83.25±4.03
4	33.54±2.24	93.86±2.01	89.02±1.13	90.05±0.18
5	46.23±1.22	97.57±4.07	91.12±0.15	92.89±0.10
6	67.11±0.34	99.80±2.19	91.77±0.12	93.82±3.07
7	70.21±0.21	99.84±0.02	93.15±0.10	95.02±0.02
8	70.71±2.09	99.95±0.02	93.04±0.07	96.73±0.03
9	71.13±0.01	99.95±0.01	95.66±0.05	96.81±0.01
10	71.32±0.00	99.95±0.00	96.03±0.56	99.52±0.02

Secretion of amylase under different dilution of potato processing wastewater

Time(hr)	100%	25%	50%	75%
0	0±0.00	0±0.00	0±0.00	0±0.00
24	24.5±2.12	37±1.41	28±1.41	26.5±2.12
48	31±1.41	53.5±3.54	39.5±2.12	13±1.41
72	7.5±3.54	34±1.41	17±2.83	7±1.41
96	4.5±2.12	17.5±3.54	6.5±2.12	2.5±0.71
120	0±0	8.5±0.71	2.5±0.71	0±0.0

Appendix B

Lipid production and total soluble carbohydrate utilization by *A.oryzae* in potato processing wastewater

Days	T1 Lipid (% w/w)	T2 Lipid (% w/w)	T1 Total Carbohydrate (g/L)	T2 Total Carbohydrate (g/L)
0	0±0	0±0	37±1.2	37±2.2
1	0.146±0.1	0.15±0	36.33±2.3	28.15±5.3
2	0.198±0	0.21±0.1	29.87±3.2	20.43±4.4
3	1.78±0.3	2.2±0.6	25.95±2	20.13±3.2
4	2.52±0.4	2.78±0.4	13.57±2.4	11.06±4.2
5	2.78±0.3	3.3±0.5	11.08±2.1	10.06±2.5
6	2.76±0.5	3.5±0.3	7.13±2.4	7.73±2.6
7	2.72±0.2	3.5±0.4	7.73±3.2	7.32±2.8
8	2.72±0.2	3.48±0.5	7.77±2.1	7.32±1.5
9	2.72±0.3	3.48±0.6	7.77±2.1	7.32±1.4
10	2.72±0.2	3.48±0.6	7.77±2.1	7.32±1.2

Lipid production and total soluble carbohydrate utilization by *M.rouxii* in potato processing wastewater

Days	T1Lipid (g/L)	T2 Lipid (g/L)	T1 Total Carbohydrate (g/L)	T2 Total Carbohydrate (g/L)
0	0±0	0±0	37±1.2	37±2.1
1	0.19±0.1	0.39±0.1	25±1.2	20±2.2
2	0.32±0.2	0.42±0.2	20±2.4	17±2.1
3	0.47±0.1	2.3±0.1	5.69±3.2	2.31±1.1
4	2.4±0.3	3.4±1.2	3.27±4.2	1.89±1.1
5	3.48±1.2	4.2±2.2	5.52±4.2	1.58±0.5
6	3.6±1.2	4.1±2.1	4.58±1.2	1.45±0.6
7	2.9±1.3	3.5±1.2	4.16±1.3	1.13±0.3
8	2.6±0.6	2.9±0.8	2.81±1.2	0.39±0.1
9	1.9±0.9	2.5±0.1	2.8±1.4	0.39±0.1
10	1.8±0.8	2.18±0.2	2.79±1.2	0.39±0.1

COD removal by *A.flavus* and *M.rouxii* in potato processing wastewater

Days	COD Reduction T1 *A.flavus*	COD Reduction T2 *A.flavus*	COD Reduction T1 *M.rouxii*	COD Reduction T2 *M.rouxii*
0	40±0	40±0	40±0	40±0
1	37±2.1	35±2.5	30±1.2	31±2.1
2	37±3.2	35±3.6	21±2.1	10±1.3
3	35±1.5	30±.2.6	19±1.4	10±1.1
4	34±1.7	26±2.4	18±1.3	6±1.3
5	33±1.8	23±2.3	15±1.3	7±0.3
6	16±1.2	18±1.2	14±1.5	5±0.7
7	14±1.3	16±1.2	12±1.4	4±0.8
8	14±1.4	16±1.7	12±1.3	4±0.6
9	14±1.5	16±1.2	12±1.3	4±0.5
10	14±1.3	16±1.3	12±1.4	4±0.4

pH changes during fermentation of potato processing wastewater.

Days	Change in pH *A.flavus*T1	Change in pH A.flavus T2	Change in pH M.rouxii T1	Change in pH *M.rouxii* T2
0	6	6	6	6
1	6.1	6.1	7.2	7.8
2	6.8	6.7	7.5	7.9
3	6.2	6.6	7.3	7.8
4	5.6	4.7	7.9	7.5
5	5	4.6	8.1	8.1
6	5.3	4.3	8	8.3
7	5.3	4	8.2	8.4
8	5.3	4	8.1	8.2
9	5.3	4	8.1	8.2
10	5.3	4	8.1	8.2

TSN removal by *A.flavus* and *M.rouxii* in potato processing wastewater

Days	TSN Removal *A.flavus* T1 (mg/L)	TSN Removal A.flavus T2 (mg/L)	TSN Removal M.rouxii T1 (mg/L)	TSN Removal M.rouxii T2 (mg/L)
0	500±0.00	500±0.00	500±0.00	500±0.00
1	250±0.00	240±0.00	240±0.00	188±0.00
2	190±14.14	90±7.07	185±7.07	170±1.2
3	50±14.14	70±0.5	40±0.00	30±1.4
4	35±0.00	5±0.1	30±7.07	35±1.5
5	35±7.07	0±0	30±0.00	35±7.3
6	35±7.07	0±0	30±7.07	25±2.1
7	15±7.07	0±0	25±0.00	20±2.2
8	10±0.1	0±0	25±7.07	10±2.3
9	10±0.2	0±0	25±0.00	10±2.4
10	10±1.2	0±0	25±0.00	10±2.5

TSP removal by *A.flavus* and *M.rouxii* in potato processing wastewater

Days	TSP Removal *A.flavus* T1 (mg/L)	TSP Removal *A.flavus* T2 (mg/L)	TSP Removal M.rouxii T1 (mg/L)	TSP Removal M.rouxii T2 (mg/L)
0	246±0.0	246±0.0	246±0.0	246±0
1	135±0.0	130±7.07	125±0.0	130±2.9
2	70.5±0.0	77±0.0	115±0.42	95±3.5
3	60±0.0	45±7.0	105±0.0	50±12.4
4	55±14.1	20±0.0	95±5.12	45±3.6
5	55±14.1	20±7.07	85.5±4.2	47±7.8
6	48±2.34	20±0.0	85±3.9	45±2.4
7	44.5±7.07	20±7.0	80±4.3	45±1.8
8	40±7.07	19±0.0	35±1.1	45±1.9
9	40±7.07	19±0.0	35±7.1	45±2.5
10	40±7.0	19±0.0	35±1.2	45±4.3

Appendix B

Depletion of ammonium ions during fermentation on different carbon sources by *M.rouxii*

Days	Glucose	Starch	Starch (60g/L)	Cellulose
0	137.33±2.08	139.33±1.15	139.00±1.00	140.33±0.58
24	60.00±2.00	79.33±1.15	67.67±2.52	40.00±1.00
48	2.77±0.59	5.83±0.06	4.33±0.42	2.00±0.44
72	0.00±0.0	0.07±0.06	0.00±0.0	0.00±0.00
96	0.00±0.0	0.04±0.06	0.00±0.0	0.00±0.0
120	0.00±0.0	0.03±0.06	0.00±0.0	0.00±0.0
144	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
168	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0

Lipid production by *M.rouxii* under different carbon sources

DAYS	Glucose(30g/L)	Starch (30g/L)	Starch (60g/L)
0	0.0±0.00	0.00±0.00	0.23±0.06
1	0.0±0.01	0.26±0.05	1.70±0.10
2	0.4±0.06	1.13±0.06	3.13±0.12
3	1.4±0.10	2.43±0.06	4.17±0.15
4	2.8±0.15	3.10±0.10	5.23±0.03
5	3.5±0.16	3.50±0.10	5.73±0.12
6	4.8±0.15	3.88±0.02	5.80±0.00
7	4.6±0.10	3.47±0.06	5.73±0.12
8	4.5±0.10	3.34±0.12	5.77±0.06
9	4.3±0.06	3.37±0.06	5.70±0.10
10	4.1±0.12	3.37±0.06	5.73±0.06

Appendix B

Utilization of different carbon source by *M.rouxii*

Days	Glucose(30g/L)	Starch(30g/L)	Cellulose(30g/L)	Starch (60g/L)
0	30.7±1.15	30.00±0.58	30.33±0.00	60.00±0.58
1	20.3±0.58	28.00±2.08	27.33±2.08	52.33±1.15
2	17.3±0.58	20.00±0.58	24.67±1.73	47.00±0.58
3	12.3±0.58	17.00±0.58	24.00±0.58	36.33±0.00
4	4.7±0.58	5.00±0.58	22.00±0.58	24.33±0.00
5	0.2±0.15	2.00±0.00	19.83±0.58	19.67±0.76
6	0.2±0.06	1.89±0.23	21.17±0.58	20.33±0.76
7	0.1±0.06	1.83±0.10	20.50±1.00	19.00±0.00
8	0.2±0.02	1.83±0.04	20.17±0.58	20.33±1.04
9	0.2±0.07	1.83±0.01	20.50±1.00	21.00±0.50
10	0.2±0.06	1.83±0.01	20.33±1.00	20.00±0.29

Secretion of amylase ad cellulase by *M.rouxii* under different carbon sources

Days	Amylase A	Amylase B	Cellulase
0	0.0±0.00	0.00±0.00	0.00±0.00
1	0.3±0.03	0.47±0.02	0.03±0.06
2	0.4±0.04	0.67±0.06	0.87±0.06
3	0.5±0.01	1.20±0.01	0.18±0.00
4	0.2±0.00	0.50±0.00	0.19±0.00
5	0.1±0.00	0.20±0.00	0.03±0.00
6	0.1±0.00	0.20±0.01	0.02±0.00
7	0.1±0.01	0.20±0.01	0.02±0.00