The Production of Hydrocarbons and other Lipids by *Trichoderma koningii, Penicillium janthinellum* and their Mixed Species Culture Grown Aerobically on Four Different Carbon Substrates

By

Amarpreet Chahal

B.Sc. (Honours), Carleton University, 2010

A thesis submitted to the Faculty of Graduate Studies and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Carleton University

Ottawa, Ontario

Your file Votre référence
ISBN: 978-0-494-94290-1

Our file Notre référence
ISBN: 978-0-494-94290-1

Abstract

The fungal species, *Trichoderma koningii, Penicillium janthinellum,* and a mixture of *Trichoderma koningii* and *Penicillium janthinellum* were tested to determine their abilities to convert different carbon substrates (undecanoic acid (UDA), decanoic acid (DA), mixture of decanoic and undecanoic acid (UDA+DA), and mixture of potato dextrose broth and undecanoic acid (UDA+PDB) into different cellular metabolites. The mixed species culture showed higher biomass and carbon dioxide production on all 4 substrates compared to individual cultures. The mixed species culture showed highest biomass production of 59.5 mg/ml and cumulative carbon dioxide production of 394.2 mg after 576 hours of growth, when grown on mixture of potato dextrose broth and undecanoic acid. The intracellular accumulation of lipids in mixed species culture and single cultures was confirmed by fluorescent microscopy during stationary and exponential phase when UDA+PDB is source of carbon.

Chemical analysis of different lipids produced by mixed species culture was analyzed by Iatroscan TLC-FID to identify 15 different lipid classes produced. The highest total intracellular lipid content of 3.5 mg/ml was observed when mixed species culture was grown on PDB+UDA during the exponential growth phase. Free fatty acids were the most dominant lipid class produced intracellularly and extracellularly during both the exponential and stationary growth phases. They account for >80% of total lipids produced. Analysis of intracellular and extracellular saturated and unsaturated fatty acids by GC-FID analysis shows, out of 55 fatty acids analyzed, the most dominating fatty acids on all substrates were palmitic acid (C_{16}), stearic acid (C_{18}), oleic acid ($C_{18:1}$), and linoleic acid ($C_{18:2}$), which account for nearly 70% of total free fatty acids (FFA)

produced. The concentration of monounsaturated fatty acids and polyunsaturated fatty acids were similar, and their content ranged from 23% to 66% of the total FFA (0.007 to 0.57 mg/ml), and concentration of saturated fatty acids ranged between 33% and 75% of the total FFA (0.01 to 1.97 mg/ml) depending on substrate type and growth phase.

The analysis of fungal culture samples by pyrolysis-field ionization mass spectrometry (Py-FIMS) supported analysis by TLC-FID, and showed that fatty acid concentrations ranged between 4.4 and 6.6% of total molecular ion intensities (TIIs) depending on carbon substrate and ranged from *n-*C_{11} to *n-*C_{18}. The Py-FIMS analysis shows that the intracellular hydrocarbons including n- alkanes, alkenes and n-alkyl esters were most abundant components with total yield ranging between 7.4 and 15.7% of total ion intensities (TIIs). When fungal cells were grown on fatty acids and PDB the C-chain lengths of extracellular alkanes ranged from *n-*C_{19} to *n-*C_{34}, alkenes n-$C_{30:1}$- n$C_{40:1}$, and long chain alkyl esters from C_{41}- $C_{60:1}$. According to Py-FIMS analyses, alkanes, alkenes and alkyl esters were not observed in a chloroform extract of PDB. The latter indicates that the studied mixed fungal species catalyze the conversion of fatty acids to hydrocarbons during growth.

Acknowledgments

I would like to express my deepest gratitude to my advisors, Dr. Carlos M. Monreal and Dr. Owen Rowland for their continuous support, guidance, and encouragement throughout my study and research. They have been great mentors with their enthusiasm, understanding and willingness to help, professionally and personally. Also, a big thanks to Dr. Morris I. Schnitzer for introducing me to different concepts of chemistry and also his guidance through many of the chemical concepts of this research. I want to thank Dr. John Bissett for providing the fungal species for this research. I would also like to thank my M.Sc. graduate committee member, Dr. Myron Smith for his helpful suggestions and guidance throughout the course of this work. I would also like to thank Shea Millar for her assistance in microscopic analysis and also for teaching me several different microscopic techniques. Thanks to all my lab-mates who helped me along the way. Finally, thanks to the ecoETI program of Natural Resources Canada and Agriculture and Agri-food Canada for providing funding to this research project.

Table of contents

List of Tables

List of Figures

List of Abbreviations

AAR: Acyl-ACP reductase

ACC: Acetyl-CoA Carboxylase

AD: Aldehyde decarboxylase

ALC – Alcohols

AMF: Arbuscular mycorrhizal fungi

AMPL – Acetone mobile polar lipids

$BaCl_2$. $2H_2O$: Barium chloride dehydrate solution

BF_3MeOH: Boron triflouride-methanol solution

BTL: Biomass to liquid

BTU: British thermal unit

$CDCl_3$: Deuterated chloroform

CO_2: Carbon dioxide

DA: Decanoic acid

DG - Diacylglycerols

EE – Ethyl esters

EKET – Ethyl ketones

FabB: Keto-acyl-ACP synthase

FabD: Malonyl-CoA transacylase

FabG: Keto-acyl-ACP reductase

FabI: Enoyl-acyl-ACP reductase

FabZ: β-Hydroxyacyl-ACP dehydratase

FadA: 3-Keto-acyl-CoA thiolose

FadB: Enoyl-CoA hydratase

FadD: Acyl-CoA synthase

FadE: Acyl-CoA dehydrogenase

FAME: Fatty acid methyl esters

FFA: Free fatty acids

GC-FID: Gas chromatography with flame ionization detection

GE – Glycerol esters

GHG: Green house gases

GPD: Glyceraldehyde-3-phosphate dehydrogenase

HC – Hydrocarbons

HCl: Hydrochloric acid

HK: Hexokinase

IEA: International energy agency

IPCC: Intergovernmental panel on climate change

LCFA: Long chain fatty acids

LSD: Least significance difference test

MAK: Methyl alkyl ketone

ME – Methyl esters

Mha: Million hectares

MKET – Methyl ketones

MUFA: Mono-unsaturated fatty acids

NMR: Nuclear magnetic resonance

OleT: A cytochrome P450 enzyme

PDA: Potato dextrose agar

PDB: Potato dextrose broth

PFK: Phosphofructose kinase

PGI: Phosphoglucose isomerase

PGK: Phosphoglycerate Kinase

PGM: Phosglycerate Mutase

PK: Pyruvate Kinase

PL – Phospholipids

PUFA: Poly-unsaturated fatty acids

Py-FIMS: Pyrolysis field ionization mass spectrometry

SE/WE - Steryl esters/wax esters

SFA: Saturated fatty acid

SPSS: Statistical package for the social science

ST – Sterols

TesA: Acyl-ACP thioesterase

TG – Triacylglycerols

TIIs: Total ion intensities

TLC-FID: Thin layer chromatography with flame ionization detection

TW: Terawatts

UDA: Undecanoic acid

UDA+DA: Mixture of undecanoic acid and decanoic acid

UDA+PDB: Mixture of undecanoic acid and potato dextrose broth

WES: Wax-ester synthase

List of Appendix

Figures

Tables

Chapter 1: General introduction

1.0 Introduction

Mankind is currently facing three major concerns: hunger, energy shortages, and the deterioration of the environment. Any one of them by itself has the ability to wipe out our civilization (Escobar *et al.*, 2009). These concerns are inter-related with origins from rapid industrialization and the implementation of modern economic systems. Energy fuels our life and energy consumption correlates strongly with our standards of living (Escobar *et al.*, 2009). The developed world has become accustomed to cheap and plentiful supplies. Recently, many of the developing world populations are striving for the same, and taking steps towards securing their future energy needs (Dermirbas, 2009). Competition over limited supplies of conventional fossil fuel resources is intensifying, and more challenging environmental problems are springing up (Demirbas, 2009).

1.1 Impact of fossil fuels

According to International Energy Agency (IEA), the world consumption of energy is 440 EJ/year. The power capacity of the world according to IEA was 14 TW in 2003 which is expected to reached over 50 TW by the end of 21st century (Ghoniem 2011). Fifty seven percent of fossil fuels are consumed in the transport sector, thus making transport sector the biggest consumer of fossil fuel and a major contributor of adverse environmental impacts (Ghoniem 2011). Fossil fuel reserves are distributed irregularly throughout the world; mostly located in the Middle East. In the past couple of decades, inefficient use of fossil fuels to fulfill high-energy demands of some developed and other developing countries has lead to the exhaustion of some of these oil reserves (Ghoniem, 2011). In developing countries, for example India and China, the energy consumption has increased by 6.4% and 5.3 %, respectively, in the past decade (IEA

report, 2003). In 2008, the United States petroleum consumption reached 37.1 quadrillion BTU, 71% of this consumption was in the transportation sector (Connor and Astumi, 2010).

Over the past century, the considerable reduction in fossil fuel reserves, through intensive and low-efficient use to provide human energy needs, is expected to result in the prognosis of fossil fuel exhaustion within the next decades. This phenomenon of exhaustion of fossil oil reserves is known as 'Peak oil'. This phenomenon is most likely to be characterized by the reduction in the world's oil production, which certain studies show might have already started in 2010 (Escobar *et al.*, 2009). There is a unanimous agreement regarding the era of cheap energy being long gone (Escobar *et al.*, 2009)

The consumption of fossil fuels results in the emission of air pollutants and green house gases (GHG) emissions. Therefore, most of the GHG accumulation in the environment is associated with emissions from the transport sector (Eurostat, 2007). The GHGs tend to excessively elevate the temperature of the planet. The GHGs include carbon dioxide, methane, nitrous oxide and aerosols. Other pollutants include sulfur oxide, ash, droplets of tar and other organic compounds (Barbir *et al.*, 1990). These fossil fuels pollute the environment during their extraction, transportation, refining and combustion (Hoffert *et al.*, 1998). Several studies have shown that these pollutants cause diseases in humans and increased death rate, damage to aquatic ecosystems, reduction of crop production and increased deforestation (Barbir *et al.*, 1990). The remediation of these damages caused by fossil fuel use leads to a total cost of well over 2000-billion dollars/ year (Barbir *et al.*, 1990).

The main GHG is carbon dioxide, which is produced during fossil fuel combustion and has been directly related to global warming. The temperature of the earth's surface and near surface has risen by slightly more than 1 °C in the past century (Abrahamson, 1989). Greenhouse gases present in the atmosphere absorb the radiation bouncing back from earth's surface thus blocking the radiation from escaping and leading to a further increase in the temperature of the earth's surface (Uherek *et al.*, 2010). A 2007 report from the Intergovernmental Panel on Climate Change (IPCC) of the United Nations stated that the current carbon dioxide levels in atmosphere are at 455 ppm CO_2-^{e}, which has already exceeded the threshold level of amount of carbon dioxide in atmosphere (450ppm). If the consumption of fossil fuels continues in the same way the CO_2 content in the atmosphere will increase by 50% by the end of 21st century (IEA report, 2004; Hoffert *et al.*, 1998). This increase is expected to lead to an increase in temperature of earth's surface by 5-6 °C by the end of 21st century (Ghoniem, 2011; Zeebe *et al.*, 2009). These CO_2 concentrations and temperature will be the highest it has ever been in the last 420,000 years (Ghoniem, 2011).

1.2 Importance of biofuels

Biofuels are important because they can replace petroleum-derived fuels. There are several different benefits for using biofuels over traditional fuels: greater energy security, possibly reduced environmental impacts, foreign exchange saving and social-economic issues related to the rural sector (Demirbas, 2009). The technology behind the development of biofuels from renewable biomass is relevant to both developing and developed countries (Demirbas, 2009).

Economic advantages of a biofuel industry would include: value added to the feedstock, increased income taxes, revenues, investment in power plants and equipment purchasing and utilization of local goods and services, reduced reliance by countries on crude oil imports and sustainability. There are also expected increases in employment because labor is directly employed in the construction and operation of biofuel plants. The sustainability criterion is aimed to ensure that minimum GHGs are released and avoid loss of cropland biodiversity and carbon stock from land. Also, to ensure that forest, highly bio-diverse grassland or nature protection areas are not used raw material for production of biofuels (Pupan, 2002; Demirbas, 2009).

The effect of biofuels on diversity could be beneficial or detrimental depending on previous land use and biofuel crops. With increasing demand of biofuels use of tropical forest or other croplands to grow biofuels crops such as corn only decreases biodiversity. If appropriate biofuel crops such as sugarcane, grassland perennials or Jatropha are grown in suitable areas such as degraded or marginal lands through conversion of these lands can be beneficial to biodiversity or not have any further negative impact.. Increased plantation of corn and plam oil for production of current biofuels have negative impact on biodiversity through soil degradation, transportation, refining and pollution. The use of natural land for biofuel production is detrimental therefore production of advanced biofuels is required in order to prevent decrease in biodiversity (Groom *et al.*, 2008; Tilman *et al.*, 2006)

Environmental advantages of using biofuels will include reduced GHG emission due to displacement of fossil fuels, reduction in air pollution due to the lower sulfur and

aromatic content in biofuels, biodegradability, improved land and water use and carbon sequestration (Escobar, 2009).

Energy securities include a reliable supply of biofuels from renewable biomass, provide additional market for agriculture products, biomass, and other domestic crops and lead to diversification of fuel sector (Pupan, 2002; Nigam and Singh, 2011; Demirbas, 2009).

1.3 Different biofuels present

Three different generations of biofuels has been developed so far. The first generation biofuels includes bioethanol, biodiesel and biogas. The first generation biofuels are presently produced on a scale useful for the transportation sector. Approximately 10% of the world's primary energy demand is presently generated from biomass (Antoni *et al.*, 2007). The first generation bioethanol is mainly generated from cornstarch and sugarcane, whereas biodiesel is mainly generated from vegetable oils such as rapeseed, soybean, sunflower, palm oil and safflower (Lang *et al.*, 2001). Biogas is mainly produced from anaerobic digestion of solid and liquid waste, and is mainly used as an alternative to compressed natural gas.

The second-generation biofuels include cellulosic ethanol, biohydrogen, biobutanol, biomass to liqud (BTL)- diesel, and pyrolytic liquids. These second-generation biofuels are mainly produced from non-edible sources such as jatorpa, cotton, tallow, animal fats, agricultural and crop residue, grass and other sources (Ladislao and Turrion-Gomez, 2008; Naik *et al.*, 2010). Second-generation biofuels are expected to reduce net carbon emission, increase energy efficiency and reduce energy dependency, potentially overcoming the limitations of first-generation biofuels (Ladislao and Turrion-

Gomez, 2008).

The third-generation biofuels include use of microorganism for production of components of biofuels or act as an enzyme to carry out catalytic reactions. The third-generation biofuels include algae and fungal produced fuels. The main process involved in the production of third-generation biofuels is isolation of long chain fatty acid or fatty alcohols from microbes and then their transestrification to produce fatty acid methy-esters (Antoni *et al.*, 2007; Nigam and Singh, 2011).

1.4 Disadvantages of current biofuels

The first generation biofuels being produced industrially on large scale are not fully carbon-neutral, since there is net production of GHG. However, the GHG emissions are highly reduced compared to the GHG emissions from combustion of fossil fuels (Streets and Waldhoff, 1999). The second-generation cellulose ethanol has the potential to produce 75% less CO_2 than normal petrol, whereas corn or sugar beet ethanol reduces CO_2 levels by just 60%. As for diesel, BTL technology could lower CO_2 emissions by 90%, compared with 75% for currently available biodiesel (Ladislao and Turrion-Gomez, 2008). Although these biofuels provide an initial platform for renewable fuel development there are current limitations. Most bioethanol is not compatible with the current fuel infrastructure. Bioethanol has highly corrosive and hygroscopic characteristics; as well it has only 70% of the energy content of gasoline (Lee *et al.*, 2008). Biodiesel has energy content of 11% lower than petro diesel (Connor and Atsumi, 2010). Biodiesel has high cloud and pour points, which makes it impossible to be transported through the pipelines (Lee *et al.*, 2008). Because both bioethanol and biodiesel are currently being produced from crops that are used by humans for food, there

is much concern. A study by Chisti (2007) shows that 0.53 billion m^3 of biodiesel is required annually to meet the current transportation needs in the United States alone. To produce 0.53 billion m^3 from the current feedstock of biodiesel production, 61% (111 million hectares (Mha)) of agricultural land would be required for the growth of plants for biodiesel using current methods of production. Western Canada has 60 Mha of cultivate land, out of which 10-15 Mha of agricultural land will be required to produce crops to be used to produce significant amount of biofuels (SCOPE, 2010), which makes it not a feasible method for producing current biofuels, as it will generate a food crisis and the cost of food items will rise tremendously (Chisti, 2008). The USA alone is responsible for 70% of the world's export of corn. As the oil prices go up, the production of biofuels out of agricultural products is more profitable, and therefore there is a risk that the price of raw material used for biofuel production will be more than the price offered by the food industry. As more grains are being destined for fuel energy purposes, the inventories available for food consumption are dropping, which causes the warnings in relation to the availability of food and the rise in the price (Nigam and Singh, 2011). In 2006, the price of wheat and corn reached the highest levels over the past 10 years (Escobar, 2009). As the current technologies used to produce biofuels could lead to a food crisis and the quality of biofuels produced is not compatible with the current infrastructure, it has become necessary to develop more advanced biofuels

1.5 Development of new advanced biofuels

The development of new advanced biofuels requires adaptation of engines to biofuels, development of biofuels that have all the physical and chemical properties of the fossil fuel and can directly be used in current infrastructure, and also blending of these

biofuels with the fossil fuels leading to advantageous modification of fuel properties (high octane rating). These adaptations will establish a transition period to replace fossil fuels by prolonging the period of fossil fuel availability (Antoni *et al.*, 2007).

When designing new biofuels, many relevant factors have to be considered, such as energy content, octane number, cetane number, volatility, freezing point, odor and toxicity. Energy content is the amount of energy produced during combustion. The number of C-H and C-C bonds in a molecule is a good indication of how much energy a particular fuel will produce (Lee *et al.*, 2008). Octane number is the measurement of its resistance to knocking, which occurs when the fuel/ air mixture spontaneously ignites before it reaches the optimum pressure and temperature for spark ignition (Lee *et al.*, 2008). Cetane number is the measurement of the combustion quality of fuel during compression ignition. A shorter ignition delay, which is the time period between the start of the injection and the start of combustion of fuel, is preferred. The ignition delay is indexed by the cetane number (Lee *et al.*, 2008). The major components of gasoline, petro-diesel and jet fuel are shown in Table 1 (pp. 17) along with different biosynthetic components present to replace these biofuels.

Gasoline, petro-diesel and jet fuels are three fossil fuels with complex mixtures of hydrocarbons of varying lengths (Table 1), mainly consisting of linear, branched and cyclic alkanes. Alkanes account for 85% of the overall composition of these fuels. Therefore to generate biofuels with similar combustion properties requires that they have a large number of alkanes, especially short chain alkanes (C_4 to C_{12}).

1.6 Chemical composition of current fossil fuels

Current biofuels does not have the same chemical composition as the fossil fuels, making these fuels not compatible to be used with the current transportation fuel infrastructure. Their chemical composition often makes them hard to transport, store, and use in the current vehicles (Somerville, 2007). Bioethanol is mainly composed of short-chain alcohols generated from carbohydrates (Wackett, 2008). Biodiesel is mainly composed of long-chain fatty acid methyl esters (C_{15}-C_{25}) generated from transestrification of fats isolated from biomass (Rashid *et al.*, 2008). Biogas is mainly composed of methane; it can be used to replace natural gas, but also can be used as transportation fuel (Yadvika, 2004). The biggest difference between biofuels and fossil fuels is the oxygen content. Petroleum essentially has no oxygen, while biofuels have 10% to 45 % oxygen content depending upon the source of biomass thus making the chemical properties of biofuels very different than petroleum. However, the presence of oxygen gives them the advantage of being more efficient in combustion and reduced release of hydrocarbon in exhaust. Still, oxygen content gives some disadvantageous chemical properties to current biofuels: lower fuel stability during storage, less energy content, lower energy density, coerciveness, low flame luminosity, lower vapor pressure, miscibility with water and toxicity to ecosystems (Demirbas, 2009). Advanced biofuels will ideally have low oxygen content, similar to that of gasoline. These new biofuels should also be enriched in alkanes and have similar physical, chemical and thermodynamic properties as gasoline.

1.7 Sources of hydrocarbons

Alkanes are found in all living organisms and they are widely distributed in organisms such as bacteria, yeast algae, higher plants and insects (Templier *et al.*, 1990). Hydrocarbons are known to retain their original architecture over a long period of time because these are one of the most stable naturally occurring compounds (Ladygina *et al.*, 2006).

1.8 Chemical and biochemical methods that are used for producing biofuels

The formation of alkanes can be achieved by chemical methods or by biochemical methods. The major precursors for production of alkanes from chemical or biochemical methods are fatty acids.

Microbes through enzymatic pathways can produce alkanes. Microorganisms are the most dominant and diverse group of organisms on the planet. Studies have shown that intracellular and extracellular hydrocarbons are produced from species isolated from mesophilic, thermophilic, psychrophilic, acidophilic, alkalinophilic and halophilic environments under aerobic or anaerobic, autotrophic or heterotrophic conditions (Lee *et al.*, 2008; Christi, 2007; Ladygina, 2006). Microorganisms act as great biocatalyst, where they can take up different carbon sources and convert them to hydrocarbon through different metabolic pathways.

The hydrocarbons in the organisms are synthesized through two hydrocarbon synthesis pathways from fatty acids, head-to-head condensation of fatty acids and elongation-decarboxylation that occurs mostly in eukaryotes. The head to head condensation is primarily found in prokaryotes. In this process the bond is formed between carboxyl carbon of one fatty acid that is initially oxidized to a β-keto acyl-CoA

and α-carbon of second fatty acid in the form of fatty acyl-CoA through the process of decarboxylative claisen condensation reaction. This reaction is catalyzed ketoacyl-ACP synthase, which was observed in *E. c oli*. The resulting long chain fatty acids are then subsequently decarboxylated to form hydrocarbons with 1 carbon less that the total number of carbons from two fatty acids. Recent studies showed that the result of condensation form β-keto thioester, which undergoes reduction and dehydration reactions, catalyzed by cluster of genes *oleABCD* to form hydrocarbon. The genes were isolated from *Micrococcus luteus* and their homologs have been observed in other bacterial species. (Sukovich *et al.*, 2010; Beller *et al.*, 2010; Brown and Shanks, 2012; Han *et al.*, 1969; Bird and Lynch, 1974).

The second pathway involved in hydrocarbon synthesis is elongation-decarboxylation, which is found in cyanobacteria, plants, algae and insects. This pathway involves elongation of short and medium chain fatty acids to long chain fatty acids. This pathway envisages *de novo* fatty acid synthesis pathway. In this pathway the fatty acids are elongated through continuous addition of C2 units derived from malonyl-CoA forming long chain fatty acid, which are subsequently converted to alkanes through the removal of carboxyl group (Brown and Shanks, 2012; Schirmer *et al.*, 2010; Banerjee *et al.*, 2002; Han *et al.*, 1969).

Previously, it was considered that there is the involvement of decarboxylase enzymes that directly decarboxylate fatty acid to alkanes. The direct evidence for the presence of decarboxylase enzymes in microbes has not been elucidated yet (Ladygina *et al.*, 2006). Direct decarboxylation requires an electron-withdrawing group adjacent to the α-carbon to lose the carboxyl group of the fatty acid (Brown and Shanks, 2012; Dennis

and Kolattukudy, 1992). The elimination of CO_2 from the carboxylic acid creates a negative charge that has to be activated by a β-substituent to stabilize the negative charge and this requires a lot of energy. Thus, suggests that process of direct decarboxylation is not feasible (Brown and Shanks, 2012; Dennis and Kolattukudy, 1992).

Recent studies have shown that aldehyde acts as immediate precursors of hydrocarbons and involves reduction-decarbonyation mechanisms. This process has been suggested to occur in cyanobacteria, plants and algae, several enzymes involved in this process have been isolated (Samuels *et al.*, 2008; Bernard *et al.*, 2012; Banerjee *et al.*, 2002). This mechanism involves the formation of aldehydes through the reduction of fatty acids and, in the second step, the aldehydes are converted to alkanes with the release of carbon monoxide (Figure 5 of Chapter 3) (Schirmer *et al.*, 2010). The enzymes involved in this pathway are an aldehyde forming fatty acyl-CoA reductase and an aldehyde decarbonylase (Schneider-Belhaddad and Kolattukudy, 2000; Metzger and Largeau, 2005). This pathway was observed in the algae *Botryococcus braunii,* in which decarbonylation of fatty aldehyde leads to formation of alkanes and carbon monoxide, and it has been suggested to occur in other species (Metzger and Largeau, 2005; Banerjee *et al.*, 2002). The major evidence for the occurrence of these two pathways is the presence of fatty acyl-CoA reductase in a variety of organisms and also purification of aldehyde decarbonylase enzyme from *B.braunii* (Figure 5 of Chapter 3) (Brown and Shanks, 2012; Samuels *et al.*, 2008; Bernard *et al.*, 2012; Banerjee *et al.*, 2002; Dennis and Kolattukudy, 1991).

A recent study showed involvement of new pathway in synthesis of alkanes from fatty acids. This pathway has recently been found in *Vibrio furnissi* in which

hexadecanoic acid is reduced to hexadecanal, which is then oxidized to hexadecanol and finally to hexadecane (Figure 5 Chapter 3) (Park, 2005). This study showed insight into production of even carbon chain alkanes by microbes (Park, 2005). Some of the microorganisms that are known to produce high quantities of alkanes through these pathways are *V. furnissi, Aspergillus* species, *Bacillus* species, and some yeasts. The main biosynthetic pathway observed behind the synthesis of branched alkanes is the head-to-head condensation of fatty acid. These are mainly substituted fatty acids with methyl groups on both ends of the molecule (Albro and Dittmer, 1970; Bird and Lynch, 1974). It has also been known that photosynthetic bacteria mainly produce cyclic hydrocarbons. Conversely, long-chain linear hydrocarbons are seen to be predominant in fungal species (Ladygine *et al.*, 2006).

The long chain fatty acids (LCFA) found in the biomass from plants, microbes or animal waste are decarboxylated to alkane through chemical catalysis. The chemical decarboxylation of LCFA takes place in the presence of inorganic catalyst at temperatures between 250°C and 400°C (Aulich *et al.*, 2008), more preferably towards the higher end. The pressure during this process ranges between 50 psi to about 200 psi, mostly near 150 psi. This process takes place in the presence of hydrogen. Some of the inorganic catalysts used in this process are palladium, platinum, nickel, silver, gold, copper or mixed metals (Aulich *et al.*, 2008). The long chain alkanes formed from the chemical decarboxylation are further cracked down to short and medium chain alkanes because most of the useful hydrocarbons found in fuels involve short and medium chain alkanes. The catalytic cracking involves presence of acid catalyst and high temperature between 400- 460 °C leading to formation of two unstable ions which undergo

rearrangement of carbon chain. These catalytic processes are highly energy intensive. Additionally, the catalysts used are very expensive and cannot be recovered after the reaction. This makes chemical catalysis of alkanes from biomass very costly and unable to compete with the low pricing of petroleum fuels.

1.9 Designing new biofuels

Due to the growing demand for biofuels and the ability of some microorganisms to produce alkanes, there is a great potential for development of new biofuels. Biosynthetic pathways may be engineered to produce fossil-fuel replacements, including short-chain, branched-chain, and cyclic alcohols, alkanes, alkenes, esters and aromatics.

The potential limitations of the biochemical approach have to do with the development of cost-effective and energy-efficient processes to convert lignocellulose or seed oil fatty acids into fuels. The production potentials are also limited by the low activity of certain pathway enzymes and the inhibitory effect of byproducts from the upstream biomass processing steps on microorganisms that are responsible for producing fuels. With the progress of recent advances in synthetic biology and metabolic engineering there is potential to overcome these difficulties and engineer microorganisms for the cost effective production of biofuels from cellulosic biomass or residual fatty wastes (Lee *et al.*, 2008).

There are some proposed techniques that can be used to design advanced biofuel of the third generation. One of techniques that could lead to development of advanced biofuels involves refining 2nd generation biofuels through biocatalytic decarboxylation of fatty acids present in the fuels. One of the raw materials used for generating new biofuels is bio-oil produced from agricultural residues (Figure 1). Bio-oil is a dark brown liquid,

which is generated from waste biomass such as forestry biomass, crop residue, and animal manure and produced through a thermochemical process known as fast pyrolysis (Das *et al.*, 2009). Using advanced thermochemical processes such as direct combustion, gasification, anaerobic digestion and fast pyrolysis, the waste generated from the livestock can be treated to produce biofuels such as raw bio-oil (Figure 1) (Schnitzer *et al.*, 2006). The products of biomass pyrolysis are mainly bio-oil 65-72%; small amount of bio chars 15-20%, and non-condensable gases around 12-18% (Bradly, 2006). The production of bio oil increases the energy density of the original biomass stock. The chemical composition of bio-oil is dependent on the biomass residues from which they are produced (Das *et al.*, 2009). A study by Das *et al* (2009) shows that bio-oil derived from chicken manure contains hydrocarbons with a large fraction of short, medium and long chain fatty acids, N-hetrocyclic compounds and many others. It is believed that the production of bio oil will increase rapidly reaching nearly 5 million tons by 2012. In comparison with petroleum, bio-oil has a higher heating value and lower content of nitrogen and sulphur, it is carbon neutral, and unlike petroleum it is renewable (Schnitzer *et al.*, 2007). In order to increase the yield of the alkanes from fatty acids in the bio oil, a new biocatalyst is required for formation of alkanes from these fatty acids.

1.10 Importance of this study

Microorganisms, which work at room temperature and under the acidic conditions of bio-oil, offer to act as a potential biocatalyst for conversion of fatty acids to alkanes through different metabolic processes. This study investigates the use of two fungal species to determine their capacity to grow on fatty acids as carbon and energy sources and determine allocation of fatty acids under controlled growth conditions of

temperature. Studies have shown that fungal species can produce n-alkanes ranging from C_{12}- C_{31} (Ul-Hassan *et al.*, 2012; Ladygina *et al.*, 2006; Strobel *et al.*, 2008; Gianoulis *et al.*, 2012). The fungal species, chosen for this experiment can grow on carbon sources that are similar to fatty acids. These fungal species, originally isolated from soils, will act as a biocatalyst that can be used in the refining of bio oil produced via pyrolysis of agricultural residues. If these fungal species can use fatty acid as carbon and energy sources, it will show that they have enzymes systems that can decarboxylate fatty acid and produce alkanes. Assuming that fungal species adapt and grow well in acidic environments, these fungal species can lead to the production of new advanced biofuels through increasing the yield of alkanes in the bio oils.

The experiments carried out in this research project addressed three main questions; 1) Do the fungal species *Trichoderma koningii, Penicillium janthinellum* and their mixture have an ability to use fatty acids as a sole source of carbon and energy? 2) Which species or mixture of species has the highest biomass production and ability to produce carbon dioxide by utilizing fatty acids? 3) How are the substrate fatty acid transformed and allocated intracellularly and extracellularly into lipids and what is the chemical composition of these lipids?

Table 1: The important components and properties of current fossil fuels and their alternative biofuels.

Fuel type	Major compounds	Alternatives Biofuels
Gasoline	C5 – C13 n- alkanes C4 – C13 branched alkanes C6, C7 and C8 cyclo alkanes C6 – olefins Aromatic (benzene, toluene, xylene, ethylbenzene and C3, C4- benzenes and other). Anti-knock additives	Alcohols- bioethanol, biobutanol, bio-isobutanol, 3-methyl-1-butanol Short chain alkanes
Diesel	C9-C23 hydrocarbons Linear, branched, cyclic alkanes and aromatic Anti-freeze additives	Biodiesel, Farnesene, ethyl hexadecanoate, pentadecane Fatty alcohols (bioethanol, biobutanol), alkanes, terminal alkenes. Linear or cyclic isoprenoids (isolated from different biomass)
Jet fuel	C4 – C15 hydrocarbons Linear branched, cyclic alkanes and aromatic Anti-freeze additives	Alkanes Biodiesel (seed oil) Linear or cyclic isoprenoids (isolated from different biomass)

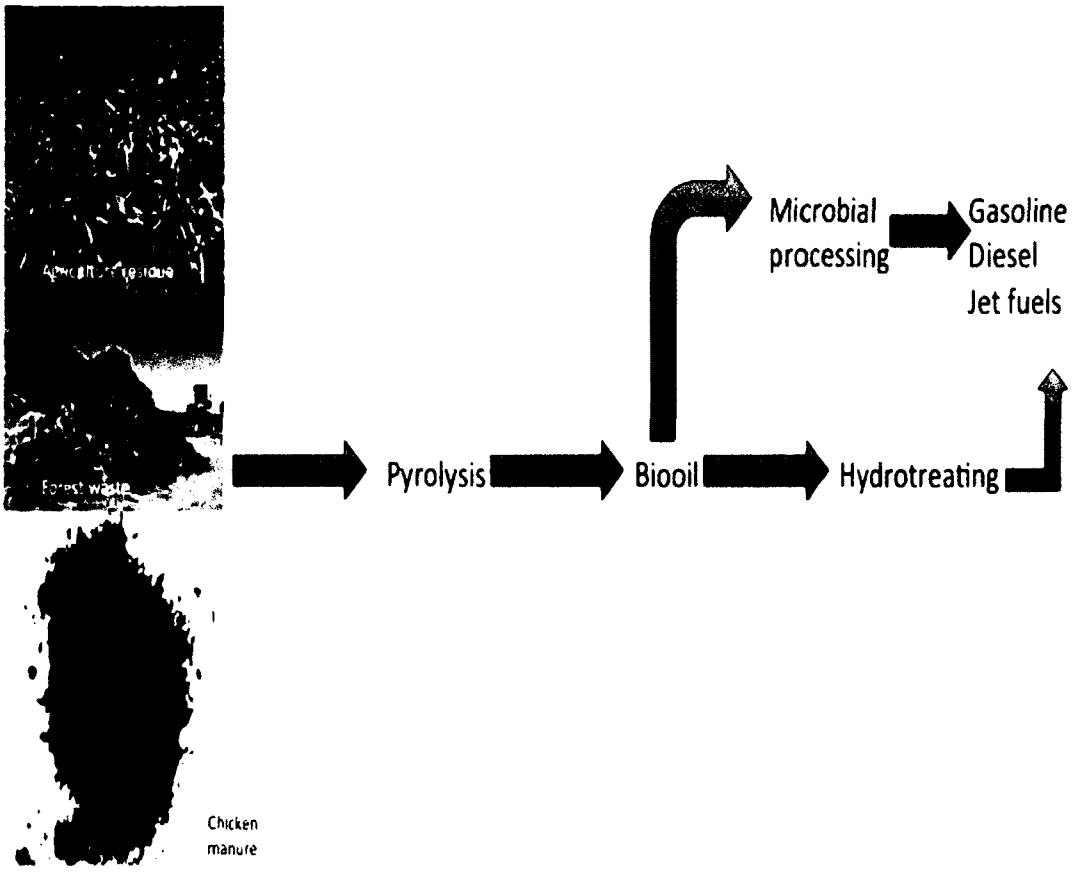


Figure 1: Process for production of advanced biofuels by fast pyrolysis and hydrotreatment

Chapter 2

Biomass production and decarboxylation activity of *Trichoderma koningii*, *Penicillium janthinellum* and their mixed species culture growing on different carbon substrates

2.1 Abstract

Fatty acids in waste products such as agricultural residues or food wastes could be used for development of new biofuels through removal of carboxyl group leading to formation of alkanes by microorganisms. Two filamentous fungal species, *T. koningii* and *P. janthinellum* and a mixture of these two species were cultivated on four different carbon substrates (Undecanoic acid (UDA), decanoic acid (DA), mixture of decanoic and undecanoic acid (UDA+DA) and a mixture of UDA+ potato dextrose broth (PDB)) to compare their abilities to use these carbon sources as sources of carbon and energy. All three fungal cultures grew on all fatty acid sources. The mixed culture of both species showed higher biomass production and decarboxylation activity on all 4 substrates compared to individual cultures of *T. koningii* and *P. janthinellum*. *T. k oningii* and *P. janthinellum* when grown alone on UDA, DA and a mixture of UDA+DA showed very similar biomass production and utilization of fatty acids. The mixed culture, when grown on UDA as the source of carbon and energy, showed the significantly higher biomass (51.5 mg/ml) and cumulative carbon dioxide (352 mg) production than in DA and UDA+DA after 576 hours of growth. The mixed species culture showed highest biomass production of 59.5 mg/ml and cumulative carbon dioxide production of 394 mg after 576 hours of growth when grown on a mixture of UDA+PDB. The mixed culture showed 97% of that of the COOH in fatty acid was decarboxylated to CO_2, when grown on UDA. The mixed culture of *T. koningii* and *P. janthinellum* has ability to produce alkanes that can be used for biofuels.

2.2 Introduction

Fungi are known to produce a broad range of metabolites that can be used for commercial development and in general have diverse characteristics and abilities that form the basis of much industrial research (Ul-Hassan *et al.*, 2012). Fungi grow in extreme environments, which reduces contamination and reduced control of culture conditions when grown on large scale (Griffiths and Harrison, 2009). Their large cell size reduces the cost of harvesting as cells can be easily removed from the culture through filtration, floatation or gravity sedimentation (Domingues *et al.*, 2000). They have a higher tolerance of shear force and contamination by other microbial species, thus allowing potential growth in polluted environments and they also possess a tendency to reduce auto-inhibition of growth at higher biomass densities (Rodrigues *et al.*, 2009). The selection of fast growing productive strains optimized for desired growing conditions is of fundamental importance for the production of valuable products.

Some fungal species have been known to accumulate oil under certain cultivation conditions, which is a very important characteristic for the production of new advanced biofuels (Ladygina *et al.*, 2006; Christi, 2008). The two fungal species that have been known for their production of a great number of metabolites and degrade complex substrates are *Trichoderma* and *Penicillium* species (Rodrigues *et al.*, 2009; Borjesson *et al.*, 1990). These two species are found in many different ecosystems. Two strains of these species from the National Fungal Collection of Agriculture and Agri-food Canada were selected for these studies are, *Trichoderma koningii* and *Penicillium janthinellum.* These species were selected based on their ability to use fatty acid and glycerol as the carbon and energy sources (Bisset. J, personal communication). These two fungal species

can degrade complex substrates such as cellulose, chitin, xylan, lignin and poly-aromatic hydrocarbons (Rodrigues *et al.*, 2009; Black and Dix 1976). These species also have proteolytic activity thus making these two fungal genus's of high biotechnological potential (Rodriguez-Kabana *et al.*, 1978).

The latter two fungal species are organoosmotrophic organisms, meaning they are able to take up carbon and nutrients in dissolved form (Gessner and Newell, 2002). The two most important compounds that are required for the growth and development of fungal species are a carbon and a nitrogen source. Other required compounds include minerals and vitamins (Miles and Chang, 1997).

Studies show that fungal species have a tendency to grow better when grown in a mixture of carbon sources. Studies also show that co-metabolism among fungal species (Wilson and Wilson, 1985). Co-metabolism is a process in which two different compounds are degraded simultaneously (Wendisch *et al.*, 1999). As fungal species are known to grow in extreme environmental conditions of pH, oxygen and carbonaceous complex substrates, the two fungal species; *T. koningii* and *P. janthinellum* in this study were grown on short-chain fatty acids, decanoic acid (C_{10}) and undecanoic acid (C_{11}) as source of carbon and energy. As these fungal species are organoosmotrophic (Gessner and Newell, 2002), these short chain fatty acids were preferred due to their low melting points (below 34 °C) therefore these fatty acids are able to dissolve in the media and thus are probably easy to be digested by the two fungal species. These experiments used potato dextrose broth as the positive control substrate treatment.

2.2.1 Research objectives

Specifically, *Trichoderma koningii*, *Penicillium janthinellum* and a mixture of *Trichoderma koningii, and Penicillium janthinellum* were used to determine their growth and capacity to oxidize five different carbon sources: undecanoic acid ($C_{11:0}$), decanoic acid ($C_{10:0}$), a mixture of both fatty acids, mixture of undecanoic acid and potato dextrose broth (UDA+PDB) and PDB over a period of 576 hours.

2.3 Material and Methods

2.3.1 Chemicals

Pure decanoic acid (CH_3- $(CH_2)_8$- COOH) and undecanoic acid (CH_3- $(CH_2)_9$- COOH) were obtained from Fisher Scientific (Table 2); Potato dextrose broth (PDB) was obtained from Sigma Aldrich (Table 5) and was used as a carbon source for fungal growth. All other chemicals used, were obtained from Sigma Aldrich (Table 2, 3, 4).

Table 2: Composition of the basal medium

Chemicals	**(g/500 ml)**	**Commercial source**
Carbon source 1) decanoic acid, 2) undecanoic acid and 3) Mixture of decanoic and undecanoic acids	5.0 g of one of the substrates / 500 ml of medium	Fischer Scientific
Sodium nitrate ($NaNO_3$)	1.0 g	Sigma Aldrich
Ammonium sulphate ($(NH_4)_2SO_4$)	1.0 g	Sigma Aldrich
Potassium phosphate dibasic (K_2HPO_4)	3.5 g	Sigma Aldrich
Potassium phosphate monobasic (KH_2PO_4)	1.0 g	Sigma Aldrich
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.15 g	Sigma Aldrich
Sodium chloride (NaCl)	0.25 g	Sigma Aldrich
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	0.05 g	Sigma Aldrich
Mineral solution (see Table 3)	0.5 ml	
Chloramphenicol solution (see Table 4)	0.5 ml	

Table 3: Composition of the Mineral solution

Mineral solution	**Amount of salt in media to make mineral solution of which 0.5 ml is added to basal media (mg/ 100 ml)**	**Commercial source**
Manganese sulphate ($MnSO_4.5H_20$)	6 mg	Sigma Aldrich
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	30 mg	Sigma Aldrich
Copper sulphate ($CuSO_4.5H_20$)	4 mg	Sigma Aldrich
$FeCl_3.6H_2O$	25 mg	Sigma Aldrich
H_3BO_3	6 mg	Sigma Aldrich
$(NH_4)6Mo_7O_{24}.\ 4H_20$	2.5 mg	Sigma Aldrich
KI	10 mg	Sigma Aldrich

Table 4: Composition of the Chloramphenicol solution[b]

Chloramphenicol solution	**Amount of antibiotic/ 100 ml media**	**Commercial Source**
Chloramphenicol	1g	Sigma Aldrich
Ethanol	100 ml	Fisher Scientific

Table 5: Composition of Potato dextrose broth media (control)

	Amount of potato dextrose broth/ litre	**Commercial source**
Potato dextrose broth (PDB)	10g	Sigma Aldrich
Ultra pure water	1000 ml	Millipore

2.3.2 Fungal species

The two fungal species used in this study were *T. koningii (strain # 216475)* and *P. janthinellum (strain # 96M-111),* which were provided by Dr. John Bissett from the National fungal collection of Agriculture and Agri-food Canada. The preserved *T. koningii* and *P. janthinellum* species were reconstituted by mixing frozen inoculum with ultrapure water. The solutions containing *T. koningii* and *P. janthinellum* were plated out and grown on two separate Potato dextrose agar (PDA, Difco) plates, one for each species. The plates were incubated for 168 hours at 30 °C and were determined to be free of microbial contaminants by observation of growth using microscope. Spores produced on these plates were used to inoculate media.

2.3.3 Carbonaceous substrates

The ability of these fungal species to grow on different substrates was studied using change in absorbance reading and increases in dry weight of fungus. Also, metabolism of these different carbon sources by three fungal cultures is studied through measurements of evolved carbon dioxide.

Three sets of studies were conducted at the same time using the three carbonaceous substrates. The first set includes use of fatty acids (UDA, DA and UDA+DA) as sole source of carbon. The second set includes mixture of fatty acid (UDA) and potato dextrose broth (PDB). The third set includes PDB as a sole source of carbon. For the first set of studies, the complete growth media for both fungal species included the basal mineral and antibiotic media described in Tables 2, 3 and 4. For the second set of studies, the growth media included the mixture of undecanoic acid medium (Table 2) and PDB medium (Table 5). A third set of studies involved PDB growth medium (Table

5) and it was used as a positive control. One flask with each carbon substrate without inoculation of fungal species was used as negative controls.

2.3.4 Growth and cultivation of fungal species in batch culture

First, the spores from *T. koningii* were collected from the agar plates to inoculate the media to minimize cross-contamination from *P. janthinellum,* by swirling 1 ml of basal medium on the agar plate and the sterile inoculation loop was used to lift the spores off the plate. The spore suspension from *T. koningii* and *P. janthinellum* plates was used to inoculate two 250 ml Erlenmeyer flasks containing 100 ml of PDB. To inoculate the mixture of *T. koningii* and *P. janthinellum* 1 loop of sterile spore suspension was taken from *T. koningii* plate and another loop of sterile spores suspension was taken from the *P. janthinellum* plate, to inoculate each of two 250 ml Erlenmeyer flasks containing 100 ml of PDB media. The inoculated cultures were grown on an oscillator shaker (120 oscillations/min) at 34 +/- 0.5°C for 192 hours.

After 192 hours, a 2 ml aliquot homogenized in a sterilized blender cup; for 30 seconds to minimize the variability of the inoculum size, of *T. koningii* grown in PDB was used to inoculate 100 ml of sterile media with undecanoic acid; a second aliquot of 2 ml was used to inoculate 100 ml of sterile media with decanoic acid; a third aliquot of 2 ml was used to inoculate 100 ml of sterile media with the mixture of decanoic acid and undecanoic acid (mixed fatty acid media); a fourth aliquot of 2 ml was used to inoculate 100 ml of the sterile media with mixture of undecanoic acid media and PDB (UDA+PDB) and a fifth aliquot of 2 ml was used to inoculate 100 ml of sterile PDB media as a positive control, in sterile 250 ml Erlenmeyer flasks. The 2 ml aliquots collected from *P. janthinellum* and the mixture of *T. koningii* and *P. janthinellum*

growing in PDB medium were used to inoculate the five types of growth substrate media. The inoculated cultures were grown on an oscillator shaker (120 oscillations/min) at 34 +/- 0.5°C for 360 hours. Once the inoculated cultures had produced a large amount of greenish color spores after 360 hours in the media, the cultures were diluted with 100 ml of sterile ultrapure water to each culture growing in 250 ml flasks and the whole solution was homogenized in a sterilized blender cup; for 30 seconds to minimize the variability of the inoculum size.

2.3.4.1 Adaptation to new carbon source

A homogenized aliquot of 4 ml of each fungal species were then used to inoculate 150 ml of the complete growth media with the appropriate carbon source and in a sterile 250 ml flask. Each fungal culture was also grown in 1 flask of the PDB growth media. The inoculated flasks with each species were placed on an oscillating shaker at 120 oscillations per minute at 34 ± 0.5°C for 576 hours to define growth curves.

2.3.4.2 Growth of two fungal species and their mixed culture

Homogenized aliquots of 4 ml of each fungal species treatment were then used to inoculate 250 ml glass bottles with screw cap each containing 150 ml of the complete growth media with the appropriate carbon source. Each fungal species in each of the carbon sources; UDA, DA, UDA+DA and UDA+PDB were grown in triplicates. Each fungal species was grown in separate flasks with PDB media as a positive control. For the negative control, five flasks, each containing a different carbon source were used without inoculation of any fungal species. All the flasks were placed on an oscillating shaker at 120 oscillations per minute at 34 +/- 0.5°C for 576 hours to define growth curves and carbon source oxidation activity for all the three species.

2.3.5 Experimental design and statically analysis

The batch culture studies were designed as a factorial experiment with fungal species, carbon source and time of incubation as the main factors. Every fungal cultures growing in the different substrates were replicated three times in separate flasks for each sampling time. The batch cultures in the 250 ml screw cap bottles were sampled every 48 hours for 576 hours.

The analysis of variance was used to test the variation in production of biomass and carbon dioxide due to several different factors which include carbon source, fungal species, time and interaction between these factors. This analysis was done to observe that if these factors have significant effect on production of biomass and carbon dioxide. Analysis of variance was conducted with a multivariate general linear hypothesis model of Statistical package for the social sciences (SPSS). General linear hypothesis model was used to test the effect of fungal species, carbon source, and time. The source of variation included fungal species, carbon sources, time, fungal species × carbon sources × time interaction, carbon source × species interaction, carbon source × time interaction and species × time interaction. One- way ANOVA was also used to observe significant difference in the means of three replicates for biomass production by three fungal species and also for means for biomass production on different. If the overall ANOVA was significant least significant difference (LSD) analysis was performed to test the pairwise comparisons at same level of significance between biomass – fungal species, biomass-carbon source, carbon dioxide- fungal species and carbon dioxide- carbon source to understand biomass and carbon dioxide production differ in three fungal species and five

carbon sources. Analysis for correlation between biomass and carbon dioxide production, bivariate 2- tailed Pearson correlation model of SPSS was also used (Zar, 1999).

2.3.6 Absorbance readings during fungal growth

An aliquot of 2.5 ml was taken from each flask placed into the disposable spectrophotometer cuvettes. The absorbance reading for all the growth media samples was taken at 770 nm on a Beckman DU series and their values recorded at each sampling time from the same flask throughout the incubation period. The absorbance 770 nm was used to measure the increase in the fungal cell number and to reduce the influence of the fatty acids present in the media on the absorbance reading for fungal cells, as lipid absorbance range between 480 and 600 nm. To minimize the variability in absorbance readings the samples from each flask were read within 30 seconds after taking the culture sample from flasks. After 336 hours, when the growth of fungal species was high and clumps of mycelia were formed in the culture, 150 ml of the cultures were blended for 30 second and then a homogenized aliquot was taken to measure absorbance. Once the absorbance reading was taken the flasks were placed back on the oscillating shaker at 120 rpm at 34 +/- 0.5°C. When taking the absorbance reading, the spectrophotometer was calibrated with each blank substrate media before taking the reading of optical density of three fungal species in each substrate.

2.3.7 Biomass determination

Once the absorbance readings were done, the cuvettes were covered with parafilm and shaken for 30 seconds. An aliquot of 500 µl was taken from each cuvette using a 1000 sterile pipette and micropipette tips. This 500 µl aliquot was then added to a preweighed 1.5 ml glass vial + screw caps, and frozen at -20 °C for 24 hours. After 24

hours, the glass vials were kept frozen in a box with dry ice. The glass vials without the screw caps were then transferred to a freeze-dryer (Lablonco lyophilizer) for a 24-hour period. After 24 hours the dried vial samples were removed from the freeze-dryer and immediately sealed with the screw caps. The biomass weight was determined by the difference in weight measured on a Mettler Toledo VS205 micro analytical balance. Fungal biomass measurements were taken every 48-hour interval from each flask during the 576 hours. Similar to absorbance samples, homogenized samples were taken for biomass reading after 384 hours of incubation. The biomass of each culture was calculated by using following formula: Biomass/ml = (weight of the freeze-dried vial with sample+ screw caps – weight of the empty vial+ screw caps) × 2× 0.5 ml.

2.3.8 Chemical methods

2.3.8.1 Oxidation of fatty acids and other carbon substrates

To measure carbon dioxide produced by these fungal species, long-term measurements of carbon dioxide evolution method was used (Stotzky, 1965). It is one of the oldest and simplest methods for carbon dioxide evolution measurements. In this method, alkali of defined concentration is placed in an open cylinder over the culture. Once CO_2 has developed in the culture it is harvested in the cylinder and confined until the time when it can diffuse and be absorbed by the alkali. The alkali is then removed after a measured period of time and the unreacted portion is determined by titration with hydrochloric acid (HCl). The amount of CO_2 that combines with the alkali can then be determined by means of subtraction (Stotzky, 1965). If an increased rate of carbon dioxide production is observed, it can be predicted that these fungal species are able to metabolize these complex substrates.

To determine the oxidation and estimate the decarboxylation activity of fungal species, carbon dioxide traps were inserted into 250 ml screw cap glass bottles containing culture of *P. janthinellum*, *T. koningii* and a mixed culture of both species growing in each of the five substrates. The carbon dioxide traps were taken out every 48 hours and replaced with new carbon dioxide traps to measure the release of carbon dioxide for the period of 576 hours.

2.3.8.2 Reagents for measurement of carbon dioxide evolved.

To measure carbon dioxide production, the following reagents were prepared: A sodium hydroxide solution (NaOH) (1N) was prepared by adding 40 g of NaOH pellets into 1000 ml volumetric flasks making the volume up to 500 ml using sterile ultra pure water. The 1.5 N barium chloride dehydrate solution ($BaCl_2.2H_2O$) was prepared by adding 183.21 g of $BaCl_2.2H_2O$ to the 500 ml sterile ultra pure water. The 1 N HCl solution was obtained directly from Fisher scientific. Phenolphthalein indicator, 100 ml solution was prepared by dissolving 1 g of phenolphthalein in 100 ml of 95% ethanol.

2.3.8.3 Capturing CO_2 evolved from Fungal Activity

A carbon dioxide trap was prepared by pipetting 5 ml of 1 N NaOH into sterile 15 ml polypropylene tubes, and placed inside each 250 ml VMR glass bottles with fungal cultures and controls. The polypropylene tubes were suspended above the culture to ensure that the culture media did not go into the polypropylene tubes or vice-versa. The VMR glass bottles were capped and then placed onto the oscillating shaker at 120 oscillation per minute and incubated at 34 ± 0.5 °C. After 48 hours, the 15 ml polypropylene tubes with sodium hydroxide were taken out of the glass bottles under sterile conditions. The 15 ml polypropylene tubes were immediately covered with

parafilm to prevent diffusion of atmospheric carbon dioxide. New sterile 15 ml polypropylene tubes filled with 5 ml of 1N NaOH were placed back into each VMR glass bottles. The 250 ml screw cap bottles with fungal culture were then tightly capped and placed back in the oscillating shaker under same conditions and sampled every 48 hours until the end of the incubation study.

2.3.8.4 Quantifying the oxidation/decarboxylation of carbon substrates

The NaOH solution from 15 ml polypropylene round bottom tubes was transferred quantitatively into 50 ml Erlenmeyer flasks. These collected sodium hydroxide solutions including those of the positive and negative controls, and the fungal cultures were then back titrated with 1N HCl as follows: The 50 ml Erlenmeyer flask containing the excess sodium hydroxide was placed on a plate with a magnetic stirrer and a small magnetic bar placed in the flask. To these flasks 10 ml of 1.5 N $BaCl_2$. $2H_2O$ was added to precipitate barium carbonate collected as sodium carbonate. Two to 3 drops of the Phenolphthalein was then added as an indicator. The unreacted NaOH was titrated with 1 N HCl slowly from a 50 ml burette. The volume of HCl needed to titrate the unreacted NaOH was noted.

2.3.8.5 Measurement of carbon dioxide evolved

The following formula was used to calculate the amount of carbon dioxide evolved from the fungal cultures and collected in CO_2 traps (Stotzky, 1965).

Milligrams (in 150 ml culture) of carbon dioxide = (B-V) × N× E

Where

B = Volume (milliliters) of HCl needed to titrate the sodium NaOH in the 50ml Erlenmeyer flask from the control jar with no fungal species to the endpoint.

V = Volume (milliliters) of HCl needed to titrate the NaOH in the 50ml Erlenmeyer flask from the jar exposed to the fungal species to the endpoint.

N = Normality of the HCl and

E = Equivalent weight (mg) of carbon dioxide, where E= 22

2.4 Results

2.4.1 Fungal biomass production

To evaluate the increase in number of cells of the three fungal species, two methods were used to measure absorbance and dry weight. Results from the absorbance readings are shown in the Appendix (Figure 21). These data are presented in the appendix as they were similar in trends to those present in my B.Sc thesis. The three fungal treatments showed very different growth pattern when grown on different substrates for a period of 3 weeks. The mixture of *T. koningii* and *P. janthinellum* showed the highest biomass production in all the different media culture, except for the positive control, PDB, when compared to individual species grown at the same temperature during 576 hours. The total dry biomass production of all the species growing on different experimental substrates ranged between 4.2 mg/ml and 59.5 mg/ml over 576 hours.

The biomass production curves showed the three distinct growth phases (lag, exponential and stationary) for three fungal cultures on all the different substrates. After the lag phase, a rapid increase in the biomass production of biomass was observed during the exponential phase for the single and mixed species between 72 hours and 384 hours. Beyond 384 hours, no significant change in biomass production was observed during the stationary phase for all the species grown on different carbonaceous substrates, except for positive control where rapid increase was observed from 12 hours to 432 hours. The

mixed cultures showed about 1.5 times higher biomass production than the two single cultures (Figure 2A). The single cultures of *T. koningii* and *P. janthinellum* showed similar increases in biomass production but much lower than that of the mixed culture (Figures 22 in appendix). The mixture of UDA+PDB produced the highest amount of biomass compared to the other substrates. The maximum amount of measured biomass observed was in mixed culture grown on this substrate was measured to be 59.5 +/- 0.702 mg/ml culture (8.9 +/- 0.11 g/ 150 ml culture) at 432 hours (Figure 2A). These differences were statistically different at $p< 0.001$. These results show that the mixed culture of two species when grown on mixture of UDA+PDB enhances the growth of fungal species. The biomass production in the rest of the carbon substrates, UDA, DA and UDA+DA was much lower (Figures 22 in appendix). These results show that the co-culture of two fungal species is able to utilize the mixture of fatty acid (UDA) and PDB much more efficiently than fatty acids alone. The one way ANOVA test (Table 6.1) shows that means of biomass production by three fungal species are significantly different. The LSD test (Table 6.2) shows that there is significant difference in production of biomass by mixed species culture compared to *T. koningii* and *P. janthinellum*. There is no significant different in biomass production between *T. koningii* and *P. janthinellum*

Interestingly, the production of biomass was highest when all fungal cultures were grown in the PDB alone (Figure 2B). Statistical analysis (ANOVA) show that the individual fungal species and their mixed culture growing on PDB were able to produce more biomass than the fatty acids sources. The results obtained for the production of biomass are highly significant as the R^2 for the ANOVA model is 0.999 (Table 6). The

three fungal cultures produced almost twice the amount of biomass on potato dextrose broth compared to all the other substrates. Table 6.4 shows that fungal species produce significant different biomass when PDB is the carbon source and also when UDA+PDB is the carbon source the biomass production is significantly different the when UDA+DA is the carbon source. Other substrates show no significant effect of biomass production. All three fungal cultures showed a longer exponential phase between 24 hours and 384 hours (Figure 2B). Noteworthy, biomass production for the three fungal cultures growing on PDB was the same and ranged between 75.2 and 76.4 mg/ml at 384 hours (Figure 2B). These results show that the individual and mixed fungal cultures are able to utilize all three sources of fatty acids (UDA, DA and UDA+DA) as sole source of carbon and energy, but highest amounts of biomass produced when PDB is the carbon substrate (UDA+PDB or PDB).

Table 6: Analysis of variance for testing potential statistical differences between biomass production by three fungal species; *Trichoderma koningii*, *Penicillium janthinellum* and mixed culture of *Trichoderma koningii* and *Penicillium janthinellum* growing on different carbon substrates over a period of 576 hours.

Tests of Between-Subjects Effects					
Dependent Variable: Biomass (mg/ml)					
Source	Type III Sum of Squares	df	Mean Square	F	Significant
Carbon source	2.62	4	.66	5561.74	<0.001
Species	.35	2	.17	1478.00	<0.001
Time	35.80	12	2.98	25338.15	<0.001
Carbon source * Species	.06	8	.01	66.51	<0.001
Carbon source * Time	2.66	48	.06	470.28	<0.001
Species * Time	.09	24	.004	30.59	<0.001
Carbon source * Species * Time	.23	96	.002	20.08	<0.001
Error	.04	312	.000		
Total	993.05	507			

a. R Squared = .999 (Adjusted R Squared = .999)

b. Corrected model and intercept values are also significant

c. df- degree of freedom (df = N−1)

d. F = F- test ratio (Mean square/ mean square error)

Table 6.1: One-way ANOVA showing the biomass means to differ significantly when fungal species are fixed factor.

Biomass (mg/ml)					
	Sum of Squares	df	Mean Square	F	Significant
Between Groups	2807.04	2	1403.52	5.36	.005
Within Groups	132033.64	504	261.97		
Total	134840.68	506			

a. Significance level – 0.05

b. df- degree of freedom (df = N−1)

c. F = F- test ratio (Mean square/ mean square error)

Table 6.2: Least significant difference post hoc comparisons of biomass (mg/ml) when the fixed factor is fungal species.

Dependent Variable: Biomass (mg/ml), Fixed factor: Fungal species						
(I) Fungal species	(J) Fungal species	Mean Difference (I-J)	Std. Error[a]	Sig[b]	95% Confidence Interval	
					Lower Bound	Upper Bound
Trichoderma koningii	Penicillium janthinellum	.66	1.76	.71	-2.80	4.12
	Mixed culture	-4.63*	1.76	.01	-8.09	-1.17
Penicillium janthinellum	Trichoderma koningii	-.66	1.76	.71	-4.12	2.80
	Mixed culture	-5.29*	1.76	.00	-8.75	-1.83
Mixed culture	Trichoderma koningii	4.63*	1.76	.01	1.17	8.09
	Penicillium janthinellum	5.29*	1.76	.00	1.83	8.75

a. Standard error
b. Significant
c. The mean difference is significant at the 0.05 level.

Table 6.3: One-way ANOVA showing the biomass means to differ significantly when fungal species are fixed factor.

Dependent variable: Biomass (mg/ml)					
	Sum of Squares	df	Mean Square	F	Significant $p < 0.05$
Between Groups	20976.11	4	5244.03	23.12	<0.001
Within Groups	113864.57	502	226.82		
Total	134840.68	506			

a. df- degree of freedom (df = N−1)
b. F = F- test ratio (Mean square/ mean square error)

Table 6.4: Least significant difference (LSD) post hoc comparisons of biomass (mg/ml) when the fixed factor is Carbon source.

Dependent variable: Biomass (mg/ml), Fixed factor: Carbon source						
(I) Carbon source	(J) Carbon source	Mean Difference (I-J)	Std. Error[b]	Sig[c].	95% Confidence Interval	
					Lower Bound	Upper Bound
UDA	DA	.26	1.97	.90	-3.61	4.13
	UDA+DA	1.55	1.97	.43	-2.31	5.42
	UDA+PDB	-2.65	1.97	.19	-6.52	1.21
	PDB	-23.71*	2.78	.00	-29.18	-18.24
DA	UDA	-.26	1.97	.90	-4.13	3.61
	UDA+DA	1.29	1.97	.51	-2.57	5.16
	UDA+PDB	-2.91	1.97	.14	-6.78	.96
	PDB	-23.97*	2.78	.00	-29.44	-18.50
UDA+DA	UDA	-1.55	1.97	.43	-5.42	2.31
	DA	-1.29	1.97	.51	-5.16	2.57
	UDA+PDB	-4.21*	1.97	.03	-8.08	-.34
	PDB	-25.26*	2.78	.00	-30.73	-19.79
UDA+PDB	UDA	2.65	1.97	.18	-1.21	6.52
	DA	2.91	1.97	.14	-.96	6.78
	UDA+DA	4.21*	1.97	.03	.34	8.08
	PDB	-21.06*	2.78	.00	-26.53	-15.59
PDB	UDA	23.71*	2.78	.00	18.24	29.18
	DA	23.97*	2.78	.00	18.50	29.44
	UDA+DA	25.26*	2.78	.00	19.79	30.74
	UDA+PDB	21.06*	2.78	.00	15.59	26.53

a. The mean difference is significant at the 0.05 level.
b. Standard error
c. Significant

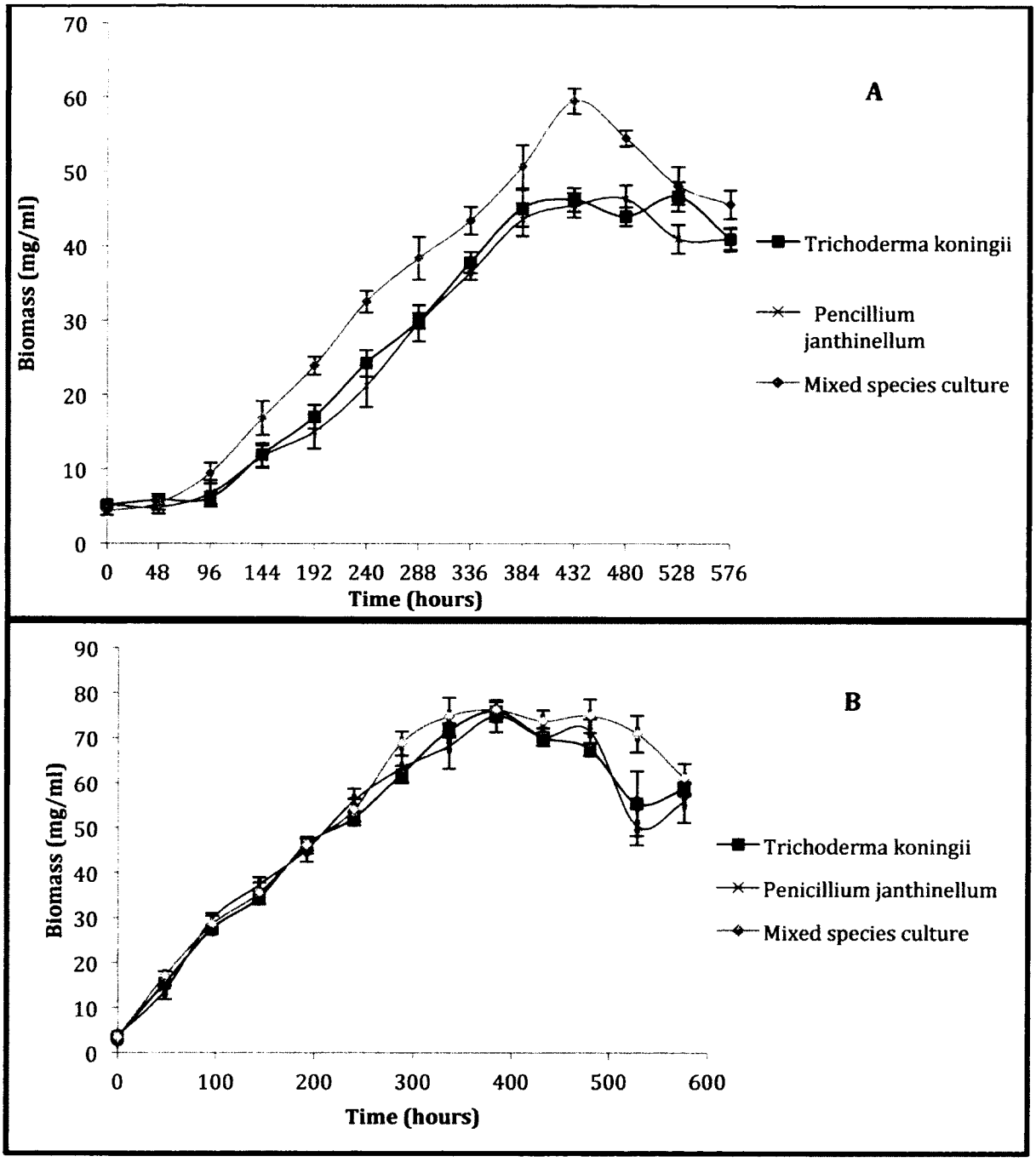


Figure 2: The biomass growth curves of three fungal species; *Trichoderma koningii (Blue)*, *Penicillium janthinellum (red)* and mixed culture of *Trichoderma koningii*, *Penicillium janthinellum (green) A)* growing on mixture of potato dextrose broth and undecanoic acid; B) potato dextrose broth as the sole sources of carbon and energy source in a batch culture at 34 °C +/ - 1 °C over a period of 576 hours. All the cultures were grown in triplicates, thus values presented are an average of 3 readings. Vertical bars represent standard deviation values.

The mixed culture of *T. koningii* and *P. janthinellum* always showed the highest biomass production on all substrates containing fatty acids (Figures 22 in Appendix). Biomass size was lower in the fatty acid treatments than the positive control (PDB). Growing on fatty acids as the sole source of carbon and energy, *T. koningii* showed slightly higher biomass production than *P. janthinellum*. The three fungal cultures were able to grow best on mixture of UDA+PDB followed by UDA> UDA+DA> DA. The growth rate of the fungal species were not determined but the production of biomass was proportional to CO_2 production (Table 8), thus its likely that the growth rates are similar to CO_2 production rates.

2.4.2 Decarboxylation of fatty acids

The decarboxylation of fatty acids was measured by collection of CO_2 from the fungal cultures. If it is assumed that the production of CO_2 will result from the preferential removal of the carboxyl group of the fatty acids. During fermentation and respiration, almost all the CO_2 evolved by microorganisms is produced by decarboxylation of organic acids (Ochoa, 1951). Thus the amount of CO_2 produced indicates the degree of decarboxylation of the fatty acids. It is expected that the remaining aliphatic moiety of fatty acids will be converted by fungal species into hydrocarbons and other intermediate metabolites.

2.4.2.1 Evolution of carbon dioxide from fungal species growning on different carbon substrates.

Figure 3 shows the cumulative CO_2 evolved during the incubation study. The increase in production of carbon dioxide over time shows similar trends as those observed for the amount of biomass production by the three fungal cultures. The

evolution of CO_2 was highest in the mixed culture for each of the fatty acids containing substrate, except for the positive (PDB) control (Figure 3). Overall, the mixed species culture growing on all substrates, except PDB, produced twice the amount of CO_2 compared to single species. Among the different substrates, the three fungal cultures produced the highest amount of CO_2 when grown in a mixture of UDA+PDB. Amongst single cultures, *T. koningii* produced more carbon dioxide than *P. janthinellum* growing in UDA and the mixture of UDA+PDB. On DA media and UDA+DA media, both species produced the same amount of CO_2. The highest amount of CO_2 was produced by the mixed culture grown on UDA+PDB. The total cumulative carbon dioxide produced by this mixed culture was 394.2 +/- 23.2 mg/150 ml culture after 576 hours (Figure 3A). Similarly, total CO_2 produced by *T. koningii* was 323.7 +/- 17.4 mg/ 150 ml culture, and the least amount of CO_2 was produced by *P. janthinellum* was 299.4 +/- 11.2 mg/ 150 ml culture after 576 hours. Among the different substrates, the mixed culture produced the least amount of CO_2 when grown in decanoic acid alone.

The total CO_2 produced by individual fungal species and mixed species culture growing on PDB is 2 to 3 times higher compared to the other experimental substrates (UDA, DA, UDA+DA and UDA+PDB) (Figure 3B). The total carbon dioxide produced by mixed culture of *T. koningii and P. janthinellum* on PDB was 624.3 +/- 30.8 mg/150 ml culture over a period of 576 hours. Similarly, total carbon dioxide produced by individual cultures of *T. koningii* and *P. janthinellum* growing on PDB was 613.4 +/- 25.7 and 611.9 +/- 21.1 mg/150 ml culture during 576 hours.

The average carbon dioxide production was highest from the mixed species culture. The mixed culture oxidized the mixture of UDA+PDB to a larger extent than the

UDA alone (Figure 3 and Figure 23 in Appendix). The results for production of carbon dioxide are highly significant with R^2 of 0.997 ($p < 0.001$) (Table 7). The cumulative carbon dioxide produced by three fungal species growing in PDB (positive control) over 576 hours is presented in Figure 3B. In the case of the positive (PDB) control, the three fungal species showed no significant difference in the carbon dioxide production.

The one-way ANOVA and LSD (Table 7.1 and 7.2) test shows that there is significant difference in the means of cumulative carbon dioxide production of three fungal species. The pairwise comparison shows that the mixed species culture produces significantly different amount of carbon dioxide that individual species, but between *T. koningii* and *P. janthinellum* there is no significant different is production of carbons dioxide. In case of pairwise comparison with carbon source shows that all the carbon sources significantly effect the production of carbon dioxide and fungal species produce significantly different carbon dioxide on all carbon substrates (Table 7.3 and 7.4).

The rate of CO_2 evolution from each culture growing on the mixture of UDA+PDB is presented in Figure 4A. The rate of CO_2 evolution for all three species (mixed species and single cultures) was highest when grown in the mixed (UDA+PDB) substrate compared to the fatty acids alone. Among the three cultures growing on all the fatty acid containing substrates, the production of carbon dioxide, was highest in the mixed culture > *T. koningii* > *P. janthinellum*. In the case of all three fungal cultures, the rates of CO_2 evolution increased gradually after 96 hours reaching a maximum at 192 hours, decreasing gradually thereafter. The rate of CO_2 was lowest and remained constant beyond 384 hours. The maximum rate of CO_2 evolution for the mixed culture, *T. koningii* and *P. janthinellum* were 17.4, 15.7 and 15.2 mg CO_2/mg biomass/day respectively, at

192 hours. These CO_2 results, when compared with the biomass production of the three fungal species growing in a mixture of UDA+PDB had similar trends. The decarboxylation activity of fungal species, mixed culture, *T. koningii* and *P. janthinellum* growing on UDA produced higher amount of CO_2 than other fatty acids substrates (DA, UDA+DA). The decarboxylation activity by the mixed species culture, *T. koningii* and *P. janthinellum* was calculated to be 97.14%, 82.1% and 59% respectively, of the total COOH in UDA after 576 hours of incubation.

The rates of carbon dioxide evolution for the three fungal cultures growing on PDB are presented in Figure 4B. These fungal cultures metabolized PDB rapidly, after 24 hours. The three cultures showed very similar rates of CO_2 and reached a maximum after 72 hours of growth. Thereafter, the rate of carbon dioxide production decreased constantly until 432 hours of the incubation. The maximum decarboxylation rates for the mixed species culture, *T. koningii* and *P. janthinellum* were 15.4, 14.3 and 13.8 μg CO_2/mg biomass/day respectively after 96 hours of incubation. Even though the maximum rates of CO_2 evolution found in the PDB positive control are slightly lower than in the other treatment fatty acid substrates, the fungal species growing in PDB produced higher total amount of carbon dioxide during 576 hours of growth (Figures 3 and 4).

Table 7: Analysis of variance for testing potential statistical differences between carbon dioxide production by three fungal species; *Trichoderma koningii*, *Penicillium janthinellum* and mixed culture of *Trichoderma koningii* and *Penicillium janthinellum* growing on different carbon substrates over a period of 576 hours.

Tests of Between-Subjects Effects					
Dependent Variable: Carbon dioxide					
Source	Type III Sum of squares	df[c]	Mean Square	F[d]	Significance $p<0.05$
Carbon source	1388042.20	4	347010.55	6761.20	<0.001
Fungal Species	164319.51	2	82159.76	1600.81	<0.001
Time	3252682.30	12	271056.86	5281.31	<0.001
Carbon source * Species	36930.50	8	4616.31	89.95	<0.001
Carbon source * Time	430505.22	48	8968.86	174.75	<0.001
Species * Time	47503.84	24	1979.33	38.57	<0.001
Carbon source * Species * Time	46446.86	96	483.82	9.43	<0.001
Error	16013.02	312	51.32		
Total	13352348.59	507			

a. R Squared = .997 (Adjusted R Squared = .995)
b. Corrected model and intercept values are also significant
c. df- degree of freedom (df = N−1)
d. F = F- test ratio (Mean square/ mean square error)

Table 7.1: One-way ANOVA showing the Cumulative carbon dioxide means to differ significantly when fungal species are fixed factor.

Dependent variable: Cumulative Carbon dioxide (mg)					
	Sum of Squares	df[b]	Mean Square	F[c]	Sig[d].
Between Groups	495845.35	2	247922.68	12.35	<0.001
Within Groups	10117060.50	504	20073.53		
Total	10612905.85	506			

a. Significance level – 0.05
b. df- degree of freedom (df = N−1)
c. F = F- test ratio (Mean square/ mean square error)
d. Significant

Table 7.2: Least significant difference (LSD) post hoc comparisons of Cumulative carbon dioxide (mg) when the fixed factor is fungal species

Dependent Variable: Cumulative carbon dioxide (mg) , Fixed factor: Fungal species

(I) Fungal species	(J) Fungal species	Mean Differen -ce (I-J)	Std. Error[a]	Sig[b]	95% Confidence Interval	
					Lower Bound	Upper Bound
Trichoderma koningii	Penicillium janthinellum	22.12	15.41	.15	-8.16	52.40
	Mixed culture	-52.45*	15.41	.00	-82.73	-22.17
Penicillium janthinellum	Trichoderma koningii	-22.12	15.41	.15	-52.40	8.16
	Mixed culture	-74.57*	15.41	.00	-104.86	-44.29
Mixed culture	Trichoderma koningii	52.45*	15.41	.00	22.17	82.73
	Penicillium janthinellum	74.57*	15.41	.00	44.29	104.8

a. Standard error
b. Significant
c. The mean difference is significant at the 0.05 level.

Table 7.3: One-way ANOVA showing the Cumulative carbon dioxide means to differ significantly when carbon sources are fixed factor.

Dependent variable: Cumulative Carbon dioxide (mg)					
	Sum of Squares	df[b]	Mean Square	F[c]	Sig[d].
Between Groups	2758681.92	4	689670.48	44.08	<.001
Within Groups	7854223.93	502	15645.86		
Total	10612905.85	506			

a. Significance level – 0.05
b. df- degree of freedom (df = N–1)
c. F = F- test ratio (Mean square/ mean square error)
d. Significant

Table 7.4: Least significant difference (LSD) post hoc comparisons of Cumulative carbon dioxide (mg) when the fixed factor is Carbon source

Dependent variable: Cumulative carbon dioxide, Fixed factor: Carbon source						
(I) Carbon source	(J) Carbon source	Mean Difference (I-J)	Std. Error[b]	Sig[c].	95% Confidence Interval	
					Lower Bound	Upper Bound
UDA	DA	75.19*	16.35	<0.001	43.06	107.32
	UDA+DA	36.61*	16.35	.026	4.48	68.75
	UDA+PDB	-18.03	16.35	.271	-50.16	14.10
	PDB	-221.48*	23.13	<0.001	-266.92	-176.04
DA	UDA	-75.19*	16.35	<0.001	-107.32	-43.06
	UDA+DA	-38.58*	16.35	.019	-70.71	-6.44
	UDA+PDB	-93.22*	16.35	<0.001	-125.35	-61.09
	PDB	-296.67*	23.13	<0.001	-342.11	-251.23
UDA+DA	UDA	-36.61*	16.35	.026	-68.75	-4.48
	DA	38.58*	16.35	.019	6.44	70.71
	UDA+PDB	-54.64*	16.35	.001	-86.77	-22.51
	PDB	-258.09*	23.13	<0.001	-303.53	-212.66
UDA+PDA	UDA	18.03	16.35	.271	-14.10	50.16
	DA	93.22*	16.35	<0.001	61.09	125.35
	UDA+DA	54.64*	16.35	.001	22.51	86.77
	PDB	-203.45*	23.13	<0.001	-248.89	-158.01
PDB	UDA	221.48*	23.13	<0.001	176.04	266.92
	DA	296.67*	23.13	<0.001	251.23	342.11
	UDA+DA	258.09*	23.13	<.001	212.66	303.53
	UDA+PDB	203.45*	23.13	<.001	158.01	248.89

a. The mean difference is significant at the 0.05 level.
b. Standard error
c. Significant

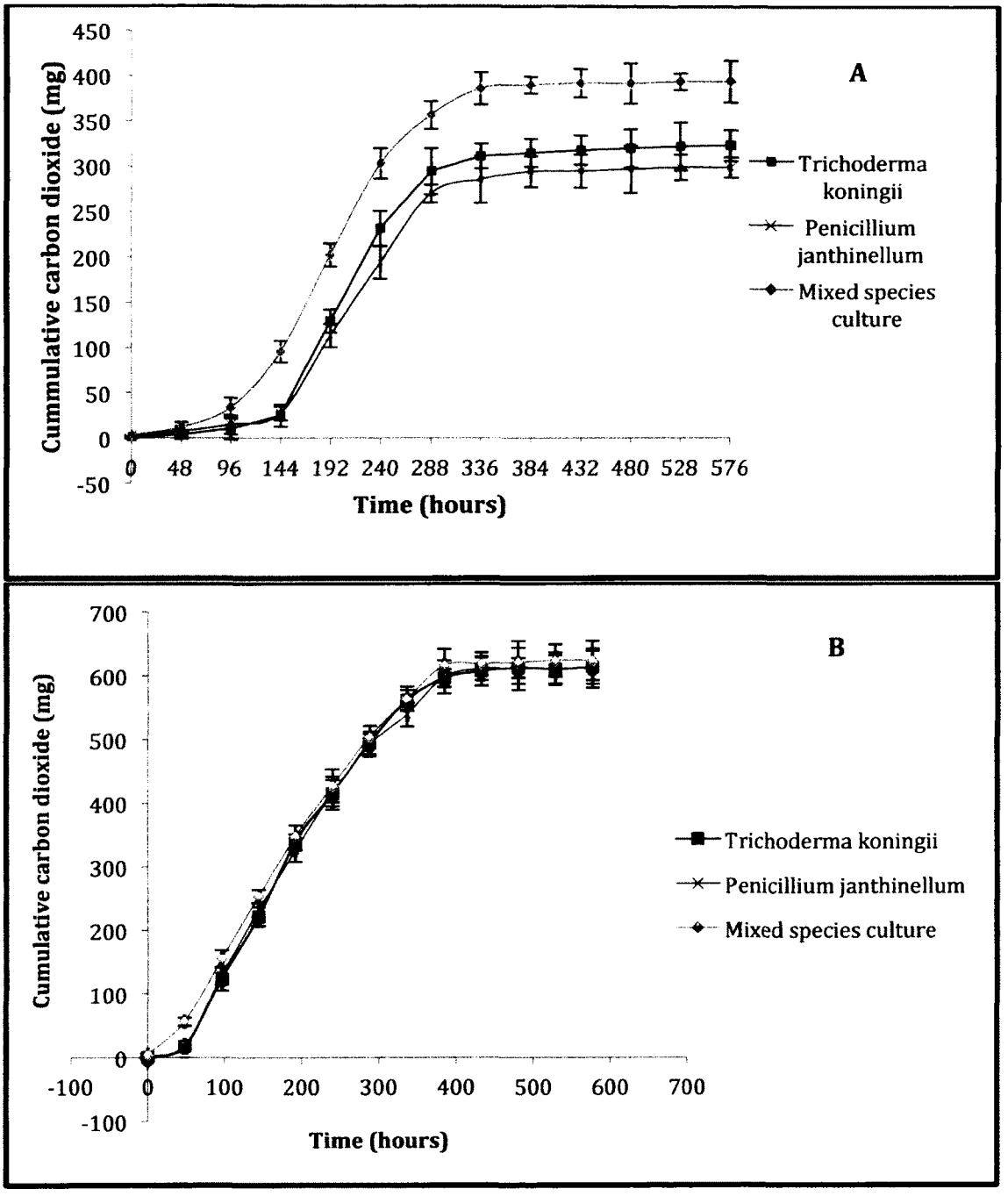


Figure 3: Cumulative carbon dioxide evolved from 150 ml of culture during the growth of three fungal species; *Trichoderma koningii (blue)*, *Penicillium janthinellum (red)* and mixed culture of *Trichoderma koningii* and *Penicillium janthinellum (green)* growing in a A) mixture of UDA+PDB; B) PDB as the sole source of carbon and energy in a batch culture at 34 °C +/ - 1 °C during 576 hours. All the cultures were grown in triplicates, thus values presented are an average of 3 readings. Vertical bars represent standard deviation values.

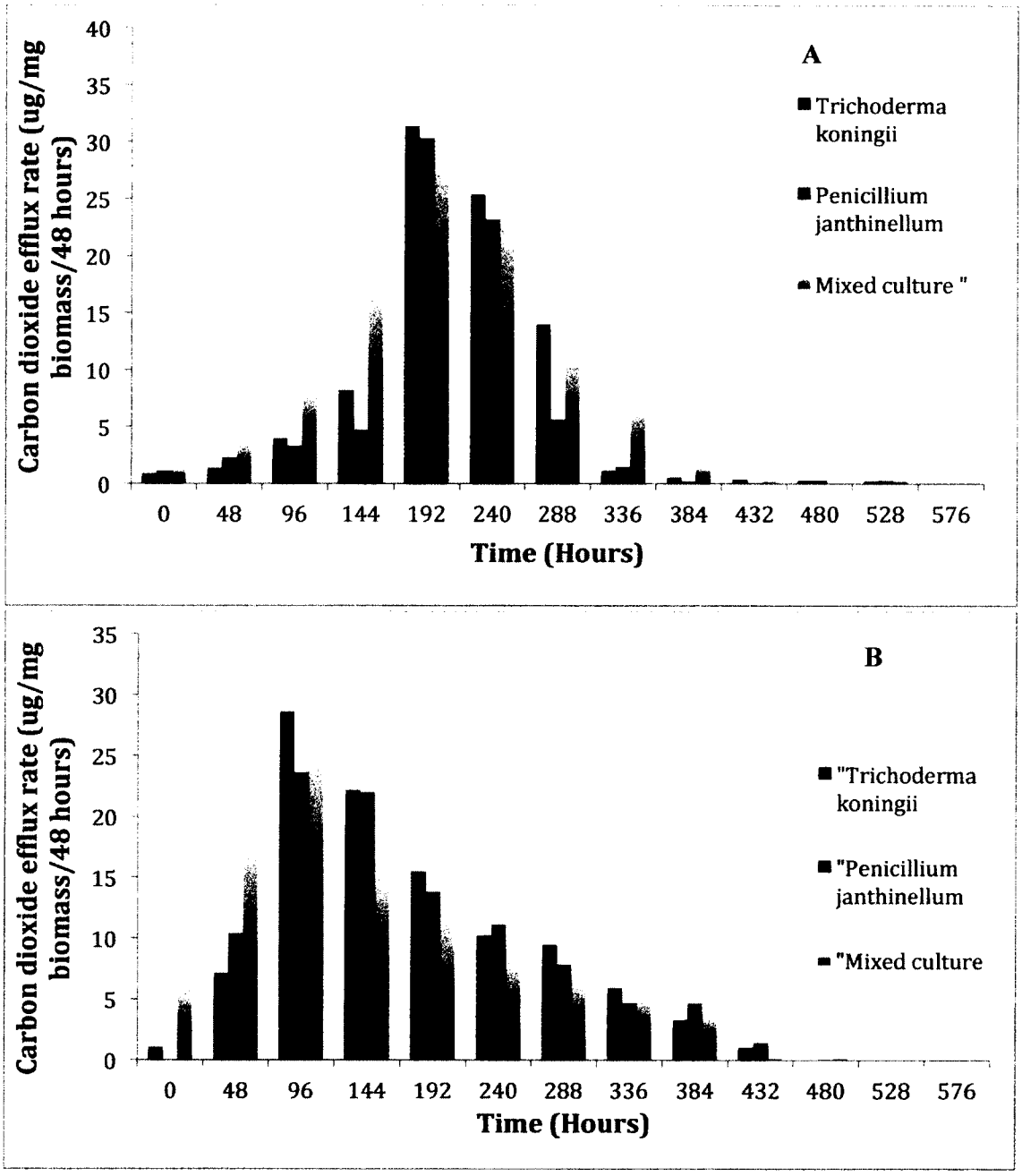


Figure 4: The average rate of carbon dioxide evolution by 150 ml of mixed culture (green), *T. koningii* (blue) and *P. janthinellum* (red) growing in the A) mixture of UDA+PDB and B) PDB as the sole carbon and energy source at 34 °C +/ - 1 °C in batch culture during 576 hours. Among the two compounds (UDA and PDB) which is being metabolized to produce CO_2 was not determined.

Table 8: Summary of analysis of correlation between biomass and carbon dioxide produce by three fungal species grown on different substrates over a period of 576 hours.

Correlations			
		Biomass (mg/ml)	Carbon dioxide (mg)
Biomass (mg/ml)	Pearson Correlation	1	.859**
	Sig. (2-tailed)		<0.0001
	N**	507	507
Carbon dioxide (mg)	Pearson Correlation	.859**	1
	Sig. (2-tailed)	<0.0001	
	N	507	507

*Correlation is significant at $p < 0.0001$ level (2-tailed).
** N- number of observations

The production of biomass is proportional to the production of carbon dioxide as shown by the Pearson correlation of 0.86 (Table 8). In summary, data from the biomass and carbon dioxide production measurements from fungal cultures studies confirm that the fungal species are able to use fatty acids as a source of carbon and grow best in a mixed species culture growing on the mixture of UDA+PDB.

2.5 Discussion

Fungi are one of the most diverse classes of microorganisms, and some produce metabolites of high biotechnological importance. Several studies have shown the ability of fungal species to grow in a diverse environment and utilize many different complex molecules as the sole source of carbon and energy. A study by Radwan and Soliman (1988) showed that several species from genera *Aspergillus, Penicillium, Fusarium, Paecilomyces, Trichoderma, Cladosporium* and *Stachybotrys* can use fatty acids as the sole source of carbon. A similar study by Musallam and Radwan (1990) showed that wool colonizing fungal species, *Chrysosporium keratinophilum, Malbranchea,*

Aspergillus fumigatus, A. flavus, and *Scopulariopsis candida* when fed fatty acid found in wool, were able to efficiently utilize them as a source of carbon and energy. Fatty acids are an important part of fungal cells and their growth, and are produced and metabolized naturally by fungi. A study by Aarle and Olesson (2003) showed that species of arbuscular mycorrhizal fungi (AMF) can produce large amounts of lipids consisting of mostly neutral fatty acids and store them as lipid droplets in spores and hyphae. Fungi metabolize the intracellular lipid droplets when the environmental conditions become adverse. In nutrient depleted environments these lipids are catabolized to provide carbon and energy requirements for the fungus (Bago *et al.*, 2003). The fungal metabolism of fatty acids can produce a wide array of metabolites such as hydrocarbons, as microorganisms have a tendency to synthesize alkanes from fatty acids. In a recent study by Park (2005), it was shown that the bacteria *V. furnissii* could produce high amounts of intracellular and extracellular hydrocarbons. This latter study shows a pathway in which fatty acids are converted to alkanes through formation of fatty aldehydes and alcohols.

Trichoderma and *Penicillium* species, obtained from Agriculture and Agri-Food Fungal Collection Centre, were used in this study. I show that these two fungal species can grow on C_{10} and C_{11} fatty acids as their sole source of carbon and energy. In addition *Trichoderma* species can produce ketoalkanes, such as methylalkylketones (MAKs) by removing the carboxyl groups from keto-fatty acids by keto-fatty acid decarboxylase. These keto-fatty acids are formed from conversion of fatty acid to keto -fatty acyl-CoA and inhibition of β-oxidation leads to conversion of keto-fatty acyl-CoA to keto- fatty acid (Yagi *et al.*, 1989). Thus suggesting that these fungal species might be able to convert these fatty acids in alkanes, which is researched in the following chapters. The

two fungal species are mesophiles and grew well at temperatures of 34 °C. The fatty acids, which served as the carbon and energy yielding substrates, may result also in the formation of useful secondary metabolites (Silliker and Rittenberg, 1951) that were not studied as part of this project.

A distinct lag phase was observed when these fungal species were first transferred from potato dextrose agar to the fatty acid medium, as fungi require adaptation to the new environmental conditions. Therefore, in the growth experiments, the two fungal species were conditioned to adapt to the fatty acids as their new sole source of carbon and energy. A mixture of *T. koningii* and *P. janthinellum* grew on even and odd chain fatty acids, as well as a mixture of these two fatty acids.

The results obtained in this study showed little variability as indicated by the standard deviation determined from data obtained from the three replicates for each culture (Figure 2A). An important factor to reduced biomass data variability was the use of blended inoculum from each fungal species, thus allowing similar amount of fungal cells in each culture. The cultures were combined and blended for 30 seconds before inoculating the culture media (Montgomery *et al.*, 2000). The variability in absorbance data was also reduced by reading the absorbance within 30 seconds after taking a sample from the 150 ml bottle of the shaker incubator. The cross contamination with atmospheric carbon dioxide was not significant. Each culture had a blank, with no fungal inoculation, titration of these blank should give amount of atmospheric CO_2 in the cultures. Theoretically, 5 ml HCl should be required to titrate 5 ml NaOH if there is no contamination. In this study the minimum amount of HCl required for the titration was 4.8 ml. Thus, account for 4.4 ml of CO_2in the blanks allowing the contamination of less

than 4%. Since amount of HCl required to titrate blank is used to calculate amount of CO_2 produced in the cultures, the amount of atmospheric CO_2 in the cultures should be almost negligible. A study by Haney *et al.*, 2008 shows that this method of CO_2 measurement is accurate ($R^2 = 0.84$) and several other factors along which atmospheric CO_2 including care in titration and accurately hitting the end point does not effect CO_2 measurements significantly.

Relative to *P. janthinellum*, *T. koningii* showed significantly higher decarboxylation activity when grown on UDA+PBD. These results are comparable with other published studies, as strains from both species have been shown to utilize fatty acids as sole sources of carbon and energy. A study by Radwan and Soliman (1988) showed that several species of *Penicillium* and *Trichoderma* were able to utilize short chain fatty acid such as caprylic, myristic, palmitic and stearic acids as a carbon source. In their study, the fungal species grew more slowly on fatty acids than in glucose, but presented higher accumulation of arachidonic acid when grown on fatty acids. Results obtained from our study and those of others show that *T. koningii* and *P. janthinellum* have ability to metabolize fatty acids.

Results form this experiment showed that CO_2 production paralleled biomass production. In this study, the mixture of two species showed higher biomass production and decarboxylation activity than the single species grown in all 5 different fatty acid and PDB substrates. When fatty acids were used the mixed species, cultures showed high metabolic activity as indicated by the oxidation of substrates to CO_2.

These results are in agreement with studies showing that mixotropic microorganisms can efficiently utilize and degrade complex molecules. It is common in

nature for colonization of habitats by mixtures of microbial populations (Bael *et al.*, 2011). Studies show that such mixtures of organisms are involved in a wide range of active physical and metabolic interactions. These interactions are essential for the growth and survival of species at a site, and also allow for organisms to exist in hostile environments (Brenner *et al.*, 2008). These interacting mixtures of microorganisms are known as microbial consortium interactions (Brenner *et al.*, 2008). The consortium interactions between bacteria and fungi or two fungal species or a mixture of many microbial species have been observed in degradation of complex carbon sources such as fatty acids, poly-aromatic hydrocarbons (Brenner *et al.*, 2008). This technique of consortium interactions is widely used in bioremediation in which a mixture of microbe is used to degrade poly-aromatic hydrocarbon or other pollutants (Aislabie *et al.*, 2006). During consortium interactions, one species breaks down the long substrate into a short chain carbon source, which is utilized by a second species as a carbon source and degrades into carbon dioxide and water (Brenner *et al.*, 2008). Consortium interactions enhance the growth rate of the microbial species and the chances of survival in the harsh environments with the complex carbon sources. In our research, the experimental results suggest that consortium interactions may be a possible utilization mechanism when the mixed species culture metabolized single or mixture of fatty acids or UDA+PDB, but which species is utilizing the fed fatty acids was not investigated.

The mixed culture produced the highest amount of biomass of 59 mg/ml in 150 ml culture (Figure 2A) when grown in a mixture of UDA+PDB. Similarly, the highest carbon dioxide evolution of 394 mg/150 ml culture (Figure 3A) was also observed in the same growth media. The single species showed higher biomass production and

decarboxylation activity in a mixture of UDA+PDB than in the fatty acids alone. These results are similar with other studies dealing with the use of microbes for bioremediation (Aislabie *et al.*, 2006). Cometabolism is a process in which there is a simultaneous degradation of two compounds. In the case of the mixed substrate media (mixture of UDA+PDB), one compound is a more easily digestable carbon source and the other is a less readily digestible. It is hypothesized, that in the case of the mixed substrate, with mixed species, the two fungal species use PDB as the preferred substrate to produce energy, proteins, cofactors and enzymes, before utilizing the fatty acid sources. Further research is required to prove that these fungal species prefer one substrate to the other. Cometablism and consortia interactions together could be responsible for the highest biomass and CO_2 production by the mixed species culture in a mixture of UDA+PDB. Future research is required to elucidate the preferred mechanism of substrate utilization and the fungal species interactions.

In this study, all three fungal cultures showed different production of biomass and CO_2 production. This study showed the ability of the two fungal species to grow on fatty acids as the sole source of carbon and energy, and opened up the doors to study the quantitative production of different lipids in the fungal cell cultures. Because fatty acids are an immediate precursor for the production of alkanes, and other metabolites these fungal species may be able to act as biocatalysts in the development of a new generation biofuels.

The next two chapters present and discuss the transformation of simple species of fatty acids into other classes of lipids (Chapter 3) and alkanes (Chapter 4).

2.6 Conclusion

Both the objectives of this research project were achieved. The two fungal species, *T. koningii* and *P. janthinellum* and their mixed culture grow on fatty acids as their sole source of carbon and energy. Relative to the single species the mixed species culture showed the highest biomass production on all the carbonaceous substrates. The two fungal species (alone or in mixed culture) were able to utilize odd-number (C11) fatty acids more biomass and carbon dioxide production than the even number (C10) fatty acids. The mixed culture showed the highest growth and decarboxylation activity when grown on the mixture of UDA+PDB, and the lowest growth in decanoic acid alone. Fungal species grown in PDB produced higher amounts of biomass and CO_2 than when grown on the fatty acid containing substrates.

Chapter 3

Conversion of fatty acids into intracellular and extracellular classes of lipids by the mixed culture of *Trichoderma koningii* and *Penicillium janthinellum*

3.1 Abstract

The microbial lipid molecules such as alkanes, alkenes, esters, alcohols and fatty acids have the potential to serve as a source of significant quantities of transportation fuels. Microscopic analysis of intracellular lipids produced by mixed species culture of *T. koningii* and *P. janthinellum* on all different carbon substrates were analyzed using nile blue dye. The fluorescent intensity corresponds to the accumulation of intracellular lipids. The highest accumulation of lipids was observed during exponential phase when fatty acids are present as a source of carbon. The cell size of fungal mycelia is also larger during exponential phase than compared to stationary phase. When PDB is sole source of carbon, the high fluorescent intensity was observed during stationary phase, thus corresponding to high accumulation of lipids. The comprehensive and comparative measurement of lipid classes and fatty acids in lipid fractions extracted from mixed fungal species culture of growing on different carbon substrates was conducted. Analyses included lipid extracts from exponential and stationary phase. Lipid classes were analyzed by Iatroscan thin-layer chromatography with flame ionization detection (TLC-FID) and fatty acid composition by gas chromatography with flame ionization detection (GC-FID). Different lipid classes determined include hydrocarbons, free fatty acids, phospholipids, sterols, acetone mobile polar lipids, diacylglycerols, triacylglycerols, steryl esters/wax esters, and alcohols. The highest total lipid content of 518 mg were observed to be accumulated intracellularly when mixed species culture was grown on UDA+PDB during the exponential growth phase. The fatty acids were the most dominant lipid class produced intracellularly and extracellularly during both growth phases, which account for >80% of total lipids produced. When PDB is the sole source of carbon,

triacylglycerols are the most dominating lipid class produced by mixed species culture. The fungal mixed culture grown on different carbon sources show highest amount of hydrocarbons when grown in a mixture of UDA+PDB, where hydrocarbons account of 4% for the total lipids produced. The saturated fatty acids (SFA) accounts for 75 % of total free fatty acids (FFA) produced during exponential phase and the concentration of mono- unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) were similar, and their content ranged from 23% to 66% of the total FFA, depending on substrate and growth phase. Out of 55 fatty acids analyzed, the most dominating fatty acids on all substrates were palmitic acid (C16), stearic acid (C18), oleic acid (C18:1), and linoleic acid (C18:2), which account for nearly 80% of FFA produced.

3.2 Introduction

Fungi are one of the least explored microorganisms with regards to the extent of their biodiversity, properties and biotechnological abilities (Ul-Hassan *et al.*, 2012). Fungal species are known to produce a wide variety of secondary metabolites. Intracellular and extracellular lipids are some of the secondary metabolite produced by the fungi (Ul-Hassan *et al.*, 2012). Fungal species synthesizing extracellular hydrocarbons can be used for the production of biofuels. Global climate change and decreasing fuel reserves are driving the development of biofuels via microbial catalysis (Glanoulis *et al.*, 2012). Several studies have shown that the potential production of hydrocarbons by fungi has not been fully explored due to the presence of cryptic biosynthetic pathways. Cryptic biosynthetic pathways include a cluster of genes that are present at distal ends of chromosomes and they are silent under normal laboratory growth conditions. These cryptic biosynthetic pathways are active under harsh environmental conditions or during symbiosis (Bok *et al.*, 2006). Several studies have shown that many different fungal species can synthesize hydrocarbons and store other lipids depending on the environmental conditions (Ul-Hassan *et al.*, 2012; William *et al.*, 2008). Many fungal species that have a high rate of synthesis and accumulation of lipids are endophytes such *as Hypoxylon* species, *Ascocoryne sarcoides, Plantago lanceolata* and many others (Ul-Hassan *et al.*, 2012; Aarle and Olsson, 2003). It has been suggested that plant-fungal interactions could activate these cryptic pathways that lead to overproduction of lipids and other secondary metabolites (Strobel, 2006). Studies have shown that there is a high synthesis of lipids when grown under limited oxygen during culture conditions (Glanoulis *et al.*, 2012). Lipids produced are of great importance to fungal species: these

are stored as fatty acids to be used for energy in growth limiting conditions leading to production of other short-chain lipids. Fatty acids are also incorporated into the cell wall where they play a protective function (Aarle and Olsson, 2003). LCFA stored inside the fungal cells are energy-rich compounds that support the growth of microorganisms that thrive in environments with a low nutrient availability (Olsson and Johansen, 2000; Aarle and Olsson, 2003). Similar to fatty acids, hydrocarbons are arranged at the fungal cell wall surface and control the physiochemical properties of cytoplasmic membranes (Weete, 1972). The hydrocarbons also act as source of carbon and energy. Formation of capsules on the surface of the cell wall by hydrocarbons protects them from high concentration of acids and from other microbes (Bagaeva and Zinurova, 2004).

3.2.1 Fatty acid metabolism

Microorganisms use sugars as a starting material for the formation of fatty acids. The overall process of formation of fatty acids from sugars is presented in Figure 5. The sugars undergo glycolysis, where sugars such as glucose or sucrose are converted to pyruvate through the action of several enzymes presented such as in Figure 5. Pyruvate produced either goes through a transhydrogenase cycle, where it is converted into malate or citrate, which in turn are recycled back into pyruvate (Ratledge, 2004). The pyruvate produced directly during glycolysis or indirectly through the transhydrogenase cycle is dehydrogenated into acetyl-CoA by pyruvate dehydrogenase (Ratledge, 2004). Malate and citrate produced during transhydrogenase cycle could also be converted to acetyl-CoA through involvement of enzyme ATP:citrate lyase (Ratledge, 2004). Acetyl- CoA acts as the precursor for fatty acid synthases, which lead to the production of fatty acids (Rude and Schirmer, 2009). Acetyl- CoA is converted to malonyl-CoA by acetyl-CoA

carboxylase, which is converted to Acyl-ACP through several enzymatic reactions (Figure 5). The Acyl-ACP is then converted into fatty acids (Ratledge, 2004). Fatty acids undergo Claisen condensation reaction leading to elongation of fatty acid chain length through the addition of malonyl-CoA which adds two carbons to the fatty acid chain. The Claisen condensation mechanism is a commonly used mechanism in biological system to synthesize fatty acids, polyketides and steroids (Wackett, 2010). The mechanism behind this reaction involves two reactants: acyl-CoA and - carboxylate of alkanoic acid. Decarboxylation of alkanoic acid generates a carbanion that attacks the carbonyl carbon of acyl- CoA leading to the formation of long-chain fatty acids. This mechanism is seen in microbes, plants and animals for biosynthesis of fatty acids (Wackett, 2010).

Fatty acids are further catabolized into several other metabolites and carbon and energy sources during nutrient depleted conditions. Stored fatty acid can be converted back to carbohydrates (glucose) to be used as source of carbon and energy source through β-oxidation. In β-oxidation the fatty acids are converted back into low molecular weight compounds such as acyl-ACP, which are oxidized back into acetyl-CoA. This acetyl-CoA formed goes through TCA cycle to form pyruvate, which is converted back to glucose, which is used as carbon and energy source (Figure 5).

These fatty acids are also incorporated into, triacylglycerol, fatty-aldehydes, fatty alcohols, fatty acid methyl esters, alkanes and also alkenes under certain environmental conditions by many different pathways as presented in Figure 5 (Zang *et al.*, 2011). The fatty acid methyl esters are produced from fatty acids through the process of transestrification. The fatty aldehydes are produced through the reduction of fatty acids by enzyme fatty acyl-CoA reductase. The fatty alcohols are formed through the reduction

of fatty aldehydes by acyl-CoA reductase. The alkanes are formed through decarboxylation of fatty acids or through decarbonylation of fatty aldehyde formed fatty acids (Strobel *et al*, 2012; Ladygina *et al.*, 2006). The exact mechanism through which alkane formation occurs in fungi is not known.

Figure 5 show that the formation of alkenes from fatty acids involves several steps. Alkanes and alkenes are of particular interest for development of new biofuels (Zang *et al.*, 2011). As long chain fatty acids act as a direct precursor for the formation of short- medium- and long-chain alkanes, the manipulation of fungal biocatalysis for alkane fuel production is promising. This research project was conducted to better understand how fatty acid substrate sources are metabolized by the two fungal species reported in Chapter 1.

3.2.2 Research objectives

The overall objective of this study was to determine the allocation and conversion of fatty acids by fungi into physical and chemical cell components. Specific objectives were:

1. Demonstrate the fatty acids conversion by fungi into intracellular and extracellular lipids, using fluorescent microscopy.
2. Determine the fungal conversion of single fatty acids into several intracellular and extracellular chemical classes of lipids.

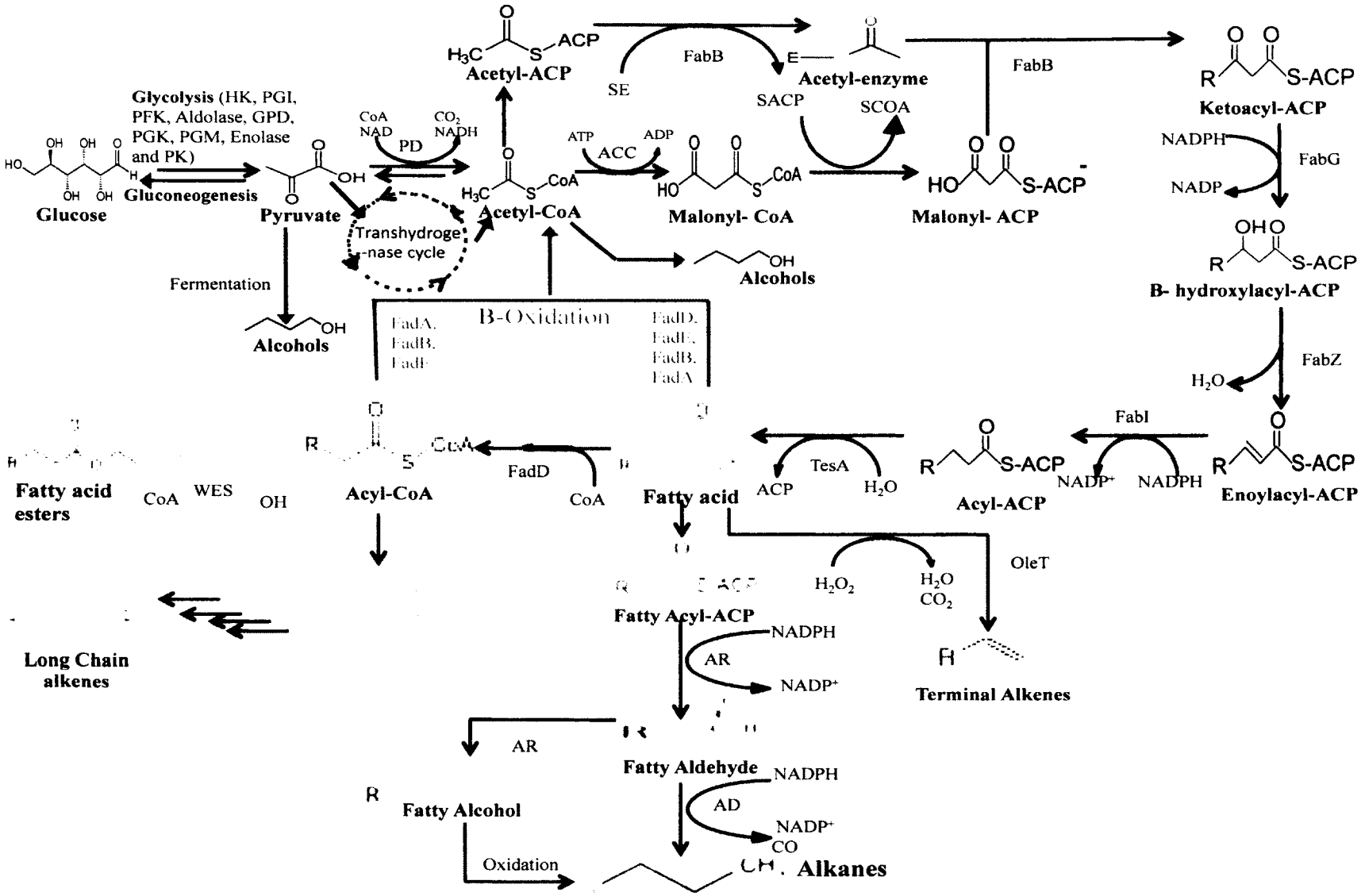

Glycolysis (HK, PGI, PFK, Aldolase, GPD, PGK, PGM, Enolase and PK)
Gluconeogenesis
Glucose
Pyruvate
CoA
NAD
PD
CO_2
NADH
Transhydroge-nase cycle
Fermentation
Alcohols
Acetyl-ACP
Acetyl-CoA
ATP
ACC
ADP
Malonyl- CoA
Malonyl- ACP
SE
FabB
Acetyl-enzyme
SACP
SCOA
Ketoacyl-ACP
NADPH
NADP
FabG
B- hydroxylacyl-ACP
FabZ
H_2O
Enoylacyl-ACP
FabI
NADP+
NADPH
Acyl-ACP
TesA
ACP
H_2O
FadA, FadB, FadI
B-Oxidation
FadD, FadE, FadB, FadA
FadD
CoA
Acyl-CoA
Fatty acid
Fatty acid esters
WES
OH
Long Chain alkenes
OleT
H_2O_2
H_2O
CO_2
Terminal Alkenes
Fatty Acyl-ACP
AR
Fatty Aldehyde
AD
CO
Fatty Alcohol
Oxidation
Alkanes

Figure 5: Biofuel production pathways showing the production of fatty acid from sugars and further conversion of fatty acids into alkanes, alkenes and fatty acid methyl esters. Pathway showing the conversion of stored fatty acids back into sugars. (The different enzymes involved in these pathways- HK-Hexokinase, PGI- Phosphoglucose isomerase, PFK- Phosphofructose kinase, GPD-glyceraldehyde-3-phosphate dehydrogenase, PGK- Phosphoglycerate Kinase, PGM- phosglycerate Mutase, PK- Pyruvate Kinase, ACC- Acetyl-CoA Carboxylase, FabD- malonyl-CoA transacylase, FabB- Keto-acyl-ACP synthase, FabG- keto-acyl-ACP reductase, FabZ- B-hydroxyacyl-ACP dehydratase, FabI- enoyl-acyl-ACP reductase, TesA- acyl-ACP thioesterase, , AR- acyl-ACP reductase, AD-aldehyde decarboxylase, OleT- a cytochrome P450 enzyme, WES- Wax-ester synthase. β- oxidation pathway: FadD- acyl-CoA synthase FadA- 3-keto-acyl-CoA thiolose, FadB- enoyl-CoA hydratase, FadE- acyl-CoA dehydrogenase). This picture is modified from picture in Zang et al., 2011, Current topics in Biotechnology

3.3 Material and Methods

Fluorescent microscopic analysis was performed using Nile blue dye to observe the intracellular and extracellular allocation of fatty acid. The Nile blue dye is used because of it ability to specifically bind to lipid classes such as fatty acids, alkanes, alkenes and other lipids and show the change in fungal lipids over a span of time. Under optical microscope the nile blue gave a blue color to lipids. The same culture samples were also observed under optical microscope because during the lag phase the fluorescence was low; and during the exponential phase the high intensity of fluorescence made it difficult to observe the whole cells and the formed lipid bodies.

3.3.1 Reagents

The stock solution of fluorescent nile blue dye was obtained from Sigma Aldrich, HPLC grade 99% chloroform and methanol was obtained from VWR. Hexane 99% (v/v) and 10% HCl (v/v) was used for cleaning all the glassware used during extraction.

3.3.2 Fluorescent microscopy analysis

The samples used for fluorescent microscopic analysis and those used for the extraction of lipids were taken from the same culture during the experiments described in Chapter 1. Microscopic observations involved culture samples taken from two replicates of each of the three fungal species culture (*T. koningii, P. janthinellum* and a mixture of *T. koningii* and *P. janthinellum),* growing in each of the fatty acid substrates (undecanoic acid (UDA), decanoic acid (DA), mixture of decanoic and undecanoic acid (UDA+DA) and mixture of undecanoic acid and Potato dextrose broth (UDA+PDB). A positive control consisted of each fungal species growing in PDB. An aliquot (0.5 ml) from each

of these cultures was taken in a microcentrifuge tube at 6 different sampling times during the lag phase (0 and 96 hours), exponential phase (192 and 288 hours), and stationary phase (432 and 576 hours). Three hundred microlitres of nile blue dye was then added to each liquid culture sample and was let to sit for 5 minutes at room temperature. After 5 minutes, 2-3 drops from the nile blue dyed culture sample were placed on a glass slide that was covered with a coverslip and viewed under the fluorescent microscope. Microscopic photographs were taken with a Zeiss Axio Imager M2 fluorescent microscope using a 490 nm excitation filter, a 505-nm diachronic mirror and a 520-nm barrier filter with 40X objective lens (Kimura *et al.*, 2004).

3.3.3 Lipid chemical analysis

3.3.3.1 Lipid extraction

For the extraction and analysis of lipids two replicates of the mixed culture of *T. koningii* and *P. janthinellum* growing in UDA media, UDA+PDB media and PDB media (+ve control) were used. Only the mixed fungal culture was used because mixed fungal culture produced highest biomass and carbon dioxide than single species. Also the mixed fungal culture showed higher lipid accumulation than the single species as observed in the microscopic analysis, thus chemical analysis was performed using mixed fungal culture. For lipid extraction, the Bligh and Dyer method was used (Bligh and Dyer, 1959). Briefly, 5 ml aliquots were taken from each of the fungal cultures and placed in 15 ml polypropylene tubes, at 4 different sampling times: lag phase (96 hours), exponential phase (192 and 288 hours), and stationary phase (432 hours). To isolate the fungal mycelia, the fungal aliquot were transferred from the polypropylene tubes to 10 ml glass tubes and were centrifuged at 4500 RPM for 10 minutes in Hettich Zentrifugen

Rotina 420 R centrifuge at 4 °C to separate the liquid supernatant from the mycelia pellet locate at the bottom of the glass tube. The supernatants were then placed into new separate 10 ml glass tubes.

3.3.3.2 Intracellular lipid extraction

The mycelial pellet obtained from each of the samples in the 10 ml glass tubes was then used to extract the intracellular lipids. To each pellet, 1 ml of sterile, ultrapure water was added. The pellet was vigorously mixed in a vortex and then sonicated for 3 minutes using a Sonicator 5-4000 MISONIX, Ultrasonic Liquid processor. Sonication was conducted using amplitude of 35 out of 100, alternating pulse on for 10 seconds, and off for 10 seconds during the 3 minutes. A dry-ice jacket was used to keep the pellet cold. After the 3 minutes sonication, the pellet was centrifuged at 4500 RPM for 12 minutes at 4 °C in the same tube. The cloudy supernatant containing media was removed using Pasteur pipette and placed in a sterile mortar and pestle. The pellet obtained after centrifugation was re-suspended with another 1 ml of sterile deionized water and then sonicated again for 3 minutes as described earlier. This sonicated suspension which was not centrifuged because it contains the cell wall layer with its lipids, was added to the same mortar and pestle containing the previous supernatant phase. To the mortar and pestle suspension, 2 ml of chloroform and 4 ml of methanol were added, making the over all ratio of chloroform to methanol to water equal to 1:2:1. The latter mixture was then homogenized with the pestle for 1 minute. Additional 2 ml of chloroform and 1.6 ml of water was added to the mixture making the overall chloroform: methanol: water ratio of 2:2:1.8. The mixture was further homogenized with pestle for an additional 30 seconds. This mixture was then transferred from the mortar to a 100 ml separatory funnel. The

mortar and pestle was washed with 1 ml chloroform and the suspension added to the separatory funnel. The suspension in the separatory funnel was allowed to sit at room temperature for 30 minutes to 1 hour until the three layers (chloroform –bottom, water-middle and methanol-top) separated out. The chloroform layer containing the lipids was then removed into 10 ml glass tube. These tubes were kept in water bath at 40 °C under a stream of nitrogen gas until all the chloroform evaporated. To the 10 ml glass tube, 500 µL of chloroform was added to mix the lipid extract, which was mixed and then transferred into sterile 1.5 ml screw cap vials and was stored until chemical analysis.

3.3.3.3 Extracellular lipid extraction

The 5 ml supernatant obtained in (3.3.3.1) was placed in separate 10 ml glass tubes until lipid extraction. For extraction, the tubes were placed in a water bath at 45 °C for 24 to 48 hours until the volume left in the tubes was about 1 ml. The 1 ml supernatant solution was then transferred into a sterile mortar and pestle. The tubes were washed with 0.5 ml of chloroform and the washed liquid added to the mortar. An additional 0.5 ml of chloroform and 2 ml of methanol was added to the mortar and pestle, making the overall concentration of chloroform to methanol to water equal 1:2:1. The mixture was then homogenized using the mortar and pestle for 1 minute. An additional 1 ml of chloroform and 800 µl of water was added to the mixture making the overall ratio of chloroform: methanol: water equal to 2:2:1.8. The mixture was further homogenized using the mortar and pestle for an additional 30 seconds. The homogenized mixture was then transferred into 10 ml sterile screw cap glass tubes. Subsequently and after the mortar and pestle was washed with 1 ml of chloroform, the wash chloroform was transferred to the 10 ml glass tube which were then shaken on high speed for 1 minute using the shaker. After shaking

the mixture was transferred to a 100 ml separatory funnel. The 10 ml tubes were washed with 1 ml chloroform, added to the same separatory funnel, that was let sit at room temperature for 30 minutes to 1 hour until the three layers (chloroform –bottom, water-middle and methanol- top) separated out. The chloroform layer containing the lipids was then removed and added to a sterile 10 ml glass tube. The tube was kept in water bath at 40 °C under a stream of nitrogen until all the chloroform evaporated. To the dry residue in the 10 ml glass tubes, 500 μl of chloroform was added to solubilize and mix the lipids. The mixture was transferred into sterile 1.5 ml screw cap vial and was stored until chemical analysis.

3.3.4 Chemical analysis of fungal lipid

The 500 μl stored chloroform extract samples were used for qualitative and quantitative analysis lipid using Iatroscan and GC-FID.

3.3.4.1 Analysis of Lipid classes by Iatroscan

Fifteen different lipid classes were determined using the method by Parrish (1987) at the laboratory of Ocean Sciences Centre of Memorial University of Newfoundland. TLC-FID with a MARK VI Iatroscan was used to analyze 15 lipid classes: hydrocarbons, free fatty acids, phospholipids, sterols, acetone mobile polar lipids, diacylglycerols, triacylglycerols, steryl esters/wax esters, alcohols, ethyl esters, methyl esters, ethyl ketones, methyl ketones and glycerol ethers. First, the Chromarods were cleaned and activated by hydrogen flame through the two blank scan, before analysis of each sample to remove impurities, allowing the Chromarods to be used repeatedly. Then, 10 μ l of intracellular and extracellular lipid extracts were spotted on silica gel coated Chromarods and a three-stage solvent development system was used to separate lipid classes. The first

separation solvent system consisted of two developments, first for 25 minutes and second for 20 minutes, carried out in 99.95:1:0.05 (v/v/v) hexane/diethyl ether/formic acid. The second separation solvent system consisted of only one development of 40-mins in 79:20:1 (v/v/v) hexane/diethyl ether/formic acid. The last separation solvent stage consisted of four development stages. The first two development systems, each of 15-min in 100% acetone, were followed by two consecutive developments, each of 10- min in 5:4:1 (v/v/v) chloroform/methanol/water. Before each solvent system the rods were dried in a constant humitiy chamber. After each development system the rods were scanned in the Iatroscan and the data collected using Peak Simple software (ver 3.67, SRI Inc). The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, Mo., USA). There were no internal standards used for analysis of lipid classes by Iatroscan. The Iatroscan was calibrated after every sample using the standards in table 9.1 and a known amount of sample of standards was analyzed from a known volume. The intracellular and extracellular lipid samples were analyzed and quantified using the standards (Table 9.1) (Jeanette Wells, personal communication). The sample raw data for calibration curve and intracellular lipid extract growing in UDA+PDB during exponential phase (Table 23 and 24 in Appendix).

Table 9.1: The Iatroscan standards used for calibration and analyses of intracellular and extracellular lipids extracts from mixed species culture grown on different carbon substrates.

Compound	**Lipid Class**	**Catalog number (Sigma Aldrich)**
Nanodecane	Hydrocarbon	N-4129
Cholesteryl Stearate	Steryl Ester/Wax Ester	C3549
3-Hexdecanone	Ketone	H-7504
Tripalmitin	Triacylglycerol	T5888
Palmitic acid	Free Fatty acid	P5917
Cetyl alcohol	Alcohol	C7882
Cholesterol	Sterol	C-3137
Monopalmitoyl	Acetone Mobile Polar Lipids	M1640
Phosphatidylcholine Dipalmitoyl	Phospholipids	P5911

3.3.4.2 Analysis of fatty acids by GC-FID

Transesterification with 14% Boron triflouride-Methanol solution (BF_3 MeOH) for 1.5 h at 85 °C was performed on lipid extracts to form fatty acid methyl esters (FAMEs) (Morrison and Smith, 1964). The FAME were analysed on a HP 6890 GC FID equipped with a 7683 autosampler. The GC column was a ZB wax+ (Phenomenex, U.S.A.), 30m in length and with an internal diameter of 0.32 mm. The column temperature profile was as follows: 65 °C for 0.5 min, then ramped to 195 °C at a rate of 40 °C/ min, hold at 195 °C for 15 min, and hold at 220 °C for 0.75 min after ramping at 2 °C min^{-1}. The injector temperature increased from 150 to 250 °C at 200 °C min^{-1}. Peaks were detected by flame ionization, and the detector was held at 260°C. The carrier gas

was hydrogen and flowed at a rate of 2 ml/minute. Peaks were identified using retention times from standards purchased from Supelco, 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033) and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2. A quantative standard purchased from Nu-Chek Prep, Inc (product number GLC490) is used to check the GC column about every 300 samples (or once a month) to ensure that the areas returned are as expected (Copeman and Parrish, 2004) (Jeanette Wells, personal communication)

3.4 Results

3.4.1 Microscopic analysis of fungal lipids

The intracellular lipid allocation was visualized by brief treatment of cultured cells with a dilute aqueous solution of nile blue, which produced an intense orange fluorescent stain of cell lipid (Figures 6, 7 and 8). The orangish color corresponds to binding of the dye to lipid molecules. The intensity of the nile blue fluorescence indicates quantity of lipid accumulation in the mycelium.

During the lag phase and according to the fluorescence and optical observations, there was a negligible amount of lipids allocated intracellularly by the mixed species culture growing on all the carbon substrates (Figure 6). The lipids observed during lag phase were sequestered to the cell wall of the mycelia of mixed species culture grown on all the carbon substrates. Most lipids were found to reside in the growth media when the carbon substrates were UDA and UDA+PDB. During the log phase, the fungal cells were small in length (2 μm diameter, 40 μm length) and were not found in clusters. The mixed

species culture growing in PDB shows almost no allocation of lipids intracellularly during lag phase (Figure 6C).

During the exponential phase, the intracellular lipids allocation in the mixed species culture growing on all carbon substrates, is large compared to that observed in the lag phase (Figure 7) as indicated by the fluorescent microscopic observations. When observed under the optical microscope, blue globules also indicated build up of a lipid intracellular pool. In the exponential phase, the lipid allocation inside the cells was observed in the cell walls, and in an intracellular lipid bodies as observed under the fluorescent microscope. The intracellular lipid content during the exponential phase is much higher when the substrates were UDA and UDA+PDB than when the fungal mixture was grown on PDB alone as a sole source of carbon and energy. The highest intracellular lipid content was observed when the substrate was UDA+PDB indicated by the highest fluorescence intensity. Lipid molecules residing outside the mycelia during the exponential phase is low compared to that observed during the lag phase (Figure 7). The mycelia in the exponential phase are large (6 μm diameter, 235 μm length) and the cells are found forming clusters (Figure 7) in all substrate cultures.

During the stationary phase, the mycelia form large clusters and the sizes of individual cells are much larger (5 μm diameter, 280 μm length) than in the lag and exponential phases in all the carbon substrates (Figure 8). The intracellular lipids of the mixed species culture growing on all substrates are present in the cell wall and as lipid bodies. When carbon substrates are UDA and UDA+PDB, the intracellular lipid content is still much higher than in the lag phase. Compared to the exponential phase, the latter two substrates the intracellular lipid content in mixed species culture is slightly lower as

observed by slightly lower fluorescent intensity of intracellular lipids (Figure 8). In the case of PDB grown cells and during the stationary phase, the intracellular fluorescent intensity of the mixed culture is higher than that observed in the exponential phase, which corresponds to higher accumulation of intracellular lipids. The microscopic analysis results indicate higher accumulation of lipid molecules inside the cells during the stationary phase when PDB is the carbon substrate and different than those observed in the mixed species culture growing on other substrates. Results from the microscopic analysis are consistent with data obtained from chemical analysis see this chapter 2 section (3.4.3 and 3.4.4).

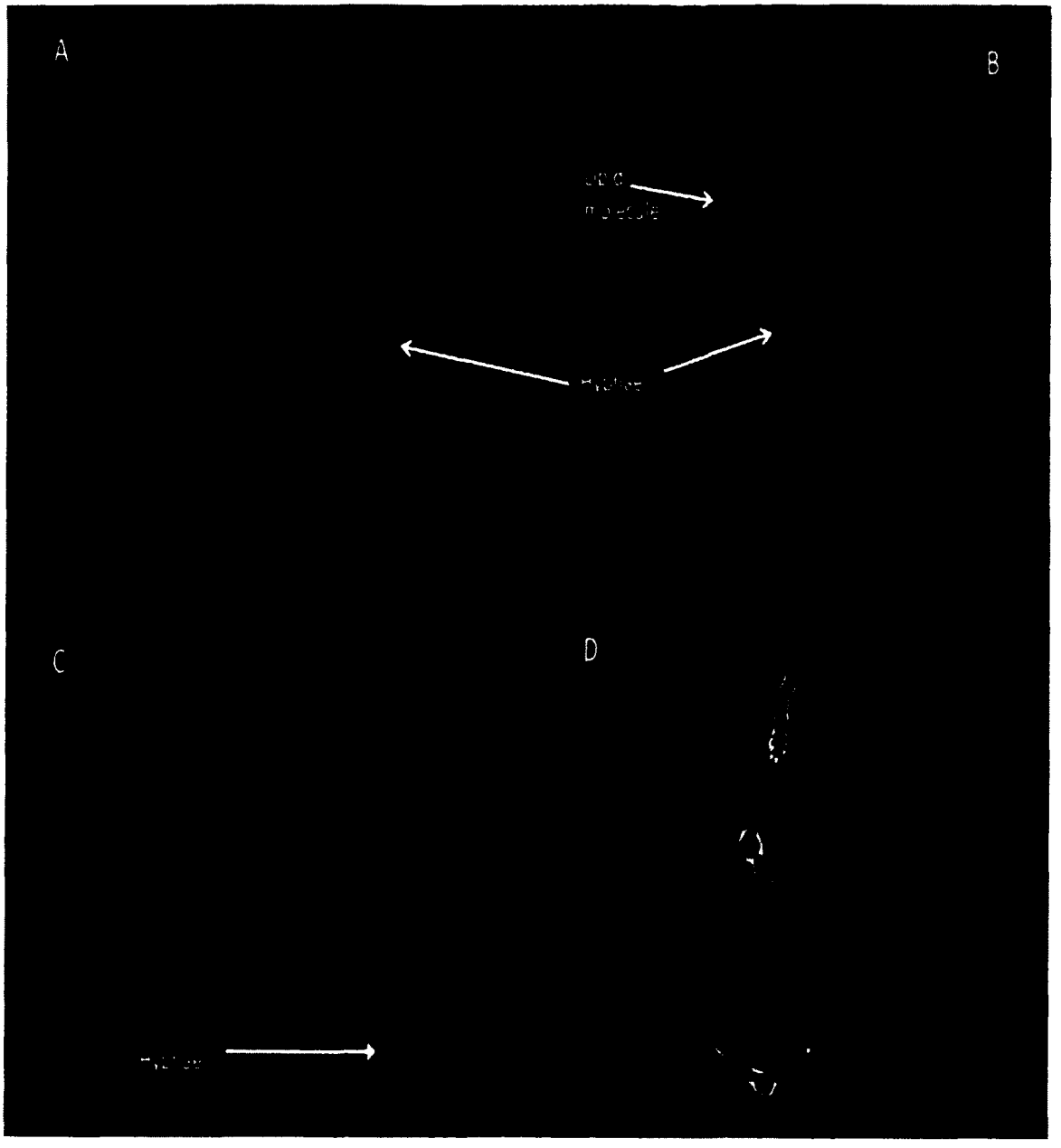


Figure 6: Fluorescent (A and B) and optical (D) microscopic analysis of mixed culture growing on UDA+PDB (A, B), UDA (C) and PDB (D) during the lag phase. The mycelia are dyed with nile blue.

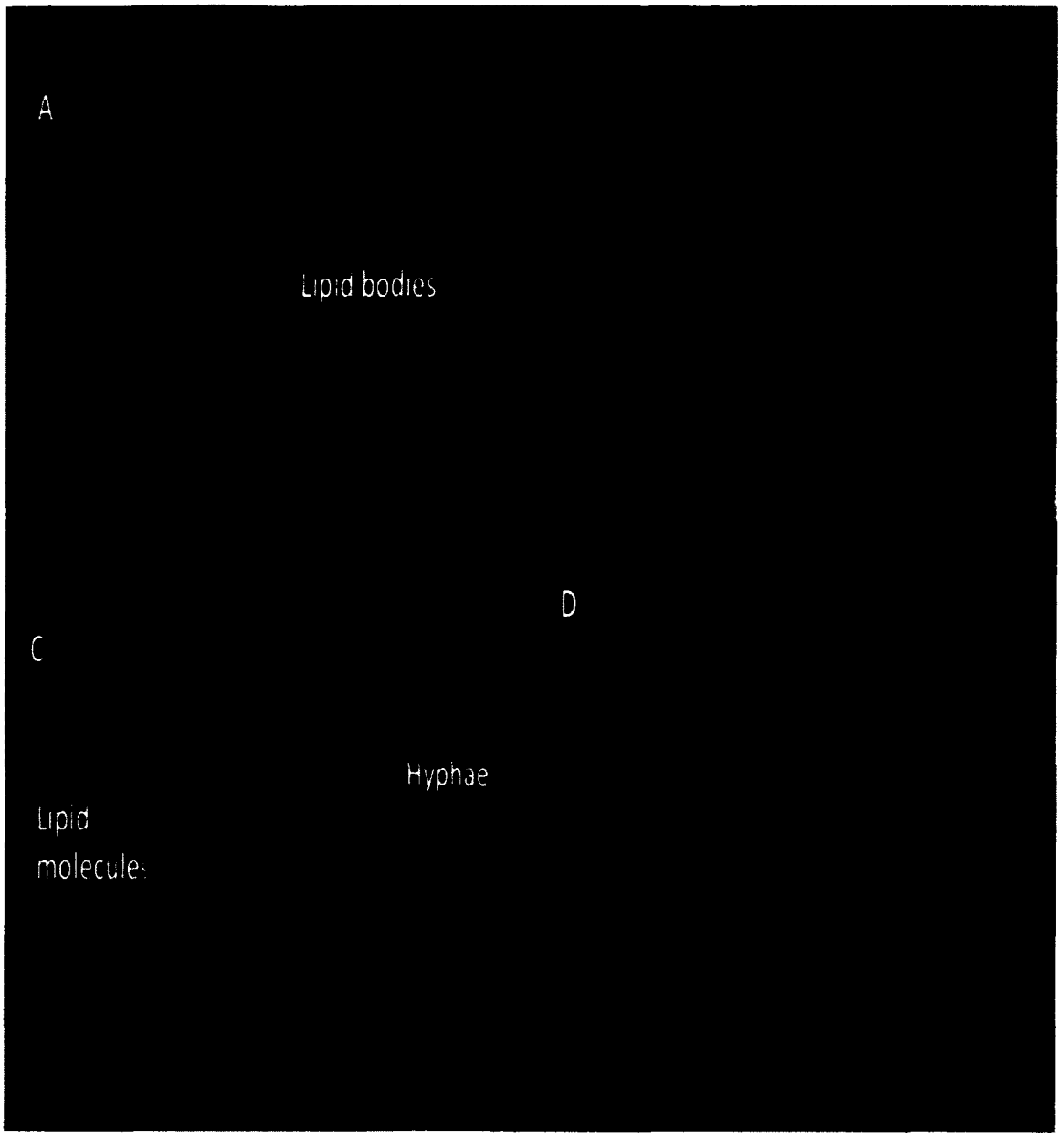


Figure 7: Fluorescent (A and C) and optical (B and D) microscopic analysis of mixed culture during the exponential phase growing on different carbon substrates. The mycelia are dyed with nile blue.

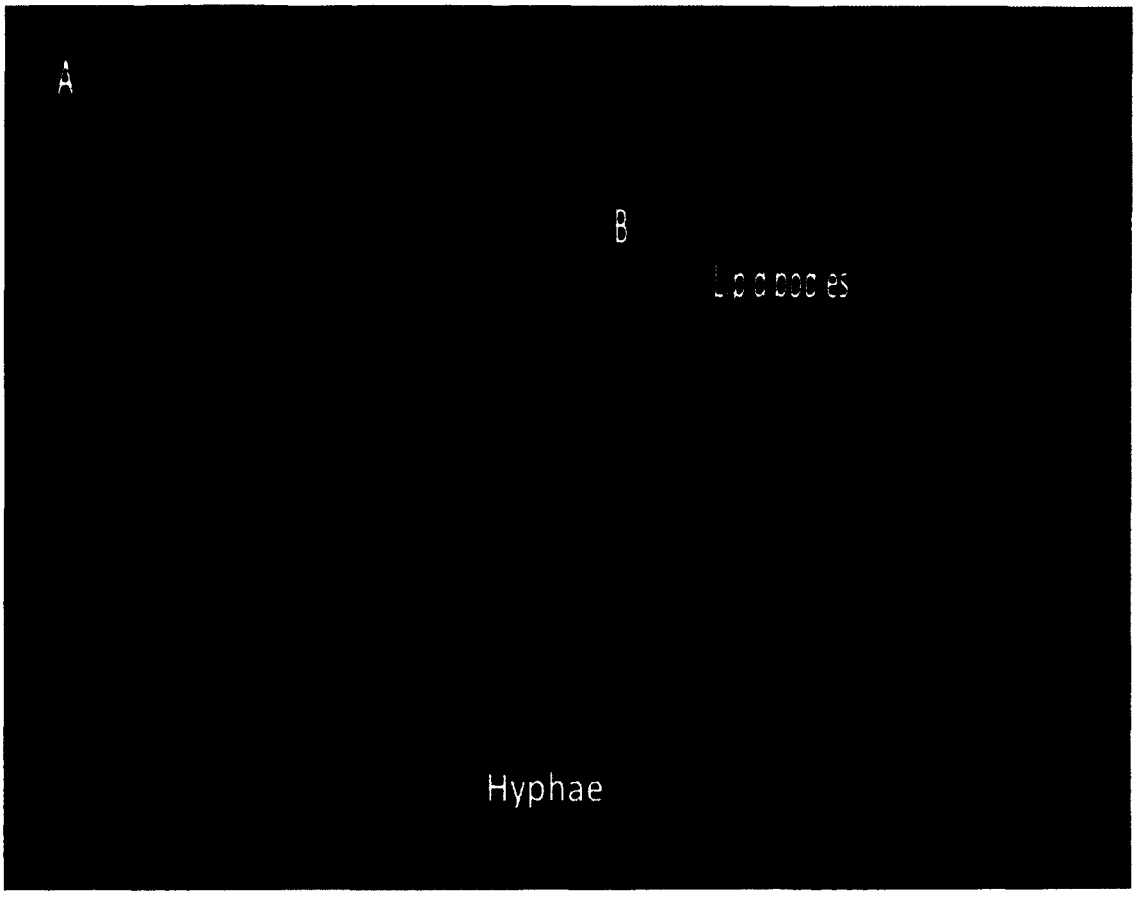


Figure 8: Fluorescent microscopic analysis of mixed culture during the stationary phase growing on UDA+PDB (A) and PDB (B). The mycelia are dyed with nile blue.

3.4.2 Analysis of chemical classes of lipids by Iatroscan

Total lipid analysis of intracellular and extracellular lipids was performed for samples taken from the mixed species culture of *T. koningii* and *P. janthinellum* growing on three different substrates: UDA, UDA+PDB and PDB during the exponential and stationary phases. Only 6 samples were selected to perform chemical analysis. The samples selected were 1 from each set explained in Chapter 2, these samples had highest production of biomass and carbon dioxide among 3 different fungal treatments. One replicate from each set was analyzed to observe the trends of the lipid production by the

mixed species culture growing on three different substrates. As the first replicate indicates the production of alkanes along with other lipid classes, further analysis of other two replicates from each set is required to observe significant production of alkanes. The mixed species culture showed highest biomass and carbon dioxide production on UDA (set 1), UDA+PDB (set 2) and PDB (set 3). Chemical analysis was performed on these samples during the exponential and stationary growth phases. The extracellular samples were only selected from exponential phase of mixed culture growing on UDA+PDB and stationary phase of mixed species culture growing on PDB as these cultures produced highest amount of biomass, carbon dioxide and lipids.

3.4.3 Intracellular lipid production by the mixed fungal species culture

Table 9 shows the distribution of intracellular lipids in different chemical classes during the exponential phase. There are 15 chemical classes of lipids characterized for the mixed culture grown on all the three substrates. The most abundant lipid classes include hydrocarbons, ethyl ketones, triacylglycerols, free fatty acids, sterols, acetone mobile polar lipids and phospholipids. Steryl ester/wax esters were either absent or found in negligible amounts (<0.01 mg) and are not reported in Table 9. The total amount of lipids account for 5% to 58% of biomass depending on carbon substrate and growth phase of fungi (Table 9).

Table 9 shows that the total content of lipids allocated intracellularly was highest (518 mg) when fungal cells were grown on UDA+PDB followed by that allocated by cells when grown on UDA (201 mg). These total lipids account for 58% of total carbon produced in biomass. In comparison, the positive controls and negative controls presented close to a total of 6 mg lipids (Table 9). In general, intracellular free fatty acids

presented the largest content of all the lipid classes (>200 mg). Most of the intracellular lipids (87%) were found as free fatty acids when fungal cells were grown under UDA+PDB and UDA (Table 9). The mixed species culture produced 2.7 times more intracellular hydrocarbons when grown in UDA+PDB than when grow in UDA (Table 9). The amount of hydrocarbons allocated intracellularly was equivalent to > 2% of the total lipid content measured under the UDA+PDB or UDA substrate treatments (Table 9). When PDB is used as the carbon substrate, mixed species culture produces the same content of fatty acids and hydrocarbons (1.5 mg) during the exponential phase.

Table 9: Intracellular lipid classes extracted from the mixed species culture of *T. koningii* and *P. janthinellum* during the exponential phase after 192 hours of incubation.

Lipid classes	**Intracellular concentrations (mg/total biomass in 150 ml culture) during exponential phase**				
	-ve control* (mg)	PDB** (mg)	UDA+PDB (mg)	UDA*** (mg)	Average of substrates (mg)
Hydrocarbons	**0.4**	**1.5**	**12.4**	**4.5**	**6.1**
Ethyl Ketones	0.0	0.0	29.12	0.0	9.7
Triacylglycerols	0.0	2.4	0.00	0.0	0.8
Free Fatty Acids	0.6	1.5	457.3	174.8	211.2
Sterols	0.0	0.3	0.0	0.0	0.1
Acetone Mobile Polar Lipids	0.3	0.3	15.5	10.3	8.7
Phospholipids	4.9	0.5	3.6	11.8	5.3
Total	6.2	6.5	517.8	201.4	241.9
Biomass	-	9540	4585	5649	6591

* -ve control- potato dextrose broth without fungal inoculation. **PDB- Potato dextrose broth. ***UDA- undecanoic acid. Only 1 replicate of mixed species culture grown on different substrates was analyzed to observe the trends of lipid production, further analysis of other two replicates is required to observe significant production of above lipids classes. The blank control was only analyzed for PDB, as rest of the media the carbon source is known.

Table 10 shows that during the stationary phase, the average amount of total intracellular lipids was low (73 mg) and about 1/3 of that extracted from fungal mycelia during exponential growth. The largest pool was free fatty acids, which represented about 67% of total intracellular lipids. The fatty acid content was highest when UDA (120.7 mg) was used as the sole source of carbon and energy. When UDA+PDB is the substrate, the fatty acid content is reduced to 52% of all lipids during the stationary phase. When PDB was used as the carbon substrate, slight decreases in the content of fatty acids (1.1 mg) were observed (Table 10). During the stationary phase, the amount of hydrocarbons was (3.3 mg) nearly 7% of the total amount of lipid classes, but less than the amount of (6.6 mg) measured in the exponential phase, when UDA+PDB and UDA are carbon substrates (Figure 9). When PDB was used as a carbon substrate the content of hydrocarbons (2.8 mg) during the stationary phase nearly doubled in comparison to that measured in the exponential phase (Table 10; Figure 9). The content of free fatty acids were followed by that of phospholipids and acetone polar lipids > triacylglycerol > hydrocarbons. Because the amount of steryl esters/wax esters and ethyl ketones were negligible (<0.01 mg) or absent, they are not presented in Table 10.

Table 10: Intracellular lipid classes extracted from the mixed species culture of *T. koningii* and *P. janthinellum* during the stationary phase after 384 hours of incubation.

Compounds	**Intracellular concentrations (mg/total biomass in 150 ml culture) during the stationary phase**				
	-ve control*	PDB**	UDA+PDB	UDA***	Average of substrates
Hydrocarbons	0.4	2.8	4.4	2.7	3.3
Triacylglycerols	0.0	16.9	0.0	0.0	5.6
Free Fatty Acids	0.6	1.1	25.8	120.7	49.2
Sterols	0.0	2	0.0	0.0	0.7
Acetone Mobile Polar Lipids	0.3	0.8	11.0	9.1	7.0
Phospholipids	4.9	2.1	8.1	12	7.4
Total	6.2	26.1	49.2	144.4	73.3
Biomass (mg)	-	12630	8625	7729	9661

* -ve control- potato dextrose broth without fungal inoculation. **PDB- Potato dextrose broth. ***UDA- undecanoic acid. Only 1 analytical replicate of mixed species culture grown on different substrates was analyzed to observe the trends of lipid production, further analysis of other two replicates is required to observe significant production of above lipids classes.

Phospholipids are another lipid class produced by the mixed species culture growing on all three substrates during the exponential and stationary phases. The amount of phospholipids produced by the mixed species culture ranged from 0.5 to 12 mg depending on the carbon substrate and growth phase. The maximum content of phospholipids were found when the mixed species culture was grown on UDA (Table 9). Similar to free fatty acids and hydrocarbon, the content of acetone mobile polar lipids was equivalent to 3.5% of the total lipid classes and its content was highest when UDA+PDB was the carbon substrate (Table 9). Twenty-nine mg of ethyl ketone were observed during exponential phase and only when fungal cells were grown on UDA+PDB (Table 9). No ethyl ketone production was observed during the stationary phase (Table 10). Triacylglycerols and sterols were only produced under PDB.

Triacylglycerols were major lipid classes produced by mixed species culture growing on PDB. During the exponential phase, triacylglycerols account for 37% of the total lipids produced by mixed species culture grown on PDB (Table 9). Sterols account for only 5% of the total lipids and were only produced when PDB was the carbon substrate (Table 9).

In contrast to other lipid classes, the average phospholipid concentration did not decrease during stationary phase, but it rather increased as also observed under PDB during the stationary phase and ranged between 2 and 12 mg (Table 10). During the stationary phase, the concentration of acetone mobile polar lipids slightly decreased during the stationary phase when UDA+PDB and UDA are the carbon substrates (Table 10). When PDB was the carbon substrate the content of acetone mobile polar lipids increased 3 times during stationary phase compared to exponential phase (Table 10).

During stationary phase, when PDB is used as the carbon substrate, the content of triacylglycerols (16.9 mg) accounted for 65% of the total lipids produced (Table 10). The sterol content was almost absent during the exponential phase but it increased to 2 mg during the stationary phase (Table 10). In addition diacylglycerol were also detected in small amounts (0.3 mg) only during the stationary phase.

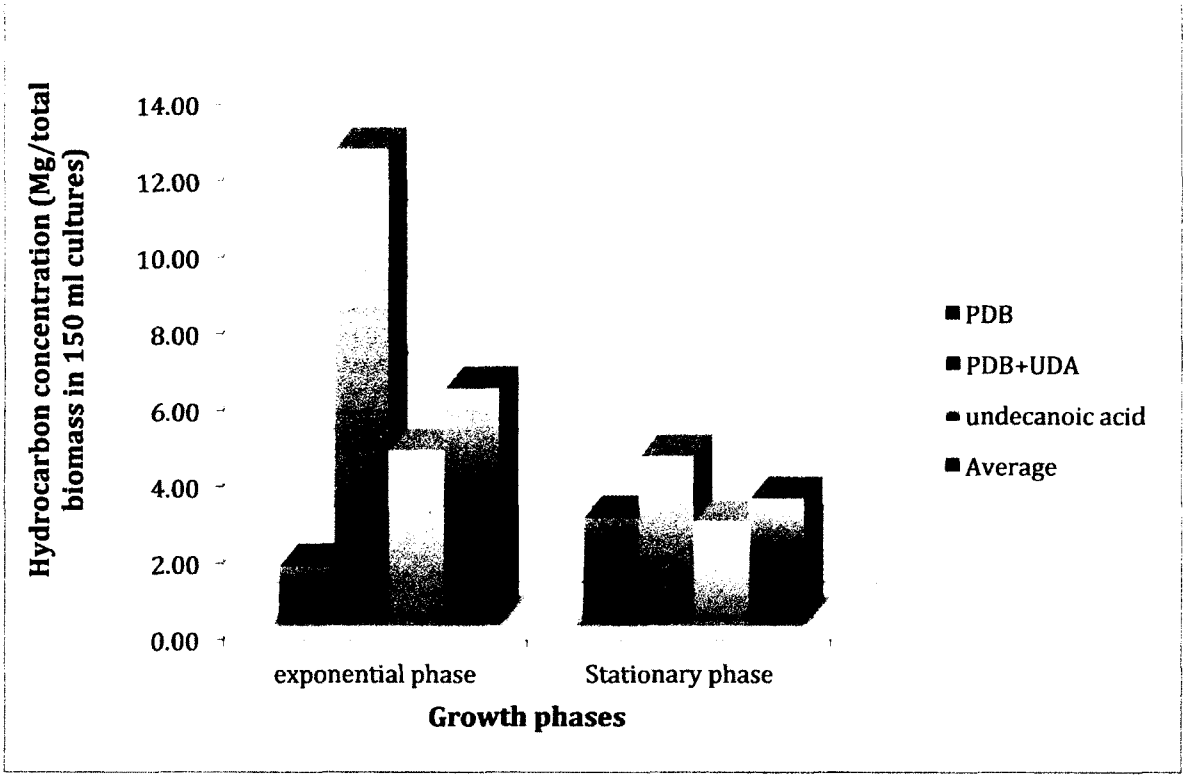


Figure 9: Comparison of total amount of hydrocarbons allocated intracellularly by mixed culture of *T. koningii* and *P. janthinellum* during exponential and stationary growth phase while growing on 3 different carbon substrates; Potato dextrose broth (blue), mixture of potato dextrose broth and undecanoic acid (red), undecanoic acid (green), average (purple).

3.4.4 Extracellular lipid production by mixed species culture

Table 11 shows the content of extracellular lipids extracted from fungal mycelia growing on PDB and UDA+PDB during the exponential and stationary phase. The extracellular lipid analysis was only performed in samples taken from the mixed species culture that produced the highest content of intracellular lipids, especially hydrocarbons. The major classes of extracellular lipids produced were, hydrocarbons > triacylglycerol > phospholipids > free fatty acids > acetone mobile polar lipids > sterols (Table 11). On average, the amount of total extracellular lipids was 13.8 mg, and it represented from 2.8 to 6.4% of that found intracellularly during both growth stages (Table 11).

The amount of hydrocarbons produced extracellularly during the exponential phase of mixed culture growing on UDA+PDB is 6.5 mg/150 ml culture, which is almost half the amount of hydrocarbon accumulated intracellularly (Tables 9 and 11). The amount of extracellular hydrocarbons produced by the mixed species culture growing on PDB are 2.8 mg, and almost double the amount produced intracellularly by this culture during the stationary phase. The results show that the total amounts of intracellular and extracellular hydrocarbons ranged from 4.5 to 6.5 mg/150 ml culture during the exponential and stationary phase respectively. These amounts represented close to 3.6% of the total amount of lipids measurement in the 150 ml culture flasks (Table 11).

The content of free fatty acids produced extracellular is extremely low in both carbon substrates: PDB (0.9 mg) and UDA+PDB (1.6 mg) much lower than intracellularly produced fatty acid (Table 11 and 9).

Table 11: Extracellular lipid classes extracted from mixed species culture of *T. koningii* and *P. janthinellum* during the exponential and stationary growth phases

Compounds	Extracellular concentrations (mg/ 150 ml media) during			
	-ve control	PDB Stationary phase	UDA+PDB Exponential phase	Average of substrates
Hydrocarbons	0.4	4.5	6.5	5.5
Triacylglycerols	0.0	6.0	0.8	3.4
Free Fatty Acids	0.6	1.0	1.6	1.3
Sterols	0.0	1.0	1.0	1.0
Acetone Mobile Polar Lipids	0.3	0.7	1.4	1.0
Phospholipids	4.9	1.6	1.9	1.7
Total	6.2	14.7	12.9	13.8

* -ve control- potato dextrose broth without fungal inoculation. **PDB- Potato dextrose broth. ***UDA- undecanoic acid. Only 1 analytical replicate of mixed species culture grown on different substrates was analyzed to observe the trends of extracellular lipid production, further analysis of other two replicates is required to observe significant production of above lipids classes.

On average, extracellular phospholipids (1.7 mg) and acetone mobile polar lipid (1 mg) is lower compared to their intracellular concentration (Table 11). When PDB was used as the carbon substrate, triacylglycerol (6 mg) is the major lipid class produced by mixed species culture during stationary phase (Table 11). Contrary to the intracellular lipid classes produced by the mixed species culture growing on UDA+PDB, triacylglycerol was produced in small amounts (0.8 mg) into the extracellular pool. Also, sterols were produced in small amounts (0.9 mg) in the extracellular pool of both substrates (Table 11).

3.4.5 Rates of lipid synthesis and turnover

Table 12 shows the rate of synthesis and turnover of different lipid classes produced by the mixed culture growing in different carbon substrates. The rate of synthesis of hydrocarbons by the mixed species culture growing in UDA in 150 ml of

culture during the exponential phase was 0.024 mg/hour, and their turnover during stationary phase was -0.0053 mg/hour. Relative to all other lipid classes the highest rate of synthesis (0.91 mg/hour) and turnover (-0.23 mg/ hour) was observed for the intracellular free fatty acid pool of the mixed species culture growing in UDA. In the case of phospholipids, no turnover was observed; they were synthesized at a rate of 0.031 mg/hour and no losses were observed during the stationary phase. The rate of synthesis and turnover of the acetone mobile polar lipids were 0.054 and -0.0050 mg/ hour respectively.

When the mixed species culture was grown on UDA+PDB, the rates of free fatty acid biosynthesis and turnover were the highest compared to all other substrates. Fatty acids were synthesized at the rate of 2.4 mg/hour during exponential growth phase and turned over at -1.8 mg/hour during the stationary phase (Table 12). The rates of hydrocarbon biosynthesis was 0.065 mg/hour during the exponential growth phase and its turnover at -0.032 mg/hour during stationary growth phase, higher than other substrates but much lower compared to fatty acid synthesis and turnover rates. The rate of biosynthesis and turnover of the acetone mobile polar lipids was 0.080 and -0.018 mg/hour respectively. Phospholipids did not get turned over, and their rate of biosynthesis was 0.019 mg/hour. The biosynthesis of ethylketones accumulated 29.1 mg (Table 9), thus their rate of synthesis was 0.15 mg/hour during the exponential growth phase. As there were no ethyl ketones detected during the stationary phase, they must have turned over before the stationary phase was detected. These ethyl ketones may be an intermediate carbon pool for the synthesis of other lipids or used as a source of energy.

Table 12: The rate of synthesis and turnover of different lipid class produced by mixed culture of *T. koningii* and *P. janthinellum* growing in different carbon substrates.

	Intracellular lipids of mixed culture growing on different substrates					
	UDA		Mixture of UDA+PDB		PDB	
Lipid classes	Synthesis **(mg/h) Exponential phase	Turnover (mg/h) Stationary phase	Synthesis (mg/h) Exponential phase	Turnover (mg/h) Stationary phase	Synthesis (mg/h) Exponential phase	Turnover (mg/h) Stationary phase
HC[a]	0.024	-0.0053	0.064	-0.032	0.0066	No turnover
FFA[b]	0.91	-0.23	2.4	-1.8	0.0080	-0.0018
EKET[c]	ND*	ND	0.15	-0.12	ND	ND
TG[d]	ND	ND	ND	ND	0.037	No turnover
ST[e]	ND	ND	ND	ND	0.0044	No turnover
AMPL[f]	0.054	-0.0050	0.080	-0.018	0.0022	No turnover
PL[g]	0.031	No turnover	0.19	No turnover	0.0046	No turnover
Biomass (mg)	5600	7700	4600	8600	9500	13000

* ND= not determined because lipid class was not produced by mixed species culture
** Synthesis and turnover rates calculated from data at 192 and 432 hours of incubation. The rate of synthesis and turnover were calculated from 1 analytical replicate of each culture. a- Hydrocarbons, b- Free fatty acids, c- Ethyl ketones, d-Triacylglycerols, e- Sterols, f- Acetone mobile polar lipids, g- Phospholipids.

When the mixed species culture is grown in PDB alone, the rate all lipid synthesis was lower than those measured for the other two substrates (Table 12). On this substrate, the rate of fatty acid biosynthesis was 0.0080 mg/hour. Relative to the exponential phase, they turned over at a much lower rate of -0.0018 mg/hour indicating slow fungal catabolism of fatty acids. The hydrocarbon synthesis rate was 0.0066 mg/hour during the exponential phase with no measurable turnover of hydrocarbon during the stationary phase. The acetone mobile polar lipids and phospholipids were biosynthesized at the rate

of 0.0022 and 0.0046 mg/hour respectively, with no turnover observed during stationary growth phase. On PDB, the mixed culture also produced triacylglycerols and sterols, which were not observed when grown on other carbon and energy substrates. Triacylglycerols and sterols were synthesized at an average rate of 0.037 and 0.0044 mg/hour respectively without any turnover during stationary phase (Table 12).

3.4.6 Chemical analysis of fatty acids by GC- FID

Table 13 shows the total concentrations of 55 free fatty acids (FFA) identified in the intracellular extracts from the mixed species culture of *T. koningii* and *P. janthinellum* during exponential and stationary phases growing on different carbon substrates. The total concentration of FFA (465.6 mg) was highest in the fungal biomass growing on UDA+PDB during the exponential phase, and 2.5 times greater than under UDA, and almost 100 times greater than under PDB. The latter amount of accumulated FFA during the exponential phase was decomposed rapidly during the stationary phase. For example, the amount of FFA decreased 13 times during the stationary phase when the mixed species were grown on UDA+PDB, and 1.5 times when they were grown on UDA. Of the 55 free fatty acids identified, the dominant saturated and unsaturated fatty acids were 16:0 > 18:0 > 18:1ω7 > 15:0 > 17:0 > 18:2 ω6 > 16:1ω7 > 18:1 ω9 when the mixed culture was grown on UDA+PDB and on the other substrates. The other fatty acid species were present in low concentrations (Table 13). The concentration of the predominant fatty acids decreased drastically from the exponential phase to the stationary phase when cells were grown in UDA+PDB.

The predominant fatty acids identified above account for 84.5% of the total FFA species. Table 13 shows that the concentrations of saturated fatty acids (SFAs) was

higher in the exponential phase than in the stationary phase when the mixed species culture was grown on UDA+PDB and UDA alone. Monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) were lower in the exponential phase than in the stationary phase. When cells were grown on UDA+PDB, the concentration of the SFAs (295 mg/150 ml) > MUFA (86 mg/150 ml) > PUFA (73 mg/ 150 ml) during exponential phase (Table 13). On average, the SFA in cells growing on UDA+PDB accounts for 63% of the total FFA during the exponential phase and 50% of the total FFA during the stationary phase. The SFA accounts for 75 % of the total FFA during exponential phase and 45 % during the stationary phase when cells were grown on UDA alone. On average, when mixed species culture is grown on PDB, the intracellular SFA account for 33% of all FFA during exponential and stationary phase. The concentration of MUFA and PUFA ranged from 23% to 66% of the total FFA, depending on substrate type and growth stage. Contrary to MUFA and PUFA, the SFAs concentration decreased from the exponential phase and to the stationary phase. The sample GC chromatograms for the fatty acids analyzed in different intracellular lipid extracts from mixed species cultures during different growth phases are shown in the appendix (Figure 26 to Figure 29).

Table 13: Intracellular free fatty acid (FFA) of mixed culture of *T. koningii* and *P. janthinellum* growing on different grow substrates analyzed during exponential and stationary phase.

	Intracellular free fatty acid Concentration (mg/total biomass in 150 ml culture) of mixed culture growing different growth substrates					
Fatty acid	PDB**-Exponential phase	PDB-stationary phase	UDA*+ PDB exponential phase	UDA+PDB stationary phase	UDA-exponential phase	UDA-Stationary phase
14:0	0.057	0.132	5.130	0.426	2.453	1.809
14:1	0.003	0.005	0.320	0.000	0.057	0.166
ι15:0	0.002	0.004	0.513	0.038	0.323	0.087
αι15:0	0.007	0.019	0.362	0.046	0.147	0.336
15:0	0.032	0.110	28.198	0.772	3.409	6.162
15:1	0.007	0.008	0.820	0.316	1.173	0.104
ι16:0	0.004	0.009	0.210	0.031	0.085	0.183
αι16:0	0.005	0.031	5.736	0.093	0.227	0.412
16:0	0.831	3.606	151.205	10.272	80.721	29.140
16:1ω11	0.000	0.008	0.694	0.000	0.000	0.258
16:1ω9	0.024	0.042	1.341	0.153	0.805	0.462
16:1ω7	0.055	0.197	17.833	0.848	3.167	6.735
16:1ω5	0.000	0.007	0.189	0.012	0.244	0.114
17:0	0.001	0.010	0.706	0.033	0.327	0.200
αι17:0	0.007	0.024	3.507	0.108	1.037	0.761
16:2ω4	0.043	0.099	3.923	0.232	1.854	0.475
17:0	0.038	0.213	23.611	0.916	3.018	6.467
16:3ω4	0.020	0.094	11.358	0.462	1.351	4.585
17:1	0.000	0.000	3.524	0.136	0.450	1.465
16:4ω3	0.012	0.020	0.812	0.134	0.263	0.137
16:4ω1	0.009	0.025	13.544	0.135	3.509	2.697
18:0	0.464	1.916	85.171	6.288	49.959	15.569
18:1ω11	0.000	0.000	1.489	0.035	0.000	0.216
18:1ω9	1.042	5.590	16.525	4.720	11.098	23.693
18:1ω7	0.026	0.087	41.810	1.390	9.125	17.313
18:1ω6	0.006	0.010	0.896	0.024	0.119	0.276
18:1ω5	0.000	0.002	0.639	0.021	0.125	0.172
18:2ω6	1.311	6.056	18.586	5.477	8.135	6.082
18:2ω4	0.000	0.000	0.000	0.000	0.000	0.000
18:3ω6	0.003	0.021	1.203	0.031	0.229	0.083
18:3ω4	0.000	0.000	1.711	0.055	0.132	0.884
18:3ω3	0.091	0.310	16.361	0.555	0.935	2.464
18:4ω3	0.020	0.015	1.169	0.299	0.142	0.246
20:0	0.019	0.092	1.064	0.186	0.501	0.273

18:5ω3	0.000	0.000	0.000	0.000	0.000	0.000
20:1ω11	0.000	0.011	0.063	0.117	0.000	0.504
20:1ω9	0.004	0.047	0.000	0.000	0.000	0.230
20:2ω6	0.000	0.017	1.468	0.303	0.297	0.429
20:4ω6	0.015	0.028	0.000	0.000	0.000	0.000
20:4ω3	0.020	0.034	1.808	0.308	0.372	0.374
22:0	0.016	0.084	0.000	0.123	0.255	0.128
23:0	0.004	0.035	0.000	0.054	0.147	0.079
22:4ω3	0.016	0.016	1.518	0.210	0.263	0.296
24:0	0.054	0.252	0.559	0.235	0.563	0.348
Total fatty acids	4.299	19.416	465.574	35.693	187.020	132.614
SFA***	1.521 (35%)	6.457 (33%)	294.938 (63%)	19.271 (54%)	141.026 (75%)	59.976 (45%)
MUFA****	1.182 (28%)	6.080 (31%)	86.142 (18%)	7.791 (22%)	26.364 (14%)	51.835 (39%)
PUFA*****	1.568 (37%)	6.781 (36%)	73.460 (16%)	8.281 (23%)	17.484 (9%)	18.824 (14%)
Biomass (mg)	9540	12630	4585	8625	5649	7729

• undecanoic acid
** potato dextrose broth
*** saturated fatty acids
**** mono-unsaturated fatty acids
***** poly-saturated fatty acids

Table 14 shows the content of extracellular FFA produced by the mixed species culture. Analysis of extracellular FFA was performed on only two samples, the mixed species culture growing in the UDA+PDB and PDB alone during the stationary phase. Experimental results indicate that the concentrations of FFA in the media are much lower than that accumulated intracellularly. The total content of FFA in the extracellular extracts were low (< 5 mg/150 ml culture) and 100 times lower than in the intracellular extracts. When grown with PDB substrates, the fungal cells produced (0.5-1.0 mg) just 5 measurable fatty acids (Table 14), and 22 measurable but very low content (<0.01 mg) of

fatty acids were produced extracellularly when grown on UDA+PDB (data not shown). The most dominant fatty acids produced extracellularly with both carbon substrates were 16:0 > 18:1ω9 > 18:0 > 18:2 ω6 > 24:0. The concentration of other saturated and unsaturated fatty acids in the extracellular pool produced and measured in both carbon substrates was low (< 0.1 mg/150 ml) and no difference in total FFA content existed between both growth phases. Interestingly, the content of SFA dominated in the PDB stationary phase (3.2 mg/ 150 ml) and UDA+PDB exponential phase (2.3 mg/150 ml). On average, extracellularly in both carbon substrates and growth phases showed similar content of MUFA (1 mg/ 150 ml) and PUFA (0.8 mg/150 ml). The amount of extracellular SFA is double than the amount of PUFA and MUFA together in both carbon substrates. The sample GC chromatograms for the fatty acids analyzed in extracellular lipid extracts from mixed species cultures growing in mixture of UDA+PDB during exponential phase is shown in the appendix (Figure 30).

Table 14: Extracellular free fatty acids (FFA) of mixed culture of *T. koningii* and *P. janthinellum* growing on different grow substrates analyzed during exponential and stationary phase.

	Extracellular free fatty acid Concentration (mg/total biomass in 150 ml culture) of mixed culture growing different growth substrates (GS)	
Fatty acid	PDB[a] - stationary phase	UDA+PDB[b]- exponential phase
16:0	1.2	1.2
18:0	0.8	0.8
18:1w9	1.1	1.0
18:2w6	0.5	0.5
24:0	0.2	0.1
Total	4.2	4.2
SFA[c]	3.2	2.3
MUFA[d]	1.0	1.1
PUFA[e]	0.8	0.7

a – Potato dextrose broth
b – Mixture of undecanoic acid and potato dextrose broth
c – Saturated fatty acid
d – Mono-unsaturated fatty acid
e – Poly-unsaturated fatty acids

3.4.7 Carbon mass balance and its dynamics in the mixed culture

Table 15 and Figure 10 presents data for a carbon mass balance and the cycling of carbon through three pools in the mixed species culture grown for 576 hours in UDA. The three physical pools were the extracellular, the fungal biomass, and the carbon dioxide evolved during the lag, exponential and stationary phases. The law of mass conversation requires that, the sum of the carbon content in all three pools at each time of sampling equals the amount of fatty acid carbon added to the fungal mixed culture at time zero. Table 15 shows that the carbon measured at different times of sampling always accounted for 100% of the carbon added at time zero. The amount of the extracellular carbon decreased rapidly during the first 192 hours of growth. This decrease is paralleled by an increase biomass-carbon and CO_2-C (Figure 10). Further, the carbon use efficiency

by the mixed culture growing on UDA during the lag phase (at 90 hours) was 53%, and it increased to 83% during the exponential phase (192 hours); and it remained about unchanged after 432 hours of growth. Extractable lipids account for 25% (wt/wt) of the total biomass during the exponential phase. The total extracted lipids decreased to 16% at the end of the stationary phase. A carbon mass balance for other two substrates was not performed, as the concentration of PDB and carbon in the three pools at different growth stages was not determined.

Table 15: Carbon mass balance of mixed culture growing in undecanoic acid as sole source of carbon. UDA was added at a rate of 1065.19 mg into 150 ml culture media.

Time (Hours)	Amount of fatty acids (mg) in culture	Carbon content (mg) in culture	Carbon content (mg) of biomass	Carbon content (mg) of Cumulative CO_2 produced	Total carbon recovered (mg)	Substrate use efficiency biomass-C (%) weight
0	1500	1065	0.000	0.000	1065	0.000
90	692.7	491.9	568.4	4.900	1065	53.00
192	170.92	121.3	891.9	52.00	1065	84.00
432	82.94	58.90	913.2	93.10	1065	86.00

- use efficiency = (biomass-C/Carbon content in culture at time 0)* 100

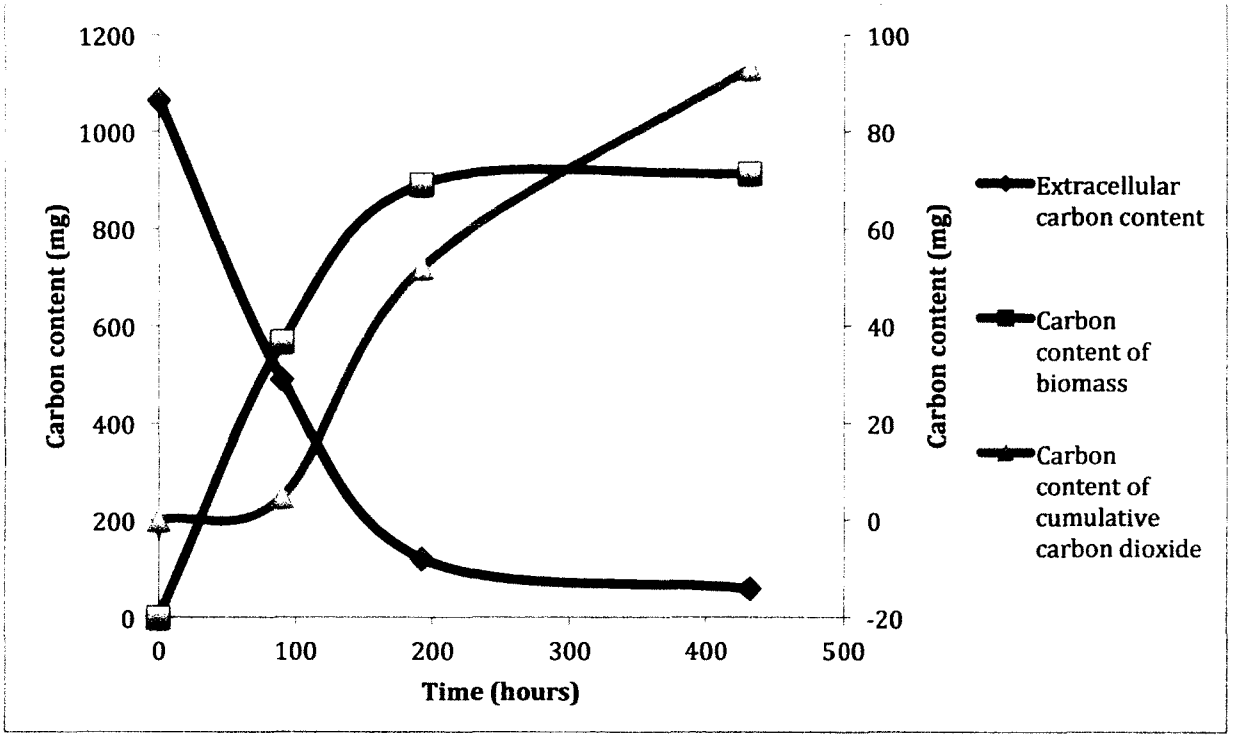


Figure 10: Carbon mass balance and its dynamics as utilized by the mixed species culture growing in undecanoic acid as the sole source of carbon and energy.

3.5 Discussion

In this research project, the intracellular allocation of lipids in a mixed culture of *T. koningii* and *P. janthinellum* was examined by microscopic observation of mycelia cells stained with nile blue that strongly fluoresces in an hydrophobic environment. A study by Greenspan *et al.* (1985) reported that this dye was very soluble in lipids and did not show any interaction with other microbial constituents. Results from the microscopic observation indicate that fungal mycelia growing in different carbon sources treated with nile blue, selectively stains lipids present in the mycelia and the growth media. During the lag phase and when the mixed species culture was grown in UDA, the low fluorescence of lipids indicated a low amount of lipids allocate in the cell walls, but a high content in the growth media. When the mixed species culture is grown in PDB or in

a mixture of UDA+PDB the same observations were made from the fluorescent microscopic analysis: small amount of lipid accumulated mostly in the cell wall. During the exponential phase, and based on fluorescence distribution, a high accumulation of lipid bodies occupied inside the mycelia when grown on all the substrates except PDB. Murphy and Vance (1999) showed that accumulated lipids in fungi are localized in the intracellular organelle, the so- called ''lipid body'' and their formation is an important processes for the lipid production in oleaginous microorganisms. These are storage lipids, which are also called oleosomes or lipid globules. These lipid globules consist of a central core of insoluble lipids surrounded by a monolayer of phospholipids; proteins are also involved in the metabolism of these lipids (Murphy, 1991; Kamisaka and Noda, 2001). Formation of such lipid globules has also been observed in several other oleogenic fungi. Bago *et al.* (2002) reports the formation and accumulation of lipid globules in arbuscular mycorrhizal fungi (AMF) and that these lipids are metabolized once nutrient conditions change. Our microscopic observations and chemical data show that, during the stationary phase, the mixed species culture still shows allocation of lipids inside the cells, but at a low fluorescent intensity and concentration when grown on UDA and a mixture of UDA+PDB. Interestingly, the fluorescence of mycelia grown on PDB alone indicated a high intensity of lipid bodies during the exponential phase. Chemical analysis of lipids, however, indicated a small content of total lipids when cells were grown on PDB (Table 9). Similar accumulation of lipids during exponential phase was also reported in a culture of *Mortierella alpine* growing on fatty acids by Hamanaka *et al.* (2001). The experimental results based on microscopic observations show higher formation of lipids during the exponential phase than in the stationary phase, as these body lipids were used

as carbon and energy sources during the stationary phase.

Over all, the microscopic observations were consistent with the chemical analysis of extracted lipids. Chemical analysis of intracellular and extracellular lipids produced by the mixed culture show that 4 lipid classes dominated the total lipid content, free fatty acids, hydrocarbons, acetone mobile polar lipids and phospholipids. When the harvested mycelia were grown on UDA+PDB, intracellular ethyl ketones were produced during the exponential phase, but they were completely utilized by the stationary phase. Growth on PDB as the sole source of carbon and energy produces small amounts of several other intracellular lipid classes such as sterols, steryl esters, and triacylglycerols. Production of lipids have been shown in other studies in which fungal species, growing on starch or glucose as the sole source of carbon, mostly as fatty acids, triacylglycerols, hydrocarbons, esters and sterols (Hare, 1988; Ladygina, 2006; Subermaniam *et al.*, 2010). Our chemical analysis of the extracted intracellular lipids also showed production of triacylglycerols and sterols along with other extracellular lipids when cells were grown on PDB. The latter compounds have several industrial uses such as triacylglycerols that serve as precursors for the production of fatty acid esters through transestrification, which are used as biodiesel. Sterols serve as precursors for the industrial production of vitamins and hormones.

Fatty acids were the most dominating lipid class produced intracellularly by the mixed species culture when grown in UDA and UDA+PDB, although their content was lower, when the fungal cells were grown on PDB alone. When cells were grown on PDB, triacylglycerol was the most dominant lipid class produced intracellularly and also extracellularly. Hare (1988) reported vesicular-arbuscular mycorrhizal fungi grown on

glucose or starch produces triacylglycerides as the most dominating lipid followed by fatty acids. When these fungi were grown on any other carbon source the most dominating lipid class produced by these fungi is fatty acids. Abraham and Srinivas (1984) shows that the species, *Aspergillus nidulans, Penicillium frequentans*, and *Fusarium lycopersicum* produces high lipid quantities when grown on a deproteinized whey medium. In the latter study, *F. lycopersicum* produced the highest content total lipid (38.6%) followed by *A. nidulans* LC-1 (16.9%) and least in *P. frequentans* (10.5%). The predominant chemical components in the neutral lipid fraction were triglycerides and phospholipids followed by diacylglycerides and free fatty acids. In another study conducted by Carreon *et al*, (1991), *Trichoderma* species accumulated neutral lipids mainly consisting of triacylglycerides, phospholipids and fatty acids when grown on carbohydrates such as glucose, xylose or sucrose. In this research, higher accumulation of fatty acid and triacylglycerides can be explained through the structure of lipid bodies and high energy content of these lipids. As explained by Kamisaka and Noda (2001), the core of lipid bodies consists mainly of neutral lipids, which include triacylglycerols and free fatty acids. These storage lipids are then used by fungal cells as carbon and energy sources when the nutrients in the media are depleted (Kamiska and Noda, 2001). The latter helps explain the rapid turnover of the fatty acids and the other lipid classes produced intracellular by the mixed species cultures during the stationary phase.

Some of the filamentous fungi have tendency to store up to 80% of their biomass as lipids depending on their growth conditions. Filamentous fungus, *Mucor rouxii* has been reported to accumulate high levels of intracellular lipids (Mamatha *et al.*, 2009). A study by Somashekar *et al.* (2002) shows that *Mucor* species grown in sesame oil, as the

sole source of carbon, they produce higher lipid content (44%) than when these species are grown in glucose (30%), as sesame oil contains a mixture of carbon sources including lipids and carbohydrates. The results from the present study are consistent with the published results by Somashekar *et al.* (2002) where the highest accumulation of lipids is observed in the mixed species culture grown in mixture of UDA+PDB where lipids accounted for over 25% of the total biomass weight at exponential phase.

In the mixed species culture grown on UDA+PDB, intracellular hydrocarbons were the class of lipids produced in relatively high (12.4 mg) quantities, and were the most dominating class produced extracellularly (4.4 mg).

The present research study also shows a high (~ 17 mg) total accumulation of hydrocarbons intracellularly and extracellularly produced by the mixed species culture grown especially in UDA+PDB and also the other carbon substrates. Where PDB was the carbon and energy source, fatty acids and hydrocarbons account for small amounts and proportions of the total lipid produced. Several fungal species including *Penicillium* species *Trichoderma virida, Aspergillus* species and other species have been known to produce long-chain alkanes in small concentrations. In other studies, hydrocarbons produced by the latter fungal species account for 0.06% to 0.7% of the dry biomass weight (Ladygina, 2006), whereas in this my study the hydrocarbons produced by mixed fungal species account for 0.5 to 2.13 % of the dry biomass weight, which is much higher than values published in the scientific literature.

The accumulation of relatively high amounts of hydrocarbons in the mixed species culture grown in UDA+PDB could be associated with the metabolic elongation and decarboxylation or head to head condensation of fatty acids leading to formation and

accumulation of alkanes. Thus, enhancing the maximum production and harvest of hydrocarbons during the exponential phase. In case of fungal species head to head condensation is not observed thus it is likely that hydrocarbons are being produced through elongation decarboxylation, further research is required to elucidate the exact pathway.

The most commonly produced fatty acids by the mixed species culture growing on the different substrates include palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and vaccenic acid (C18:1). These fatty acids account for over 80% of FFA produced intracellularly. In this study, other fatty acids were produced such as myristic acid (C14:0) and arachidic acid (C20:4). In a recent study, Fakas *et al.* (2009) reports that *Mortierella isabellina* produces high amounts of fatty acids mainly oleic acid, palmitic acid, linoleic acid, steric acid and palmitoleic acid. In an earlier study conducted by Hare (1988), several vesicular-arbuscular mycorrhizal fungi produced most fatty acids with carbon chain lengths between C14 and C18, similar to the results found in my study with *T. koningii* and *P. janthinellum*. The *Mortierella ramanniana* when grown under an optimized pH of 4 shows highest accumulation of lineoleic acid as fungal species tend to grow better in lower concentration of octadecanol and yeast extract as sole sources of carbon and energy (Subramaniam *et al.*, 2010). My study confirms that the mixed fungal species produce saturated and polysaturated fatty acid in similar and large quantities (~295 mg) as observed in the studies conducted by (Fakas *et al.* 2009; Hare. 1988; Subramaniam *et al.*, 2010; Xian *et al.*, 2002).

The ability of the two fungal species obtained from the National Fungal Collection of Agriculture and Agri-food Canada, to accumulate neutral and

polyunsaturated fatty acids and hydrocarbons have a great potential to serve as fatty acid feedstocks for producing significant amounts of biodiesel for transportation by transestrification. Alternatively, the produced fatty acids can also be converted by chemical catalysis into hydrocarbons for transportation biofuels. The availability of low cost feedstocks such as biooil derived from the pyrolysis of agricultural residues and food wastes, used together with the ability of the studied fungal species to grow on a wide range of conditions needs to be investigated, as there is a great potential to develop and produce new transportation fuels from fatty acids in biooil (Schnitzer *et al.*, 2008). The chemical catalysis of fatty acids produced by fungi growing on the fatty acids in biooil becomes an essential and complementary process in biorefineries.

3.6 Conclusion

These results show that different carbon sources have different effects on the production, accumulation and turnover of lipids and hydrocarbons. The mixed species culture of *T. koningii* and *P. janthinellum* was revealed to be a important biocatalyst for the production of lipids, from single C_{10} and C_{11} fatty acids and mixed fatty acids with PDB. The mixed species culture grown on UDA+PDB shows the highest accumulation of intracellular and extracellular lipids (58% of the biomass), which were dominated by C_{14} to C_{18} fatty acids. These latter free fatty acids account for 88% of the total lipids produced and 46% of total biomass, when UDA+PDB is sole source of carbon and energy. The intracellular and extracellular accumulated fatty acids range from C_{14} and C_{18} carbon chain length produced by the mixed species culture in all substrates. For all the carbon substrates the concentration of PUFA (between 2 and 73 mg) and MUFA (between 1 and 86 mg) increased (between 2 and 30%) from exponential to stationary phase, while SFAs

decreased from exponential to stationary phase. The highest concentration of SFA (295 mg), MUFA (86 mg) and PUFA (73 mg) was observed when UDA+PDB was the substrate during the exponential phase.

The fungal mixed culture grown on different carbon sources show the highest production of hydrocarbons when grown in UDA+PDB, where hydrocarbons account for 4% during the exponential phase. The high production and accumulation of hydrocarbons and fatty acids intracellularly and extracellularly during the exponential phase, shows a great potential for development of new advanced biofuels via fungal metabolism. The production of hydrocarbons by the mixed culture grown in undecanoic acid shows that mixed species culture of *T. koningii* and *P. janthinellum* decarboxylates this fatty acid to produce lipids, especially hydrocarbons. The intracellular lipids which accumulate rapidly (2.7 mg/hour) during the exponential phase of the cells turnover rapidly at 2.1 mg/hour during the stationary phase, especially when the mixed species culture is grown in UDA+PDB.

Chapter 4

^{1}H and ^{13}C- Nuclear Magnetic Resonance and Pyrolysis Field Ionization Mass Spectrometry of hydrocarbons and other lipids produced by mixed culture of *Trichoderma koningii* and *Penicillium janthinellum* grown on different carbon sources

4.1 Abstract

There is no information on the composition of hydrocarbons and other lipids produced by aerobic batch cultures of *Trichoderma koningii* and *Pencillium janthinellum* Hydrocarbons and other chemical classes produced by a mixed fungal species culture of *T. koningii* and *P. janthinellum* growing on different carbon substrates were characterized by ^{1}H and ^{13}C-nuclear magnetic resonance (NMR) spectrometry and pyrolysis-field ionization mass spectrometry (Py-FIMS). Data from the NMR and Py-FIMS analysis of lipid fractions extracted from the mixed fungal culture support data on the fungal production of intracellular and extracellular lipids measured by gas chromatography and thin layer chromatography with flame ionization detection Iatroscan reported in Chapter 3.

The ^{1}H and ^{13}C NMR spectra showed an abundance of aliphatic and aromatic protons and carbons. According to both types of NMR analysis, the intensity of chemical shifts for all functional groups of organic matter in the intracellular pool of the mixed culture was much higher during the exponential than the stationary phase. Py-FIMS analysis showed that the identified organic components in the intracellular and extracellular pools could be classified into the following twelve classes: (a) low molecular weight compounds (< m/z 50); (b) carbohydrates; (c) phenols + benzene type structures; (d) lignin type components; (e) alkyl aromatics; (f) N-heterocyclics; (g) n-fatty acids; (h) hydrocarbons including n-alkanes, alkenes and n-alkylesters; (i) sterols; (j) suberin type compounds and (k) high molecular weight compounds (>m/z 558). The fungal mixed species culture produced many lipids and various size hydrocarbons, when undecanoic acid (UDA), undecanoic acid + potato dextrose broth (UDA+PDB) and

potato dextrose broth (PDB) alone were used as the carbon and energy sources. As measured by Py-FIMS, the total content of intracellular and extracellular hydrocarbons produced by the mixed species of fungi, including n- alkanes, alkenes and n-alkyl esters were the most abundant components with total yields ranged from 7.4 to 15.7% of total ion intensities (TIIs), and that for fatty acid concentrations ranged from 4.4 to 6.6% of TIIs, measured for all identified compounds, depending on the carbon substrate.

The amount of lipids and hydrocarbons produced was highest in the extracellular pool when the mixed culture was grown on PDB alone. Conversely, most hydrocarbons and fatty acids accumulated in the intracellular pool when the fungal mixed culture was grown on UDA or UDA+ PDB. Fatty acids in all intracellular and extracellular samples ranged from *n-*C_{11} to *n-*C_{18}, alkanes from *n-*C_{19} to *n-*C_{34}, alkenes from n-$C_{30:1}$ to n-$C_{40:1}$ and long chain alkyl esters from C_{41} to $C_{60:1}$. The presence of long-chain hydrocarbons such as alkanes, alkenes and alkyl esters indicates the potential production of advanced biofuels from fatty acids in pyrolytic bio-oil by the mixed species culture of *T. koningii* and *P. janthinellum*.

4.2 Introduction

Efforts are being made to develop new processes and technologies to meet the growing demands for energy, fuel and synthetic chemistry feedstocks with a reduced environmental footprint, with no disruption of the food supply and enhanced economic gains. The production of renewable fuels, chemicals and food products needs to be sustainable and secure, while at the same time environmentally friendly (Stephanopoulous 2007; Rittmann 2008). The development of novel technologies for producing a new generation of biofuels requires the advancement of our present knowledge on biocatalysis and the chemical composition of biofuels derived from biomasses. The potential renewable energy carriers include biologically produced hydrocarbon molecules and their derivatives. Numerous hydrocarbons and other chemically useful industrial feedstocks are natural metabolites and part of structural portions of microorganisms (Birch and Bachofen, 1988). Fossil hydrocarbons have a high energy density on the basis of both mass and volume, which lead to production of substantial energy when these hydrocarbons are oxidized (Hong *et al.*, 2012). Current fuels, such as petroleum and its refined products, consist of a mixture of hydrocarbons of different carbon chain-length depending on their use. The core components of fuels are aromatic and aliphatic hydrocarbons, thus biologically produced hydrocarbons may be suitable as biofuels (Spormann and Widdel, 2000).

Properties of new biofuels include lower oxygen content similar to that of gasoline, low viscosity, high-energy content and few acidic components (Monreal and Schnitzer, 2011). Hydrocarbons are known to retain their original composition over a

long period of time because they are the most stable of naturally occurring compounds (Ladygina *et al.*, 2006). New biofuels that are enriched in alkane, alkenes and alcohols, will have similar properties as gasoline fuels.

Hydrocarbons are found in all living organisms and they are widely distributed in bacteria, yeast, algae, higher plants and insects (Templier *et al.*, 1990). Fungi have the potential to produce high quantities of hydrocarbons, due to their ability to use a wide variety of compounds as sources of carbon and also their ability to survive under extreme environmental conditions (Ladygina *et al.*, 2006). Accumulation of hydrocarbons is higher in algae and fungi than in other microbial species. Aliphatic hydrocarbons are synthesized from fatty acids in all the species through different pathways as explained above in the introduction (Section 1.8). In microbes, the overall pathway involved in the production of alkanes through fatty acids is shown in Figure 5 of Chapter 3.

Results reported in Chapter 3 indicate that a mixed species culture of *T. koningii* and *P. janthinellum* convert fatty acids used as carbon and energy sources into hydrocarbons and other lipids. This chapter reports on the chemical composition of hydrocarbons, lipids and other metabolites as characterized by the spectrometric analytical techniques of pyrolysis field ionization mass spectrometry (Py-FIMS) and nuclear magnetic resonance (NMR). Such detailed information helps elucidate the molecular composition of hydrocarbons, other lipids and other intracellular and extracellular fungal metabolites for potential transportation and industrial uses.

4.2.1 Research objectives

The specific objective of this study was to characterize the molecular composition of hydrocarbons and other metabolites produced by the mixed species culture of *T. koningii* and *P. janthinellum* when grown on UDA, UDA+ PDB and PDB. The fungal metabolites are characterized by Py-FIMS and NMR.

4.3 Material and Methods

The fungal mycelia and growth media samples used for chemical analysis in this study include the same samples identified in Chapter 2. Samples include intracellular and extracellular lipid pools extracted from a mixed culture of *T. koningii* and *P. janthinellum*.

4.3.1 Chemical analysis of fungal lipid

The 500 μL stored chloroform lipid fraction, obtained as described in Chapter 3, was used to characterize the lipid fraction by NMR and Py-FIMS. These fractions corresponded to intracellular and extracellular parts studied during 576 hours of fungal growth.

4.3.1.1 Analysis by Nuclear magnetic resonance (NMR)

Samples for NMR analysis were prepared by drying the chloroform extracts of intracellular and extracellular lipids. The dried samples were then dissolved in deuterated chloroform ($CDCl_3$). Proton and ^{13}C NMR spectra of the intracellular and extracellular lipids samples were recorded on a Bruker AMX spectrometer operating at 500 MHz. The ^{13}C spectra were subdivided into 6 chemical shift regions according to the types of carbon: alkyl-C (0–40 ppm), O-alkyl-C and amino acids-C (41– 60 ppm), carbohydrate-C (61–105 ppm), aromatic-C (106–150 ppm), phenolic-C (151–170 ppm) and carboxyl-C

(171–190 ppm) (Muñoz *et al.*, 2008). The ^{1}H spectra was subdivided into 3 main regions; aliphatic-H (0-2.9 ppm), carbohydrate-H and polyether-H (2.9-4.2 ppm) and aromatic-H and aromatic heterocyclic-H (6.2-8.6 ppm) (Muñoz *et al.*, 2009).

4.3.1.2 Analysis by pyrolysis field ionization mass spectrometry (Py-FIMS)

For the analysis by Py-FIMS, 5 µL of the chloroform extracts of intracellular and extracellular lipids was transferred to a quartz micro-oven which was introduced in the vacuum chamber of a double-focusing MAT 900 mass spectrometer (Finningan MAT, Bremen Germany) and then heated under high vacuum of 10^{-6} pa from 50 to 700 °C. The temperature increased at the rate of 10°C per minute. During about 16 minutes of total measurement time, 46 magnetic scans were recorded for the mass range 15 to 900 Daltons (single spectra). These scans were combined to obtain one thermogram of total ion intensity (T11) and a summed Py-FI mass spectrum (Schnitzer *et al.*, 2008).

4.4 Results

4.4.1 NMR analysis of lipid extracts of mixed culture grown on different substrates

4.4.1.1 ^{13}C NMR spectrum

The NMR spectra for the intracellular lipid extract of mixed species culture growing on UDA+PDB during exponential and stationary phases is shown in Figure 11A and 11B respectively, where notable spectral differences in intensities can be observed between the two growth phases. The most abundant carbon corresponds to 0-40 ppm (alkyl-C) and 171-190 ppm (carboxyl-C). The chemical shifts at 14 ppm observed in both the spectra are due to terminal CH_3 carbons. The resonances at 22, 24, 29, 30, 31, 33 and 34 ppm arose from straight chain aliphatic hydrocarbons. The signals observed at 178 and 179 ppm is due to the carboxyl carbons. The signals at 76 and 77 ppm are due to

$CDCl_3$, the NMR sample solvent. The spectra of both growth phases show the same chemical shifts but the intensities are different. These spectra are enriched in aliphatic CH_2 groups, showing the accumulation of alkanes and fatty acids. The intensity of the carboxyl carbon signal is lower in the stationary phase as compared to the exponential phase, which reflects a decrease in intracellular fatty acids inside as reported in the Results section of Chapter 3. Similar spectra to Figure 11A and 11B are also observed for the mixed species culture growing on other carbon substrates. The ^{13}C NMR data complements the data presented in chapter 3 showing the highest accumulation of hydrocarbons and fatty acids. Although the NMR analysis shows the presence of intracellular lipids, the presence of intracellular alkanes cannot be demonstrated unambiguously by data obtained from this spectroscopic analysis.

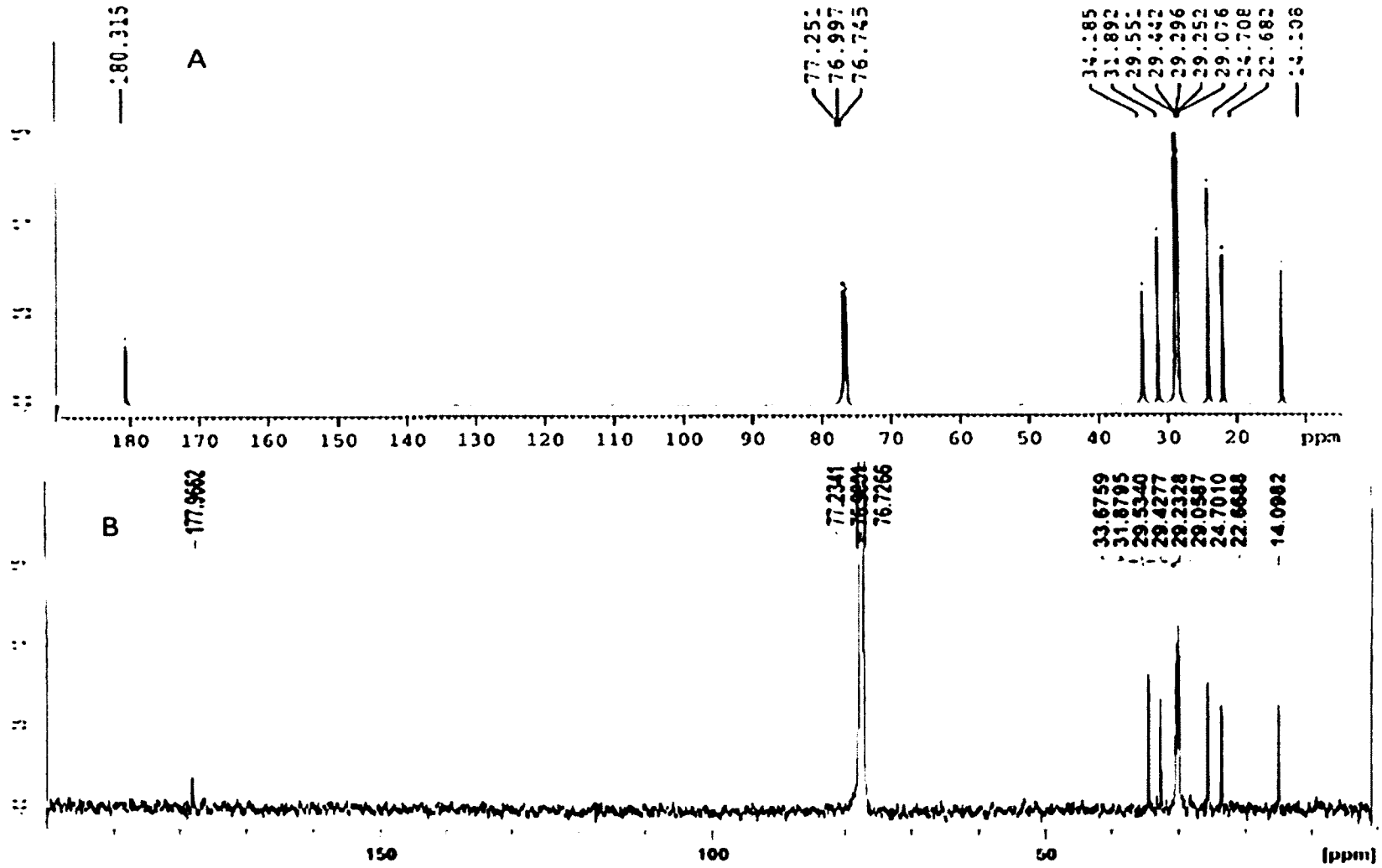


Figure 11: ^{13}C NMR spectrum of intracellular lipid extract of mixed species culture grown on mixture of UDA+PDB extracted during A) exponential phase, and B) stationary phase.

4.4.1.2 ^1H NMR spectrum

Figures 12 and 13 show the ^1H NMR spectra for the intracellular lipids extracted during exponential and stationary growth phases of mixed species culture when grown on UDA+PDB and PDB. For both substrates, strong signals were observed between $\delta = 0$ and 2.9 indicating the predominance of protons in aliphatic chains (CH_3, CH_2 and CH groups bonded to C). The signals observed between δ= 2.0 and 2.6 ppm can be assigned to protons on functional groups such as amide, carbonyl or ester groups. The signals between δ= 2.9 and 4.2 ppm indicate protons of carbohydrates and those between 5.3 and 5.7 correspond to olefinic protons. During the stationary phase of the mixed species culture growing in both carbon substrates, additional chemical shifts are observed at 4.3 and 4.4 ppm, indicating methylene protons of possibly $C\text{-}CH_2\text{-}NO_2$ (Figure 12B and 13B). The resonance observed at 7.3 ppm is due to $CDCl_3$, which is the solvent. When UDA+PDB is the substrate, the NMR spectra of the mixed species culture shows that the peak intensities during the stationary phase are much smaller compared to the exponential, which corresponds to higher concentration of aliphatic proton in the exponential phase than in the stationary phase (Figure 12). Figure 13 shows that when PDB is the carbon source, higher peak intensities for aliphatic and carboxylic regions are observed for samples taken during stationary phase than those taken during the exponential phase. These data support the results reported in Chapter 3. Figure 14 shows the ^1H NMR spectra of a chloroform extract of PDB without any fungal inoculation. The PDB spectrum shows very similar chemical shifts as those observed for the mixed culture

growing in PDB during the stationary phase, but the peak intensity of aliphatic protons is much lower in the chloroform extract of PDB.

The ^{13}C and ^{1}H NMR spectra of the mixed species culture grown on different substrates shows dominance of aliphatic protons, which reflect the dominance of hydrocarbons and other lipid components as observed in results reported in Chapter 3. The decrease in peak intensities for the aliphatic and COOH regions during the stationary phase of mixed species culture grown on UDA+PDB corresponds to the turnover of lipids produced during the exponential phase. As per the ^{13}C and ^{1}H NMR results, this analytical technique alone is not able to confirm the intracellular fungal biosynthesis of lipid classes and specifically hydrocarbons.

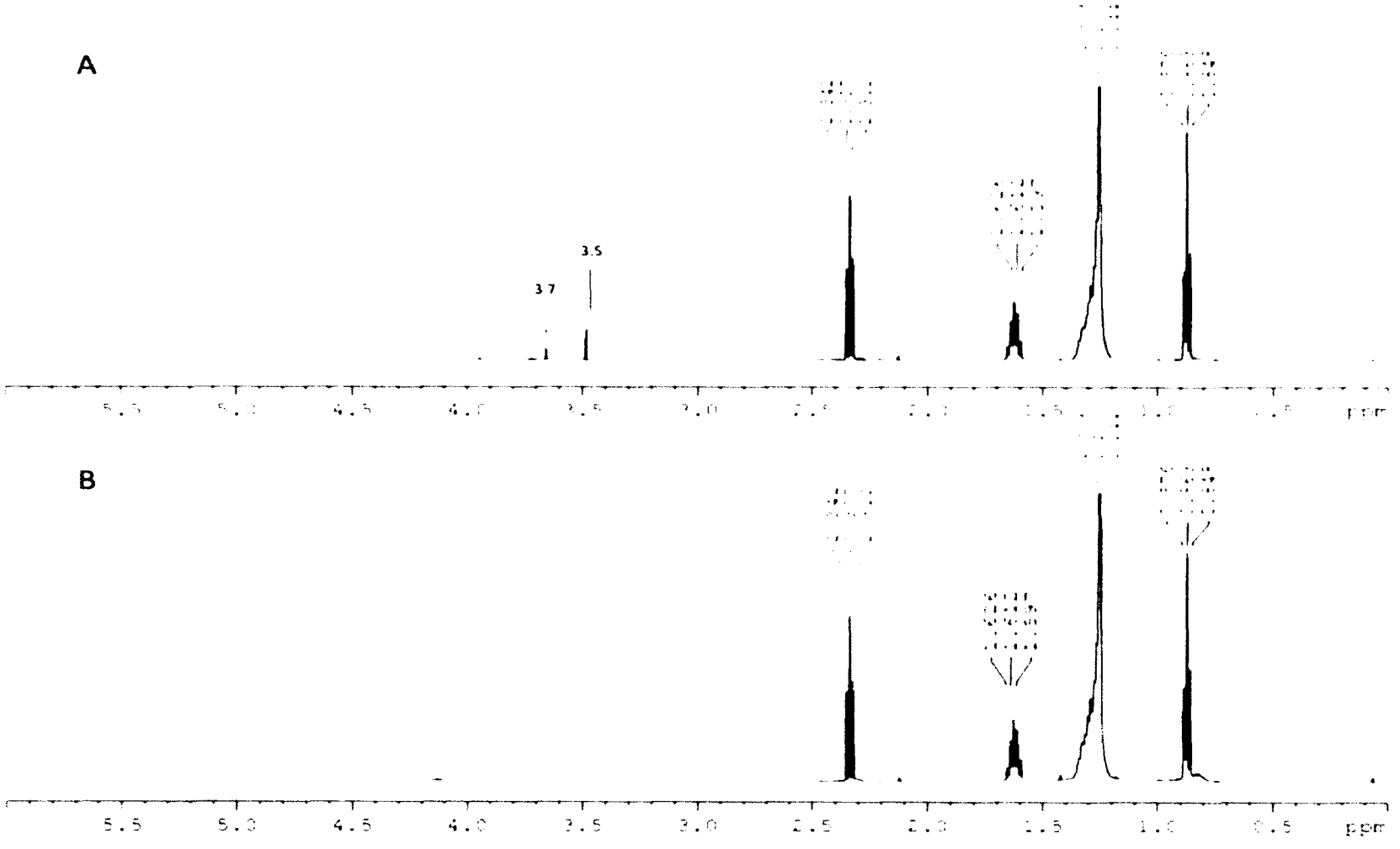


Figure 12: ^{1}H NMR spectrum of intracellular lipid extracted from the mixed species culture grown on mixture of UDA+PDB extracted during A) exponential phase, and B) stationary phase

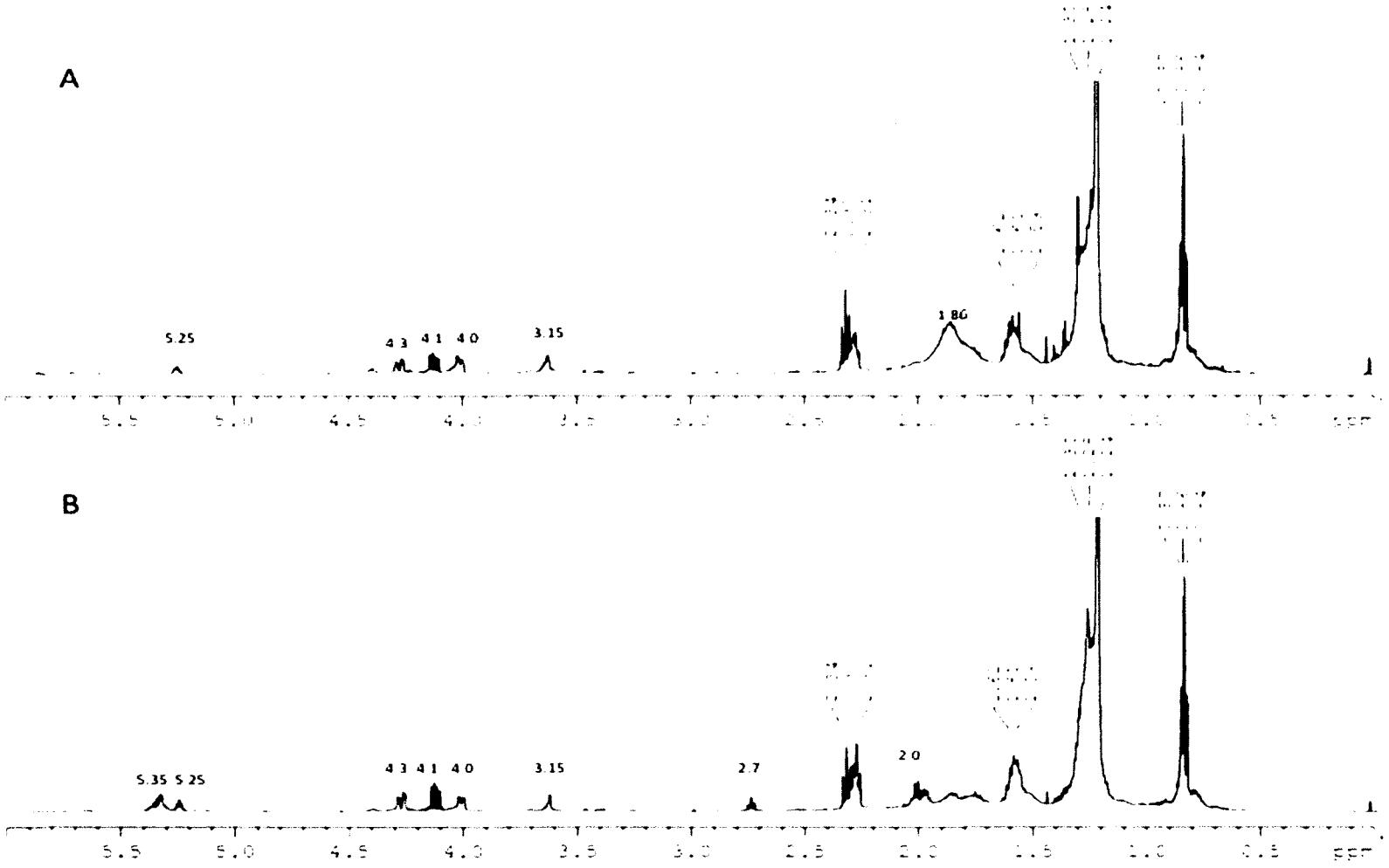


Figure 13: ^{1}H NMR spectrum of intracellular lipid extract of mixed culture grown on PDB extracted during A) exponential phase, and B) stationary phase

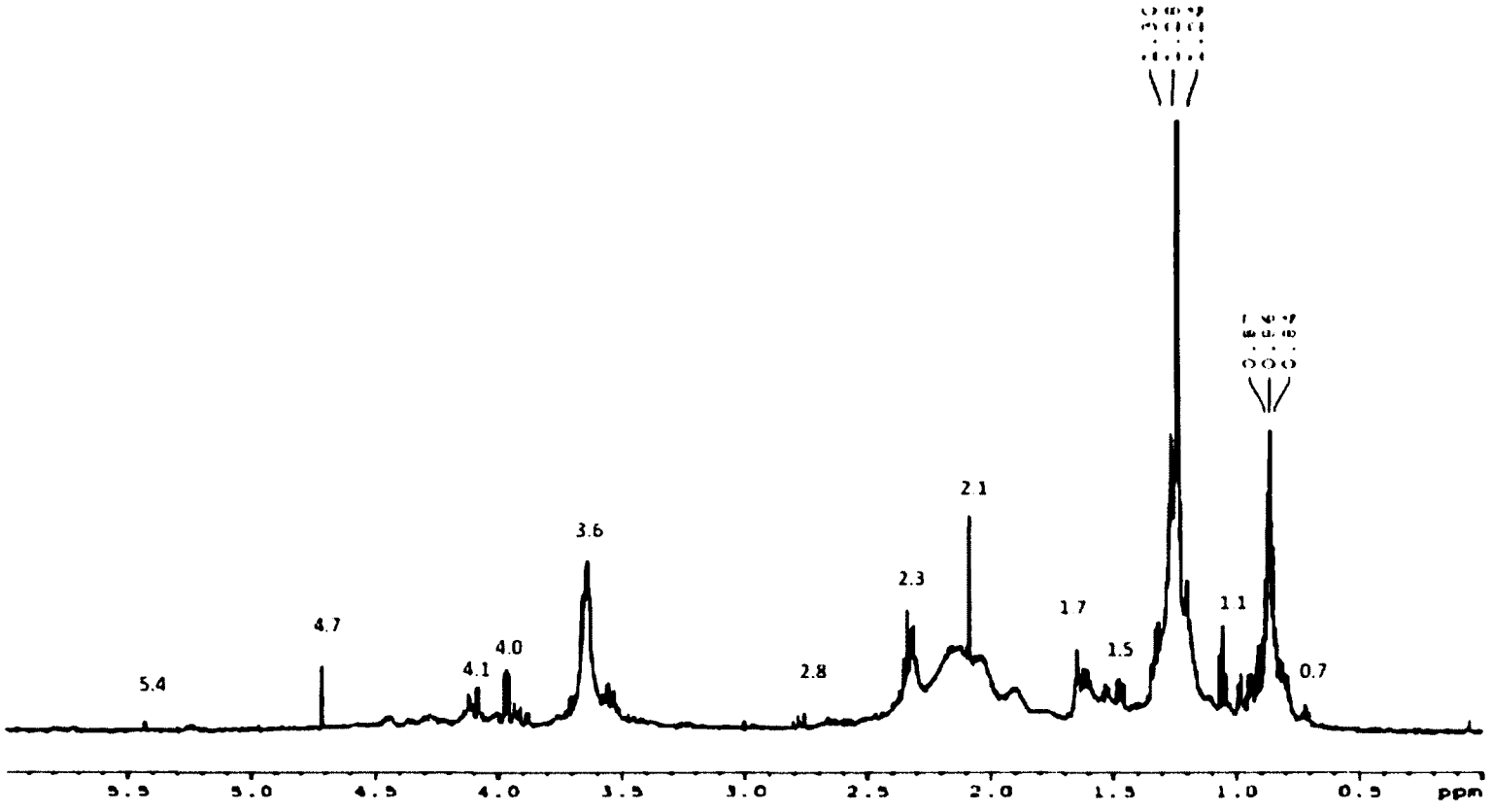


Figure 14: ^{1}H NMR spectrum of the chloroform extract of PDB.

4.4.2 Selection of samples for Py-FIMS analysis of lipid fractions

The Py-FIMS spectra of intracellular lipids extracted from the mixed species culture grown in UDA during the stationary phase is presented in Figure 17, and a mixture of PDB+UDA during the exponential phase are presented in Figure 18. Figure 19 and Figure 20 present the Py-FIMS spectra of extracellular lipid extract of the same mixed fungal species culture growing in UDA+PDB during the exponential phase, and in PDB during the stationary phase, respectively. These samples were selected based on the carbon substrate treatments that produced the highest amount of biomass and lipids as shown in Chapter 2 and 3. When UDA+PDB is the carbon and energy source, the highest accumulation of lipids and biomass production was observed during the exponential phase. Therefore intracellular and extracellular extracts from UDA+PDB media were used to characterize hydrocarbons and other lipids produced. Conversely, when PDB was the carbon source, the highest accumulation of lipids was observed during the stationary phase. In addition, the hydrocarbon concentration was higher in the extracellular lipid extract as seen in Chapter 3. Thus, this sample was used to characterize lipids by Py-FIMS. When UDA was the carbon source, the accumulated biomass was highest in the stationary phase. In addition, this substrate yielded the highest lipid content as seen in Chapter 3. The analysis of lipid extracts from the mixed species culture grown on UDA showed that the mixed species culture can use fatty acids as the sole source of carbon converting these fatty acids into different lipid classes and other compounds.

This sole replicate of selected samples were used to analyze hydrocarbons to observe the trends of hydrocarbons produced by mixed species culture grown on different carbons substrates. The assignment of the peaks are tentative, only major peaks were

assigned, the results from chapter 3 show that these samples are enriched in different lipid classes, the Py-FIMS peaks were assigned to different lipid molecules with same molecular masses as observed in the py-FIMS analysis.. The assignment of m/z values determined for analyzed samples by Py-FIMS followed the approaches by Monreal and Schnitzer, (2011) and Schnitzer et al., (2008). The original identification of compounds by Py-FIMS was derived from intensive research with plant, biooil and soil sample materials. Therefore, the assignment of m/z determined from the analysis of our samples need to be further confirmed by an analytical techniques of mass spectrometry such as liquid chromatography electronspray ionization mass spectrometry- mass spectrometry (LC – ESI- MS/MS).

4.4.2.1 Chemical composition of PDB (solid sample) and PDB extracted with $CHCl_3$

Analysis by Py-FIMS was also performed on PDB (solid sample) and PDB extracted with $CHCl_3$ to confirm that the extracellular lipids produced in the mixed fungal species culture originate from the fungal activity and not from the PDB substrate. The Py-FIMS spectrum in Figure 15 shows that PDB consists of compounds mostly with masses < 550 daltons. Ten major chemical classes of compounds were found in the solid PDB, with carbohydrates and alkylaromatics being the most abundant ones followed by phenols+ benzene type compounds, N-containing compounds (N-heterocyclics) and peptides (Table 22). Other aromatic compounds plus sterols and fatty acids were present in low concentrations (< 1%) of TIIs (Table 22). Two main carbohydrate molecules observed in the PDB were levogluosenone and anhydrohexose, which are pyrolysis products of sugars (Table 16). There was also a small concentration of low molecular weight molecules, which include several alkyl radicals. The thermogram depicted on the

upper right corner of Figure 15, shows that a thermolabile organic matter from PDB was volatilized during pyrolysis at about 220 °C, and that another more stable component was volatilized near 470 °C.

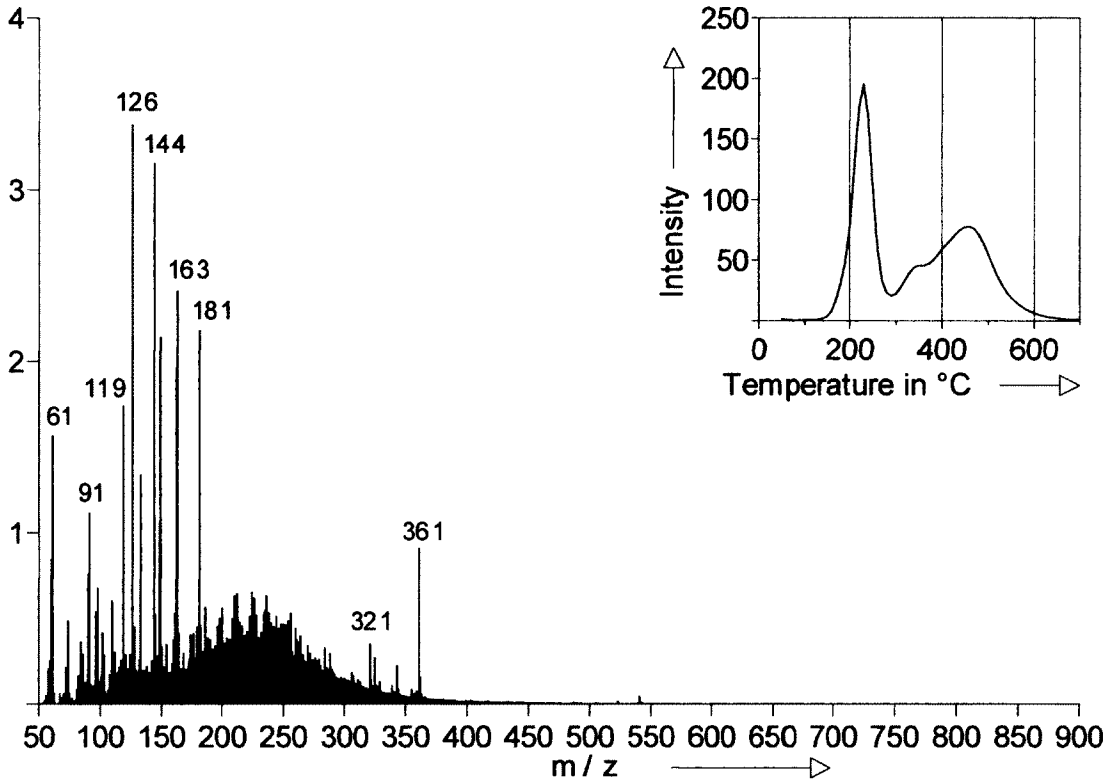


Figure 15: Py-FIMS spectra of potato dextrose broth (PDB) (Solid)

Table 16: Identities of major components of potato dextrose broth (solid).

Peak number	m/z	Identity
1	61	Protonated hydroxyacetaldehyde
2	91	Benzyl radical
3	119	4-hydroxybenzonitrile
4	126	Levogluosenone
5	144	Anhydrohexose
6	163	Hexose (Levoglusan)
7	181	Phenols+ benzene type compounds (syringyl aldehyde)
8	321	Unknown
9	361	Unknown

Figure 16 and Table 17 show the Py-FIMS spectrum and predominant organic molecules present in the chloroform extract of the liquid growth PDB medium in the absence of fungal species. This spectrum shows a few major m/z signals consisting mostly of compounds with m/z < 450 Daltons, such as alkanes, alkenes, fatty acids and lignin type components (Table 17). The main lipid molecules in the chloroform extract of the PDB liquid medium were fatty acids (m/z 256 (C_{16}) and m/z 284 (C_{18})) and alkenes (m/z 196 ($C_{14:1}$) and m/z 210 ($C_{15:1}$)). The spectrum also shows the presence of a long-chain alkane (n-C_{26}) at m/z 366 (Table 17). These lipids are present as major peaks in the liquid PDB growth media, but they were not observed as major peaks in the PDB (solid). Lipids were the major chemical class present in the chloroform extract of PDB (control, no fungi). Lipids account for 17.7 million counts/ μL (Table 22). Other major compound classes present were alkyl-aromatics, phenols and benzene type compounds and N-containing compounds which account for 8.9, 5.6 and 4.9 million counts/μL respectively. Sterols were present in small quantity with TIIs of 2.6 million count/μL (Table 22).

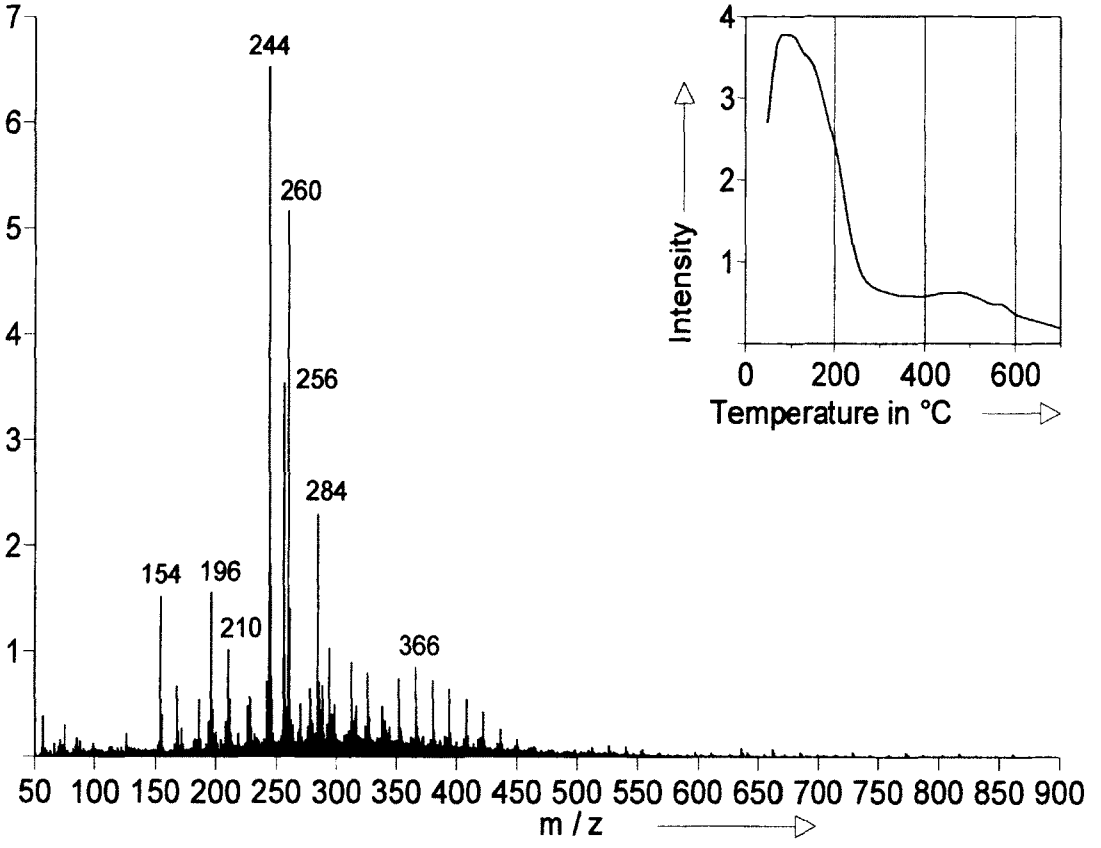


Figure 16: Py-FIMS spectra of the chloroform extract of the PDB liquid growth media in the absence of fungi.

Table 17: Identities of major components of chloroform extract of PDB.

Peak number	m/z	Identity
1	154	Phenol+ benzene type compounds (Syringol)
2	196	$C_{14:1}$ alkene
3	210	$C_{15:1}$ alkene
4	244	n- C_{15} diols
5	256	Palmitic acid (nC_{16})
6	260	Lignin type components
7	284	Stearic acid (nC_{18})
8	366	C_{26} alkane

4.4.2.2 Py-FIMS analysis of intracellular lipid extract from mixed species culture grown on UDA during the stationary phase

Figure 17 and Table 18 show the Py-FIMS spectra and identifies the major m/z signals of the intracellular lipid pool extracted from the mixed species culture growing on UDA during the stationary growth phase. The major components in this spectrum are two low molecular weight compounds (m/z < 50), ethyl aldehyde and propyl radicals. Saturated fatty acids (nC_{11}, C_{16}, C_{18}) and a long-chain alkyl di-ester (nC_{41} at m/z 636) were also observed (Figure 17). The most intense fatty acids peak shows the presence of UDA (n-C_{11}), which shows very high concentrations accumulating intracellularly. Low molecular weight components produced by the mixed species culture when growing on UDA account for only 3.2% of their total ion intensities (TIIs) as determined by PyFIMS (Table 22).

Another organic component found in the intracellular extract is heptadecylbenzene, (m/z 316) an alkylaromatic. The alkylaromatic components represent 2.0% of the TIIs (Table 22). The signal at m/z 858 was small and could not be identified (Figure 17, Table 18).

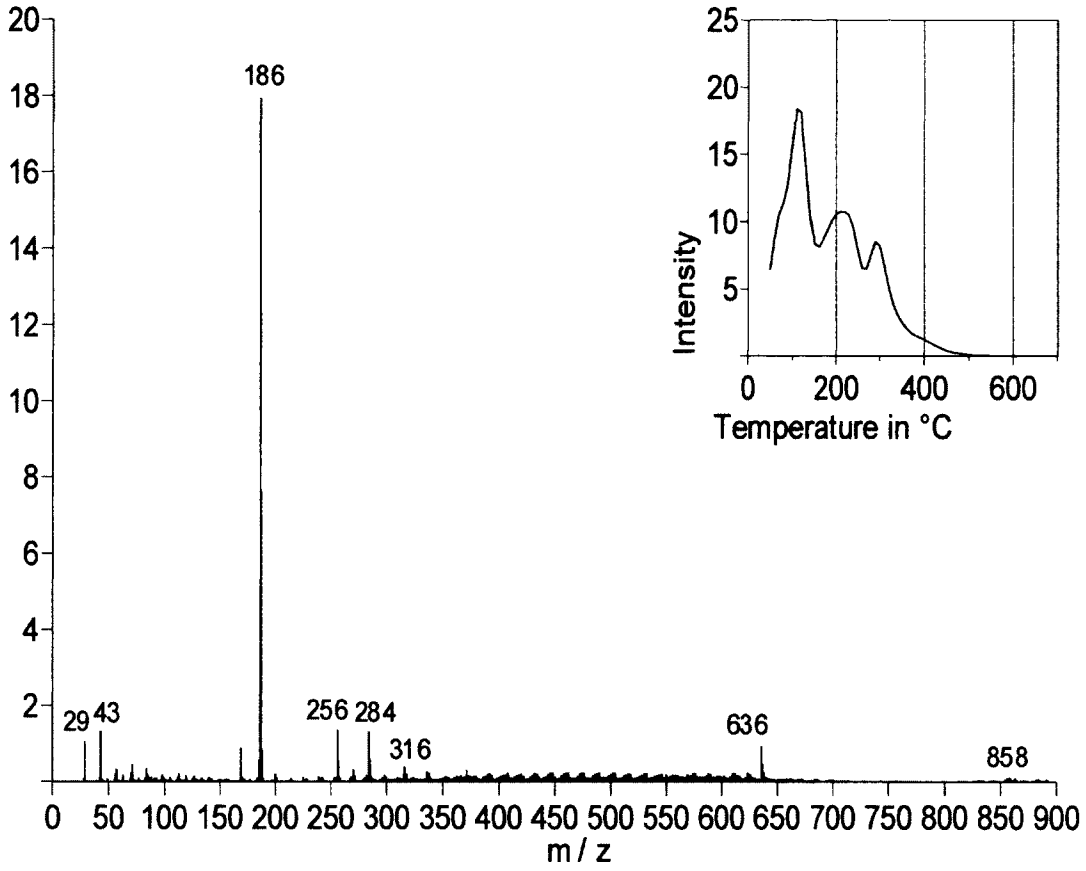


Figure 17: Py-FIMS spectra of the intracellular lipid extract obtained from the mixed species culture grown in UDA during the stationary phase.

Table 18: Identities of major components of intracellular lipids extract of mixed culture grown in UDA during stationary phase.

Peak number	m/z	Identity
1	29	Ethyl radical and aldehyde radical
2	43	Propyl radical
3	186	Undecanoic acid (nC_{11})
4	256	Palmitic acid (nC_{16})
5	284	Stearic acid (nC_{18})
6	316	Heptadecylbenzene
7	636	nC_{41} alkyl diester
8	858	Unknown

The content of intracellular lipids (alkanes, alkenes, fatty acids, n-alkyl esters) was 39.2 million count/ μL and it represented the major chemical class produced by the mixed species culture growing on UDA. Fatty acids (n-C16 to n-C34) produced by the mixed species culture on UDA account for 5.6% (16.9 million count/ μL) of their TIIs (Table 22). Alkanes, alkenes and n-alkyl diesters (m/z 636) account for 7.4% (22.3 million count/ μL) of the TIIs as analyzed by PyFIMS. The concentration of other classes of compounds such as sterols, N-containing compounds ranged from 1.6 to 2.5% of the identified TIIs (Table 22). The presence of medium-chain fatty acids, alkanes, long-chain alkyl diesters, sterols and other compound classes shows that the mixed species culture is able to metabolize UDA, and produce and accumulate the intracellular lipids and other metabolites. The latter organic compounds do not come from the growth media, as UDA was the only carbon and energy source provided to the mixed species culture.

4.4.2.3 Py-FIMS analysis of the intracellular lipid extract from the mixed species culture grown on mixture of UDA+PDB during the exponential phase

Table 19 and Figure 18 show the Py-FIMS analysis of the intracellular lipids extracted from the mixed species culture grown on UDA+PDB. The Py-FIMS spectra of Figure 18 shows the major chemical classes including low molecular weight compounds alkyl radicals (alkyl radicals m/z < 50), fatty acids, N-containing compounds and sterols. The major low molecular weight compounds in these lipid extracts are ethyl and propyl radicals (Table 19). Fatty acids are found at m/z 186 (undecanoic acid, n-C_{11}), 242 (pentadecanoic acid, n-C_{15}), 256 (palmatic acid, n-C_{16}) and 284 (stearic acid, n-C_{18}). The compound at m/z 414 in the intracellular lipid extract may be either β- sitosterol or a $C_{30:4}$ alkene. The m/z 169 appears to be a protonated form of the alkene n-$C_{12:1}$ (Table 19). The

high molecular weight compounds corresponding to m/z 636, 856 and 882 daltons are n-C_{41} alkyldiester, n-$C_{58:1}$ alkylmonoester and n-$C_{60:1}$ alkylmonoester, respectively (Table 19).

Table 22 shows that other major lipids produced intracellularly in the UDA+PDB substrate are n-alkanes, n-alkenes and n-alkyl esters which account for 14.6% of the intracellular lipid TII's. The intracellular fatty acids account for 6.6% of TIIs analyzed by Py-FIMS. Thus the total intracellular lipids account for 21.2% of the TII of all intracellular chemical components (Table 22). Alkyl aromatics represented (4.2%), phenols + benzene type compounds (1.3%), lignin type components (1.6%) and suberin type compounds (2.8 %) of the TIIs (Table 22). The latter aromatic and alkyl-aromatic compounds are associated with the intracellular synthesis of intermediates of metabolism the total content of lipids growing on UDA+PDB is 25.8 million count/ μL. In comparison the lipid content in the chloroform extract of PDB (no fungi), 17.7 million count/ μL (Table 22). Thus, the fungal culture produced intracellularly 8.1 million counts more than the lipid content present in the growth media. The latter shows that fungal biosynthesis by the mixed culture allocated significant amounts of lipids intracellularly during the exponential phase.

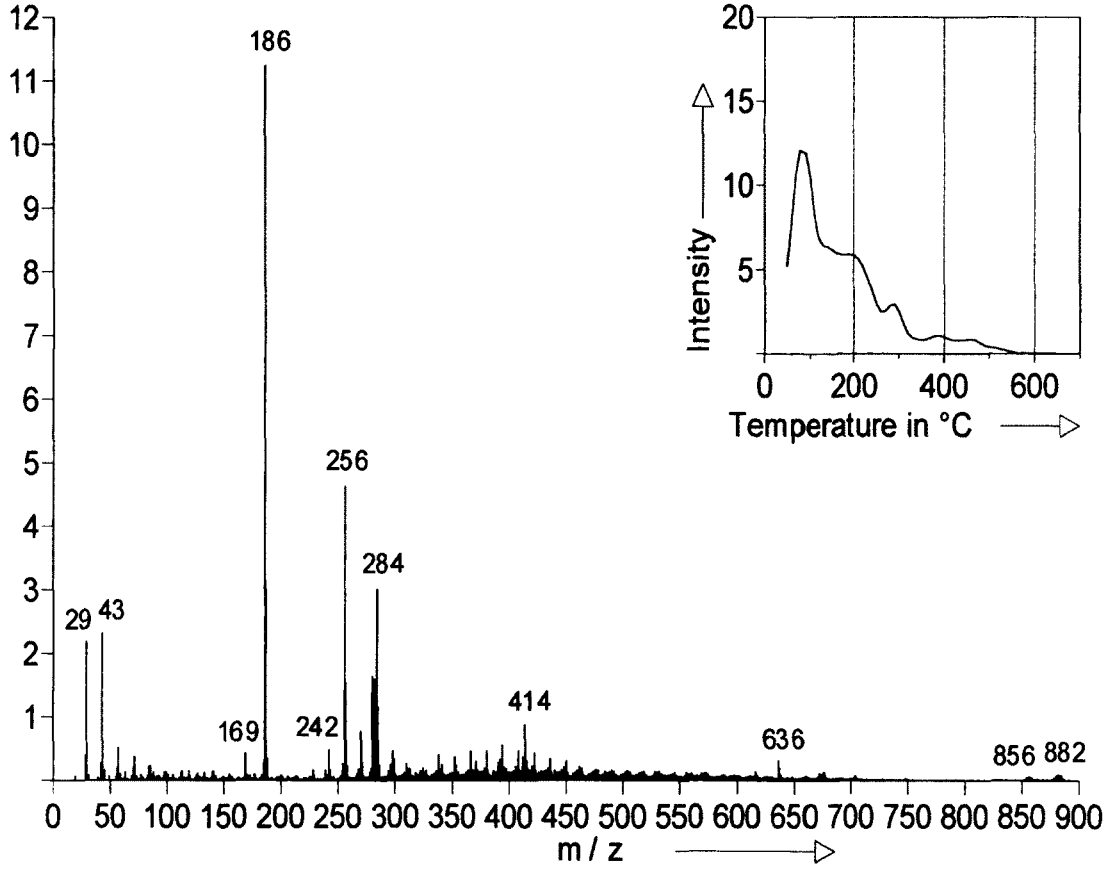


Figure 18: Py-FIMS spectra of intracellular lipids extract of mixed culture grown in mixture of UDA+PDB during exponential phase.

Table 19: Identities of major components of intracellular lipids extract of mixed culture grown in mixture of UDA+PDB during the exponential phase.

Peak number	m/z	Identity
1	29	Ethyl radical and aldehyde radical
2	43	Propyl radical
3	169	Protonated alkene $C_{12:1}$
4	186	Undecanoic acid (C_{11})
5	242	Pentadecanoic acid (C_{15})
6	256	Palmitic acid (C_{16})
7	284	Stearic acid (C_{18})
8	414	$C_{30:1}$ alkene
9	636	C_{41} alkyldiester
10	856	$C_{58:1}$ alkylmonoester
11	882	$C_{60:1}$ alkylmonoester

The Py-FIMS analysis of the intracellular lipid pool extracted from the mixed fungal species growing on UDA and UDA+PDB alone shows very similar m/z signal composition (Figure 17 and 18 and Tables 18 and 19). These m/z signals show the predominance of the same or similar alkyl radicals, fatty acids, alkenes and alkyl-esters produced intracellularly by the mixed species culture. As a proportion of TIIs, the content of intracellular lipids produced by the mixed species culture growing on UDA+PDB during the exponential phase was 25.8 million counts/µl and also to the amount produced in UDA during stationary phase (22.3 million counts/µl). Py-FIMS also shows that per microliter the content of total extracted intracellular organic components was higher under UDA in stationary phase than under UDA+PDB during exponential phase. The free fatty acids produced by mixed culture growing on UDA during the stationary phase was 5.14 million count/µL higher than that in the mixed species culture growing on PDB+UDA during the exponential phase (Table 22). These results indicate that relative to TIIs, the proportion of lipids produced under PDB. The absolute amounts of lipids, however, were similar under UDA and UDA+PDB.

4.4.2.4 Py-FIMS analysis of extracellular lipids extracted from the UDA+PDB growth media during the exponential growth phase

Figure 19 and Table 20 show the Py-FIMS spectrum and the predominant organic compounds found in the extracellular lipid extract obtained from the mixed culture grown on UDA+PDB during the exponential phase. Table 22 shows that the amount of lipids produced by the mixed species culture were twice as high in the intracellular than in the extracellular pool. Also, the amount of lipids produced extracellular by the mixed species culture grown on UDA+PDB (90.5 million/µl) is

slightly higher than lipid content of chloroform extract of PDB (no fungi). Table 20 also shows that the extracellular pool of lipids contains many fatty acids and alkanes. The signals at m/z 172, 186, 201, 256, 284 and 514 are fatty acids. The total amount of fatty acids account for 6.6% of the TIIs (Table 22). The extracellular fatty acids consist of the same fatty acids found intracellularly including n-C_{10}, n-C_{11}, n-C_{16}, and n-C_{18} and m/z 514 possibly be a derivative of the fatty acid, glutaric acid, 2,4- dichlorobenzyl hexadecyl ester (Table 20, Figure 19). The signals at m/z 338, 366, 394, 422 and 450 show presence of longer-chain alkanes, including n-C_{24}, n-C_{26}, n-C_{28}, n-C_{30} and n-C_{32} (Table 20). The signal at m/z 338 could also be $C_{22:1}$ fatty acids, as it has same weight as nC_{24} alkane. The main low molecular weight components are ethyl radicals, propyl radicals and 1-butyl radicals corresponding to m/z signal 29, 43 and 47. Methylbenzene an alkyaromatic compound at m/z 92. Vanillin a phenol is shown at m/z 152. The molecular species for the m/z 558 was not identified.

In addition, the extracellular lipids produced by mixed species culture extracellularly include low molecular weight alkyl radicals, which accounts for 6.2% (1.6 million count/μL) of the TII's produced under PDB+UDA. Lipids are the major chemical class with TIIs of 11.8 million count/μL followed by free fatty acids (4.5 million count/μL). Another major class present in extracellular pool is N-containing compounds, which accounts for 4.4 % (4.0 million count/μL) of the TIIs analyzed by Py-FIMS.

The concentration of lipids in the extracellular extract of mixed species culture growing on UDA+PDB is lower than that in the chloroform extract of PDB. These Py-FIMS results suggest indirectly that the extracellular lipids in the mixed species culture may be associated with those measured in the PDB substrate. Confirmation of the latter

needs further research. Also, free fatty acid (nC_{16} to nC_{34}) concentration in the extracellular pool is similar when the mixed species culture growing on PDB+UDA than that measured in the chloroform extract of PDB. Sterols are another compound produced extracellularly by the fungal species but its amount appears to be slightly higher than the chloroform extract of PDB.

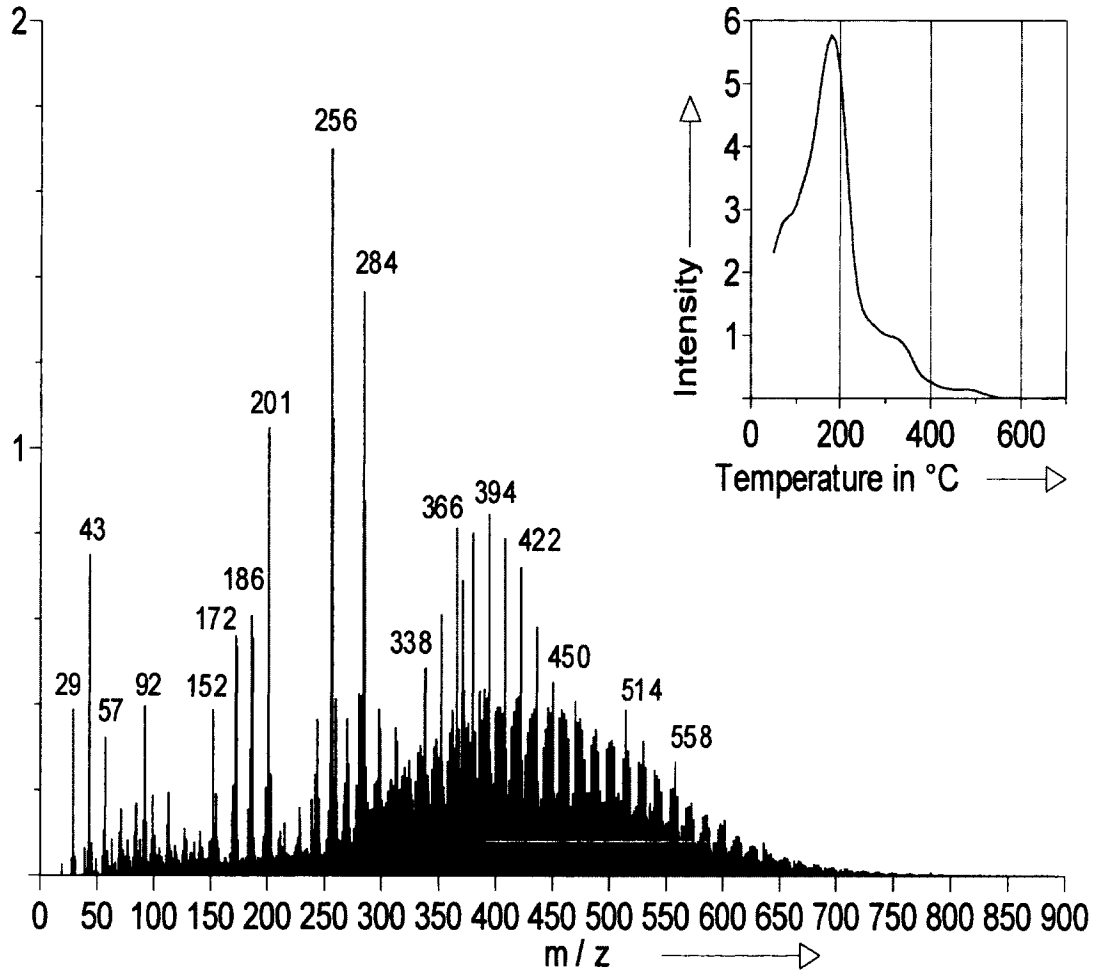


Figure 19: Py-FIMS spectra of extracellular lipids extract of mixed culture grown in mixture of UDA+PDB during the exponential phase.

Table 20: Identities of major components of extracellular lipids extract of mixed culture grown in mixture of UDA+PDB during the exponential phase.

Peak number	m/z	Identity
1	29	Ethyl radical and aldehyde radical
2	43	Propyl radical
3	57	1-butyl radical
4	92	Methylbenzene
5	152	Vanillin
6	172	Decanoic acid (C_{10})
7	186	Undecanoic acid (C_{11})
8	201	Protonated C_{12} fatty acid
9	256	Palmitic acid (C_{16})
10	284	Stearic acid (C_{18})
11	338	C_{24} alkane or $C_{22:1}$ fatty acids
12	366	C_{26} alkane
13	394	C_{28} alkane
14	422	C_{30} alkane
15	450	C_{32} alkane
16	514	Glutaric acid, 2,4-dichlorobenzyl hexadecyl ester
17	558	Unknown

4.4.2.5 Py-FIMS analysis of extracellular lipids extracted from the mixed species culture grown on PDB growth media during the stationary phase

Figure 20 and Table 21 shows the chemical composition of the extracellular lipids extracted from the mixed species culture grown on PDB during the stationary phase. The extracellular extract of mixed species culture growing on PDB has nearly identical m/z signals as those characterized in the extracellular pool by the mixed species culture grown on UDA+PDB (Table 21). Alkanes were the predominant class followed by alkyl radicals in the fungal culture grown on PDB (Table 21). The lipids components including alkanes and alkenes account for 15.7% of the TII's of all classes of compounds analyzed by Py-FIMS. The signals at m/z 268 and 324 could correspond to n-C_{19} alkane or $C_{17:1}$

fatty acid and n-C_{23} alkane or $C_{21:1}$ fatty acid respectively, as they have same molecular weight. The signals at m/z 352, 366, 394, 422 and 450 were alkanes with carbon chain lengths of n-C_{25}, n-C_{26}, n-C_{28}, n-C_{30}, n-C_{32} and n-C_{34}. The signals at m/z 532 and 560 are long chain alkenes $C_{38:1}$ and $C_{40:1}$. The fatty acids in extracellular pool of mixed species culture growing on PDB accounts for 4.4% of the TII's. Fatty acids are indicated by m/z 186 (undecanoic acid, n-C_{11}), 242 (pentadecanoic acid, n-C_{15}), 256 (palmatic acid, n-C_{16}) and 284 (stearic acid, n-C_{18}) (Figure 20). The low molecular weight alkyl radicals include ethyl and aldehyde, propyl, 1-butyl and octyl radicals. A phenol, syringol, is present at m/z 154.

Table 22 shows the TIIs of all the classes analyzed in extracellular lipid extract of mixed species culture growing on PDB. This lipid extracts showed highest amount of total ion intensities per microliter (328.1 million count/μL) compared to all other substrates. Lipids including alkanes, alkenes, alkyl-esters and short chain fatty acids were the most concentrated classes found in Py-FIMS analysis of extracellular lipid extract of mixed species culture growing on PDB. This lipid extract has 34 million count/μL more lipids compared to the chloroform extract of PDB. Similarly it has ~ 11 million count/μL more free fatty acids than the chloroform extract of PDB. The extracellular pool also contains high concentration of alkyl aromatic, N-containing compounds and sterols have TII's 9.1, 10.1 and 22.5 million count/μL (Table 22). The concentration of sterols was 8 times higher in the extracellular lipid extract of mixed species culture growing on PDB than the chloroform extract of PDB. Similarly, other compounds also had higher concentrations than the chloroform extract of PDB and also the other carbon substrates. These results indicate that presence of such high concentration of lipids and free fatty

acids extracellularly could be through the production of these lipids by mixed species culture and their excretion into the media.

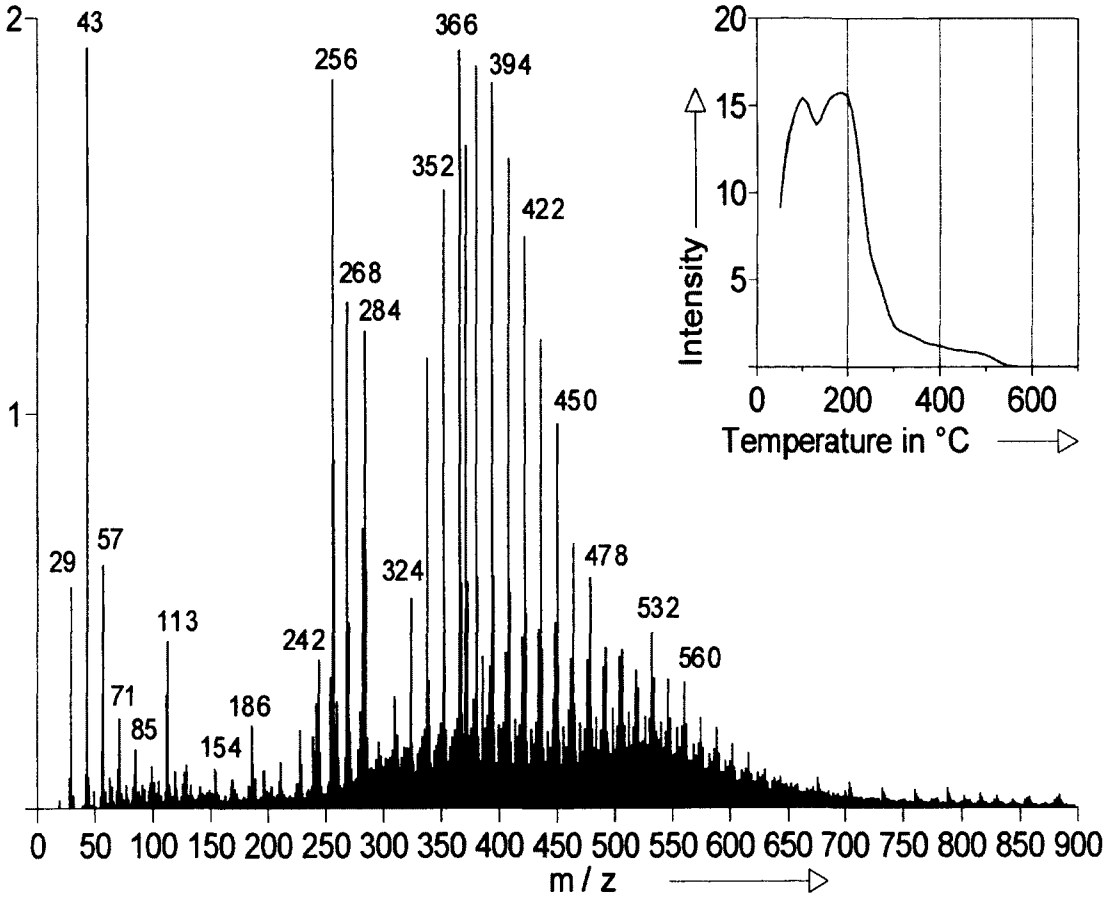


Figure 20: Py-FIMS spectra of extracellular lipids extract of mixed culture grown in PDB during stationary phase.

Table 21: Identities of major components of extracellular lipids extract of mixed culture grown in PDB during stationary phase.

Peak number	m/z	Identity
1	29	Ethyl radical and aldehyde radical
2	43	Propyl radical
3	57	1-butyl radical
4	71	Acetyl radical ($CH_3CHCOCH_3$)
5	85	C_7H anion
6	113	Unknown
7	154	Syringol
8	186	Undecanoic acid (C_{11})
9	242	Pentadecanoic acid (C_{15})
10	256	Palmitic acid (C_{16})
11	268	n-C_{19} alkane or $C_{17:1}$ fatty acid
12	284	Stearic acid (C_{18})
13	324	C_{23} alkane or $C_{21:1}$ fatty acid
14	352	C_{25} alkane
15	366	C_{26} alkane
16	394	C_{28} alkane
17	422	C_{30} alkane
18	450	C_{32} alkane
19	478	C_{34} alkane
20	532	$C_{38:1}$ alkene
21	560	$C_{40:1}$ alkene

Table 22: Summary of yields of compound classes identified by Py-FIMS in the intracellular and extracellular lipid extract of mixed culture growing on different growth substrates during stationary and exponential phases.

		Total ion intensities (TII) as million counts/ μl (%)				
Compound classes	TII (10^6/mg)		Intracellular lipid extracts		Extracellular lipid extracts	
	PDB (Solid) sample	Chloroform extract of PDB	Mixed culture growing in UDA** during stationary phase	Mixed culture growing in PDB+UDA* during exponential phase	Mix culture growing in PDB+UDA during exponential phase	Mixed culture growing in PDB ***-stationary phase
Carbohydrates	315.4 (12.6)	0.7 (1.0)*****	3.2 (1.1)	1.7 (0.9)	0.7 (0.7)	2.4 (0.7)
Phenols+benzene type compounds	184.2 (7.4)	5.6 (6.6)	2.0 (0.7)	1.6 (0.9)	1.2 (1.3)	2.5 (0.8)
Lignin type components	62.3 (2.5)	3.6 (4.2)	1.0 (0.3)	3.0 (1.7)	1.5 (1.6)	5.1 (1.6)
Lipids (Alkanes, alkenes, fatty acids, n-alkyl-esters)	121.4 (4.9)	17.7 (21.1)	22.3 (7.4)	25.8 (14.6)	11.8 (13.0)	51.7 (15.7)
Alkyl aromatics	267.6 (10.7)	8.9 (10.6)	6.0 (2.0)	3.4 (2.0)	3.8 (4.2)	9.1 (2.8)
n-containing compunds	189.6 (7.6)	4.9 (5.9)	7.3 (2.4)	7.8 (4.4)	4.0 (4.4)	10.1 (3.1)
Sterols	7.5 (0.3)	2.6 (3.1)	7.5 (2.5)	8.0 (4.5)	6.1 (6.7)	22.5 (6.9)
Peptides	85.7 (3.4)	1.4 (1.7)	4.9 (1.6)	3.0 (1.7)	1.2 (1.4)	4.4 (1.4)
Suberin type compounds	1.5 (0.1)	0.4 (0.4)	5.8 (1.9)	2.1 (1.2)	2.5 (2.8)	5.6 (1.7)
Free fatty acids n-C16-n-C34	18.5 (0.7)	3.7 (4.4)	16.9 (5.6)	11.7 (6.6)	4.5 (5.0)	14.6 (4.4)

m/z 15.56	33.1 (1.3)	2.2 (2.6)	9.8 (3.2)	11.0 (6.2)	1.6 (1.7)	10.3 (3.1)
M+H and 13C	563.3 (22.6)	12.7 (15.1)	31.3 (10.3)	19.7 (11.2)	13.3 (14.7)	37.7 (11.5)
Total TIIs**** (10^6 counts/μl)	2493.2 (100)	83.8 (100)	302.5 (100)	175.5 (100)	90.5 (100)	328.1 (100)
% volatile matter	81.1	100	100	100	100	100

* mixture of potato dextrose broth and undecanoic acid
** undecanoic acid
*** potato dextrose broth
**** total ion intensities
*****Percentage

The extracellular hydrocarbon observed could be coming from PDB the extact source of these hydrocarbon needs further analysis to confirm that these alkanes and other hydrocarbons are being produced by mixed species culture.The Py-FIMS analysis cannot distinguish between unsaturated fatty acids and alkanes as they have same molecular weight. The other signals correspond to alkanes, as fatty acid composition results in chapter 3 shows no unsaturated fatty acids higher than C22. Therefore further analysis is required to differentiate between alkanes and unsaturated fatty acids. Production of both even and odd chain alkanes have been observed by mixed species culture. Strobel *et al.,* (2008) reported the production of both even and odd chain alkanes and most of these alkanes were short or medium chain. A study by Huang *et al.*, (2012) shows predominance of *anteiso* even chain alkane over the odd chain alkanes in fungi. Also alkanes are used by fungi as source of carbon and energy, Hadibarata and Tachibana, (2009) reported that Trichoderma sp. uses even chain alkanes to odd chain fatty acid that undergo β – oxidation to produce energy for the fungi.

The overall results from Py-FIMS suggest that the mixed species culture is able to produce similar amounts of lipids intracellularly when grown on UDA and UDA+PDB. The amounts and types of lipids found in the extracellular pool of mixed species culture growing on UDA+PDB was equivalent to that found in the extracellular PDB lipid extract alone. These results also indicate that mixed species culture is able utilize fatty acids and produce several alkanes, alkenes, and alkyl esters through different metabolic pathways

4.5 Discussion

NMR analyses show accumulation of intracellular aliphatic and aromatic carbon and protons in the mixed culture of *T. koningii* and *P. janthinellum* growing on different carbon substrates. The high aliphaticity observed in the ^{13}C NMR spectra is supported by data from the Py-FIMS analysis, which shows high accumulation of medium and long-chain fatty acids, alkanes and alkenes in the intracellular pool. The intracellular production of the identified long-chain aliphatic hydrocarbons (alkanes and alkyl diesters) by fungal species has been documented in several studies, through the identification of homologous series of n-alkanes in *Piccinia striiformis* (Baker and Strobe, 1965), and also in other fungal species and their spores (Oro, 1966). Results reported by this thesis are the first to be published for *T. koningii* and *P. janthinellum*. The exact origin and location of alkanes produced by these fungi are not known. Alkanes from fungal species are of interest because of the high chain length variations. Studies show that alkanes produced by these fungal species have typical chain length between C_{16} to C_{35}, with the most dominant alkanes from C_{26} to C_{30} (Haung *et al.*, 2012; Fisher *et al.*, 1977). These long chain hydrocarbons produced by fungal species have a high potential to be used for the production of biodiesel like fuels.

Data reported in this thesis show the intracellular formation of higher chain length alkanes ranging between C_{19} to C_{34} alkanes, by the mixed species culture growing on UDA and mixture of UDA+PDB (Tables 18, 19, 22). The production of these long chain alkanes could be taking place through head to head condensation or elongation decarboxylation pathways explained earlier in section 1.8. The exact mechanism involved in the production of alkanes by mixed species culture was not determined. Most likely,

elongation decarboxylation mechanism could be involved behind production of these alkanes through elongation of odd chain fatty acid and then removal of carboxyl group. The results of this research project are also supported by other recent published studies, which show production of long chain alkanes from C_{15} to C_{35} but also short chain alkanes by both mycelia and spores of many fungal species such as *Aspergillus, Trichoderma, Gliocladium* (Ladygina *et al.*, 2006). Fungal species are able to produce a variety of hydrocarbons including alkenes, cycloalkanes, branched alkanes and others (Ladygina *et al.*, 2006; Wilkesa *et al.*, 2003). The intracellular long-chain production of alkenes was also observed in this study, where the mixed species culture growing on PDB shows the production of C_{38} and C_{40} alkenes (Table 21). These alkenes may be also formed from the carbohydrates in the media through the pathway shown in Figure 5 in Chapter 3. The presence of such long-chain alkenes with chain length over C_{36}, in fungi hasn't been reported before. Production of ethylene, propylene and isoprene by various fungal species has been reported in several studies (Ladygina *et al.,* 2006). Similarly, cycloalkanes and branched alkanes production has also been observed in fungi but not as extensively as alkanes or alkenes.

The results from this thesis indicate that the mixed species culture is able to produce lipids through metabolism of fatty acids, as a high accumulation of lipids was observed when UDA was used as a sole source of carbon and energy. No previous studies have been reported where fatty acids are used as a sole source of carbon to produce hydrocarbons and other lipids. These results provide strong indication that alkanes could be derived from fatty acids in pyrolytic biooil through the use of these fungal species to act as biocatalyst to decarboxylate fatty acids.

Py-FIMS shows that intracellular alkanes and low molecular weight radicals were produced by the mixed species culture of *T. koningii* and *P. janthinellum*. This study did not show the accumulation of lipids in the extracellular pool when cells were grown on UDA+PDB. Significant amounts of extracellular lipids were produced when fungal cells were grown in PDB (positive control). Most of the published studies have reported the trace production of extracellular hydrocarbons under normal conditions ranging from 0.2% to 0.7% of the dry biomass (Oro *et al.*, 1966; Ladygina *et al.*, 2006). The quantity and quality of alkanes produced in fungi vary among different species, growth medium and growth conditions (Wilkesa *et al.*, 2003; Fisher *et al.*, 1977). The fungal species were also reported to have a higher hydrocarbon production when grown under certain conditions such as in co-culture with other microbes (Strobel *et al.*, 2008). Under certain environmental conditions *Gliocladium* strains are able to efficiently produce hydrocarbons ranging from C_6 to C_{19} extracellularly (Ahamed and Ahring, 2011). A study by Ahamed and Ahring, (2011) shows that *Gliocladium* strains grown in co-culture with *E. coli* lead to higher accumulation of hydrocarbons extracellularly.

The production of long-chain alkyl esters in this study occurred under aerobic conditions. Earlier published studies have shown that production of such a wide range of hydrocarbons from C_6 to C_{35} depends on growth conditions (Stinson *et al.*, 2003). Short-chain alkanes are mostly produced under aerobic conditions, while long-chain alkanes were mostly produced under anaerobic conditions by fungi (Wilkesa *et al.*, 2003). Alkane accumulation was observed in the mixed species culture grown on UDA and UDA+PDB; which could be due to presence of co-culture and conditions and due to the presence of UDA or more complex carbon source (UDA+PDB). A study by Ul-hassan *et al* (2012),

shows that *Hypoxylon* sp. Cl-4 grown with a complex carbon source or oxygen limiting condition lead to activation of crytic biosynthetic pathways to produce volatile organic compounds such as hydrocarbons. Such phenomenon could be taking place when the studied mixed species culture is grown in the presence of the UDA+PDB.

The extracellular production of medium and long-chain alkanes in this study was under aerobic condition when PDB was sole source carbon. In a study by Strobel *et al*, (2008) shows that endophytic fungus, *Gliocladium roseum* produces a series of short and medium-chain volatile hydrocarbons and hydrocarbon derivatives ranging between C_6 and C_{19} extracellularly but under limited oxygen supply on different carbon sources.

The short and long-chain hydrocarbons found in the culture studies of *Trichoderma* and *Penicillium* species have great potential for producing biofuels as they consisted of C_8 to C_{34} hydrocarbons. The tendency of the fungal species, *P. janthinellum* and *T. koningii* grown on UDA to produce a wide range of hydrocarbon makes them useful biocatalysts for producing advanced biofuels from the conversion of fatty acids in biooil.

4.6 Conclusion

^{1}H and ^{13}C NMR analyses indicate that the chemical composition of intracellular lipids extracted from a mixed species culture growing on different carbon substrates and growth phases was dominated by aliphatic and aromatic components with an evidence of hydrocarbons and other lipids. Py-FIMS analysis showed that intracellular lipids of mixed species culture grown on fatty acid as the sources of carbon (UDA and UDA+PDB) are produced and accumulated intracellularly during the exponential phase. Both fatty acid containing substrates show the production of similar types of alkanes, alkenes, fatty acids and other metabolites. The alkanes produced were long-carbon chain lengths ranging between C19- C32. The fatty acids produced are short and medium-chain lengths ranging between C10- C18. When UDA+PDB is sole source of carbon highest accumulation of lipids was observed intraellularly. These intracellular lipids include short (C_{11}) and medium-chain fatty acids (C_{15}, C_{16} and C_{18}), short- chain alkenes ($C_{12:1}$) and long-chain alkyl-diesters (C_{41}, $C_{58:1}$ and $C_{60:1}$). Where UDA is only source of carbon and energy, high accumulation of lipids and production of fatty acids between C16 and C18 indicates that mixed species culture is able to metabolize and decarboxylate fatty acids to produce different lipids and other metabolites.

Results indicate that extracellular lipids observed when UDA+PDB was substrate are probably originating from lipids in the PDB and not through fungal synthesis. Further analysis is required to confirm extracellular production of lipid by mixed species culture in the presence of fatty acid as a source of carbon and energy. The lignin and suberin type compounds are also most likely originating from PDB. Extracellular lipids were produced through biosynthesis when the mixed species culture was grown on PDB. Extracellular

alkanes and long chain alkenes ($C_{38:1}$- $C_{40:1}$) were produced by the mixed species culture grown on PDB. Highest lipid accumulation was observed extracellularly when PDB was the carbon source where lipids account for 52 millions counts/µl When UDA and UDA+PDB were source of carbon the intracellular lipid content of 22 and 26 millions counts/µl respectively almost half of the extracellular lipid content of mixed species culture grown on PDB. The concentration of lipids were much higher in the intracellular pool of UDA and UDA+PDB and extracellular pool of PDB of mixed species cultures than the chloroform extract of PDB (no fungi) indicates the production of hydrocarbons and fatty acids by mixed species culture. Further analysis is required to confirm the formation of alkanes by fungi.

5. General Discussion

Due to insecurities of supply, and the decreasing total reserve of petroleum-based fuels and chemicals, the need to discover renewable liquid fuels, as well as commodity chemicals, has escalated. Decreased fossil fuel reserves have caused a rise in the price of crude oil. These depleting fossil oil reserves have adverse impacts on the economy (Demirbas, 2009; Gouveia and Olivera, 2009). Therefore, biofuels have received elevated attention in recent years. Throughout history, fuels generated from biomass have been used. Most of these fuels were produced by fermentation of plants derived from sugars or from plant oils. These were not only used for combustion purposes but also as solvents, greases, cleaners and chemicals. Their consumption decreased when cheaper sources were found in fossil oil (Antoni *et al.*, 2007). Biofuels are a potentially carbon neutral liquid fuels. The need to overcome current limitations of biofuels has led researchers to design new alternative fuels. Alkanes and other hydrocarbons are the major component that will make alternative biofuel to replace the currents fossil fuels. Hydrocarbons are detected in most plants, animals and microorganism. Microbial alkanes and other hydrocarbons provide great potential to act as replacement fuels. The main source of these hydrocarbons produced in microorganisms is from fatty acids (Templier *et al.*, 1991).

The results from this study show that the fungal species *T. koningii* and *P. janthinellum* are able to metabolize fatty acids alone or in the presence of potato dextrose as a carbon and energy source. The mixture of fungal species *T. koningii* and *P. janthinellum* showed the highest biomass production, carbon dioxide emissions and

production of lipids. This study shows that the consortium interactions between fungal species lead to higher production of lipids and other metabolites. The results from all three studies were consistent and compliment other results. The two major classes of chemical compounds produced from single fatty acids include several other fatty acids and hydrocarbons. This indicates that these fungal species are able to convert short-chain fatty acids into medium-chain fatty acids and medium to long-chain alkanes, alkenes and alkyl diesters. Similar results have been observed for algae (Chatzifragkoul *et al.*, 2010; Radwan and Soliman, 1988). The analysis of intracellular and extracellular lipid fractions extracted from mixed species culture showed that fatty acids ranged from *n-*C_{11} to *n-*C_{18}, alkanes from *n-*C_{19} to *n-*C_{34}, alkenes n-$C_{30:1}$- n-$C_{40:1}$ and long chain alkyl esters from $C_{41}$to C $_{60:1}$. Even though fatty acids account for over 80% of all identified lipids, the biosynthesis of long-chain alkanes, alkenes and alkyl esters, show that the mixed fungal species have the ability to produce hydrocarbons which can be used for production of new biofuels through the development of new biocatalytic processes. The new process can be associated with the refining of third generation biofuels such as bio-oil, which is enriched in fatty acids (Monreal and Schnitzer, 2011), to produce advanced biofuels that will be enriched in alkanes.

6. Future Directions

Further analysis of lipid fractions is required using GC-MS, Py-FIMS and other MS/MS techniques to quantify (moles/litre or μg/ml) and verify the production of alkanes and alkenes by these two species. The quantitative production of single hydrocarbons and other lipids need to be complemented by studies on biosynthetic pathways, enzymes and genes involved in the production of hydrocarbon and fatty acids by the mixture of *T. koningii* and *P. janthinellum*. Future work also needs to consider growing of the mixed species culture of *T. koningii* and *P. janthinellum* in pyrolytic biooil as the sole source of carbon and energy and observe the increase in biomass, carbon dioxide production and production of hydrocarbons from the fatty acid pool found in biooil. More specifically analyze the change in the chemical composition of biooil and determine which fatty acids in the biooil are utilized by mixed species cultures and through different metabolic pathways are converted into alkanes, alkenes and other lipids or different organic compounds. Isolation and encapsulating the enzymes, involved in the fungal synthesis of hydrocarbons, may be directly used with the biooil to carry out the conversion of fatty acids to alkanes and other hydrocarbons, which would be a significant step in the quest to produce novel, renewable biofuels.

7.0 References

Aarle, I.M., and Olsson, P.M. (2003). *Fungal lipid accumulation and development of mycelia structures by two arbuscular mycorrizal fungi.* Applied Environmental Microbiology. 69: 6762-6767.

Abraham, M.J., and Srinivasan, R.A. (1984). *Lipid and Fatty Acid Composition of Selected Fungi Grown on Whey Medium.* Journal of Food Science. 49: 950-951.

Abrahamson, D.E. (1989). *Global warming; the issue, impacts, responses in D.E Abrahamson* (ed.). The Challenges of Global Warming. 3-34.

Antoni , D., Zverlov, V.V., Schwarz, W.H. (2007). *Biofuels from microbes.* Applied Microbiology Biotechnology. 77:23–35.

Ahamed, A., and Ahring, B. K. (2011). *Production of hydrocarbon compounds by endophytic fungi Gliocladium species grown on cellulose*. Bioresource Technology. 102: 9718–9722.

Aislable, J., David, J., Saul, A.E., and Foght, J.M. (2006). *Bioremediation of hydrocarbon-contaminated polar soils.* Extremophiles. 10:171–179.

Albro, P. W., and Dittmer, J. C. (1970). *Bacterial hydrocarbons: occurrence, structure and metabolism.* Lipids. 5: 320–325.

Aulich, T.R., Wocken, C.A., Timpe, R.C., Pansegrau, P. (2008). *Optimal energy pathway to renewable domestic and other fuels.* US patent Application. 60/822637.

Bagaeva, T.V., and Zinurova, E.E. (2004). *Comparative characterization of extracellular and intracellular hydrocarbons of clostridium pasteurianum.* Biochemistry. 69: 427–428.

Bago, B., Zipfel, W., Williams, R.W., Jun, J., Arreola, R., Lammers , P. J., Pfeffer , P. E., and Hill, Y.S. (2002). *Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis.* Plant Physiology. 128: 108–124.

Baker, K. and Strobel, A. (1965). *Lipids in the cell wall of Puccinia Striiformis uredospores.* Proceeding of Montana Academy of Sciences. 25: 83-86.

Banerjee, A., Sharma, R., Chisti, Y., and Banerjee, U. C. (2002). *Botryococcus braunii: a renewable source of hydrocarbons and other chemicals.* Critical Reviews Biotechnology. 22: 245- 279.

Barbir, F., Veziroglu, N. and Plass Jr, H.J. (1990). *Environmental damage due to fossil fuel use.* International journal of hydrogen energy. 15: 739-749.

Beller, H.R., Goh, E. B., and Keasling, J. D. (2010). *Genes Involved in Long-Chain Alkene Biosynthesis in Micrococcus luteus.* Applied and Environmental Microbiology. 76: 1212-1223.

Bernard, A., Domergue, F., Pascal, S., Jetter, R., Renne, C., Faure, J.D., Haslam, R. P., Napier, J. A., Lessire, R., and Joubes, J. (2012). *Reconstitution of Plant Alkane Biosynthesis in Yeast Demonstrates That Arabidopsis ECERIFERUM1 and ECERIFERUM3 Are Core Components of a Very-Long-Chain Alkane Synthesis Complex.* American Society of Plant Biologists. 24: 3106-3118.

Bligh, E.G. and Dyer, W.J. (1959*). A rapid method for total lipid extraction and purification.* Canadian Journal of Biochemistry and Physiology. 37: 911-917.

Birch, L., Bachofen, R. (1988). *Microbial production of hydrocarbons.* In: Rehm H. J (Ed) Biotechnology. 6: 71–99.

Bird, C.W., and Lynch, J.M. (1974). *Formation of hydrocarbons by microorganisms.* Chemical Society Review. 3: 309- 328.

Black, R.L.B. and Dix, N.J. (1976). *Utilization of ferulic acid by microfungi from litter and soil.* Transations of the British Mycologial Society. 66: 305-311.

Bok, J. W., Hoffmeister, D., Maggio-Hall, L. A., Murillo, R., Glasner, J. D. and Keller, N. P. (2006). *Genomic mining for aspergillus natural products.* Chemistry and Biology. 13: 31–37.

Borjesson, T., Stollman, U., and Schnurer, J. (1990). *Volatile metabolites and other indicators of penicillium aurantiogriseum growth on different substrates.* Applied and Environmental Microbiology 56: 3705-3710.

Bradly, D. (2006). *European market study for biooil (pyrolysis oil).* Climate change and solutions.

Brenner, K., You, L., and Arnold, F.H. (2008). *Engineering microbial consortia: a new frontier in synthetic biology.* Trends in Biotechnology 26: 483-489.

Brown, M. and Shanks, J. (2012). *Linear Hydrocarbon Producing Pathways in Plants, Algae and Microbes.* In Gopalakrishnan, K., Leeuwan, J., and Brown, R. (Eds), *Sustainable Bioenergy and Bioproducts.* p.p 1-11. Springer London.

Carreon, S.L., Hathout, Y., Bensoussan, M., and Belin, J.M. (1992). *Lipid accumulation in trichoderma species.* Federation of European Microbiological Societies Microbiology Letters. 93: 181-7.

Chatzifragkoul, A., Fakas, S., Galiotou-Panayotou, M., Komaitis, M., Aggelis, G., and Papanikolaou, S. (2010). *Commercial sugars as substrates for lipid*

accumulation in Cunninghamella echinulata and Mortierella isabellina fungi. European Journal of Lipid Science and Technology. 112: 1048-1057.

Chisti, Y. (2007). *Biodiesel from microalgae.* Biotechnology Advances. 25: 294–306.

Chisti, Y. (2008). *Biodiesel from microalgae beats bioethanol.* Trends in Biotechnology. 26: 126–131.

Connor, M.R., and Atsumi, S. (2010). *Synthetic biology guides biofuel production.* Journal of Biomedicine and Biotechnology. 2010: 1- 9.

Copeman, L.A., and Parrish, C.C. (2004). *Lipids classes, fatty acids, and sterols in seafood from gilbert bay, southern labrador.* Journal of Agricultural Food Chemistry. 52: 4872–4881.

Das,D.D., Schnitzer, M.I., Monreal, C.M., and Mayer, P. (2009). *Chemical composition of acid–base fractions separated from biooil derived by fast pyrolysis of chicken manure.* Bioresource technology. 100: 6524-6532.

Demirbas, A. (2009). *Political, economic and environmental impacts of biofuels: a review.* Applied energy. 86: 108-117.

Dennis, M., and Kolattukudy, P. E. (1991). *Alkane biosynthesis by decarbonylation of aldehyde catalyzed by a microsomal preparation from Botryococcus braunii.* Archives of Biochemistry and Biophysics. 287: 268-275.

Dennis, M., and Kolattukudy, P. E. (1992). *A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO.* Proceedings of National Academy of Sciences of United States of America. 89: 5306–5310.

Domingues, F.C., Queiroz, J.A., Cabral, J.M.S., and Fonseca, L.P. (2000). *The influence of culture conditions on mycelia structure and cellulose production by trichoderma reesei Rut C-30.* Enzyme Microbiology Technology. 26: 394-401.

Escobar, J.C., Lora, E.S., Venturini, O.J., Yanez, E.E., Castillo, E.F. and Almazan, O. (2009). *Biofuels: environment, technology and food security.* Renewable and Sustainable Energy Reviews. 13: 1275-1287.

Eurostat. (2007). *Online database of the European Union: 2920 Luxembourg.* [http://epp.eurostat.ec.europa.eu].

Fakas, S., Makri, A., Mavromati, M., Tselepi, M., and Aggelis, G. (2009). *Fatty acid composition in lipid fractions lengthwise the myce- lium of mortierella isabellina and lipid production by solid state fermentation.* Bioresource Technology. 100: 6118–6120.

Fisher, D. J., Brown, G., and Holloway, P. J. (1978). *Influence of growth medium on surface and wall lipid of fungal spore.* Phytochemistry. 17; 85-89.

Gessner, M.O. and Newell, S.Y. (2002). *Biomass, growth rate and production of filamentous fungi in plant litter.* Manual of Environmental Microbiology. 2: 390-408.

Ghoniem, A.F. (2011). *Needs, resources and climate change: clean and efficient conversion technologies.* Progress in Energy and Combustion Science. 37: 15-51.

Gianoulis, T. A., Griffin, M. A., Spakowicz, D. J., Dunican, B. F., Alpha, C. J., Sboner, A., Sismour, A. M., Kodira, C., Egholm, M., Church, G. M., Gerstein. M. B., Strobel, S. A. (2012). *Genomic analysis of the hydrocarbon producing, cellulolytic, endophytic fungus Ascocoryne sarcoides.* Public Library of Sciences Genetics. 8: 1-12.

Gouveia, L. & Oliveira, A. C. (2009). *Microalgae as a raw material for biofuels production.* J Ind Microbiol Biotechnol. 36: 269–274.

Graham, J.H., and Linderman, R.G. (1980). *Ethylene production by ectomycorrhizal fungi, fusarium oxysporum f.sp. pini, and by aseptically synthesized ectomycorrhizae and fusarium-infected douglas-fir roots.* Canadian Journal of Microbiology. 26: 1340–7.

Greenspan, P., Mayer, E.P., and Fowler, S.D. (1985). *Nile red: a selective fluorescent stain for intracellular lipid droplets.* Journal of Cell Biology. 100: 965–973.

Griffiths, M. J. and Harrison S.T.L. (2009). *Lipid productivity as a key characteristic for choosing algal species for biodiesel production.* Journal of Applied Phycology. 21: 493–507.

Groom, M.J., Gray, E.M., and Townsend, P.A. (2008). *Biofuels and Biodiversity: principles for creating better policies for biofuel production.* Conservation Biology. 22: 602-609.

Hadibarata, T., and Tachibana, S. (2009). *Microbial degradation of n-eicosane by filamentous fungi.* In: Obayashi, Y., Isobe, T., Subramanian, A., Suzuki, S., and Tanabe, S. (Eds.), Interdisciplinary Studies on Environmental Chemistry - Environmental Research in Asia. Terrapub, Tokyo, pp. 323-329.

Hamanaka, T., Higashiyama, K., Fujikawa, S., and Park, E.Y. (2001). *Mycelial pellet intrastructure and visualization of mycelia and intracellular lipid in a culture of Mortierella alpine.* Applied Microbiolology and Biotechnology 56: 233–238.

Han, J., Chan, H. W. S., and Calvin, M. (1969). *Biosynthesis of alkanes in Nostoc*

muscorum. Journal American Chemical Society. 21: 5156–5159.

Haney, R.I., Brinton, W.H., and Evans, E. (2008). *Estimating soil carbon, nitrogen, and phosphorous mineralization from short term carbon dioxide respiration.* Communications in Soil Science and Plant Analysis. 39: 2706-2720.

Hare, S. S. (1988). *Lipid and fatty acid profiles of some vesicular-arbuscular mycorrhizal fungi: contribution to taxonomy*. Mycologia 80: 622–629.

Heath, R.J., and Rock, C.O. (2002). *The claisen condensation in biology*. National Product Review. 19: 581-596.

Hoffert, M.I., Caldeira, K., Jain, A.K., Haites, E.F., Danny Harvey, L.D., and Potter, S.D. (1998). *Energy implication of future stabilization of atmospheric CO2 contents.* Nature. 395:881.

Hong, Y., Zurbriggen, A. S., Melis, A. (2012). *Isoprene hydrocarbons production upon heterologous transformation of Saccharomyces cerevisiae*. Journal of Applied Microbiology. 113: 52–65.

Huang, X., Xue, J., and Guo, S. (2012). *Long chain n-alkanes and their carbon isotopes in lichen species from western Hubei Province: implication for geological records*. Frontiers of Earth Science.6: 95-100.

Huub H. J., Cox, J., Houtman, H.M., Doddema, H.J. and Harder, W. (1993). *Enrichment of fungi and degradation of styrene in biofilters*. Biotechnology Letters. 15: 737-742.

International Energy Agency. (2004). *World energy outlook*. http://www. worldenergyoutlook.org.

IPCC. (2007). *Intergovernmental panel on climate change 'AR4 synthesis report'*. [www.ipcc.ch].

Kamisaka, Y., and Noda, N. (2001). *Intracellular transport of phosphatidic acid and phosphatidilcholine into lipid bodies in an oleaginous fungus, mortierella ramanniana var. an- gulispora*. Journal of Biochemistry. 129: 19–26.

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

Kolattukudy, P. E. (1967). *Biosynthesis of paraffins in Brassica oleraceae: fatty acid elongation–decarboxylation as a plausible pathway*. Phytochemistry. 6: 963–975.

Ladislao, B.A. and Turrion-Gomez, J.L. (2008). *Second-generation biofuels and local bioenergy systems.* Biofuels Bioproducts and Biorefining. 2:455–469.

Ladygina, N., Dedyukhina, E. G., Vainshtein, M. B. (2006). *A review on microbial synthesis of hydrocarbons.* Process Biochemistry. 41: 1001-1014.

Lang, X., Dalai, A. K., Bakhshi, N. N., Reany, M. J., and Hertz, P. B. (2001). *Preparation and characterization of biodiesel from various biooils.* Bioresource Technology. 80: 53–62.

Lee, S.K., Chou, H., Ham, T.S., Lee, T.S., and Keasling, J.D. (2008). *Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels.* Current Opinions in Biotechnology. 19: 556-63.

Mamatha, S.S., Ravi, R., and Venkateswaran, G. (2009). *Medium opti- mization of gamma linolenic acid production in mucor rouxii cfr–g15 using rsm.* Food Bioprocess Technology. 1: 405–409.

Marsh, P.D. and Bowden, G.H.W. (2000). *Microbial community interactions in biofilms. in community structure and co-operation in biofilms.* Cambridge University Press. 167-198.

Metzger, P., and Largeau, C. (2005). *Botryococcus braunii: a rich source for hydrocarbons and related ether lipids.* Applied Microbiology and Biotechnology. 66: 486-496.

Miles, P.G and Chang, S.T. (1997). *Mushroom Biology: Concise Basics and Current Developments.River Edge*, NJ: World Scientific, 36-53

Monreal, C. M., and Schnitzer, M. (2011). *Production of a refined bio-oil derived by fast pyrolysis of chicken manure with chemical and physical characteristics close to those of fossil fuels.* Journal of Environmental Science and Health. 46; 630-637.

Montgomery H.J., Monreal, C.M., Young, J.C., Seifert, K.A. (2000). *Determination of soil fungal biomass from soil ergosterol analysis.* Soil Biology and Biochemistry. 32: 1207-1217.

Morrison, W. R., and Smith, L. M. (1964). *Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride methanol.* Journal of Lipids Research. 5: 600-608.

Munoz, C., Monreal, C., Schnitzer, M., and Zagal, E. (2009). *Analysis of soil humic acids in particle size fractions of an alfisol from a Mediterranean-type climate.* Global Journal of Soil Science (Geoderma). 151: 199-203

Munoz, C., Monreal, C., Schnitzer, M., and Zagal, E. (2008). *Influence of Acacia caven (Mol) coverage on carbon distribution and its chemical composition in soil organic carbon fractions in a Mediterranean-type climate region.* Global Journal of Soil Science (Geoderma). 144: 352–360.

Murphy, D.J. (1991). *Storage lipid bodies in plants and other organisms.* Progress in Lipid Research. 29: 299–324.

Murphy, D.J., and Vance, J. (1999). *Mechanisms of lipid-body formation.* Trends in Biochemical Sciences. 24, 109–115.

Musallam, A. A. and Radwan, S.S. (1990). *Wool-colonizing micro-organisms capable of utilizing wool-lipids and fatty acids as sole sources of carbon and energy.* Journal of Applied Bacteriology. 69(6): 806-813.

Naik, S.N., Goud, V.V., Rout, P K. and Dalai, A.K. (2010). *Production of first and second generation biofuels: a comprehensive review.* Renewable and Sustainable Energy Reviews. 14: 578–597.

Nigam, P.S. and Singh, A. (2011). *Production of liquid biofuels from renewable resources.* Progress in Energy and Combustion Science. 37: 52-68.

Ochoa, S. (1951). *Biological mechanisms of carboxylation and decarboxylation.* Physiological Reviews. 31: 56.

Olsson, P.A., and Johansson, A. (2000). *Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages.* Mycological Research. 104: 429–434.

Oro. J., Laseter, J. L., and Weber, D. (1966). *Alkanes in fungal spores.* Science. 154: 399–400.

Park, M.O. (2005). *New Pathway for Long-Chain n-Alkane Synthesis via 1-Alcohol in Vibrio furnissii M1.* Journal of Bacteriology. 187: 1426-1429.

Parrish, C.C. (1987). *Separation of aquatic lipid classes by chromarod thin-layer chromatography with measurement by Iatroscan flame ionization detection.* Canadian Journal of Fisheries and Aquatic Sciences. 44: 722- 731.

Pinches, S.E., and Apps, P. (2007). *Production in food of 1,3-pentadiene and styrene by trichoderma species.* International Journal of Food Microbiology. 116: 182-185.

Puppan, D. (2002). *Environmental evaluation of biofuels.* Periodica. Polytechnica. 10: 95–116.

Radwan, S.S. and Soliman, A.H. (1988). *Arachidonic acid from fungi utilizing fatty acids with shorter chains as sole sources of carbon and energy.* Journal of General Microbiology. 134: 387-393.

Rasheva, T., Kujumdzieva, A., and Hallet, J.N. (1997). *Lipid production by monascus purpureus albino strain.* Journal of Biotechnology. 56: 217–224.

Rashid, U., Anwar, F., Moser, B.R., and Knothe, G. (2008). *Moringa oleifera oil: a possible source of biodiesel.* Bioresource Technology. 99: 8175–8179.

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

Rittmann, B. E. (2008) *Opportunities for renewable bioenergy using microorganisms.* Biotechnology and Bioengineering. 100: 203–212.

Rodriguez-Kabana, R.; Kelley, W.D. and Curl, E.A. (1978). *Proteolytic activity of trichoderma viride in mixed culture with sclerotium rolfsii n soil.* Canadian Journal of Microbiology. 24: 487-490.

Rodrigues, B.C.R., Brochetto-Braga, M.J., Tauk-Tornisielo, S.M., Carmona, E.C., Arruda, V.M. and Netto, J.C. (2009). *Comparative growth of trichoderma strains in different nutritional sources, using bioscreen automated system.* Brazilian Journal of Microbiology. 40: 404-410.

Rude, M.A., and Schirmer, A. (2009). *New microbial fuels: a biotech perspective.* Current Opinion in Microbiology 12: 274-281.

Samuels, L., Kunst, L., and Jetter, R. (2008). *Sealing plant surfaces: Cuticular wax formation by epidermal cells.* Annual Reviews Plant Biolology. 59: 683–707.

Sarpal, A. S., Kapur, G. S., Mukherjee, S., and Tiwari, A. K. (2001). *PONA analyses of cracked gasoline by 1H NMR spectroscopy Part II.* Fuel. 80: 521–528.

Schirmer, A., Rude, M. A., Li, X., Popova, E., Cardayre, S. B. (2010). *Microbial biosynthesis of Alkanes.* Science. 329: 559-567.

Schneider-Belhaddad, F., and Kolattukudy, P. (2000). *Solubilization, partial purification, and characterization of a fatty aldehyde decarbonylase from a higher plant, Pisum sativum.* Archives of Biochemistry and Biophysics. 377: 341–349.

Schnitzer, M.I., Monreal, C.M., Facey, G.A., and Fransham, P.B., (2007). *The conversion of chicken manure to biooil by fast pyrolysis I. analysis of biooils by FTIR and NMR spectroscopy.* Journal of Environmental Science and Health, Part B 42, 1532.

Schnitzer, M.I., Monreal, C.M., and Jandl, G. (2008). *The conversion of chicken manure to bio-oil by fast pyrolysis. III. Analyses of chicken manure, bio-oils and char by Py-FIMS and Py-FDMS.* Journal of Environmental Science and Health Part B. 43: 81-95.

Shapiro, J.A. (1998). *Thinking about bacterial population as multicellular organisms.* Annual Reviews Microbiology. 52: 81-104.

Silliker, J.H. and Rittenber S.C. (1951). *Studies of aerobic oxidation of fatty acids by bacteria.* Journal of Bacteriology. 61: 653-659.

Somerville, C. (2007). *Biofuels.* Current topics in Biotechnology. 17: 115-119

Spormann, A. M., Widdel, F. (2000). *Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria.* Biodegradation. 11: 85–105.

Somashekar, D., Venkateshwaran, G., Sambaiah, K., and Lokesh, B.R. (2002). *Effect of culture conditions on lipid and gamma-linolenic acid production by mucoraceous fungi.* Process Biochemistry. 38: 1719–1724.

Streets D.G. and Waldhoff S.T. (1999). *Greenhouse-gas emissions from biofuel combustion in asia.* Energy. 24: 841-865.

Souse, D.Z., Balk, M., Alves, M., Schink, B., McInerney, M, J., Smidt, H., Plugge, C.M., and Stams, A.J.M. (2010). *Degradation of long-chain fatty acids by sulfate-reducing and methanogenic communities.* In Handbook of Hydrocarbon and Lipid Microbiology. New York, Berlin

Stephanopoulos, G. (2007). *Challenges in engineering microbes for biofuels production.* Science. 315: 801–804.

Stinson, M., Ezra, D., Hess, W. M., Sears, J., and Strobel, G. A. (2003). *An endophytic Gliocladium sp. of Eucryphia cordifolia producing selective volatile antimicrobial compounds.* Plant Science. 165: 913–922.

Stotzky, G. (1965). *Microbial respiration in methods of soil analysis: part 2 chemical and microbiological properties.* American Society of Ag-Bodenk. 56: 26–38.

Strobel, G. (2006). *Harnessing endophytes for industrial microbiology.* Current Opinions in Microbiology. 9: 240–244.

Strobel, G.A., Knighton, B., Kluck, K., Ren, Y., Livinghouse,T., Griffin, M., Spakowicz, D., Sears, J. (2008). *The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus gliocladium roseum.* Microbiology. 154: 3319–3328.

Subramaniam, R., Dufreche, S., Zappi, M., and Bajpai, R. (2010). *Microbial lipids from renewable resources: production and characterization.* Journal of Industrial Microbiology and Biotechnology. 37:1271–1287.

Sukovich, D. J., Seffernick, J. L., Richman, J. E., Gralnick, J. A., and Wackett, L. P. (2010). *Widespread Head-to-Head Hydrocarbon Biosynthesis in Bacteria and Role of OleA.* Applied and Environmental Microbiology. 76: 3850-3862.

Templier, J., Largeau, C., Casadevall, E. (1991). *Non-specific elongation-decarboxylation in biosynthesis of cis and trans alkadienes by Botryococcus braunii.* Phytochemistry. 30: 175-183.

The International Energy Agency. (2003). World energy outlook. http://www.worldenergyoutlook.org/.

Tilman, O., Hill, J., and Lehman, C. (2006). *Carbon-negative biofuels from low input high diversity grassland biomass.* Science. 314: 1598-1600.

Tressl, R., Bahri, D., and Engel, K.H. (1982). *Formation of eight-carbon and ten-carbon components in mushroom (Agaricus campestris).* Journal of Agriculture and Food Chemistry. 30: 89-93.

Uherek, E., Halenka, T., Borken-Kleefeld, J., Balkanski, Y., Berntsen, T., Borrego, C., Gauss, M., Hoor, P., Juda-Rezler, K., Lelieveld, J., Melas, D., Rypdal, K. and Schmid, S. (2010). *Transport impacts on atmosphere and climate: land transport.* Atmospheric Environment. 44: 4772- 4816.

Ul-Hassan, S. R., Strobel, G. A., Booth, E., Knighton, B., Floerchinger, C., and Sears, J. (2012*). Modulation of volatile organic compound formation in the Mycodiesel-producing endophyte Hypoxylon sp. Cl-4.* Microbiology. 158: 465-473.

Ulfig, K. (2005). *Effect of sewage sludge alkalization and acidification on keratinolytic and keratinophilic fungi.* Journal of Environmental Studies. 14: 647-653.

Van Bael, S.A., Estrada, C. and Wcislo, W.T. (2011). *Fungal-fungal interactions in leaf-cutting ant agriculture.* Psyche, Article ID 617478, 2011:1-9.

Wackett, L.P. (2008). *Microbial-based motor fuels: science and technology.* Microbial Biotechnology. 1: 211-225.

Wackett, L.P. (2010). *Aliphatic hydrocarbons, carbon-carbon bond formation.* Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg. 370-373.

Wang, X., and Kolattukudy, P. E. (1995). *Solubilization and purification of aldehyde generating fatty acid acyl-CoA reductase from green alga Botryococcus braunii.* Federation of European Biochemical Societies Letters. 370: 15-18.

Weete, J. D. (1972). *Review article: aliphatic hydrocarbons of the fungi.* Phyto-Chemistry. 11: 1201–1205.

Wendisch, V.F., De graaf, A.A., Sahm, H. and Eikmanns, B. J. (2000). *Quantitative determination of metabolic fluxes during coutilization of two carbon sources: comparative analyses with corynebacterium glutamicum during growth on acetate and/or glucose.* 182: Journal of Bacteriology. 3088–3096.

Wilkesa, H., Kuhnerb, S., Bolmc, C., Fischerd, T., Classenc, A., Widdelb, F., and Rabus, R. (2003). *Formation of n-alkane- and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil.* Organic Geochemistry. 34: 1313–1323.

Williams, R. B., Henrikson, J. C., Hoover, A. R., Lee, A. E., and Cichewicz, R. H. (2008). *Epigenetic remodeling of the fungal secondary metabolome.* Organic and Biomolecular Chemistry. 6: 1895–1897.

Wilson, J. T. and Wilson, B.H. (1985). *Biotransforamtion of trichloroethylene in soil.* Applied and Environmental Microbiology. 29: 242-243.

Xian, M., Kang, Y., Yan, J., Liu, J., Bi, Y., and Zhen, K. (2002). *Production of linolenic acid by mortierella isabellina grown on octadeca- nol.* Current topics in Microbiology. 44:141–144.

Yadvika, S.S., Sreekrishnan, T.R., Kohli, S., and Rana, V. (2004). *Enhancement of biogas production from solid substrates using different techniques—a review.* Bioresoure Technology. 95: 1–10.

Yagi, T., Kawaguchi, M., Hatano, T., Fukui, F., and Fukui, S. (1989). *Production of ketoalkanes from fatty acid esters by fungus trichoderma.* Journal of Fermentation and Bioengineering. 68: 188-192.

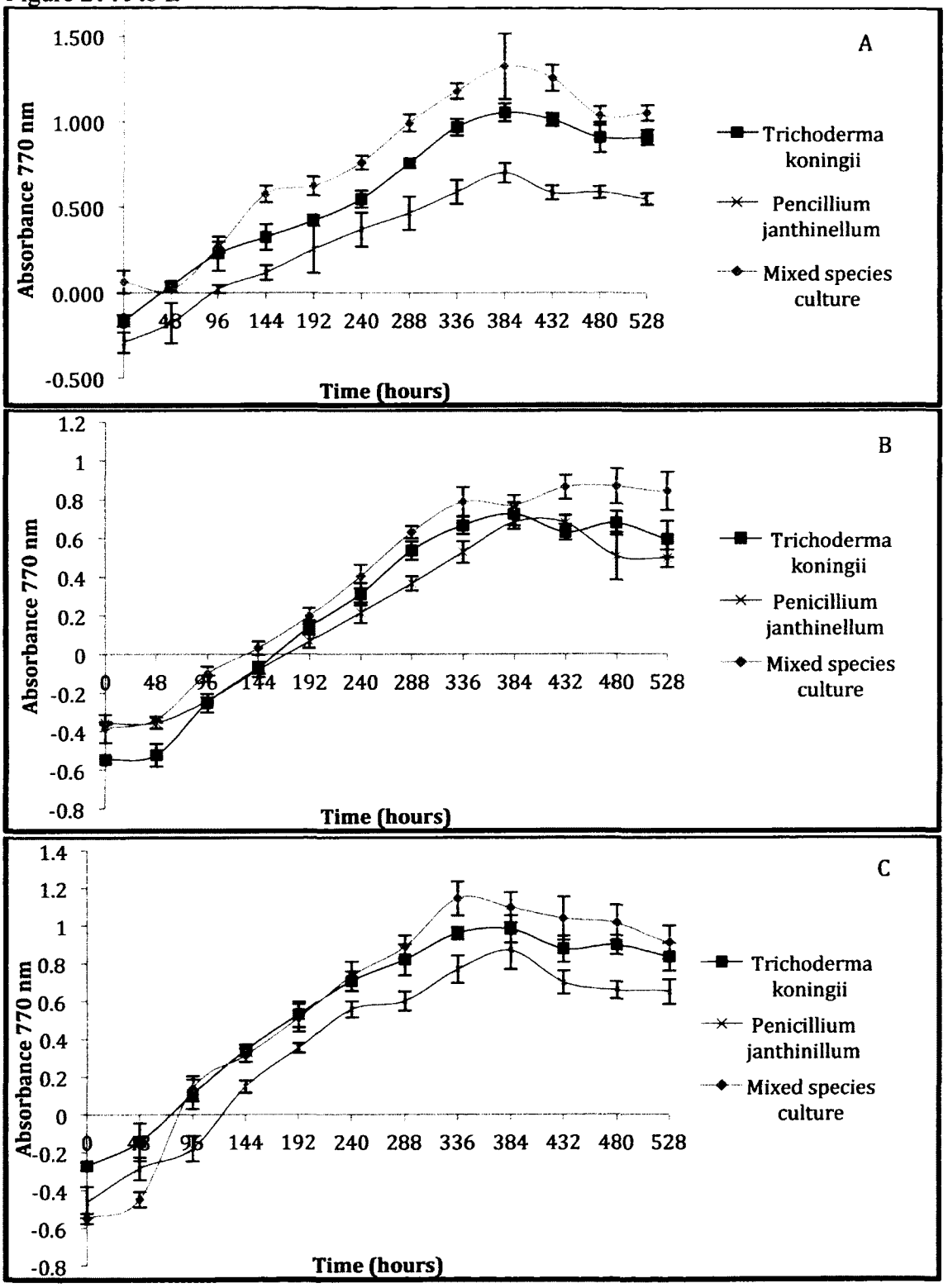
Zang, F., Rodriguez, S., and Keasling, J.D. (2011). *Metabolic engineering of microbial pathways for advanced biofuels production.* Current Opinion in Biotechnology. 22: 775–783.

Zar, J. H. (1999). *Biostatistical analysis.* 564. Upper Saddle River, NJ: Prentice hall.

Zeebe, R.E., Zachos, J, C. and Dickens, G. R. (2009). *Carbon dioxide forcing alone insufficient to explain Palaeocene–Eocene Thermal Maximum warming.* Nature Geoscience. 2: 576 – 580.

8.0 Appendix

Figure 21 A to E



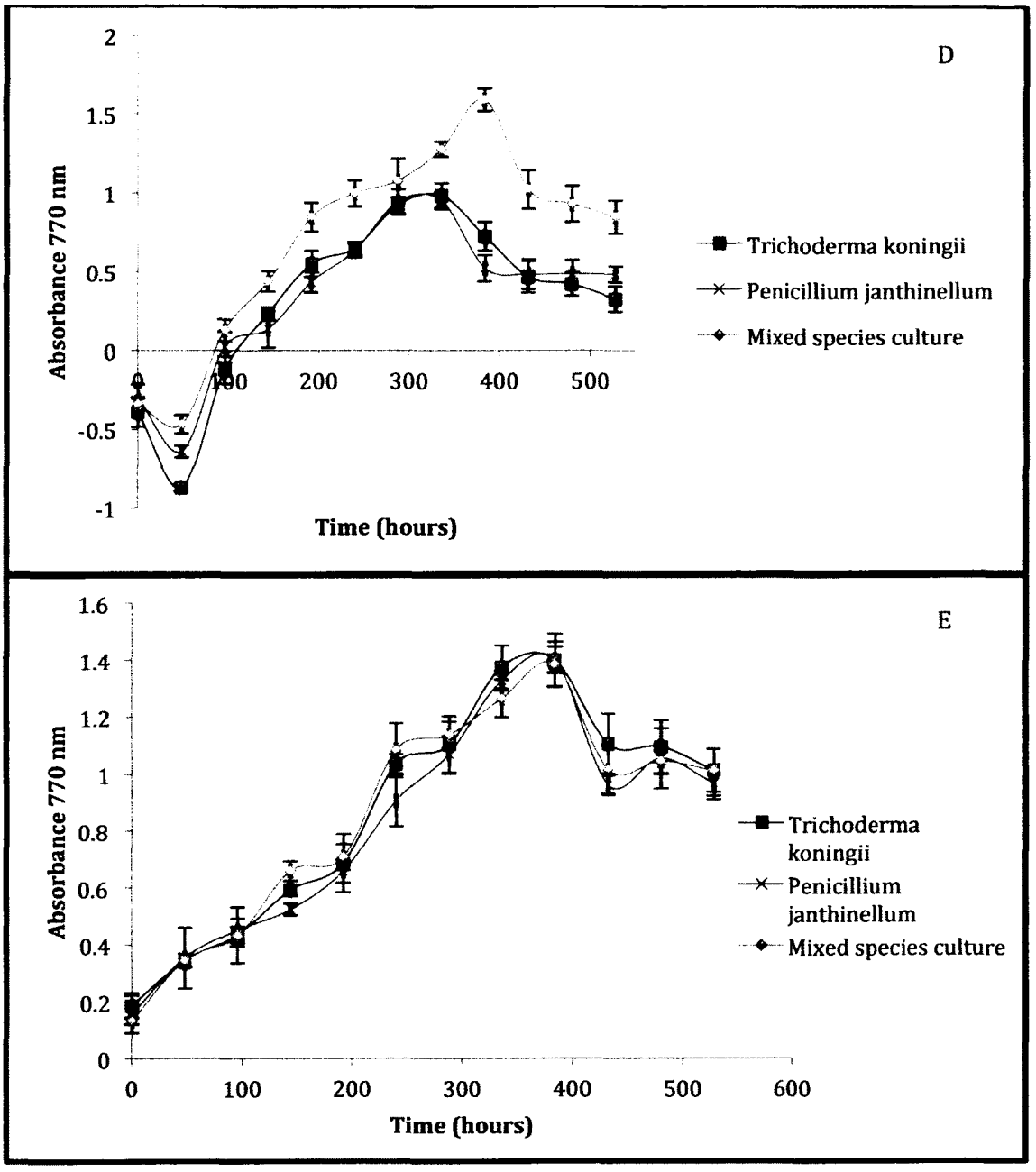
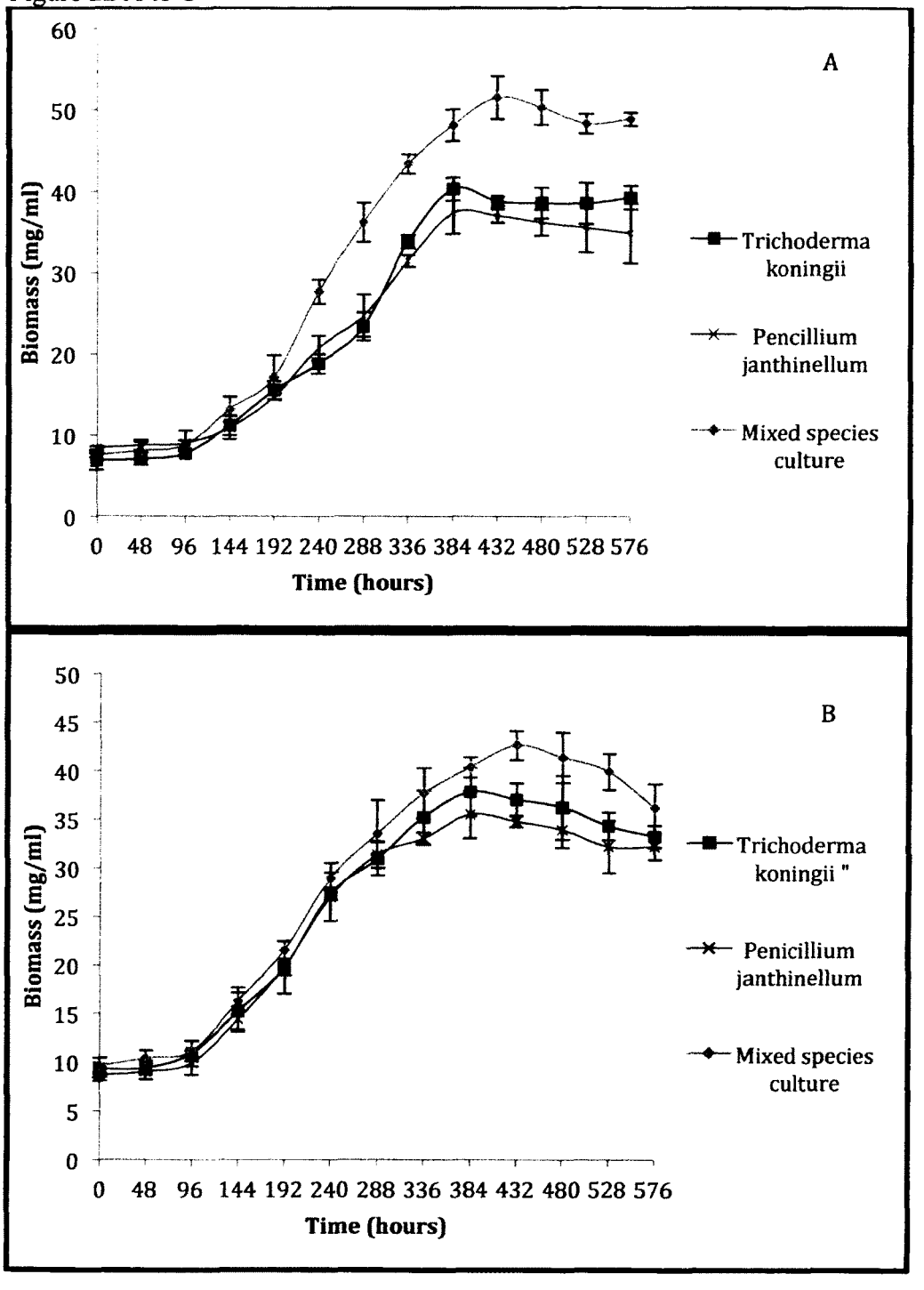


Figure 21: The absorbance growth curves of three fungal species; *Trichoderma koningii (Blue)*, *Penicillium janthinellum (red)* and mixed culture of *Trichoderma koningii*, *Penicillium janthinellum (green)* growing on A) undecanoic acid; B) decanoic acid; C) mix fatty acid; D) mixture of undecanoic acid and potato dextrose broth; E) potato dextrose broth as the sole sources of carbon and energy source in a batch culture at 34 °C +/ - 1 °C over a period of 576 hours. Vertical bars in represent standard deviation values.

Figure 22 A to C



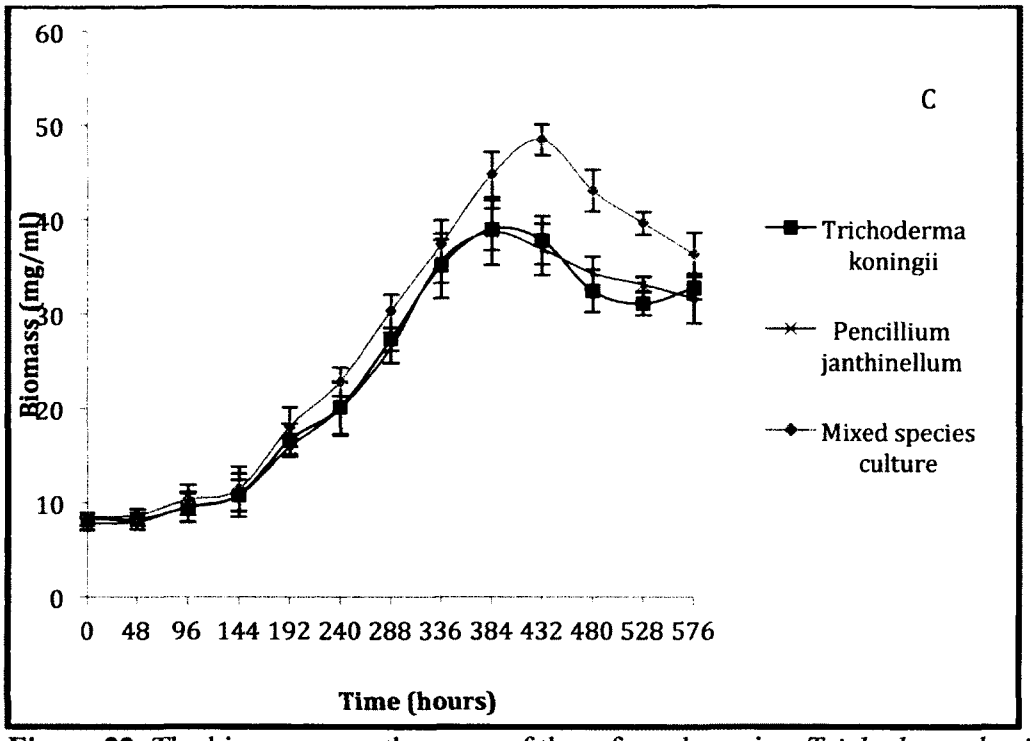
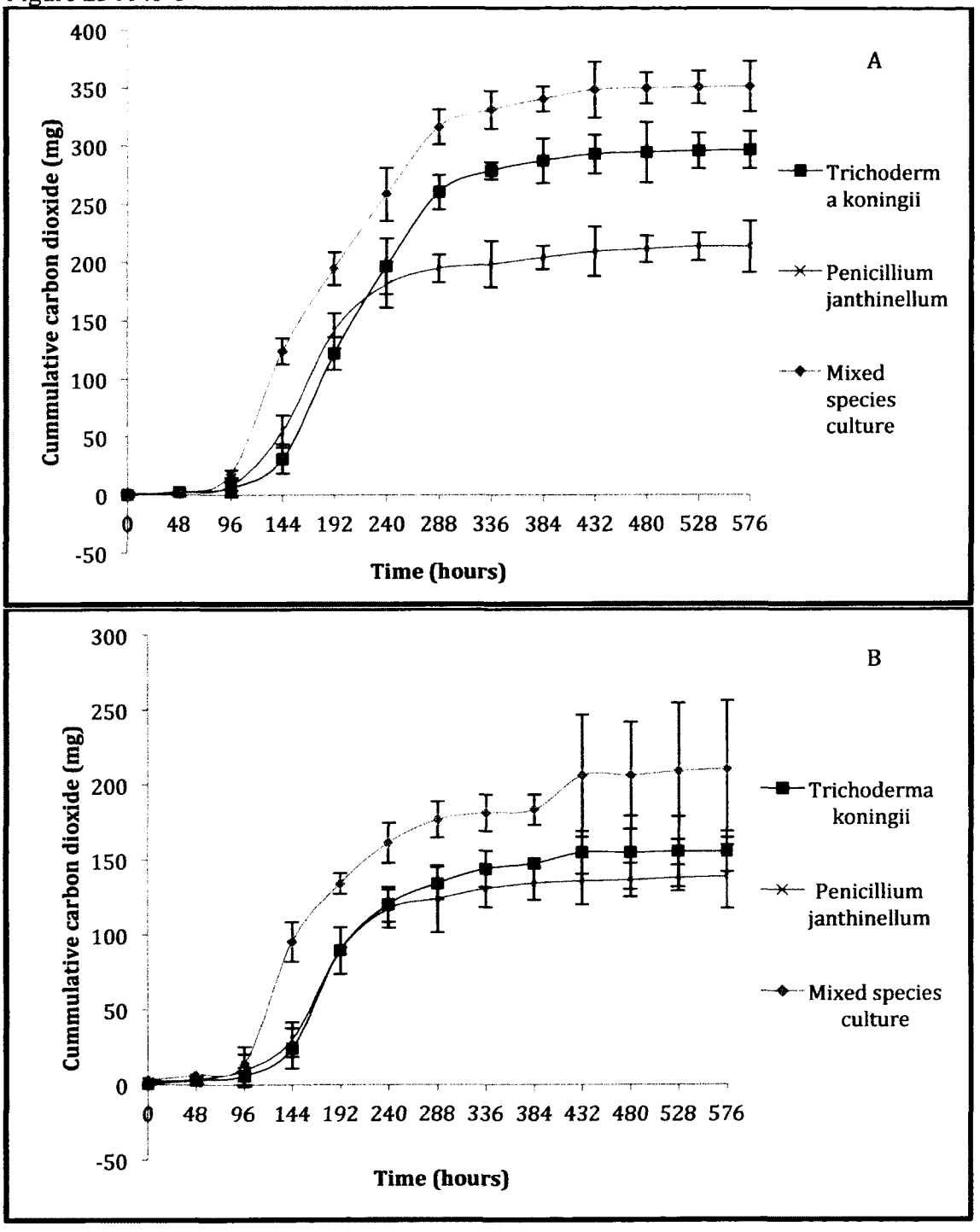


Figure 22: The biomass growth curves of three fungal species; *Trichoderma koningii (Blue)*, *Penicillium janthinellum (red)* and mixed culture of *Trichoderma koningii*, *Penicillium janthinellum (green)* growing on A) undecanoic acid; B) decanoic acid C) mix fatty acid as the sole sources of carbon and energy source in a batch culture at 34 °C +/ - 1 °C over a period of 576 hours. Vertical bars in represent standard deviation values.

Figure 23 A to C



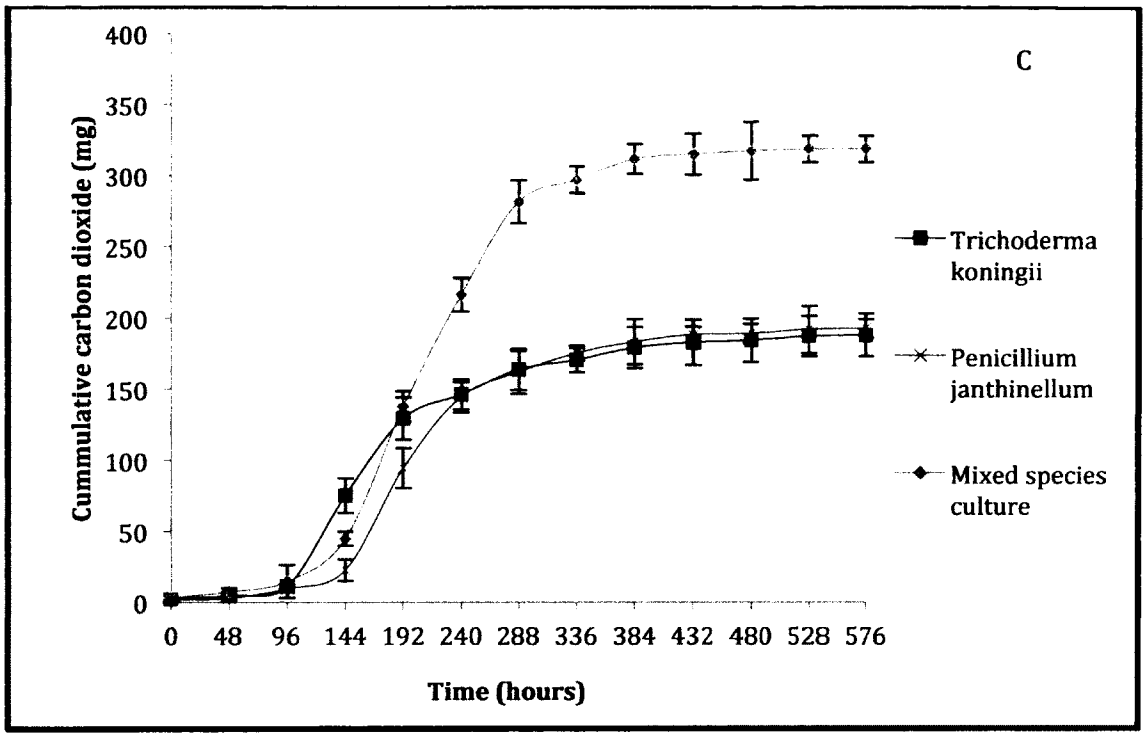
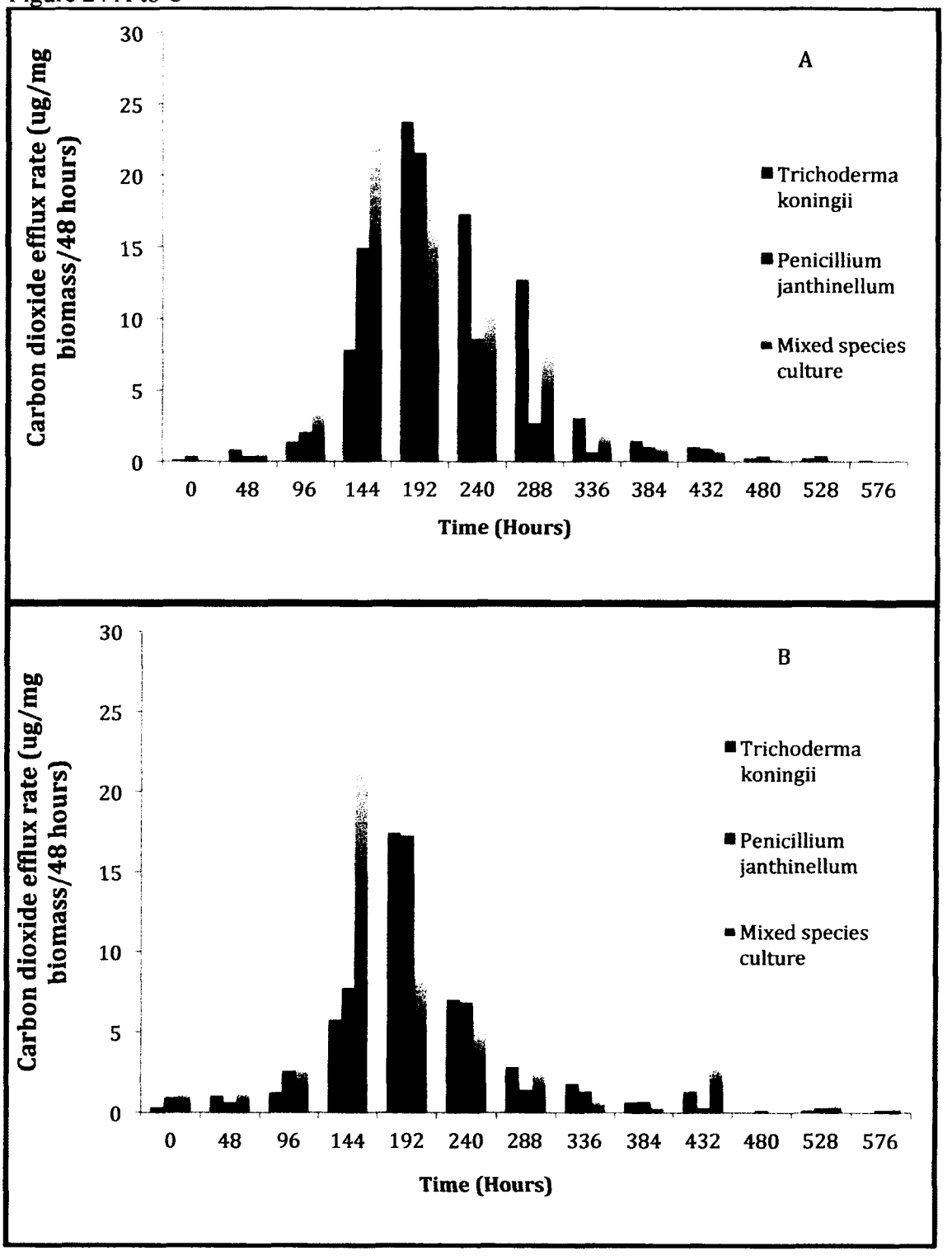


Figure 23: Cumulative carbon dioxide evolved from 150 ml of culture during the growth of three fungal species; *Trichoderma koningii (Blue)*, *Penicillium janthinellum (red)* and mixed culture of *Trichoderma koningii* and *Penicillium janthinellum (green)* growing in a A) undecanoic acid; B) decanoic acid; C) mixed fatty acid as the sole source of carbon and energy in a batch culture at 34 °C +/ - 1 °C during 576 hours. The vertical bars in represent standard deviation values of three replicates

Figure 24 A to C



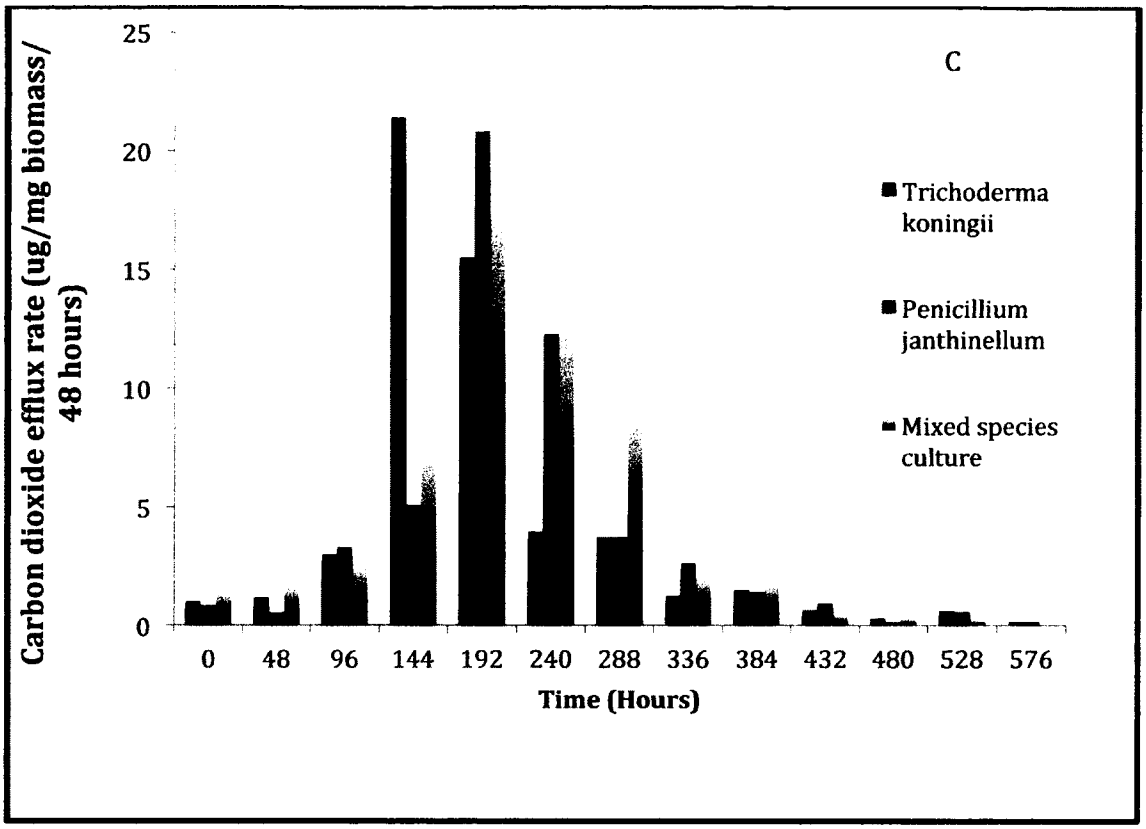
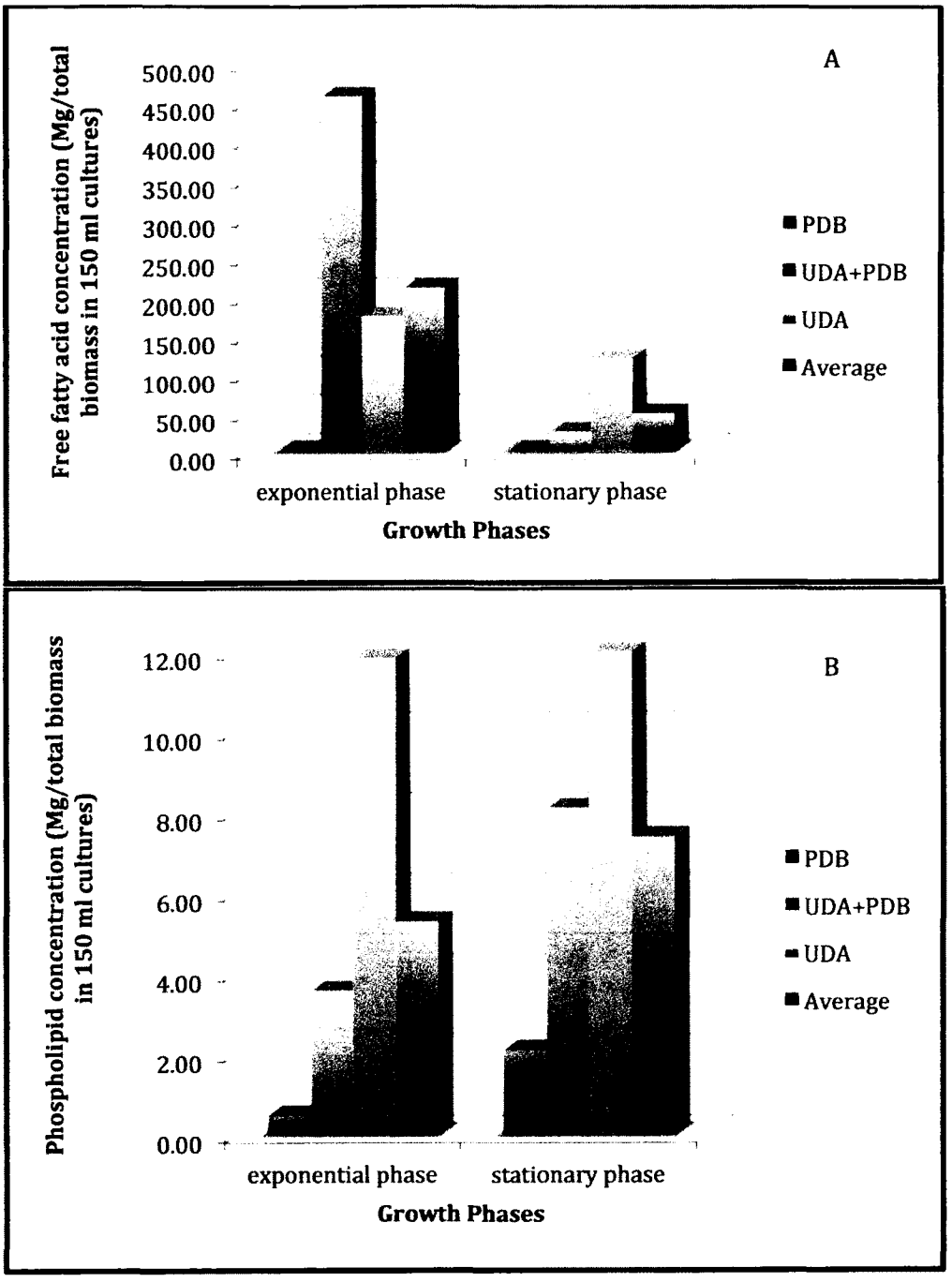


Figure 24: The average rate of carbon dioxide evolution by 150 ml of mixed species culture (green), *T. koningii* (blue) and *P. janthinellum* (red) growing in the A) undecanoic acid B) decanoic acid C) mixed fatty acids as the sole carbon and energy source at 34 °C +/ - 1 °C in batch culture during 576 hours of incubation.

Figure 25 A to D



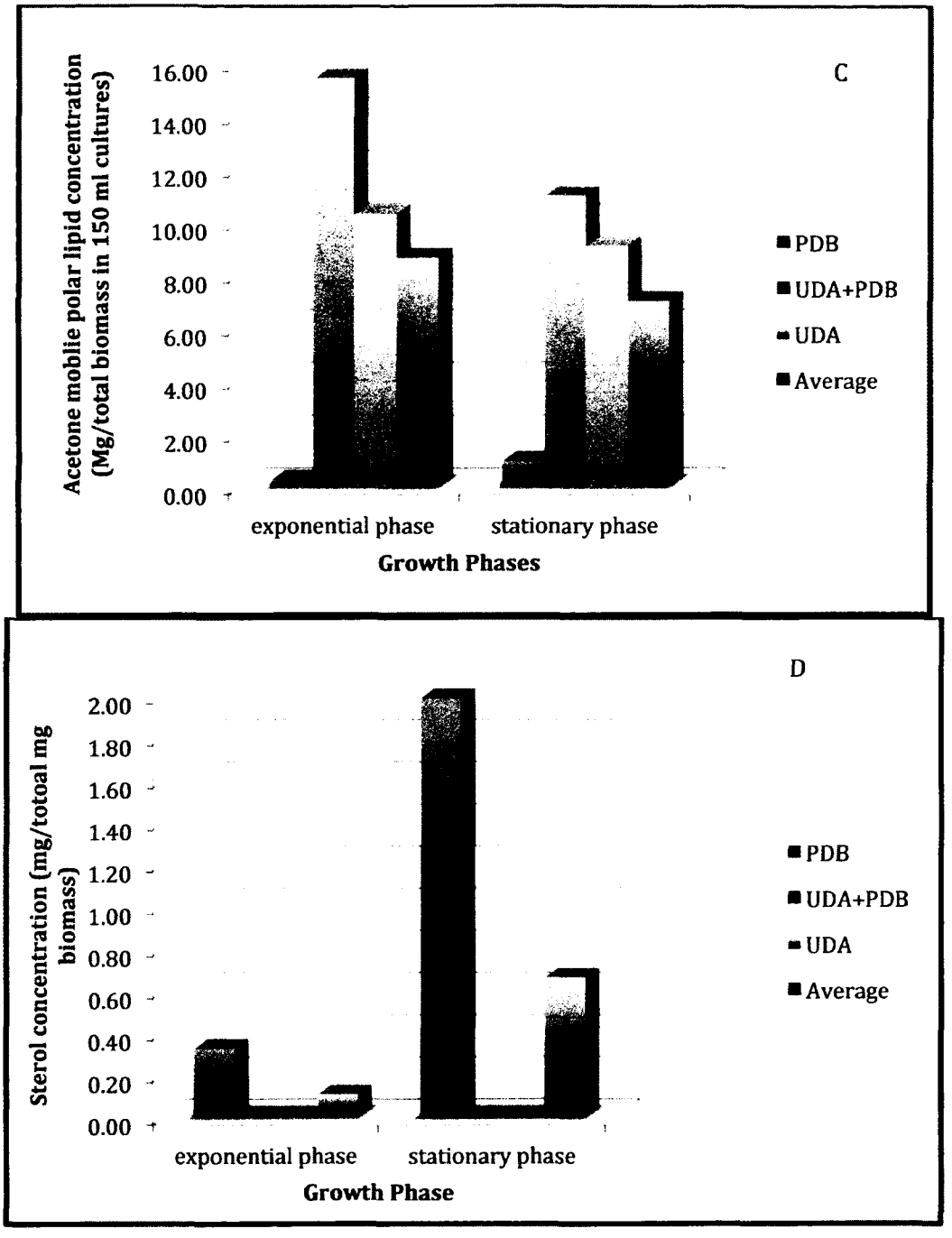


Figure 25: Comparison of total amount of A) free fatty acids B) phospholipids C) acetone mobile polar lipids D) sterols allocated intracellularly by mixed culture of *T. koningii* and *P. janthinellum* during exponential and stationary growth phase while growing on 3 different carbon substrates; Potato dextrose broth (blue), mixture of potato dextrose broth and undecanoic acid (red), undecanoic acid (green), average (purple).

Table 23: Sample data for the calibration curve – 4 of Iatroscan using the standards in Table 9.1

Compounds	Area X	X-coefficient A	Constant B	Amount Y=(X-B)/A	Lowest Calibrant	Smallest area	½ Area	Largest Area	2* largest area	y0	a	b
HC[a]	0.434	4.4717	-0.9994	0.15	0.5274	1.5756	0.79	19.356	38.71	0.0442	0.2882	-0.0034
SE/WE[b]	0	6.3424	-3.0038	0.00	0.6010	1.6311	0.82	30.593	50.00	0.2146	0.2251	-0.0023
EE[c]	0	4.8770	-2.4622	0.00	0.9678	2.6308	1.32	36.504	73.01	0.3368	0.2368	-0.0009
ME[d]	0	4.8770	-2.4622	0.00	0.9678	2.6308	1.32	36.504	73.01	0.3368	0.2368	-0.0009
EKET[e]	0	4.8770	-2.4622	0.00	0.9678	2.6308	1.32	36.504	73.01	0.3368	0.2368	-0.0009
MKET[f]	0	4.8770	-2.4622	0.00	0.9678	2.6308	1.32	36.504	73.01	0.3368	0.2368	-0.0009
GE[g]	0	4.0007	-1.7517	0.00	1.2398	2.9783	1.49	28.437	56.87	0.4883	0.2431	0.0002
TG[h]	0	4.0007	-1.7517	0.00	1.2398	2.9783	1.49	28.437	56.87	0.4883	0.2431	0.0002
FFA[i]	2.218	3.0389	-0.3908	0.86	0.5276	0.9826	0.49	9.4179	18.84	0.3022	0.2278	0.0096
ALC[j]	0	5.2137	-3.5516	0.00	0.6258	0.9608	0.48	25.157	30.00	0.2790	0.3254	-0.0054
ST[k]	0	3.5550	-1.6210	0.00	0.4784	0.5496	0.27	13.033	20.00	0.2787	0.3945	-0.0090
DG[l]	0	3.5550	-1.6210	0.00	0.4812	1.7077	0.85	16.397	32.79	0.2787	0.3945	-0.0090
AMPL[m]	1.267	4.4229	-0.3549	0.37	0.4812	1.7077	0.85	16.397	32.79	0.1725	0.1952	0.0017
PL[n]	1.502	6.0672	-2.4352	0.27	1.0718	5.9638	2.98	54.287	70.00	-0.365	0.2583	-0.0017

a- Hydrocarbons, b- Steryl esters/wax esters, c- Ethyl esters, d- Methyl esters, e- Ethyl ketones, f- Methyl ketones, g- Glycerol ethers, h- Triacylglycerols, i- Free fatty acids, j- Alcohols, k- Sterols, l- Diacylglycerols, m- Acetone mobile polar lipids, n- Phospholipids

Table 24: Sample data of lipid classes analysis for intracellular lipids extracted from the mixed species culture growing on mixture of UDA+PDB during exponential phase

Lipid Classes	Amount on Rod (µg)	Amount in Extract (µg)	Corrected amount in extract (µg)	Amount per µl (µg/µl)	% Wet weight (g/g)	% Lipid composition
HC[a]	0.14	412.62	412.62	0.83	0.00	2.40
SE/WE[b]	0.00	0.00	0.00	0.00	0.00	0.00
EE[c]	0.00	0.00	0.00	0.00	0.00	0.00
ME[d]	0.00	0.00	0.00	0.00	0.00	0.00
EKET[e]	0.32	970.74	970.74	1.94	0.00	5.62
MKET[f]	0.00	0.00	0.00	0.00	0.00	0.00
GE[g]	0.00	0.00	0.00	0.00	0.00	0.00
TG[h]	0.00	0.00	0.00	0.00	0.00	0.00
FFA[i]	5.08	15241.60	15241.60	30.48	0.00	88.31
ALC[j]	0.00	0.00	0.00	0.00	0.00	0.00
ST[k]	0.00	0.00	0.00	0.00	0.00	0.00
DG[l]	0.00	0.00	0.00	0.00	0.00	0.00
AMPL[m]	0.17	514.90	514.90	1.03	0.00	2.98
PL[n]	0.04	119.37	119.37	0.24	0.00	0.69
Total Lipids	5.75	17259.23	17259.23	34.52	0.00	100

a- Hydrocarbons, b- Steryl ester/was esters, c- Ethyl esters, d- Methyl esters, e-Ethyl ketones, f- Methyl ketones, g- Glycerol ethers, h- Triacylglycerols, i- Free fatty acids, j- Alcohols, k- Sterols, l- Diacylglycerols, m-Acetone mobile polar lipids, n- Phospholipids.

Table 25: Sample data for fatty acid analysis for intracellular lipids extracted from the mixed species culture growing on mixture of UDA+PDB during exponential phase

Fatty acids	Molecular weight	Area	Weight % Fatty acids	Mole Equivalent Fame	Mole % Fame	Fatty acid (μg/μl)
14:0	242.4000	0.122	1.10	0.00	1.27	0.34
Trimethyltridecanoic acid (TMTD)	270.5100		0.00	0.00	0.00	0.00
14:1	240.3840	0.0076	0.07	0.00	0.08	0.02
ι15:0	256.4270	0.0122	0.11	0.00	0.12	0.03
αι15:0	256.4270	0.0086	0.08	0.00	0.08	0.02
15:0	256.4270	0.6706	6.06	0.02	6.61	1.88
15:1	254.4110	0.0195	0.18	0.00	0.19	0.05
ι16:0	270.4540	0.005	0.05	0.00	0.05	0.01
αι16:0?	270.4540	0.1364	1.23	0.00	1.28	0.38
16:0	270.4540	3.5959	32.48	0.12	33.63	10.08
16:1ω11	268.4380	0.0165	0.15	0.00	0.16	0.05
16:1ω9	268.4380	0.0319	0.29	0.00	0.30	0.09
16:1ω7	268.4380	0.4241	3.83	0.01	4.00	1.19
16:1ω5	268.4380	0.0045	0.04	0.00	0.04	0.01
ι17:0	284.4810	0.0168	0.15	0.00	0.15	0.05
αι17:0	284.4810	0.0834	0.75	0.00	0.74	0.23
16:2ω4	266.4220	0.0933	0.84	0.00	0.89	0.26
17:0	284.4810	0.5615	5.07	0.02	4.99	1.57
16:3ω4	264.4060	0.2701	2.44	0.01	2.58	0.76
17:1	282.4650	0.0838	0.76	0.00	0.75	0.23
16:4ω3	262.3910	0.0193	0.17	0.00	0.19	0.05
16:4ω1	262.3910	0.3221	2.91	0.01	3.11	0.90
18:0	298.5080	2.0255	18.29	0.06	17.16	5.68
18:1ω11	296.4920	0.0354	0.32	0.00	0.30	0.10
18:1ω9	296.4920	0.393	3.55	0.01	3.35	1.10
18:1ω7	296.4920	0.9943	8.98	0.03	8.48	2.79
18:1ω6	296.4920	0.0213	0.19	0.00	0.18	0.06
18:1ω5	296.4920	0.0152	0.14	0.00	0.13	0.04
18:2	294.4760		0.00	0.00	0.00	0.00
18:2	294.4760		0.00	0.00	0.00	0.00
18:2ω6	294.4760	0.442	3.99	0.01	3.80	1.24
18:2ω4	294.4760		0.00	0.00	0.00	0.00
18:3ω6	292.4610	0.0286	0.26	0.00	0.25	0.08
19:00	312.5350		0.00	0.00	0.00	0.00
18:3ω4	292.4610	0.0407	0.37	0.00	0.35	0.11
18:3ω3	292.4610	0.3891	3.51	0.01	3.37	1.09
18:4ω3	290.4450	0.0278	0.25	0.00	0.24	0.08

18:4ω1	290.4450		0.00	0.00	0.00	0.00
20:0	326.5620	0.0253	0.23	0.00	0.20	0.07
18:5ω3	288.4270		0.00	0.00	0.00	0.00
20:1ω11	324.5460	0.0015	0.01	0.00	0.01	0.00
20:1ω9	324.5460		0.00	0.00	0.00	0.00
20:1ω7	324.5460		0.00	0.00	0.00	0.00
20:2α	322.5300		0.00	0.00	0.00	0.00
20:2β	322.5300		0.00	0.00	0.00	0.00
20:2ω6	322.5300	0.0349	0.32	0.00	0.27	0.10
20:3ω6	320.5140		0.00	0.00	0.00	0.00
21:00	340.5890		0.00	0.00	0.00	0.00
20:4ω6	318.4980		0.00	0.00	0.00	0.00
20:3ω3	320.5100		0.00	0.00	0.00	0.00
20:4ω3	318.4980	0.043	0.39	0.00	0.34	0.12
20:5ω3	316.4830		0.00	0.00	0.00	0.00
22:0	354.6160		0.00	0.00	0.00	0.00
22:1ω11(13)	352.6000		0.00	0.00	0.00	0.00
22:1ω9	352.6000		0.00	0.00	0.00	0.00
22:1ω7	352.6000		0.00	0.00	0.00	0.00
22:2NIMDa	350.5840		0.00	0.00	0.00	0.00
22:2NIMDb	350.5840		0.00	0.00	0.00	0.00
21:5ω3	330.5100		0.00	0.00	0.00	0.00
23:0	368.6430		0.00	0.00	0.00	0.00
22:4ω6	346.5520		0.00	0.00	0.00	0.00
22:5ω6	344.5370		0.00	0.00	0.00	0.00
22:4ω3	346.5520	0.0361	0.33	0.00	0.26	0.10
22:5ω3	344.5370		0.00	0.00	0.00	0.00
24:0	382.6700	0.0133	0.12	0.00	0.09	0.04
22:6ω3	342.5210		0.00	0.00	0.00	0.00
24:1	380.6540		0.00	0.00	0.00	0.00
Total		11.07	100.00	0.36	100.00	31.04
Bacterial			14.62			4.54
Sum of Saturated FA			63.35			19.66
Sum of MUFA			18.50			5.74
Sum of PUFA			15.78			4.90
Sum of ω3			4.65			1.44
Average Chain Length					16.78	
Average Double Bonds					0.64	
Average Fatty Acid Methyl Ester Molecular Weight					280.06	
Average Fatty Acid Molecular Weight					266.06	

* 1 analytical replicate of intracellular lipid extract from mixed species culture growing on UDA+PDB

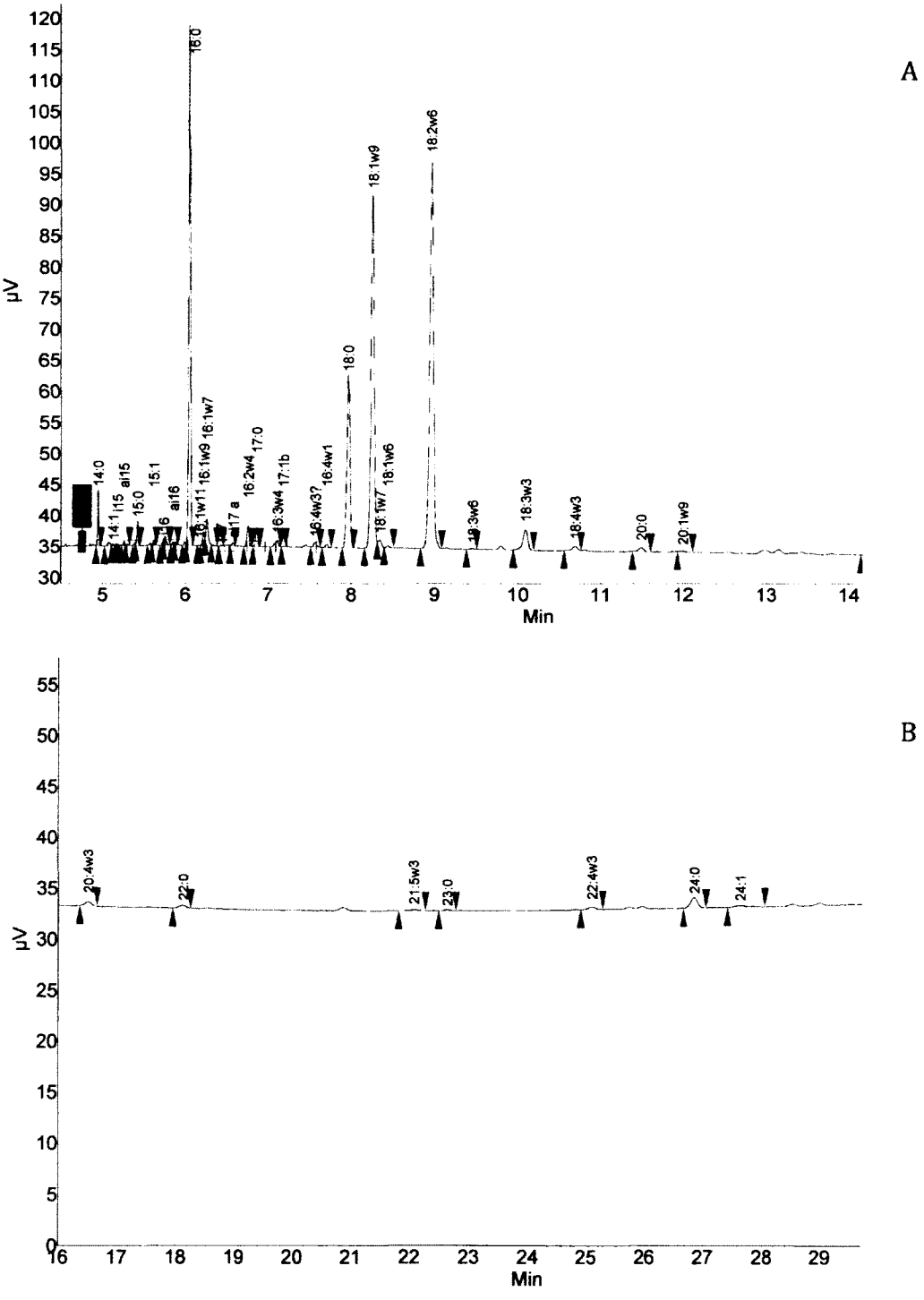


Figure 26: GC chromatogram of fatty acids in intracellular lipids extract of mixed species culture grown in PDB during exponential phase, a) retention time 0 to 16 minutes and b) retention time 16 to 32 minutes.

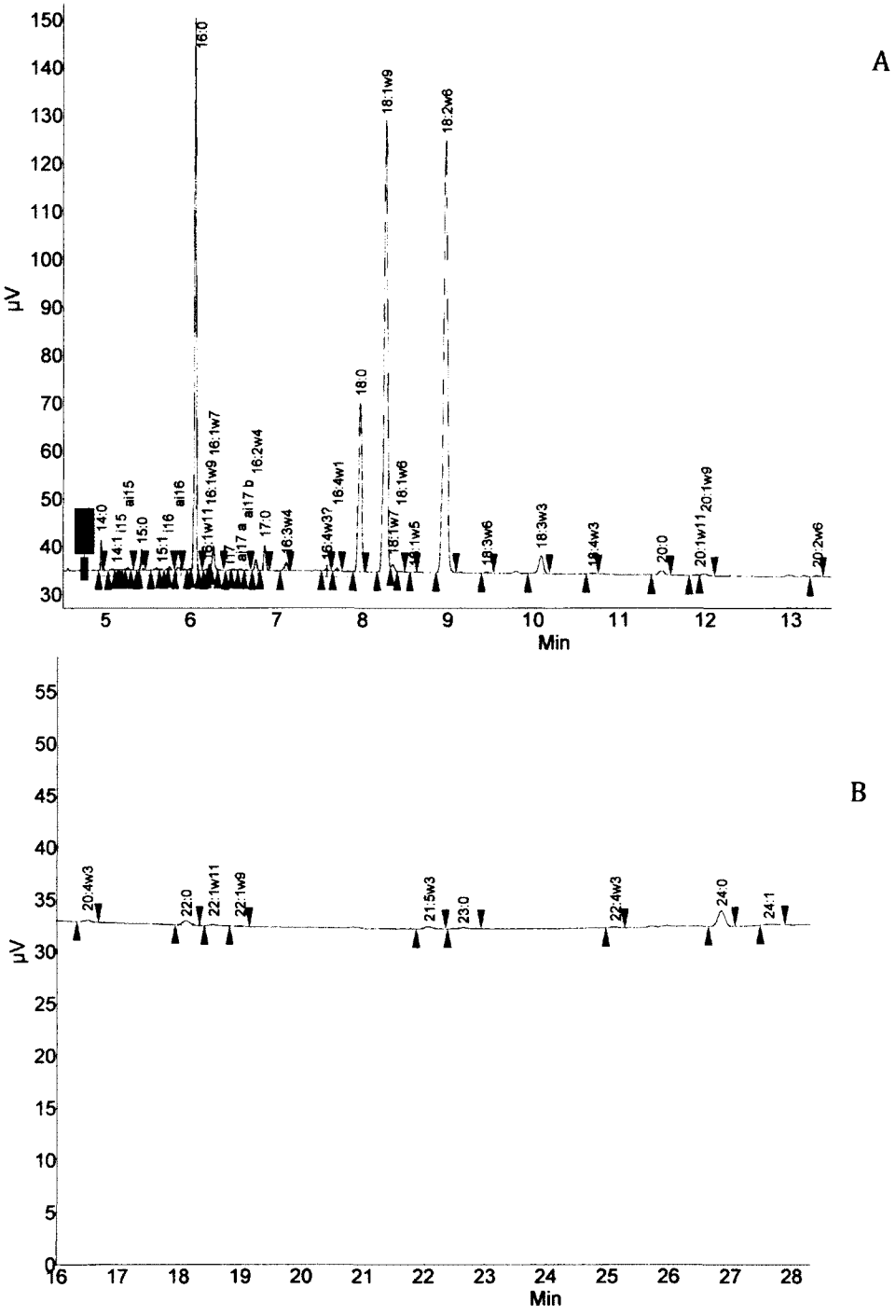


Figure 27: GC chromatogram of fatty acids in intracellular lipids extract of mixed species culture grown in PDB during stationary phase, a) retention time 0 to 16 minutes and b) retention time 16 to 32 minutes.

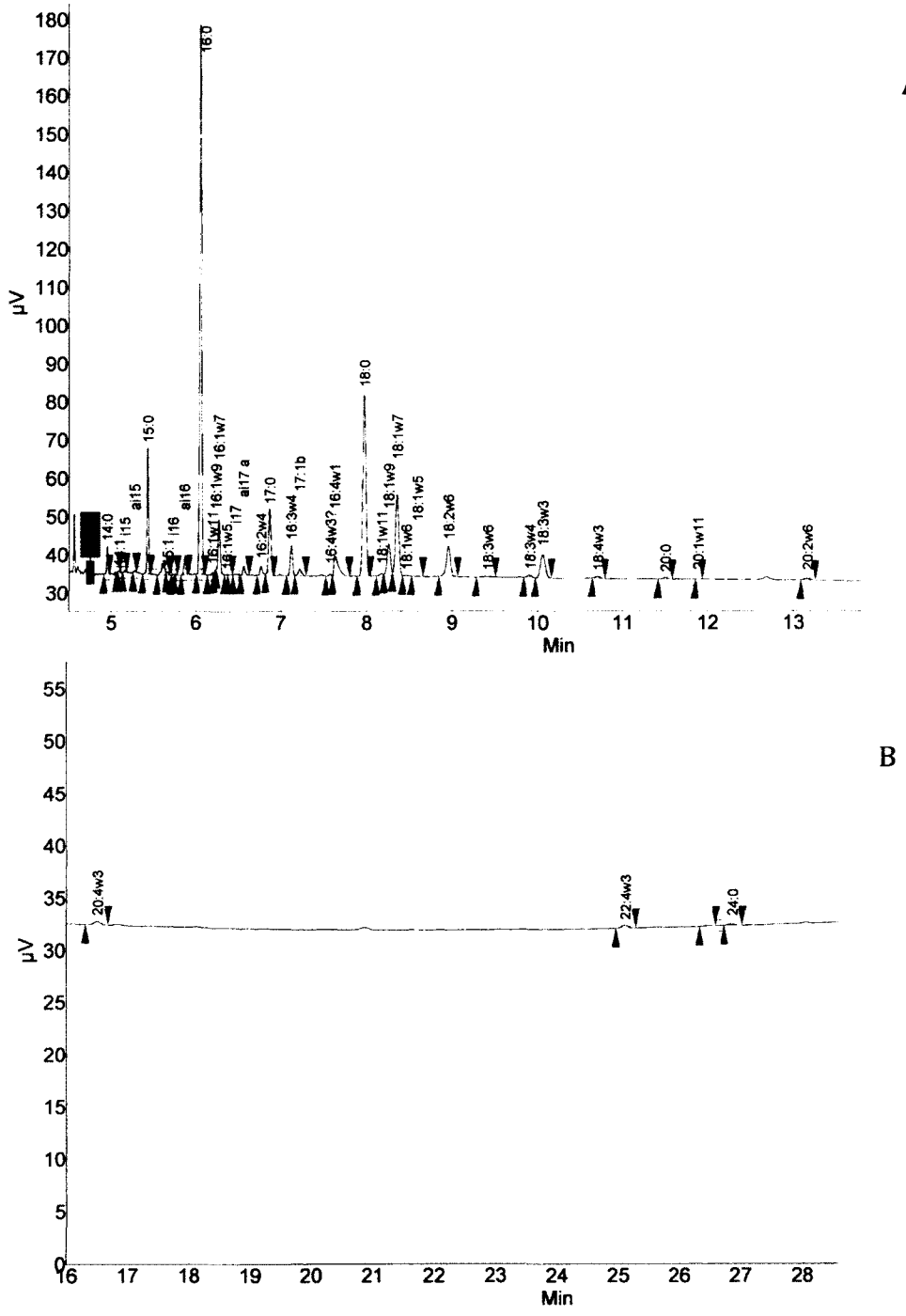


Figure 28: GC chromatogram of fatty acids in intracellular lipids extract of mixed species culture grown in mixture of UDA+PDB during exponential phase, a) retention time 0 to 16 minutes and b) retention time 16 to 32 minutes.

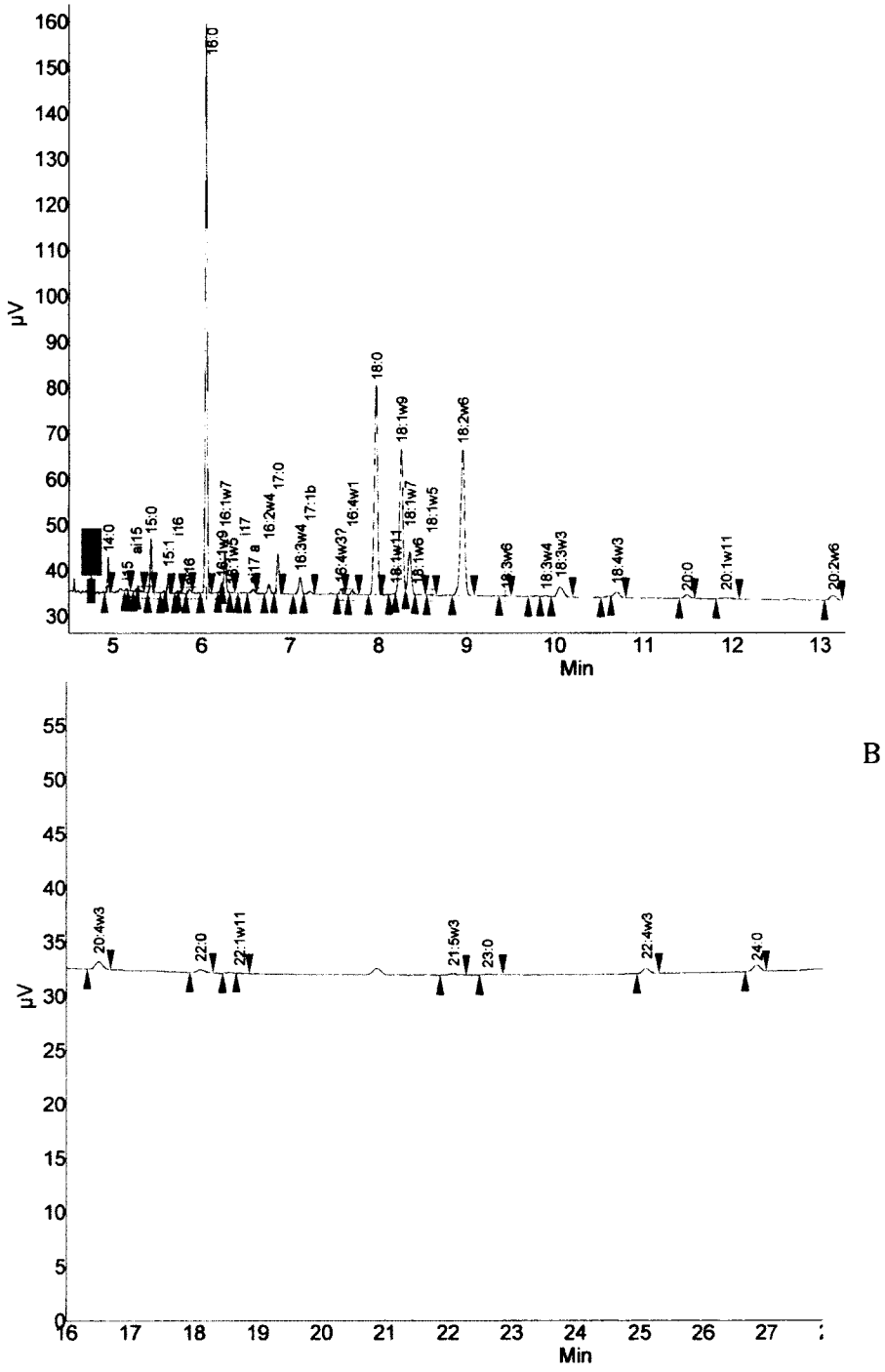


Figure 29: GC chromatogram of fatty acids in intracellular lipids extract of mixed species culture grown in mixture of UDA+PDB during stationary phase, a) retention time 0 to 16 minutes and b) retention time 16 to 32 minutes.

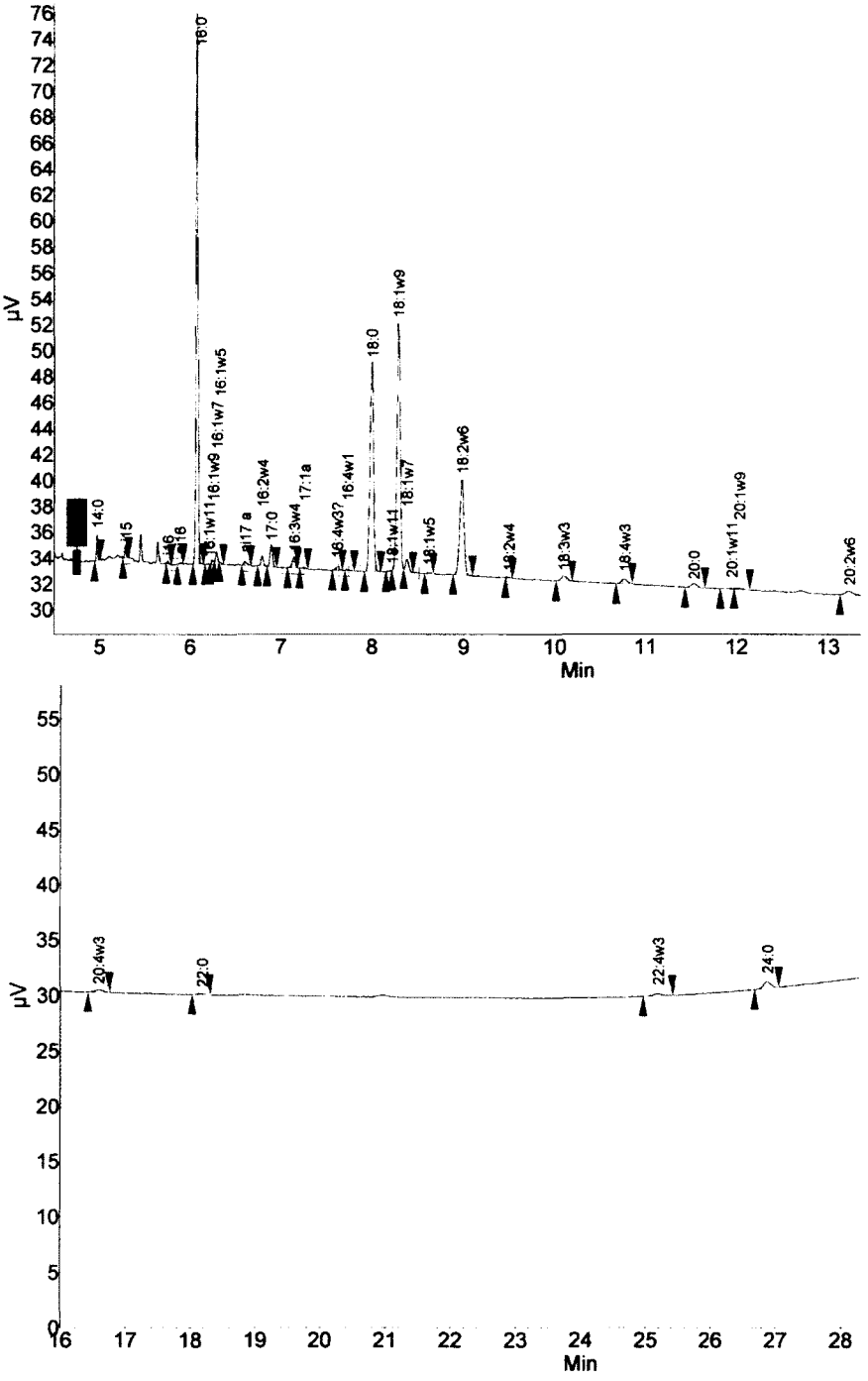


Figure 30: GC chromatogram of fatty acids in extracellular lipids extract of mixed species culture grown in mixture of UDA+PDB during exponential phase, a) retention time 0 to 16 minutes and b) retention time 16 to 32 minutes.