LIPIDS PRODUCTION FROM OLEAGENOUS MICROBES USING AGRICULTURAL RESIDUES AS A FEEDSTOCK

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2016

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

IN

ENVIRONMENTAL SCIENCE

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CERTIFICATE

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DEDICATED…..!!!

To my parents

ACKNOWLEDGEMENTS

All praises to my Allah Almighty for His incalculable blessings and support throughout my life. I am grateful to Him, the most beneficent and the benevolent whose protection is sorted by all and who bestowed me all the mental abilities and wisdom from His unfathomed treasure to complete this "research work". Peace and blessings of Allah be upon our last prophet Muhammad (S.A.W).

I greatly acknowledge contributions of my thesis supervisor Dr Muhammad Arshad. His useful suggestions and comments helped me in learning new concepts in the field of research. I would like to thank my guidance and examination committee members Dr. Imran Hashmi and Dr. Faraz Bhatti for their tremendous help during my research at IESE, NUST, Islamabad.

I am thankful to IESE and ASAB staff members for providing us resources for research and for keeping environment amiable for research and study.

I would like to dedicate my thesis work to my beloved parents. My deepest gratitude goes to my father for his everlasting enlightened vision and always been a great source of inspiration for me in making my career, special thanks to my mother for always believing in me and supporting me with her love and patience.

I would like to express my deepest appreciations to my brother and sisters for their love and affection. In the end, thanks to all my friends for making my way well oriented. Thanks for cherished memories and everlasting friendship.

Bushra

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LIST OF ABBREVIATIONS

CMC	Craboxymethyl Cellulose
CGW	Cotton Gin Waste
GDP	Gross Domestic Product
HDIP	Hydrocarbon Development Institute of Pakistan
LCW	Lignocellulose Waste
MTOE	Million Tones of Oil Equivalent
PUFs	Poly-unsaturated Fatty Acids
T	Temperature
Tg	Teragrams

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ABSTRACT

Energy crisis, food insecurity, global warming, fossil emissions, and open burning of agricultural residues are growing challenges across the world. Such state of affairs has forced scientists and engineers to introduce green biotechnology for reliable production of biodiesel. Current interest in oil accumulation by oleaginous strains is highly appreciable for converting agricultural residues into lipids as an alternative renewable energy resource. The objectives of present work were to optimize oleaginous microbial growth and to produce lipids through oleaginous fungal strain(s) using agricultural waste as a feedstock. The lipids may further be utilized for bio-energy generation. Chemical pretreatment (acid and base) followed by etherification process was used to synthesize carboxymethyl cellulose (CMC) from lignocellulosic cotton ginning waste (CGW). Fungal growth rate was determined through point inoculation technique. Bligh and Dyer, lipids protocol was preferred method for lipids extraction from dry fungal biomass. Different pH levels i.e. 5.5, 6.0, 6.5, 7.0, & 7.5 were tested to achieve maximum growth of fungal strain. Mean radial growth rate of *Fusarium oxysporum* was determined for six days on CMC carbon source derived from CGW. *Alternaria arborescence*, *Alternaria alternatae* and *Asperigillus flavous* fungal strains had negative growth response towards CMC energy source. The growth and morphological characteristics of *Fusarium oxysporum* on different carbon sources (PDA, SDA, CMC-A, and CGW-CMC) were also compared. The maximum colony diameter was 85 mm in response to CGW-CMC after 6 days of incubation period. Optimized growth conditions were 6.5 pH and 28 °C temperature. General morphology of strain in response to energy sources and substrate composition represented velvety surface and color altered from orange to white. On average 36 milligram fungal bio-mass was harvested from 100 mL of fermentation media. Estimated annual production of CGW in Pakistan is 0.3 million tons with 50% cellulose recovery

efficiency in the course of acid-base chemical pretreatment. Keeping in view the annual production of agricultural waste, *Fusarium oxysporum* through enzymatic hydrolysis may produce 540 tons/year of lipids by consuming 0.15 million tons/year of CMC as a growth substrate. Furthermore, 0.15 million tons/year of cellulose may also produce 52500 tons/year of bio-ethanol. The future scope of the study signifies that the cotton waste recovery for bio-fuel production in Pakistan will escort to establish multi-dimensional scientific models and engineering technology for energy production.

Chapter 1

INTRODUCTION

1.1 Background

Economic challenges around the globe have increased energy demand with an exertion on conventional energy resources. In 2013, the global renewables energy supply mix constituted a major portion of fossil fuels (78.3%), followed by renewables (19.1%) and nuclear power (2.6%) (REN21, 2015*).* Industrial countries consume a lot of fossil energy and are greatly involved in global emissions with an approximately share of 61% (Baumert *et al.,* 2005). Similarly, energy supplies of Pakistan are also dominated by fossil fuels (87.1 % in 2014), with small contribution from hydropower (11.4%) and nuclear energy (1.9%) (HDIP, P.E.Y. (2014).

In 2012, government intended to extract energy from lignite coal reserves located in Southern Sindh to increase its share in energy supply mix. However, technology investments or true economic cost measurement is an utmost requisite to use indigenous energy source (GoP, 2012-13). Even, if these coal reserves cannot meet the required fuel demand of growing population, Pakistan will increase its crude oil import by casting a huge financial burden on its destabilized economy. In 2014, estimated annual loss in Gross Domestic Product (GDP) was 7% because of frequent disruptions in productive activities owing to load shedding and power shutdowns. There are many reasons behind energy shortfall which include increasing energy demand, inefficient use of energy resources, unsatisfactory energy supply, poor energy sector performance, policy failure, and circular debt (GoP, 2014-15). There is no other option either to add clean energy technology to the system or to shift conventional trend towards renewable energy resources. Existing scenario has originated complex issues in Pakistan with adverse effects on environment and economy which includes;

1. Energy crisis resulting in an economic instability
2. Unremitting collapse of fossil reserves
3. Food insecurity
4. Increase in GHG emissions by burning fossil fuels and agricultural residues.

It has been estimated that burning of biomass emits 1100 teragrams (Tg) of CO_2, 3.1 Tg of CH_4, 0.37 Tg of SO_2, 2.8 Tg of NOx, and 67 Tg of CO in Asian countries. Emissions from agricultural residues contribute 250 Tg out of total 730 Tg of GHG. Burning of crop residues in an open field is a common practice contributing 379 Tg of CO_2, 0.68 Tg of CH_4, 0.10 Tg of SO_2, 0.96 Tg of NOx, and 23 Tg of CO (Gadde *et al.,* 2009; Streets *et al.,* 2003). There are four major sources of biomass in Pakistan such as; municipal waste, animal waste, forest residues and agricultural residues. The potential to generate power from biomass among renewable energy resources such as solar, wind, geothermal and hydro is quite exceptional. Biomass stores energy efficiently which may be converted into liquid, solid or gaseous fuel collectively termed as bio-fuels (Javaid *et al.,* 2011). Bio-fuel is acquired from biomass and may substitute petroleum based transportation fuels in four ways; firstly, composition is similar to the petro-diesel, secondly engine needs little or no further modification, thirdly it may reduce dependency on depleting fossil fuels and fourthly it may contribute in lowering greenhouse gas emissions (Bessou *et al.,* 2011). They are classified into two major forms; conventional (1st generation) and advanced bio-fuels (2nd, 3rd and 4th generations). The 2nd generation bio-fuels are obtained from crop residues such as husks, stalks, leaves, stems and non-food crops like jatropha or switch grass (Janda *et al.,* 2012).

1.2 Agricultural residues

Agricultural residues are composed of lignocellulose. Lignocellulosic biomass is a plant material with an intricate composition of cellulose, hemicellulose and lignin. Lignocellulose waste (LCW) requires pretreatment (chemical, biological, or physical) to break the cell walls into monosaccharide (Bessou *et al.,* 2009). These monomers are consumed by microorganisms to produce supplementary valuable energy products. The value added products acquired from LCW are generally summarized as; industrial chemicals, bio-ethanol, biodiesel, bio-hydrogen, biogas, bio-fertilizers, food, medicines, bio-sorbents etc (Mtui, 2009). Enzymatic hydrolysis of cellulose or cellulose derivatives using oleaginous fungal microorganisms may produce lipids. Lipids may further be utilized for bio-diesel production.

1.3 Cotton ginning waste

Global production of cotton gin waste is approximately 3.23 million tons per year (Sahu & Pramanik, 2015). Pakistan is rich in cotton production and generates huge quantity of CGW. The disposal methods associated with cotton waste is either it's burning in open field or is used as a fuel in brick kilns. Identified problems related to cotton gin waste at ginning mills engross allocation of space, fire hazard, and expensive disposal treatments. To resolve these issues, researchers, organizations and communities are finding solutions to use cotton gin waste as an alternative renewable energy fuel (Mahar, 2009).

1.4 Pretreatment of lignocellulose cotton ginning waste

The cotton ginning waste constitutes majorly cellulose which has tight binding with hemicelluloses and lignin oriented in a complex pattern (Ingram *et al.,* 2008). Therefore, pretreatment/delignification is an important step to release hemicellulose and cellulose (Sahu & Pramanik, 2015). Lignocellulosic biomass requires efficient pretreatment technologies to convert

complex polymers into simpler constituents. Generally pretreatment methods are classified into four major groups; physical, chemical, biological and physico-chemical pretreatment (Sun and Chen, 2002). Chemical pretreatment (Acid/Alkali) may extract cellulose from cotton gin waste. Isolated cellulose further needs two processes; mercerization and etherification for transformation into carboxymethyl cellulose (Haleem *et al.,* 2014). Carboxymethyl cellulose is a carbon substrate which can be consumed by oleaginous fungal strains for lipids production (Li *et al.,* 2012).

1.5 Oleaginicity and lipids production

Microbial oil industry has fascinated world attention to produce biodiesel to meet energy demand for growing population. All microorganisms may not be considered as rich sources of fats and oils, like rest of the living cells. Lipids are essential macronutrient to perform proper functioning of cell membranes and membranous structures. Those which may accumulate more than 20% of their biomass as lipids are known as "oleaginous". Of the some 60,000 different fungal species, only 50 may accumulate more than 25% lipids (Ratlege, 2001). Among heterotrophic microorganisms, oleaginous fungi (molds and yeast) have been extensively reported as fine producers of triacylglycerides. Oleaginous filamentous fungi may produce poly unsaturated fatty acid (PUFA), such as γ-linolenic acid and arachidonic acid. Famous *Mortierella alpine* has accounted finest biotechnological application for producing oils containing n-1, n-3, n-4, n-6, n-7 and n-9 PUFAs (Sakuradani & Shimizu, 2009). *Fusarium species* has been reported for producing lipids using analytical grade carboxymethyl cellulose as a carbon substrate (Li *et al.,* 2012). These strains may also produce ethanol from chemically pretreated cotton gin waste.

1.6 Enzymatic hydrolysis of cellulose and carboxymethyl cellulose

Cellulose and related oligosaccharides derivatives are hydrolyzed by a group of enzymes called cellulase (Berry *et al.,* 1990). Cellulase enzyme is produced by numerous microorganisms, mostly by bacteria and fungi. The cellulase complex includes C_1 [(EC3.2.1.9), cotton lyase, CMase (carboxymethyl cellulase), C_x (EC 3.2.1.4), pectinase, C_b (EC 3.2.1.21), and b-glycosidase] which is generally used in simultaneous scarification fermentation system. Filamentous fungi are considered to be more preferable for commercial production of important enzymes, as quality of enzymes acquired from fungal cultures is higher than production from bacteria. Saccharification and fermentation are linear steps for converting cellulose into sugars and subsequently into ethanol. Sacchrification process may be achieved by acid or enzymatic hydrolysis. Enzymatic hydrolysis is preferable during reaction because it produces fewer by-products and proceeds under milder conditions (Mandels *et al.,* 1974).

Many studies have focused on bio-methane or bio-ethanol production as compared to lipids production from cotton gin waste. This study has multi-dimensional scientific approach to emphasize on the choice of carbon substrate, chemical pretreatment method and types of oleaginous fungal microbes that have been used for economical production of lipids. Chemically pretreated cotton gin waste was used as a carbon substrate for lipids accumulation in fungal strains. Cotton residues were selected as energy source because of its availability in Pakistan with highest percentage ($\geq$ 85%) of cellulose among lignocellulosic crop residues.

1.7 Objectives

Keeping in view the information available from the literature and data sources, the objectives of present work were;

1. Optimization of oleaginous microbial growth
2. Lipids production from oleaginous microbes using agricultural residues as a substrate

1.8 Scope of the study

One of the major advantages of this study will be utilization of lignocellulose from agricultural waste for lipids/ethanol production to retrieve renewable energy resource. Oleaginous filamentous fungal strains have colossal industrial application. This research may be effective in future for providing an environmental friendly solution to meet energy demand, to reduce emissions from crop burning and to minimize the food security risk from the cultivation of arable land for bio-fuel crops.

Chapter 2

LITERATURE REVIEW

2.1 Pakistan geography and energy scenario

Pakistan is geographically located between the regions of Middle East, central Asia and south Asia with an area of 796,096 square kilometer. Topography and terrain of Pakistan has a blend of assorted flavors including lush green forests, flat plateaus, fertile cultivating area, Indus river plain, hilly mountains, hot deserts, fresh waterfalls, and lofty glaciers. Such a blend of landscape in the region crafts variations in its climatic conditions from tropical to temperate to grow variety of crops. Pakistan has extraordinary diversity in its natural resources; however it severely requires appropriate resource allocation and wise policy governance to achieve sustainable economic growth. To grow sustainable economy of the state, energy acts as a driving force. The net primary energy supply of Pakistan stood at 66.8 Million Tons of Oil Equivalent (MTOE) in FY 2014. Natural gas and crude oil shared 85 percent to the total 66.8 MTOE of energy mix. The energy mix consisted of major energy sources with share of 46.8 percent (30. 98 MTOE) of natural gas, followed by oil 34.4 percent (23.01 MTOE) , electricity (Nuclear, hydro, Imported)13.3 percent (8.92 MTOE), coal 5.4 percent (3.59 MTOE), and LPG 0.5 percent (0.36 MTOE). In FY 2014, primary energy consumption efficiency by all energy reliance sectors was 39.8 MTOE; remaining energy was either transformed (23.6 MTOE) into other forms or was used for non energy purpose (3.4MTOE) (HDIP, P.E.Y. (2014).

In recent years, Pakistan is in the firm grip of energy crisis which has caused serious effects on socio-economic development. The rising gap between energy demand and energy supply has neither being reduced nor stayed constant. Pakistan is entirely dependent upon oil and natural gas energy sources to satisfy its 70 percent energy demand in all economic sectors. In

order to curb oil import from Middle East, government is devising policies to attract private investments for tracking their way into the energy system. Current energy scenario urges, to use indigenous energy resources such as hydro, coal and renewable source for diversified energy mix.

2.2 Agriculture

Pakistan is the sixth most populous country in the world, with an estimated population of 191.71 million. About, sixty two percent of our population lives in rural areas and directly or indirectly has reliance on agriculture for their source of income. Agriculture sector of Pakistan plays decisive role in earning foreign exchange. During 2014-15, it has contributed 20.9 percent to the Gross Domestic Product (GDP). This sector has conventionally sustained rational growth to pledge food security for our immense growing population. It has four sub-sectors: crops, fisheries, livestock and forestry. The sub-sector crop is further alienated into other crops, important crops, and cotton ginning. Important crops such as sugarcane, wheat, maize and cotton play vital role in supporting annual economic budget. Pakistan is the fourth largest cotton producing country in the world. During 2014-15, cotton production stood at 13,983 thousand bales over 2,961 thousand hectares (GoP, 2014-15). This dependency on agricultural sector indicates massive availability of agricultural residues in the country.

2.3 Composition of cotton ginning waste

Cotton ginning waste is an appropriate lignocelluloses biomass, because of sustainability, availability and cost effective. CGW is very vital to the cotton producers, researchers and environmentalists due to the massive production of waste and disposal problems. Raw cotton generates cotton gin residue, which consists of burs, leaves, cotton lint, cotton seed, immature

balls, sticks and dirt. With reference to 218 kg of cotton fiber produce 68-91 Kg of CGW. Ginning process of one bale (227 kg) generates 37 to 147 kg of waste. Global production of CGW is approximately 3.23 million tons per year. The composition of CGW is cellulose, which binds tightly with hemicelluloses and lignin forming a complex material (Sahu & Pramanik, 2015).

2.3.1 Cellulose

Cellulose is a linear polysaccharide polymer of anhydroglucopyranose-molecules, connected by β-1, 4- glycosidic bonds. The cellulose chains are crammed by hydrogen bonds in so-called 'elementary microfibrils'. These fibrils are connected to each other by hemicelluloses, pectin and enclosed by lignin. The microfibrils are linked in the form of bundles. This complicated structure makes cellulose resistant to both chemical and biological treatments (Ha *et al.,* 1998; Delmer and Amor, 1995; Morohoshi, 1991).

2.3.2 Hemicellulose

Hemicellulose, is the second main heterogeneous polymer in lignocellulosic residues which consists of sugar acids, hexoses (including less glucose, mannose and galactose) and pentoses (mainly arabinose and xylose) (Saha & Cotta, 2007).

2.3.3 Lignin

Lignin is the third most abundant heterogeneous polymer in lignocellulosic biomass, consisting of three aromatic alcohols including sinapyl, coniferyl and p-coumaryl. Lignin gives structural strength and acts as a barrier for enzymes and solutions (Zaldivar *et al.,* 2001; Hamelinck *et al.,* 2005).

2.4 Pretreatment methods

Explicitly pretreatment is for lignin removal, decrystallizing cellulose, reducing polymerization of cellulose, removal of acetyl group from hemicellulose, extraction of hemicellulose, structural expansion to increase internal surface area and pore value, so that hydrolysis of carbohydrate fraction into monomers can be acquired quickly with higher yields (Aita and Kim, 2010). Pretreatment which meets all these goals are mostly expensive, so mainly pretreatment targets on acquiring few value-added products. It is confirm, that different methods of pretreatment affect biomass in different ways (Mosier *et al.,* 2005). Some of the delignification techniques affect the biocompatibility of the process. As an advantage, cotton and viscose waste due to lack of lignin content in their cellulosic configuration (Miranda *et al.,* 2007) won't face such troubles. If the technique is not efficient, the substrate is not easily hydrolysable via cellulose enzymes and if it is severe, it will produce toxic substances to hinder microbial metabolism (Kodali and Pogaku, 2006). Generally pretreatment methods are classified into four major groups; physical, chemical, biological and physico-chemical pretreatment (Sun and Chen, 2002). Various techniques have been developed for cellulose isolation from biomass i.e. chlorine free method (Sun *et al.,* 2005), ionic liquid method (Jiang *et al.,* 2011), steam explosion (Chen and Liu, 2007), pooled chemical and enzymatic extraction (Reddy and Yang, 2006) and organosolv process (Sun *et al.,* 2004).

2.4.1 Chemical pretreatment

Pure cellulose with high yield can be extracted through acid pretreatment (removal of hemicellulose) followed by alkali pretreatment (removal of lignin) (Taherzadeh & Karimi, 2008; Wingren *et al.,* 2003). This method has been used in this study for the cellulose isolation from cotton gin waste.

2.4.1.1 Acid pretreatment

Acid pretreatment involves the use of diluted and concentrated acids to disrupt the firm structure of the lignocellulosic biomass. Dilute sulphuric acid (H_2SO_4) has been used commercially for pretreatment of a wide variety of biomass example, corn stover, soft wood, switch grass, and poplar. A large volume of concentrated sulfuric acid is usually required to allow strong acid for complete degradation of biomass into sugar. The treatment method results in the production of an inhibitory byproduct furfural (Goldstein and Easter, 1992). On the other hand, dilute acid requires high temperature with reduced acid concentrations and gives furural. The major problem to acid pretreatment is the requirement to neutralize the acid after treatment and cost of the acid (Bhatia *et al.,* 2012).

2.4.1.2 Alkaline pretreatment

Alkali pretreatment involves the use of bases, such as potassium, ammonium, sodium, and calcium hydroxide for breaking ester and glycosidic side chains causing cellulose swelling, lignin structural alteration, partial solvation of hemicellulose (Sills & Gossett, 2011; McIntosh & Vancov, 2010) and partial decrystallization of cellulose (Ibrahim *et al.,* 2011; Cheng *et al.,* 2010; McIntosh & Vancov, 2010). Mostly conditions for alkali pretreatment are less severe than any other pretreatments. This method needs ambient condition where duration of the process exceeds otherwise require higher temperature. The lime pretreatment is cost-effective among alkaline methods. Mostly NaOH and $Ca(OH)_2$ pretreatment methods are preferred. For many years sodium hydroxide pretreatment has been studied which dismantle biomass lignin structure by enhancing enzymes accessibility to hemicellulose and cellulose (Zhao *et al.,* 2008; Soto *et al.,* 1994; Macdonald *et al.,* 1983). Complete removal of lignin reduces adherence of enzymes onto

lignin and permits effective interactions with cellulose (Aswathy *et al.,* 2010). The process results in the elimination of all lignin and components of hemicellulose and enhancing cellulose reactivity in further hydrolysis steps (Hamelinck *et al.,* 2005).

2.5 Carboxymethyl Cellulose

Cellulose is insoluble in water. It needs conversion into cellulose derivatives such as carboxymethyl cellulose to broaden its applications. It has wide range of industrial applications in food industry, ceramics, paper, textile, cosmetics, adhesives, detergents, pharmaceuticals (Yang and Zhu, 2007) and is also used as a food source for lipids producing microbes. The diversity of oleaginous microorganisms varies with habitats. Filamentous fungi can use CMC as a carbon source for biodiesel production example *Gibberelle fujikuroi, Fusarium sp.*, and *Backusella ctenidi* have the strongest carbon utilization capability (Li *et al*., 2012; Li *et al*., 2011). CMC is a cellulose derivative with β-D- glucopyranose 2-O-(carboxymethyl)-monosodium salt and β-D-glucose connected through β-1,4-glycosidic bonds. It acts as an emulsifier, thickener, viscosity modifier (Ninan *et al.,* 2013) and as a food source for microorganisms. Species utilizing cellulose / derivatives as a carbon and energy sources are filamentous fungi, actinomycetes and basidiomycetes, bacteria, anaerobic, aerobic, thermophilic and mesophilic (Lednicka *et al.,* 2000; Leschine, 1995). They have extracellular cellulose degrading enzymes such as cellobiase, exoglucanase and edoglucanase which act synergistically to produce glucose (Maheshwari *et al.,* 1990).

2.6 Carbon substrates for lipids production

Microorganisms can accumulate and grow on variety of carbon sources. It is interesting to note that the composition of lipids accumulation better reflects the composition of carbon substrate. The substrate utilization efficiency determines the ability of an organism in terms of an economic lipid producer. Many fungi are screened, specifically for utilization of cheap industrial waste products. The straight chain hydrocarbons like C-10 to C-20 (n-alkanes) support the microbial growth where long-chain alkanes and short chains are poorly utilized. It has been observed single organism cannot utilize all these sources of carbon as growth substrates. The excessive growth leads to the increase in cell lipid content. Therefore, lipid content generally increases at slow growth rates. Olive waste, whey, orange peel, or wheat/rice extracts are used as carbon sources for growth and lipids production (Akpinar-Bayizit, 2014).

2.7 Lipids

Fats and oils are classed as lipids, materials of animal and vegetables origin which is found in nature. Lipids are soluble in organic solvents such as benzene, chloroform, ethers, hydrocarbons and alcohols (Voet and Voet, 2011; Gunstone *et al.*, 1994). They have various metabolic roles, for instance are responsible for maintaining structure of cell membrane, work as storage material in plants, animals, and microbial cells, and act as a barrier against environmental influences and other physical parameters e.g. cold. One of the significant physiological roles of lipids is stand-in as a precursor of hormones-like compounds example PUFAs (Akpinar-Bayizit, 2014, Sprecher, 2000). Although, human body can synthesis mono-unsaturated and saturated fatty acids from food particles but it cannot produce polyunsaturated fatty acids. In short, PUFAs are known as essential fatty acids and its requirement is fulfilled through external intake of specific food such

as fish and leafy vegetables (Suddaby,1992). About 80% of the world's fat and oil requirement is satisfied from agricultural products and rest is acquired from marine and animal sources. It is necessary to find new oil resources to reduce the negative effects of global warming and introducing biotechnology via development of microorganisms on commercial scale for fuel and chemicals production. The option of cultivating microorganisms on large scales for single cell protein production has realized that microbes can struggle on equal conditions with economical plant products given that the degree of operation is satisfactory. Hence, it can be a reason why a progression for microbial fats and oil production must not compete with non-renewable energy resources in an effort to produce cost effective and best commodity (Papanikolaou *et al.,* 2007). Traditionally, the theme of microbial lipids being used as additional sources of fats and oil has fascinated attention during the First World War because of the disruption of supplies and trade. However, none of the oil produced was used for technology or edible purposes. It has been investigated that microbial cell lipids may vary in type and in amount depending upon the conditions for microbial growth and growing stage (Akpinar-Bayizit, 2014).

2.7.1 Classification of lipids

Biological substances are classified as minerals, carbohydrates, proteins and lipids. The major difference of lipids from other natural products is their solubility in organic solvents for example benzene, chloroform, ether etc and is insoluble in water. Lipids are amphipathic tiny molecules which may be formed in part or wholly by carbocation-based condensation of isoprene units (sterols, prenols etc) or thioesters (polyketides, fatty acids etc). Categories of lipids are sterols and sterol esters, phospholipids, waxes, hydrocarbons, fatty acids, sphingolipids, etherglycerides, fat soluble vitamins (A,D,E,K) and acylglycerols (Harvey and Ferrier, 2011; Larsson *et al.,* 2006). Lipids can be divided into two broad categories; simple and complex lipids. Simple lipids

yield two forms of primary products per mole on hydrolysis; whereas complex lipids are defined as those that on hydrolysis yield three or more primary products per mole. The complex lipids are considered best in requisites either the glycolipids (holding polar carbohydrate moiety) or the glycerophospholipids (holding polar phosphorus moiety). The most important lipid classes comprised of fatty acids connected by an ester bond to the trihydric alcohol- glycerol or to other alcohols like cholesterol or linked by amide bonds to act as sphingoid bases or to other amines. Fatty acids yield huge quantities of ATP when metabolized as a rich source of energy. They have chain of even number from 4 to 28 carbon atoms. The length varies from 2 to 26 of the aliphatic chain or can be short as glycerol. Triacylglycerols (lipids) consists of three fatty acids connected to 3 carbon, their characteristics vary from translucent liquids (oil) to hard waxy solids at ambient temperature (fats) (Christie, 2003). Saturated fatty acids are without double bonds and unsaturated fatty acids have double bonds or can have more than one double bonds (Gurr *et al.,* 2008).

2.7.2 Oleaginicity

All microorganisms cannot be considered as rich sources of fats and oils, like rest of the living cells. Lipids are essential macronutrient to perform proper functioning of cell membranes and membranous structures. Those which can accumulate more than 20% of their biomass as lipids are known as “oleaginous”. Of the some 60,000 different fungal species, only 50 can accumulate more than 25% lipids (Ratledge, 2001).

2.7.3 Fungal lipids

Fungus is a heterotrophic eukaryote that digests food externally and assimilates nutrients directly via cell walls. They have microscopic tubular cells known as, hyphae and reproduce by spores.

Some fungi obtain nutrients from dead animals, or plants are called saprobes and those acquire nutrients from living host are called biotrophs. Few infect and kill host cells, these fungi are called nectrophs. Currently, fungi have been classified under life kingdom as eukaryotes. Lipids in fungi are found as key constituents in membrane systems and as storage substance in cell wall. They are being observed as lipids bodies or as extracellular products in some cases. The complexity and huge cell size is generally supported by matching diversity and multiplicity of lipid components. The types and amounts of lipid vary from organism to organism, environmental and nutritional conditions, stages of development and with progressive age (Weete and Weber, 1980). Therefore; by varying culture conditions fungal species lipid content can easily be manipulated. Hence, total lipid content records are considered to be of little value until the growth parameters are precise. Information of total lipids composition is available due to its simple analysis and preparation. The fraction of lipids from different moulds, demonstrated broad spectrum of values for both neutral and polar lipids. Triacylglycerols are storage lipids that may be used for carbon skeletons and energy during growth and development. Squalene, sterols and other hydrocarbons are considered to be the major fraction of lipids of oleaginous moulds. Study has depicted sterols exhibit liquefying or condensation effect on acyl lipids based upon lipids physical state. Polar lipids like glycolipids and phospholipids are involved in the active movement of ions across membranes (Cohen, 2011; Sancholle *et al*., 2003). The lipid components relative proportions vary according to the fungal age, conditions, and development stages. Majority of the fungal sp. contain, palmitoleic acid (C16:1) linolenic acid (C18:3), stearic acid (18:0) as minor ones with palmitic acid (C16:0), linoleic acid (C18:2) and oleic acid (C18:1) as the major acids.

2.7.4 PUFAs

Early studies have explored that algae has produced wide range of PUFAs with fungi containing linoleic acid. Recently, interest has focused on less- advanced phylogenetically fungi (*Mortierella spp., Mucor* and *Phytium*) for oil production due to long chain PUFAs in the oil.

2.7.5 Mechanism of lipids accumulation

The definition of oleaginicity in biochemistry is based on the presence of one of the chief enzymes like ATP: citrate lyase (ACL) for lipogensis. Malic enzyme has key involvement in lipid accumulation. ATP: citrate lyase supplies acetyl units for fatty acid biosynthesis where malic enzyme produces NADPH through which acetyl units are reduced and used as supporting backbone for the fatty acids. Culturing conditions of oleaginous microbes determine the amount of lipids accumulation (Wynn *et al.,* 2001). Lipid accumulates in case of excess of carbon availability to the microbial cells at stage when another nutrient is essential for cell proliferation is consumed from the medium (Ratledge, 2001). The sudden metabolic consequence in the drop of AMP concentration reduces activity of NAD+ isocitrate dehydrogenase. This activity of enzyme is completely based upon the AMP presence. As a result, via citric acid cycle the isocitrate cannot metabolise making citric acid and isocitrate to accumulate. The citrate accumulates in first stage following the nitrogen reduction from media. When citric acid accumulates it moves across the membrane of mitochondria in exchange for malate. The malate performs both as counter-ion to transport out citrate or to uptake pyruvate into the mitochondrion. The oleaginicity exists in the capacity of oleaginous microbes for citrate accumulation and also dealing with it. Hence, the key role to oleaginicity is the control of ATP: citrate lyase (Akpinar-Bayizit, 2014).

2.8 Fungal cultivation for lipids production

Lipids accumulate in fungus when growth is limited by some other substrate and C-source is available sufficiently. N-source is usually exhausted, where lipid accumulation by the limitation of other nutrients has been elucidated (Gill *et al.,* 1977). A systematic chain of biochemical events escorts to lipid accumulation in oleaginous fungi in N-source limitation. It was explained in detailed by Wynn et al in 2001. In this process key enzymes were identified (Wynn *et al.,* 2001), however it didn't moved so far to formulate high producing strains through genetic modification (Beopoulos *et al.,* 2011) Lipid accumulation has mainly been studied in oleaginous yeasts and fungus as these microorganisms produce PUFAs (Certik and Shimizu, 1999). For instance, the only commercially available process uses the fungus *Mortierella alpine* to produce arachidonic acid (C20:4) (Ratledge, 2004).Recently, fungal lipids have gained importance for biodiesel production (Feofilova *et al.,* 2010; Meng *et al.*, 2009; Li *et al*., 2008). The use of renewable energy substrates has become more vital in research, these days (Vamvakaki *et al.,* 2010; Angerbauer *et al.,* 2008; Peng and Chen., 2008; Papanikolaou *et al.,* 2007). The most studied biofuels are bio-diesel and bio-ethanol. First-generation biofuels are primarily produced from edible crops such as vegetable oil, corn, grain which brings in food competition. Second-generation uses agricultural residues or non-edible biomass as a feedstock (Walker, 2011). Solid-state and submerged fermentation are used for cultivating fungi; both culture systems are described below.

There are two methods of fermentation; Solid-state fermentation (SSF), Submerged fermentation (SmF).

2.8.1 Solid-state fermentation

In these fungal culture techniques few factors are important such as environmental parameters, substrate and species of organisms. Solid- state fermentation is a type of fermentation process in which moulds usually a filamentous fungus, can grow on solid substrates such as fragments of stems, seeds, roots, and wood. The aim of this technology is to bring cultivated fungi to absorb maximum nutrients from substrate and have tight contact with insoluble substrate. The fungal strain grows on the substrate surface which produces enzymes to disintegrate substrate to release monomers. Both the monomers as well as the enzymes are transported by diffusion in the solid particles where oxygen is taken up form air. SSF has many advantages as compared to SmF. Many fungi produce better and grow in SSF since this culture system proves a natural habitat where products can easily be recovered as dilution is non-existed, less waste water is produced and processes are usually cheaper than SmF (Bhargav *et al.,* 2008; Holker and Lenz 2005; Pandey, 2003). The advantages of using agricultural waste as energy substrate are appreciable for exhibiting rich nutrients and easily recyclable (Subramaniyam & Vimala, 2012). The pre-eminence of SSF to the SmF could be the minimal amount of fermentation medium, required space is economical, low operating costs, higher product yields, minimal contamination due to the absence of free water, utilizing agricultural waste as key substrate and complex equipment or machinery for the process is not required. Fungi needs low moisture for its growth in a medium therefore production efficiency is higher in contrast to other microorganisms (Hosseinpour *et al*., 2012). To grow fungal strains on a petri plate is also meant for providing the solid-substrate.

SSF also has shortcomings because the substrate comprised of particles, where diffusion process causes gradient to slow down the chemical reactions. Furthermore, it is hard to remove heat produces during the cultivation due to the fact that solid particles with air in-between generally

conduct heat very poorly. The SSF is mostly carried out as a batch culture. Bioreactors are available in different types, both on large and small scale. The tray reactor is the simplest of all, which consist of a layer of solid substrate in a tray. The layer is made thin to avoid overheating as active aeration is not applied. Therefore, active aeration removes heat by using aerated packed bed reactor is more common. Other mixed bioreactors such as stirred beds and rotating drums are available (Mitchell *et al.,* 2006). Bioreactors are essential fermentation systems which provide space for the microbial cultivation. The factors effecting the growth are humidity, type of substrate, temperature, aeration, height of bed, cooling rate, and fungal morphology.SSF bioreactors are broadly classified and major differentiation lies when they are used at small and large scale.

The mathematical modeling can describe physiology of fungal cells and substrate physical and chemical properties besides that formation of fungal bio-film. Following are the steps of the process for lipids accumulation using SSF;

1. Fungus produces hydrolyzing enzymes
2. Enzymes diffusion to the substrate
3. Substrate polymer is hydrolyzed to monomers
4. Diffusion of oxygen and monomers to the fungus
5. Transformation of the substrate into lipids and biomass

Water activity, heat transfer and biomass are important growth parameters that have been studied for optimum growth conditions. Water generally effects fungal physiology in solid culture medium such as radial growth rate, cellular mechanisms and fungal orientation. It has been observed that the mycelia radial extension rate is related to the water activity. Heat generation during SSF process is proportional to the metabolic activities of the strains. It effects the fungal

sporulation, metabolites and germination. Biomass is an important parameter for characterizing fungal growth and can be estimated by various techniques, like reacting biomass with specific flourochrome probes, respiration rate of microorganisms, scanning electron microscope and reflectance infrared (IR) spectroscopy (Bhargav *et al.,* 2008).

2.8.2 Submerged fermentation

Submerged fermentation (SmF) is widely used as the process handling is easy. In SmF microorganisms are cultured in a liquid medium. Control of pH, concentrations and temperature is easy as heat and mass transport are fast. Because fungi mostly require oxygen to grow and generate lipids where oxygen is transferred to the liquid medium to reach fungi. This is possible by aeration, stirring and or by shaking (at very small scale in shaking flasks). Fungal hyphae dramatically increases the viscosity, therefore it increases energy requirements for oxygen transfer and decreases mass transfer rates (Van't Riet and Tramper, 1991). It can be avoided via growing fungus in pellets; however it causes oxygen transfer troubles inside the pellets. In a batch culture system, at an earlier stage of culture all substrate is added. Whereas, in continuous culture system substrate is added continuously, broth is harvested constantly at the same rate, to keep the culture volume constant. Once this is done for assured time with equal feed rate, fungal culture reaches a stable state, where the culture ingredients do not change in time. In that situation, continuous culture system is known as chemostat. Continuous culture is not supported because of the high capital costs, where as mostly batch fermentation is preferred for lipids accumulation.

A batch culture comprised of the three following phases:

i. Exponential growth phase

During the exponential growth phase, N-source and C-source are consumed and basic biomass along with a basal fraction of functional lipids is formed. As long as the C- substrate and N-source are available, the fungus produces functional lipids like for membranes and grows well.

ii. Lipids accumulation phase

Once the N-source is exhausted, the fungal cells stop the further growth and successively use the C-source for structure, maintenance, lipids and carbohydrates produces unless the C-source is exhausted. The path of lipids accumulation in moulds is a growth linked process. The growth rate increases then the lipid accumulation rate increases. The literature has described for acquiring maximum lipid production is not based upon the optimum growth conditions. The rate of its synthesis in reference to the other cellular products synthesis rectifies if a lipid accumulates (Anderson & Wynn, 2001).

iii. Lipid turnover phase

Once the C-source is depleted in the medium, a fungal cell utilizes the accumulated lipids for maintenance.

A statistical model can assist to structure this date and as a tool can be used to improve the lipids production. The organisms use oxygen (O), N-source (N) and C-source (S) to produce water (W), carbondioxide (C), Carbohydrates (IP), lipids (L) and cell material(X).

Chapter 3

MATERIALS AND METHODS

3.1 Washing and Sterilization of Glassware

All the glassware used in laboratory experiments was washed with tap water and then was rinsed with distilled water. After washing, glassware went through a sterilization process and was autoclaved at 121 ℃, 15 per square inch pressure for 15 min.

3.2Preparation of agar plates

3.2.1 Potato Dextrose Agar (PDA) plates

The solid media was prepared according to the manufacturer's instructions. Potato Dextrose Agar (Agar 15 g/L, dextrose 20 g/L, potato extract 4.0 g/L, Merck) of 39 g was dissolved in 1 L of de-ionized water, until powder was mixed completely. Media was autoclaved at 121 ℃, 15 psi pressure for 15 min and was poured into agar plates. Plates were made in sterilize conditions in a bio-safety cabinet. After the solidification, plates were flipped, labeled and stored in a refrigerator at 4 ℃. These plates were used for inoculums preparation and radial growth measurement.

3.2.2 Sabouraud Dextrose Agar (SDA) plates

The solid media was prepared according to the manufacturer's instructions. To prepare a Sabouraud Dextrose Agar (mycological peptone 10 g/L, glucose 40 g/L, agar 15 g/L, Oxide),

65 g was dissolved in 1 L of de-ionized water, until powder was mixed completely. Media was autoclaved at 121 ℃, 15 psi pressure for 15 min and was poured into agar plates. Plates were made in sterilized conditions of bio-safety cabinet. After the solidification, plates were flipped, labeled and placed in an incubator for 24 hours sterility test. Plates were then stored in a plastic

bag and placed in a refrigerator at 4℃. These plates were used for inoculums preparations and radial growth measurement.

3.2.3 Carbon substrate plates

Fungal growth media was prepared by adding nutrients including NH_4Cl 5 g, KH_2PO_4 2 g, $MgSO_4.7H_2O$ 0.75 g, $CaCl_2.2H_2O$ 0.05 g, $ZnSO_4.7H_2O$ 0.01 g, $FeCl_3.6H_2O$ 0.01 g, and Na_2HPO_4 1 g, in 1000 mL of distilled water as specified in Table (Li *et al.,* 2011). Solution was properly stirred to dissolve all salts absolutely. Mineral solution was further used to dissolve CGWS CMC/ CMC-A/ glucose as a carbon source. Different pH ranges i.e. 5.5, 6.0, 6.5, 7.0, & 7.5 were tested to achieve maximum growth of fungal strain. The pH was adjusted using 0.1 N sodium hydroxide (NaOH) /0.1 N Acetic Acid (CH_3COOH). Prepared media was sterilized in an autoclave for 15 minutes at 121 ℃. After the solidification of agar, plates were flipped, labeled and stored at 4 ℃ in a refrigerator.

Table 3.1 Composition of growth media

1 L media	
10g	CMC-A/CMC
5g	NH_4Cl
2g	KH_2PO_4
0.75g	$MgSO_4.7H_2O$
0.05g	$CaCl_2.2H_2O$
0.01g	$ZnSO_4.7H_2O$
0.01g	$FeCl_3.6H_2O$
1g	Na_2HPO_4
15g	Agar

Abbreviations:

CMC-A – Carboxymethyl cellulose- Analytical, CMC- Carboxymethyl cellulose

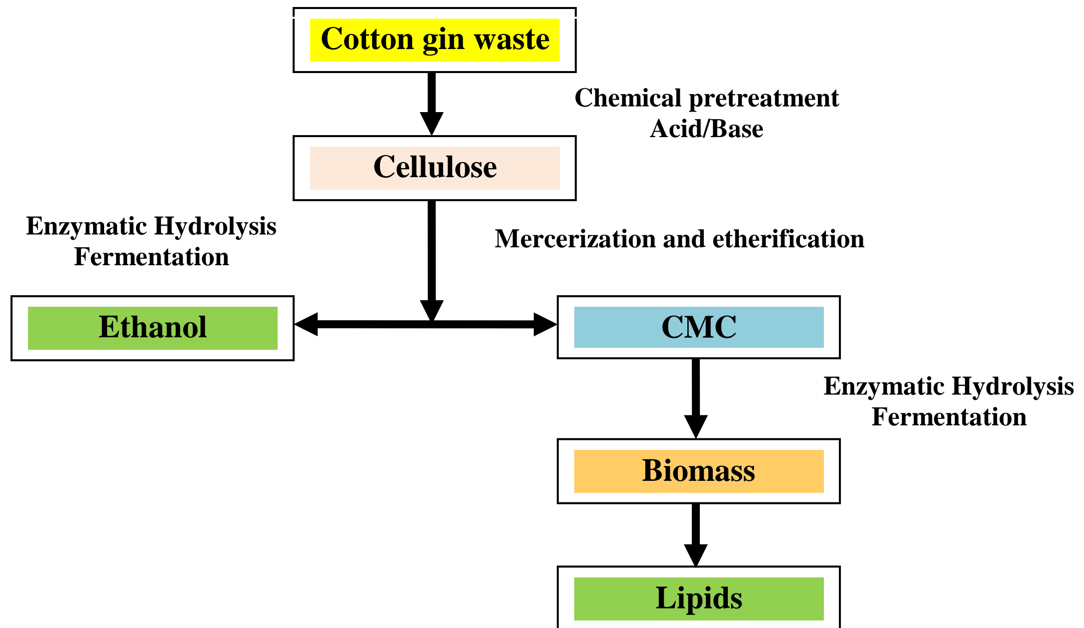

Fig 3.1 Experimental design

3.3 Quantification of agricultural residues

In 2009, a joint baseline report of Mehran University of Engineering & Technology (MUET) and United Nations Environmental Programme (UNEP) was instigated by Mahar on converting waste agricultural biomass into energy source. This report has covered various aspects including assessment of current waste management system, quantification and characterization of waste agricultural biomass in District Sanghar of Pakistan. Questionnaires were designed to evaluate total quantity of field and process agricultural residues including cotton stalks and gin waste. The data was published in International Journal of Biomass & Renewables (Mehar *et al.,* 2012).

Using the following formula quantity of agricultural waste can easily be estimated;

QPY= CY x AAC x QPY x CRR

Where, QPY= Quantity of WAB per year (tons), AAC= Annual Area Cultivated (Hec),

CY= Crop Yield (Kg/Hec), CRR= Crop to Residue Ratio

3.4 Pretreatment of cotton ginning waste

3.4.1 Cellulose Isolation

Cellulose was isolated from cotton gin waste through acid and alkali pretreatment process to remove lignin, hemicelluloses, pectin and waxes as shown in Figure 3.2 (Haleem *et al*., 2014). Cotton gin waste was chemically pretreated with dilute sulfuric acid at 80 ^{o}C for 1 hr. Sample was rinsed and it was further pretreated with sodium hydroxide for 2 hours at 80 ^{o}C by constant stirring. Alkali treated sample was washed several times with water to remove alkali. After then, sample was treated with hydrogen peroxide to remove chemicals and was rinsed properly with water. Cellulose structure was depolymerized by treating sample with 10 % sulfuric acid for 1 hour. Solution was left for decanting process and pH of slurry was adjusted (7.5). The filtered solution was oven dry at 70 ^{o}C temperature. Dried powder (cellulose) was stored in a plastic bag.

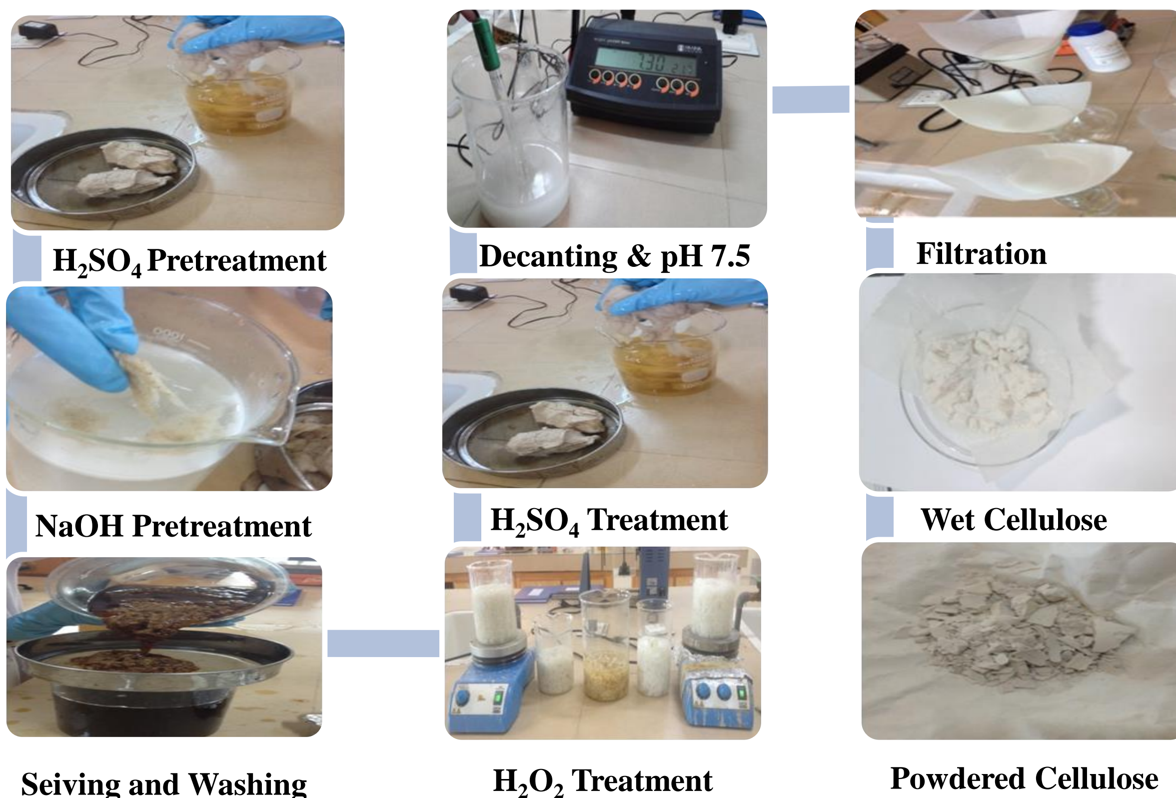

Fig 3.2 Chemical pretreatment for cellulose isolation

3.4.2 Synthesis of CMC

3.4.2.1 Alkalization

In this step, powdered cellulose was weighed and added into isopropyl alcohol. Sodium hydroxide solution was added drop wise in first 20 minutes in CGW cellulose isopropyl alcohol Slurry and stirred for 3 hours. This cellulose-sodium hydroxide activation reaction is also termed as mercerization. Mixture was prepared for the process of etherification.

3.4.2.2 Etherification

Sodium monochloroacetic acid was added into distilled water to start carboxymethylation reaction. Solution was added drop wise in first 20 minutes and stirred constantly for 3 hours at 80 °C temperature. Mixture was filtered and residue was dipped into methanol overnight. The pH was adjusted through dilute acetic acid. Mixture was filtered again and rinsed with ethanol thrice. The residue was oven dried at 80 °C for 3 hours and CMC powder was obtained (Haleem *et al.*, 2014).

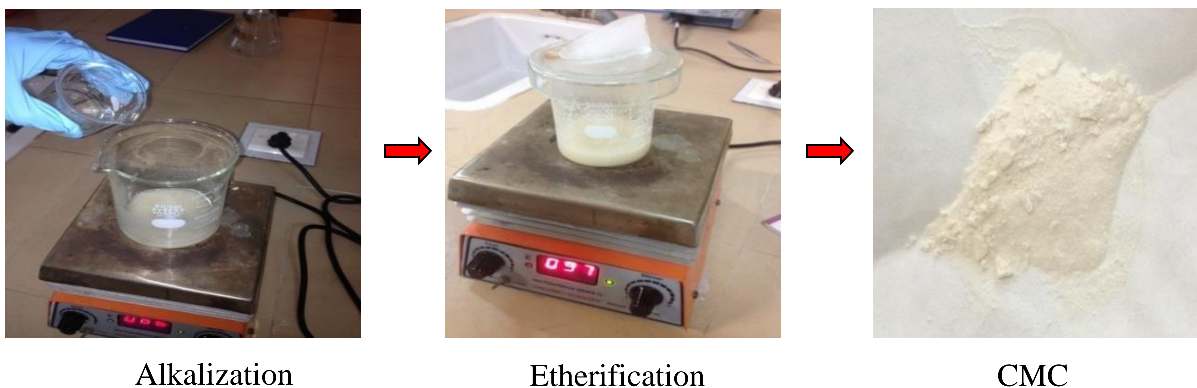

Fig 3.3 Etherification process

3.5 CMC chemical identification test (analytical and local)

Solutions of CMC-A and CGWs CMC in distilled water were made separately for its detection test. Both Solutions were autoclaved at 121 ^{o} C temperature and 15 psi for 15 minutes. CMC detection test was performed after cooling the solutions.

1 gram of powdered CMC was added into 50 ml of distilled water and stirred vigorously to mix it well. Clear solution was produced. Solutions were used for Test A and Test B (United States Pharmacopoeal Convention, 2014).

Test A: 1mL of solution was diluted with water. 5 drops of napthol test solution (TS) was added and by keeping the test tube inclined 2mL of sulfuric acid was carefully introduced. Layer was formed and at interface red purple color was developed.

Test B: 5mL of Barium Chloride test solution (TS) was added in an equal volume of autoclaved CMC solution. Fine and white precipitates were formed.

CMC was present in the prepared solution which clearly indicated CMC is not degradable after autoclave as shown in Figure 3.3

Fig 3.3 CMC chemical identification Test A and Test B (standard and local)

3.6 Molecularly identified fungal strains

All the strains were provided by the Mycoviruses Science Laboratory, Atta-ur-Rahman School of Applied Biosciences (NUST). The former study was conducted by (Mushtaq, 2014) for

identification of 50 environmental fungal isolates using light microscope and strains were further confirmed through molecular identification via amplification of internal transcribed spacer (ITS). In total, four fungal strains originated from ascomycota and basidiomycota phylums were used to evaluate their potential for CMC consumption derived from lignocelluloses CGW. Strains A*lternaria alternatae, Asperigillas flavous, Alternaria arborescensce* & *Fusarium oxysporum* were maintained on SDA plates at 4 ºC.

3.6.1 Pre-cultured fungal strains

Fungal strains were pre-cultured on SDA. 20 µL *Alternaria spp.* cultures were point inoculated using micropipette. Strains which do not produce enough spores, fungal disks of 5 mm in diameter were collected from the solid medium for inoculation. Therefore, Plug in technique was used for *Fusarium oxysporum.*

3.7 Radial growth measurement

Point inoculation technique was applied to check fungal growth on petri plates (9 cm diameter) through radial measurement. Petri plates were incubated at 28 ^{o}C temperature for optimum fungal growth. Two lines were drawn perpendicular to each other on petri plate. Fungal growth rate was recorded in millimeters at the gap of 24 hours until its complete expanded growth on petri plate. Circles were marked as the growth stretched outwards each day. *Fusarium oxysporum* growth rate was recorded for six days where 1 % of CGWs CMC was used as a carbon source. Similarly, measurements were taken for 1 % CMC-A, SDA, and PDA for six consecutive days.

3.8 Preparation of fermentation media

Fermentation media was prepared by adding minerals including yeast extract 1 g, NH_4Cl 5 g, KH_2PO_4 2 g, $MgSO_4.7H_2O$ 0.75 g, $CaCl_2.2H_2O$ 0.05 g, $ZnSO_4.7H_2O$ 0.01 g, $FeCl_3.6H_2O$ 0.01 g, & Na_2HPO_4 1 g in 1000 mL of de-ionized water. CGW CMC as a carbon source for fungal growth was added in mineral media (Li *et al.,* 2011). Flasks (250 mL) were poured with 100 mL of fermentation media each contained 1% of CGW CMC. Similarly, CMC-A, and glucose were used to determine carbon source efficiency in comparison. All experiments were carried out in triplicates.

3.9 Determination of biomass and lipids

3.9.1 Biomass

Fusarium oxysporum was pre-cultured on sabouraud dextrose agar for seven days. Fungus was inoculated into fermentation media to determine its bio-mass. Conditions for growth were optimized by keeping incubation temperature at 28 ^oC and shaking at 150 rpm for six days. Oleaginous *Fusarium oxysporum* mycelial growth was observed and poured into 50 mL of falcon tubes. Tubes were centrifuged at 4000 rpm for 20 minutes. Pellet was obtained and supernatant was removed. Pellet was rinsed with sterilized distilled water and centrifuged again. Fungal biomass was determined through filtration using whattman filter paper no 42. Wet biomass was oven dried at 80 ^oC for 24 hours and dry mass was weighed (Li *et al.,* 2011).

Table 3.2 Experimental conditions for growth on orbital shaker

Orbital Shaker	Specifications
Speed	150-200 rpm
Temperature	28 ºC
Sample collection	6 Days
Size of inoculums	5 mm Diameter
Total volumetric load	50 and 100 mL

3.9.2 Lipids

Lipids were determined through Bligh and Dyer, 1959 lipids extraction protocol. The biomass was harvested from 100 mL of liquid media by centrifugation at 4000 rpm for 10 minutes. The extracted biomass was kept for 30 minutes at room temperature after adding 15 mL of 4 M HCl solution. The freezing and thawing process was made for cells to break via dipping into liquid nitrogen and boiling water for 10 minutes respectively. Methanol/chloroform (1:1) was shacked vigorously using vortex oscillator and centrifuged for 10 minutes at 4000 rpm. The chloroform layer containing lipid was dried and weighed to obtain the lipids content.

Chapter 4

RESULTS AND DISCUSSION

The CMC was synthesized from chemically pretreated cotton gin waste as a precursor for the production of lipids. The factors affecting fungal biomass were analyzed in a batch mode and results are discussed in the following text.

4.1 Synthesis of CMC and characterization

The chemical pretreatment was a way helpful in cellulose isolation. The major advantage of the alkali/acid pretreatment was the isolation of pure cellulose from cotton gin waste. The etherification process was sustained for CMC synthesis. The chemical testing of analytical grade and CGW-CMC revealed a comparison between the solutions. The results were quite positive, screening out the red purple color at the interface and formation of white precipitates at the base. It was necessary to use carbon substrate free from impurities and toxic elements for lipids accumulation and production of fungal biomass. Once the energy source was isolated from agricultural waste it was subjected to Energy dispersive spectroscopy analysis. The EDS analysis of the samples represented the C, Na, O and Cl % composition as represented in Table 4.1.

Table 4.1 Energy dispersive spectroscopy showing elemental analysis of CMC

	C %	O%	Na%	Cl %
CMC-A	37.62	51.78	9.99	0.61
CGW-CMC	35.67	43.58	16.31	4.45
Percentage Difference	1.95	8.2	6.32	3.84

CMC-A-Carboxymethyl cellulose-Analytical; CGW-CMC-Cotton ginning waste-carboxymethyl cellulose

The overall composition of both samples was comparable. The difference was observed in Na and Cl % due to improper washing at the time of pretreatment process. The % difference of the contents couldn't affect the growth of *Fusarium oxysporum*.

4.2 Product cost

According to cost-benefit analysis, an estimated production cost of locally isolated CMC was Rs.700/kg weight against Rs.6000/kg CMC-A. The utilization of cotton gin waste may solve the problem of environmental air pollution, and disposal plan (Mahar *et al.,* 2012). Most importantly the analytical grade was a rare chemical and economically not feasible for energy sector to afford bio-fuel.

Table 4.2. Fungal strains growth response on CMC

Sources	**Strains**	**Locally isolated CMC**	**CMC-A**
Maize associated	*Alternaria arborescens*	-	-
Sesame associated	*Altenaria alternatae*	-	-
Open air	*Asperigillus flavous*	-	-
Sesame associated	*Fusarium oxysporum*	+	+

Negative: (-) symbol represented fungal strains which couldn't grow on locally as well CMC analytical grade.

Positive: (+) sign represented *Fusarium oxysporum* had utilized carboxymethyl cellulose as a carbon substrate.

To the best of our knowledge this study has been reported for the first time using locally isolated CMC from CGW for lipids production by fungal strains. In current study 3 out of 4 fungal strains such as *Alternaria arborescens, Altenaria alternatae,* and *Asperigillus flavous* could not utilize the CMC carbon substrate as shown in Table 4.2. Their sources of isolation and habitat were maize, sesame seeds and open air. Mirza and Qureshi. (1978) had reported *Fusarium oxysporum*

from Pakistan for the first time on sesame seed. There were multiple factors for not showing positive response towards energy source. The cellulolytic genes expression, different sources of fungal isolation and regulation of the bio-chemical mechanisms were quite multifaceted to understand the factors hindering fungal growth on carboxymethyl cellulose solid substrate. These strains due to the instinct genomic variations and changed habitat had shortened the utilization of complex carbon substrate at neutral pH 6-7.

Their characteristics, behavior towards energy source and enzymes production might have been suppressed or lacking the instinct quality as species were incapable owing to their genetic makeup or the ecological/ growth parameters were not identical to exhibit extraordinary growth proficiency. On the other hand, oleaginous *Fusarium oxysporum* had confirmed the positive growth response towards cellulose derivative (El-Haj *et al.,* 2015; Li *et al.,* 2012). During experiments chemical composition and utilization strength was alike for oleaginous *Fusarium oxysporrum* to grow on a solid substrate by spreading the mycelium. The color of hyphe changed from colorless to pale yellow. (Lednicka *et al.,* 2000; Leschine, 1995) studied the filamentous fungi, actinomycetes and basidiomycetes mostly utilize cellulose/derivatives as carbon and energy sources. In another study *Asperigillus flavus* and *Fusarium oxysporum* were studied extensively in their potential to produce extracellular enzymes namely exoglucanases, cellobiase, and endoglucanases that act synergistically for converting cellulose into monomers (Li *et al*., 2012; Li *et al*., 2011; Maheshwari *et al.,* 1990). Filamentous *Fusarium oxysporum* includes variety of filamentous plant pathogenic strains causing wilt disease of a broad range of ornamental and agricultural host plant species. It produces enzymes that act upon cellulose components of cell walls (El-Haj *et al*., 2015) also reported for showing positive growth response towards CMC-A, cellulose derivative.

4.3 Linear regression growth

A linear regression growth equation was used to study the fungal growth system in a simplest form on solid substrate medium. Different pH ranges i.e. 5.5, 6.0, 6.5, 7.0, & 7.5 were tested to achieve maximum growth of fungal strain on CMC-A by keeping temperature at 28 ^{o}C and other growth parameters invariable. There were several growth parameters which had to be optimized like moisture, temperature, media composition, inoculum size, C/N ratio of nutrients etc. But the fermentation system was restricted to few parameters as ascribed to the complications in biomass production, lipids and ethanol yield. According to the linear regression maximum growth rate 6.6 was observed at pH 6.5 represented in Table 4.3.

Table 4.3 Linear growth for CMC-A at different pH

pH	Growth rate (mm/hr)	R^2	Kr
pH5.5	0.287	0.997	5.8
pH 6.0	0.286	0.995	5.9
pH 6.5	0.298	0.996	6.6
pH 7.0	0.283	0.994	5.9
pH 7.5	0.291	0.991	6.4`

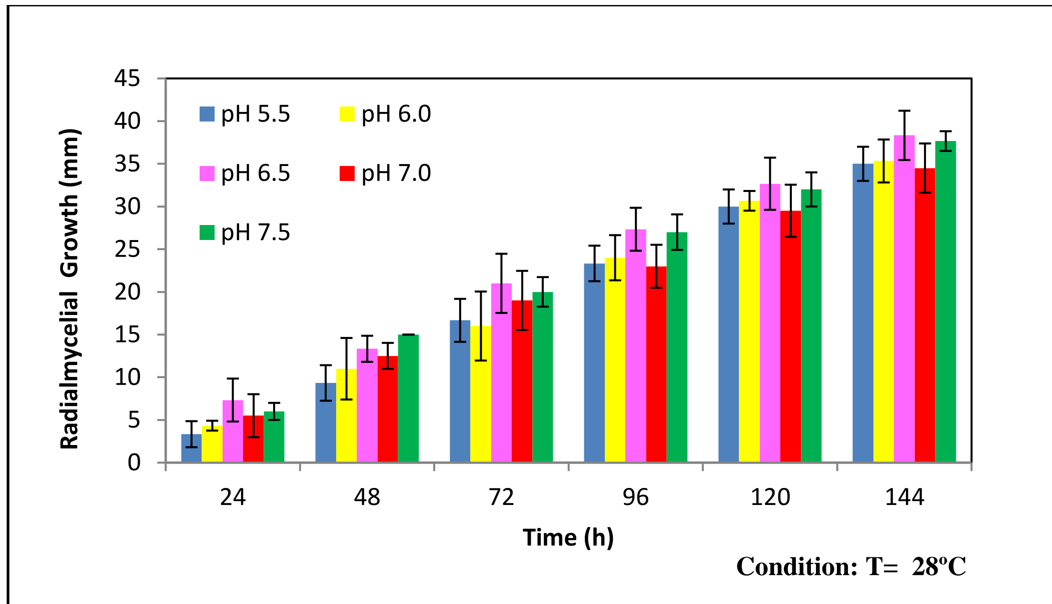

Fig 4.1 Effect of pH on mean radial growth of *fusarium oxysporum* on CMC-A

When growth was assessed using CMC-A, it gradually increases over time until the complete expansion on a petri plate of 9 cm diameter. Results related to growth are presented in Figure 4.1. As compared to growth at pH 5.5, there was % increase of 11.9 (in 24 h), 42 (in 48 h), 26 (in 72 h), 17 (in 96 h), 8 (in 120 h) and 9 % (in 144 h) in growth radii at pH 6.5. The % increase in radii growth at pH 6.5 was 22 in first 24 hours, 5 in 72 h, 1 in 96 h, 2 in 120 h and 1% in 144 h as compared to growth at pH 7.5. This % growth measurement explained the differences between the pH ranges for six days. *Fusarium oxysporum* growth at pH 7.5 was higher than pH 7.0, where the mycelia extension was observed with an increase of 9, 20, 5, 17, 8 and 9% for six consecutive days. Weigh against pH 5.5, 6.0, 7.0 and 7.5; there was a % increase of 3, 4, 5 and 1% in radii on sixth day at pH 6.5 respectively. The comparison was made between maximum and minimum % increase in growth values. In Figure 4.1, the difference in growth % was measured in 24h. In orientation to the highest growth at pH 6.5, there was % decrease of 26 at pH 5.5 and 16 % at pH 6.0. In 48 hours, the growth in acidic environment was decreased to 19%

as compared to the alkaline environment. In 72 hour, the % growth at pH 6.0 was reduced to 8% in contrast to radii growth at pH 6.5. In 120 hr, the growth was observed in a similar trend with reduced growth at pH 7.0. The general pattern had indicated that overall % growth was increased from day 1 till day 6th at 5.5, 6.0, 6.5, 7.0, and 7.5.

The radial growth was further analyzed using statistical tool ANOVA-one way analysis for the pH values 5.5-7.5 on solid substrate medium. The results exhibited p-value > 0.05 which has accepted null hypothesis. There was no significant effect of the pH factor on growth but with progressive time growth increased from 5 to 40 mm in radii. The petri plate of (9 cm diameter) was used for the growth experiment. The parameter had little variation in comparison to the pH ranges. This research finding has concluded that using CMC-A as a growth media the pH value if fluctuates between 5.5 to 7.5 the oleaginous strain *Fusarium oxysporum* may grow with full potential. The pH factor with given nutrients in medium such as NH_4Cl 5 g, KH_2PO_4 2 g, $MgSO_4.7H_2O$ 0.75 g, $CaCl_2.2H_2O$ 0.05 g, $ZnSO_4.7H_2O$ 0.01 g, $FeCl_3.6H_2O$ 0.01 g, and Na_2HPO_4 1 g may support optimum growth with three units relaxation at pH 5.5, 6.5 and 7.5. The reason behind considering pH factor was to find a compromising limit to produce ethanol and maximum biomass for lipids extraction. The cellulase enzymes are usually produced at pH 5.5 therefore it was required to generate biomass at lower values for generalizing industrial application.

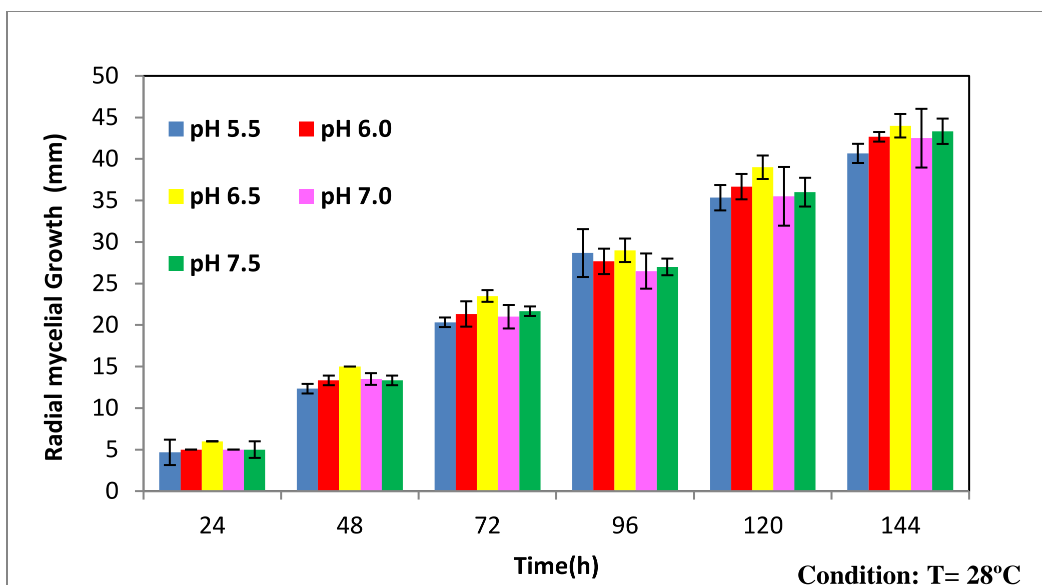

Fig 4.2 Effect of pH on mean radial growth of *Fusarium oxysporum* on locally isolated CMC

Different pH ranges i.e. 5.5, 6.0, 6.5, 7.0, & 7.5 were tested to achieve maximum growth of fungal strain on locally isolated CMC by keeping temperature at 28 ºC. Growth conditions for strain were optimized and maximum growth was observed at pH 6.5 as shown in Fig 4.2. When fungal growth was evaluated using CGW-CMC it was increased gradually over time until the complete expansion. Results related to the growth are presented in Figure 4.2 which is similar to the Figure 4.4. The CGW carbon source was utilized by oleaginous *Fusarium oxysporum.* As compared to the growth at pH 5.5, there was an increase of 8% in growth radii on 6th day at pH 6.5. The % increase in radii growth at pH 6.5 was 20 in 24 h, 12 in 72 h, 8 in 96 h, 8 in 120 h and 1% in 144h as compared to growth at pH 7.5. This % growth measurement explained the differences between pH ranges for six days. The general pattern had indicated that overall % growth was increased from day 1 till day 6th at 5.5, 6.0, 6.5, 7.0, and 7.5. According to linear regression maximum growth rate of strain 7.48 was observed at pH 6.5 as shown in Table 4.4.

Table 4.4 Linear growth for CGW-CMC at different pH

pH	R^2	Kr
pH5.5	0.989	6.87
pH 6.0	0.993	7.09
pH 6.5	0.992	7.48
pH 7.0	0.992	6.96
pH 7.5	0.992	7.08

The radial growth was further analyzed using statistical tool ANOVA-one way analysis for the pH values 5.5-7.5 on solid substrate medium. The results exhibited p-value > 0.05 which states null hypothesis has been accepted. There was no significant effect of pH factor on growth but with progressive time growth increased from 5 to 40 mm in radii.

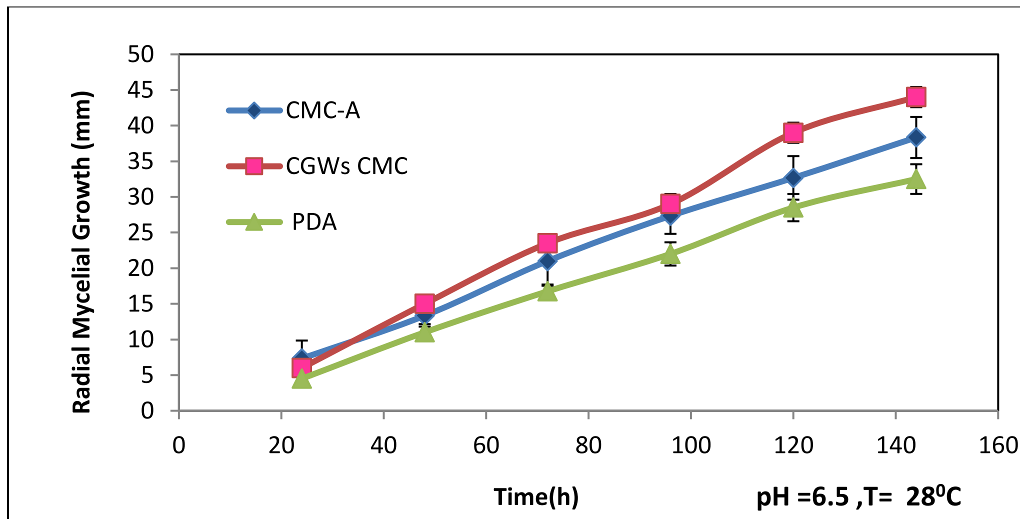

Fig 4.3 Comparison of mean radial growth of *Fusarium oxysporum* on different carbon sources

Three carbon sources PDA, CMC-A and CGW CMC were used to study fungal growth and strength of carbon substrate. Conditions remained same with pH 6.5 and temperature 28 ^{o}C. Figure 4.3 represents growth on PDA was less as compared to CMC. On day 1, growth on CGW CMC was 5 mm and on day 6th growth was eventually more than CMC-A (analytical grade).

CGW-CMC proved to be a rich source of carbon and energy for fungal growth and may compete with CMC-A. Linear regression on the bases of mathematical derivation represented the maximum growth rate 7.48 on CGW-CMC substrate for six days of incubation as shown in Table 4.5. The % increase in radii growth on CGW-CMC carbon source at pH 6.5, there was an increase of 35 and 14 % as compared to the radial growth on PDA and CMC-A on 6th day respectively.

Table 4.5 Linear growth for different media

Media	R^2	Kr
CGW-CMC	0.992	7.48
CMC-A	0.993	6.43
PDA	0.995	5.51

Abbreviation: CGW-CMC-Cotton ginning waste-carboxymethyl cellulose; CMC-A-Carboxymethyl cellulose-Analytical; PDA-Potato dextrose agar

The utilization of cotton gin waste could facilitate in filling the increasing demand for bio energy feedstock, therefore boosting its value via providing extra profits to cotton growers and cotton gins as well as offer a means of waste disposal produced during a ginning process.

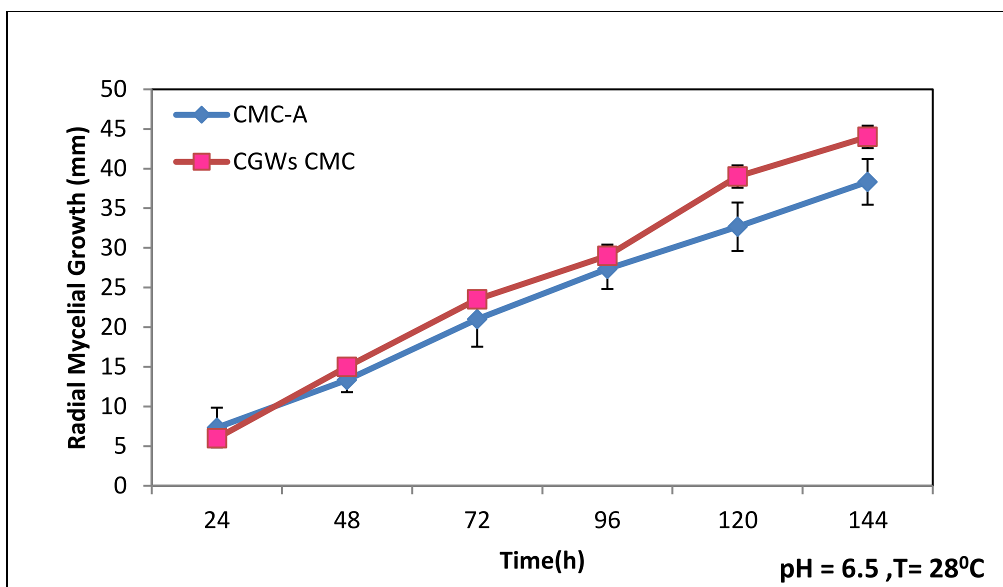

Fig 4.4 Efficiency of locally prepared CMC from cotton ginning waste as compared to analytical grade CMC

Figure 4.4, represents that the fungal growth rate at pH 6.5 and temperature 28 ^{o}C was quite linear to each other. CMC locally prepared from cotton gin waste has a potential to compete with analytical grade CMC. Toxic substances might have been produced but it couldn't affect the growth rate in locally isolated CMC. CMC degradation was assessed on 6th day by selected fungal strain *Fusarium oxysporum* until the complete expanded growth on a petri plate (9 cm diameter). The enzymatic activity of strain on CGW-CMC substrate was slightly higher on day 5th and day 6th. The % increase in radii growth rate using CGW-CMC was 12 in 48 h, 12 in 72 h, 11 in 96 h, 6 in 120 h and 19 % in 144 h as compared to the growth on CMC-A**.** According to linear regression growth rate in CGW-CMC medium was 7.48 and in CMC-A 6.58 as represented in Table 4.6.

Table 4.6 Comparison of growth rate on CMC

Media	R^2	Kr
CGW-CMC	0.992	7.48
CMC-A	0.993	6.58

Abbreviation: CMC-A-Carboxymethyl cellulose; CGW-CMC-Cotton ginning waste

The growth was further analyzed using statistical tool t-test assuming equal variances for two different mediums. The results inferred p-value > 0.05 with an acceptance of null hypothesis. There was no significant effect on growth either using locally isolated CMC from cotton gin waste or analytical grade. The locally isolated CMC may compete with an analytical grade.

Table 4.7 *Fusariun oxysporum* growth response on carbon sources

Strain	Carbon source	Growth Response	Colony Diameter (mm)	Surface	Color
Fusarium oxysporum	**PDA**	(+++)	66	Velvety	Oranges white to creamy
	SDA	(+++)	70		Orange to white
	CCGW-SA	(+)	---		White
	CMC-SA	(+)	80		
	CGW-CMC-SA	(+)	85		

Conditions: Incubation temperature 28 ºC; Incubation period 6 Days; pH 6.5; PDA Potato dextrose agar;SDA-Sabourade dextrose agar; CCGW-SA-cellulose cotton ginning waste-substrate agar; CMC-SA-Carboxymethyl cellulose-substrate agar; CGW-CMC-SA-Cotton ginning waste- carboxymethyl cellulose-substrate agar; Less Luxuriant (+), Luxuriant (++), More Luxuriant (+++), Most Luxuriant (++++)

4.4 Effects of media composition

Table 4.7 shows growth response of *Fusarium oxysporum* on different carbon sources. After incubation period of 6 days at 28 ℃, growth response on isolated cellulose, CGW-CMC, and CMC-A grade remained less luxuriant. Colony diameter on the other hand figured out maximum colony diameter at 85 mm as compared to potato-dextrose and glucose-mineral substrates. More luxuriant growth was observed on PDA and SDA. General morphology of strain in response to energy sources and substrate composition represented velvety surface and color altered from orange to white. In this study, *Fusarium oxysporum* growth on PDA was also measured after 8 days of incubation period with 85 mm diameter. The same colony diameter was reported by (Selvi and Sivakumar, 2013). On 6th day, growth was 66 mm. The color of the hyphe changed from white cotton to pale yellow which turned into purple or pink with maturation. At pH 6.5, temperature 28 ℃ and the carbon utilization of different energy resources by oleaginous strain manifested the % increase in colony diameter. The maximum growth was observed in (CGW-CMC-SA) with 85 mm colony diameter and in CMC-SA with 80 mm. The growth on CGW-CMC-SA was calculated with an increase of 6, 21 and 28 % in colony diameter from CMC-SA, SDA and PDA respectively. The minimum growth was observed in PDA with (66 mm) and in SDA with (70 mm). Colonies on cotton waste was also observed since growth was cloudy and haziness couldn't craft the plate clear for measurement. The carbon source with more positive sign depicted the luxuriant growth. It was implied that glucose rich energy source was consumed actively.

This study was carried out on the bases of radial extension and energy source utilization efficiency. This research has pointed up an argument that growth via radii measurement solely cannot determine the growth density. Thickness in fermentation media and growth mycelia

extension on solid media were independent factors to study the growth rate. This has supported the major difference between the selections of solid or liquid based fermentation media for fungal cultivation. Solid state fermentation media has two dimensional growth modeling system therefore cannot determine the multiple factors under discussion. Ramanathan *et al.* (2010) reported, studied and analyzed the enzymatic activity of *Fusarium oxysporum* on the bases of clearing zone diameter. The readings were noted on alternative days as 12, 24, 32 and 36 mm. It was calculated as follows; β-glucosidase 1.78U/ml and FPase 1.34 U/ml and CMase 1.92 U/ml.

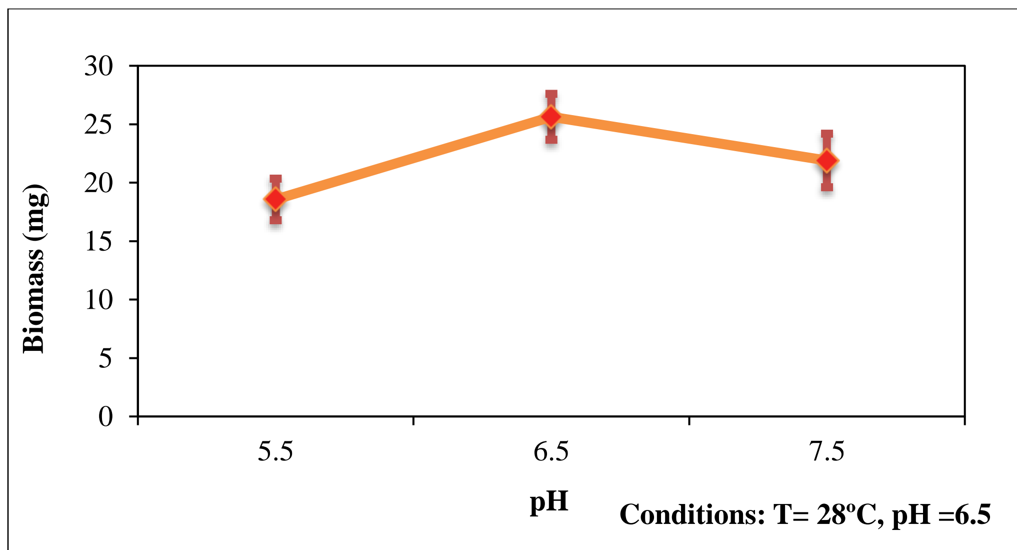

Fig 4.5 Production of biomass using CMC-A grade

The maximum biomass with an average value of 25 mg was observed at pH 6.5, and temperature 28 ºC with a shaking period of 150 rpm in 100 mL of submerged fermentation media as shown in Fig 4.5. Net decrease in biomass below pH 6 and above 7 revealed that the oleaginous strain performed better in neutral and slightly acidic pH conditions. As compared to the growth at pH 5.5, there was an increase of 38 % in biomass production at pH 6.5. The % increase in biomass at pH 6.5 was 17 % as compared to growth at pH 7.5. This % biomass growth measurement

explained the differences between the production values after six days of incubation. The overall % growth at pH 6.5 was higher supporting the possible conditions for lipids production.

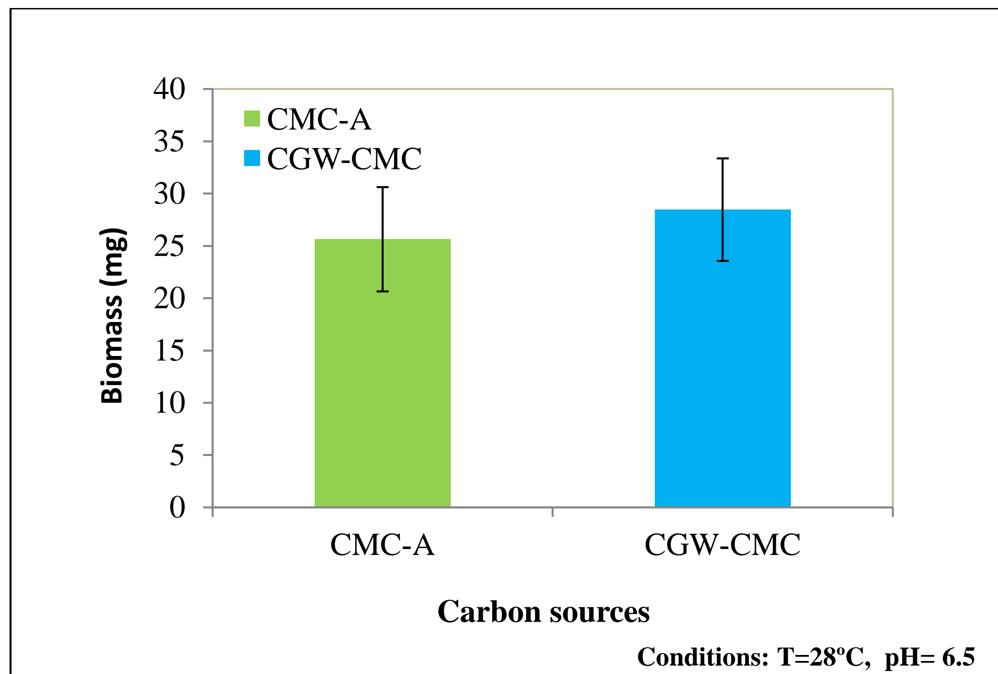

Fig 4.6 Comparison of CMC-A and CGW-CMC carbon utilization efficiency

Fig 4.6 represents that locally isolated CMC can compete with an analytical grade. Three factors of media such as composition of nutrients NH_4Cl 5 g, KH_2PO_4 2 g, $MgSO_4.7H_2O$ 0.75 g, $CaCl_2.2H_2O$ 0.05 g, $ZnSO_4.7H_2O$ 0.01 g, $FeCl_3.6H_2O$ 0.01 g, Na_2HPO_4 1 g, yeast extract (Organic nitrogen source), pH 6.5, and temperature 28 °C were made similar except the addition of carbon sources. The growth was further analyzed using statistical tool t-test assuming equal variances for two different mediums. The results displayed p-value > 0.05 with an acceptance of null hypothesis. There was no significant effect on growth either using locally isolated CMC from cotton gin waste or analytical grade. The biomass of 25 and 30 mg was produced with an increase of 5 % in fungal growth using CGW-CMC as compared to CMC-A. Therefore, advantage by utilizing agricultural waste as an energy source was appreciable for representing rich nutrients and easily recyclable constituents (Subramaniyam & Vimala, 2012). The most

concerning point of present research was to utilize cotton waste in production of fungal biomass and lipids extraction for bio-fuel.

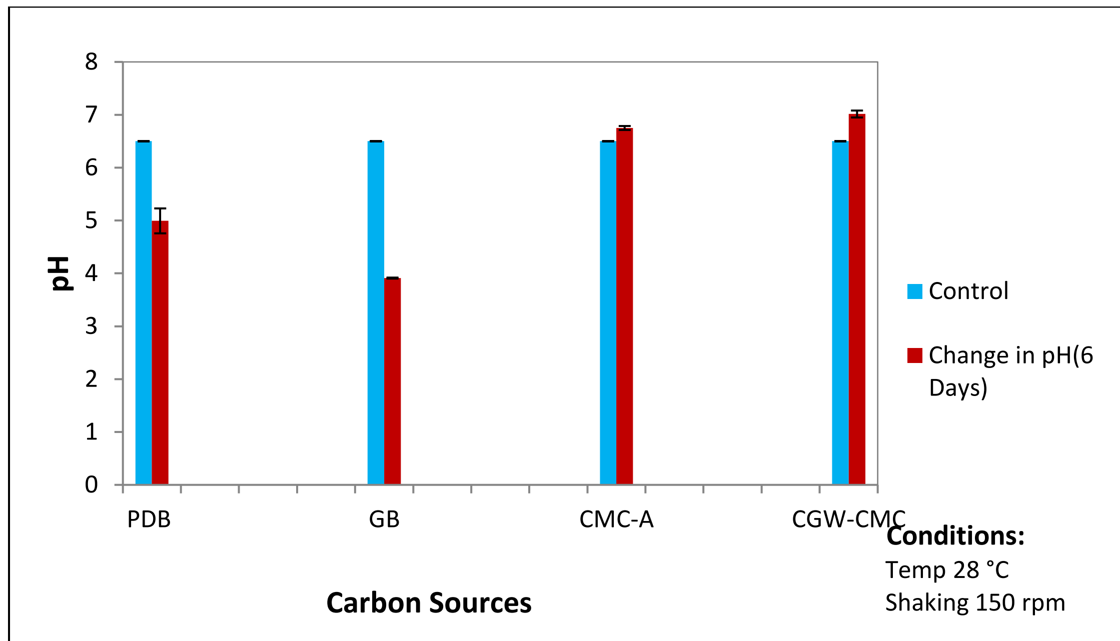

Fig 4.7 Comparison of carbon utilization efficiency at pH 6.5 on carbon sources

Fig 4.7 represents that the pH changes in the growth medium when the fungal growth continued over a period of time. Error bars based on standard deviation showed statistical difference between solution pH of control and *Fusarium oxysporum.* The pH of the potato-dextrose and glucose liquid media containing fungal strain was decreased from 6.55 to 4.99 and 3.99 respectively. On the other hand, the results construed that the pH was increased from 6.5 to 6.75 and 7 neutral pH in CMC-A and CGW-CMC fermentation media respectively. The reduction in enzymatic yield had reduced the biomass production and slight change in pH was observed towards alkaline medium. *Srivastava et al.* (2011) reported that the pH of fermentation liquid medium containing *Fusarium oxysporum* and glucose slowly decreases as the fungal growth progresses. When the residual glucose concentration was high at an initial growth phase, then the starch hydrolyzing enzymatic activity increased such as pectinase and amylases to convert

carbon substrate into simple sugars which was utilized by the fungus. Therefore, growth lingered down as the carbohydrates were consumed and strain started relying on metabolic end products for energy and growth. The extracellular enzymes were chiefly involved namely cellobiase, exoglucanases, and endoglucanase by *Fusarium oxysporum* to disintegrate carboxymethyl cellulose as illustrated by Maheshwari et al. (1990).

In recent study by Olajuyigbe *et al.* (2016). the lowest yield of β-glucosidase (68.67 U/mg; 15.12 U/mL) was produced using CMC of commercial grade when evaluated against the yield on other cellulosic carbon substrates. The findings also revealed that the production of β-glucosidase from *Fusarium oxysporum* demonstrates a wide range of pH stability. This might have resulted due to high viscosity of CMC in the culture media which restricts enzymatic degradation of the complex cellulose to produce metabolites essential for growth. The other reason which has reduced the fungal growth is a repression of cellulases by glucose increased concentration. This could be a consequence of catabolite repression as β-glucosidase production was suppressed in the presence of glucose highest concentration (Suto & Tomita, 2001). The nutrients composition and growth conditions were dissimilar as reported in literature. The results of locally isolated and CMC-A were similar with biomass production of 28.46 and 25.63 mg at pH 6.5 respectively.

CGW- CMC

Biomass

10 g of CMC in 1 liter of fermentation media may produce 0.328 g of biomass

0.15×10^{12} g of cotton gin waste may produce $0.328 \times 0.1 \times 0.15 \times 10^{12}$

$= 4.92 \times 10^{9}$ g biomass

Lipids yield

0.328 g of Biomass may produce 0.0366 g of lipids

4.92×10^{9} g fungal biomass may produce $0.0366 \times 0.328 \times 4.92 \times 10^{9}$

$= 5490 \times 10^{6}$ g Lipids

Statement: 0.3 million tons of cotton gin waste may produce 4,842 tons of fungal biomass with lipids yield 540 tons per annum in Pakistan.

Table 4.8 Lipids yield of *Fusarium sp.* on CMC carbon substrate

Accession #	Fungal Strains	Carbon substrate	Biomass g/L	Lipids yield g/L	Lipids content %	
HQ871880	*Fusarium Sp.1*	CMC-A	5.3	0.6	11	**Reference**
HQ871889	*Fusarium Sp.2*	CMC-A	2.8	0.35	12.1	Li *et al.*,2011
HQ871893	*Fusarium Sp.3*	CMC-A	3.2	0.4	12.53	
	F oxy. (CGWS-CMC)	CGW- CMC	0.32	0.036	11.8	Current study
	F oxy.(CMC-A)	CMC-A	0.28	0.034	11.8	

Abbreviation: *F oxy. Fusarium oxysporum*; CGW-CMC-cotton ginning waste-carboxymethyl cellulose; CMC-A-Carboxymethyl cellulose-Analytical

Now a day's research on oil production is mainly concentrated on glucose as a carbon source which is very costly, little work has done on hemicellulose and cellulose. The reported oil content data by Li and his co-workers in 2011 for *Fusarium spp*.1, *Fusarium spp*.2 and *Fusarium spp*.3 was used as a reference to estimate lipids yield of *Fusarium oxysporum* on CMC-A and CGW-CMC as represented in Table 4.8. The current study inferred that the lipids content and biomass was very little for oil production. This was due to the difference in nutrients composition and optimum growth at pH 7.5 as reported by Li et al. (2011). The most concerning point of present research is that isolated CMC from waste may produce lipids and may compete with standard.

Though, very limited study has been performed in Pakistan on lipid production by culturing oleaginous fungi with cotton gin waste. Thus, utilizing the fungal biomass for lipid extraction is critical to improve the process and reduce the overall production cost. My research presents cotton gin waste as a potential feedstock for biodiesel production.

The potential is 0.3 million tons of cotton gin waste may produce 4,842 tons of fungal biomass in Pakistan. Fungal biomass has been identified as a potential biodiesel source due to high lipid productivity. In Pakistan, 540 tons of lipids may produce every year which may be utilized after chemically reacting with alcohol to produce biodiesel. Biodiesel is designed to be used in diesel engines. The total global production of biodiesel from various sources had reached approximately 3.8 million tons in 2005 where 85% of biodiesel was produced by European Union. According to the figures released by EPA in 2011, production of biodiesel had reached more than 1 billion gallons. This digit had far exceeded as the target set by EPA was 800 million gallon. The estimated biodiesel production is just about 12 billion gallons for 2020.

Experimental data presented in present research shows the feasibility of using the fungal biomass to produce bio-fuel. Thus the fuel obtained from fungal biomass by employing cost effective experimental approach may serve as one of the alternative platform to compensate the Pakistan current energy crisis and future energy needs.

4.5 Bio-ethanol production from cotton gin waste using *Fusarium oxysporum*

Cotton year	Area (000Hectares)	Production (000Bales)	World cotton outlook		Yield Kgs/Hec
			Production Million tones	Consumption Million tones	
2012-2013	2,879	13,031	2.00	2.42	769
2013-2014	2,806	12,769	2.08	2.27	773
2014-2015(P)	2,961	13,983	2.30	2.31	802
Average 2012 – 2015	**2,882**	**13,261**	**2.12**	**2.33**	**781.33**

Source: Pakistan Economic Survey 2014-2015

The published data of cotton yield, production and cropped area by Government of Pakistan (2014-15) was used to estimate existing cotton gin waste generation in Punjab and Sindh provinces of Pakistan. Using the following formula quantity of cotton gin waste was estimated;

QPY = AAC x CY x YRR

PCCC data =>

i) AAC = Annual area cultivated in Hectares

Area (Sindh + Punjab)

ACC = 2895800 Hectares

ii) Crop yield = (Kg/Hectare)

Crop yield = 785.59 kg/hectare

iii) CRR = 1: 0.135 (Kg to Kg of Cotton)

QPY = AAC x CY x YRR

= 2895800 Hectares x 785.59 (Kg/Hectare) x 0.135

= 307113055.47 Kg

= 307113055.47/1000 tons

= 0.309 million tons/ year

Total production of cotton gin waste per year = 0.309 million tons / year

The two provinces Punjab and Sindh can produce an annual average of 0.329 million tons cotton gin trash for the period from 2012 to 2015. According to Panagiotou *et al*. (2005) research study *Fusarium oxysporum* strain has ethanol yield of 0.35 g/g cellulose with a volumetric productivity of 0.044 g/L/h under anaerobic conditions. The value was used to calculate total ethanol production from cotton gin waste in Pakistan annually.

4.6.1 Ethanol yield

Ethanol yield = 0.35 gm/gm cellulose

0.35g ethanol = 1 gm cellulose

1 gm cellulose = 0.35 gm ethanol

0.15×10^{12} gm cellulose = $0.35 \times 0.15 \times 10^{12}$

= 0.0525×10^{12}

= 52.5×10^{9}

= 52500 tons/year

The estimated ethanol yield using cellulose as a growth substrate was 52500 Tons/Year. The state lacks well designed and well established technologies for ethanol production from cotton residues nevertheless biomass itself is cheap. The design configuration and efficiency of strains production requires research to estimate the energy product cost. The way out to renewable

transportation based biofuels may not solely be ‘‘bio-ethanol”, but it will positively play a considerable part. The consumption and marketing of the bio-ethanol depends upon the compatibility with current technology, production and quality. It needs further research to assess product efficiency based on octane number. Since the system is in the production phase so in next phase cannot be implemented. The possible ways of its consumption can be the cotton ginning commerce where transportation cost of waste can be reduced. The efficient product will offer payback if it is consumed locally in energy systems. Khan et al. (2011) projected that in Pakistan annually there were roughly 17.86Â MT of wasted crops to produce bio-ethanol 4.91Â GL. Bio-ethanol can be consumed as partial or total replacement as a transportation fuel for gasoline. E-10 contains 10 percent ethanol and 90 percent gasoline. E- 85(Alternative Fuel) contains 15 percent gasoline and 85 percent ethanol. By taking consideration in terms of implementation stage pilot scale project was launched by Pakistan State Oil (PSO) at selected retail outlets in Islamabad, Lahore and Karachi. Hydrocarbon Development Institute of Pakistan (HDIP) conducted feasibility study to launch the system. E-10 new fuel was introduced on experimental bases as the strategy driven by government to support utilization of renewable energy resources. This project was administered in each city with 25 pre-identified automobiles for six months Mirza *et al.* (2008). This scheme had suffered failure because of lack of collaboration among stakeholder’s and flaws at development and implementation phase (Arshad, 2011).

4.5.2 Future perspectives

According to scale of economies, bio-fuels which are produced commercially prove to be at higher production costs as compared to conventional fuels. However, the production costs are

difficult to assess as they depend upon fuel processing method, commodity cost of the feedstock crop and state variations which highlights the fuel taxation and agricultural practices. Usually the high manufacturing costs may be compensated by fuel excise duty functional benefits to encourage production. Therefore, European Union states on preliminary basis encouraged zero-rated fuel duty for bio-ethanol fuels that meets agreed standards. Further research is necessary to evaluate economic feasibility for using cotton waste in bio-energy production and logistics would be needed to use cotton residues as a potential feedstock. Manufacturing costs of energy products for using cotton ginning waste is necessary for development planning and an investment decision that assessment was beyond the capacity of this study. As industrial course turn out to be commercially feasible, investment decisions and production costs can be analyzed systematically.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Based upon the data collected from experimentation it is concluded that out of four environmental fungal isolates only *Fusarium oxysporum* had the potential for CMC consumption derived from lignocelluloses CGW. *Fusarium oxysporum* strain was able to grow on carboxymethyl cellulose in fermentation medium at optimum pH 6.5. At pH 6.5, temperature 28 ºC and the carbon utilization efficiency of different energy resources by oleaginous strain manifested the % increase in colony diameter. The maximum growth was observed in (CGW-CMC-SA) with 85 mm colony diameter and in CMC-SA with 80 mm. The growth on CGW-CMC-SA was calculated with an increase of 6, 21 and 28 % in colony diameter from CMC-SA, SDA and PDA respectively. General morphology of strain in response to energy sources and substrate composition represented velvety surface and color altered from orange to white. The biomass of 25 and 30 mg was produced with an increase of 5 % in fungal growth using CGW-CMC as compared to CMC-A. There wasn't any significant effect on biomass either using locally isolated CMC from cotton gin waste or analytical grade. The strain produced 0.328g/L of biomass, with possible lipids production 0.0366 g/L. The current study inferred that the lipids content and biomass was very little for oil production. Therefore, intensive research is required for positive results in future. It was also mathematically derived that 0.3 Million tons/year of cotton ginning waste may produce lipids 540 tons/year and ethanol 52500 tons/year through enzymatic hydrolysis by *Fusarium oxysporum*.

5.2 RECOMMENDATIONS

Yeasts and molds have wide range of industrial applications especially in energy production but unfortunately fungal related research work is not supported by many research institutes and organizations due to lack of technical expertise and limited resources. This kind of study needs intensive mutual cooperation from research intellectuals. Here are few suggestions for future prospects;

- Technical expertise and financial support is inevitable for progressive experimental setup.
- Chemical pretreatment of cotton gin waste is a time consuming process at lab scale. Therefore, to increase cellulose production pilot scale investment is required.
- Cellulose extraction process also produces toxic chemicals. Comparative study of emissions will determine whether the realistic environmental friendly strategy is possible to adopt.
- In this research, growth parameters like pH and carbon sources for *Fusarium oxysporum* have been optimized. There are several other growth parameters such as; temperature, inoculum size, supplemental enzymes, salts composition, organic nitrogen source, oxygen etc which are indispensable for growth optimization.
- Development of bioreactor for submerged or solid state fermentation is recommended for possible production of fungal biomass and consequently lipids accumulation.
- Simultaneous scarification and fermentation system needs optimized growth chamber for alcohols production.
- Strains should be selected on the basis of thick growth, spores number, enzymes production, biomass and nature of fungal behavior.

- The burning of cotton gin waste emits greenhouse gases; its mathematical modeling using Standard Operating Procedures with practical experimentation is required for GHG assessments.
- Project economic assessment for alcohols is possible through HYSYS software where as biodiesel properties can be evaluated through prediction models.

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ANNEXURE I

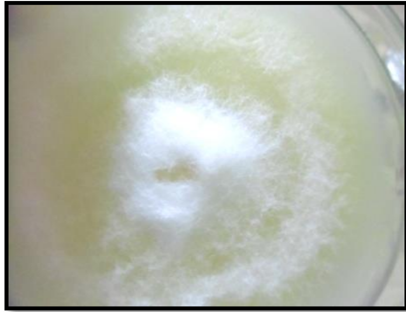
Fusarium oxysporum growth in six days on CGWs CMC substrate

		Day 1 24 hours	Day 2 48 hours	Day 3 72 hours	Day 4 96 hours	Day 5 120 hours	Day 6 144 hours
		Colony Diameter(mm)					
pH 5.5	Rep 1	7	24	40	53	75	85
	Rep 2	11	26	42	54	72	85
	Rep 3	10	22	46	58	72	90
Colony Radii (mm)							
	Rep 1	3,5	12	20	26,5	37,5	42,5
	Rep 2	5,5	13	21	27	36	42,5
	Rep 3	5	13,5	20,5	27,5	36,5	41,5
Colony Diameter (mm)							
pH 6.0	Rep 1	10	26	41	50	70	85
	Rep 2	9	27	45	56	74	85
	Rep 3	10	26	44	56	70	83
Colony Radii (mm)							
	Rep 1	5	13	20,5	25	35	42,5
	Rep 2	4,5	13,5	22,5	28	37	42,5
	Rep 3	5	13	22	28	35	41,5
Colony Diameter (mm)							
pH 6.5	Rep 1	14	30	47	59	78	85
	Rep 2	12	28	46	56	76	85
	Rep 3	14	28	46	56	78	85
Colony Radii (mm)							
	Rep 1	7	15	23,5	29,5	39	42,5
	Rep 2	6	14	23	28,5	38,5	42,5
	Rep 3	7	14	23	28,5	39	42,5
Colony Diameter (mm)							
pH 7.0	Rep 1	9	27	45	55	71	85
	Rep 2	13	30	45	57	75	85

	Colony Radii (mm)						
	Rep 1	4,5	13,5	22,5	27,5	35,5	42,5
	Rep 2	6,5	15	22,5	28,5	37,5	42,5
	Colony diameter(mm)						
	Rep 1	12	28	44	54	72	80
pH 7.5	**Rep 2**	10	24	42	52	76	80
	Rep 3	10	26	44	56	70	84
	Colony Radii (mm)						
	Rep 1	5	13,5	22,5	28	36	40
	Rep 2	5	12	21	26	38	40
	Rep 3	5	13	22	28	35	42

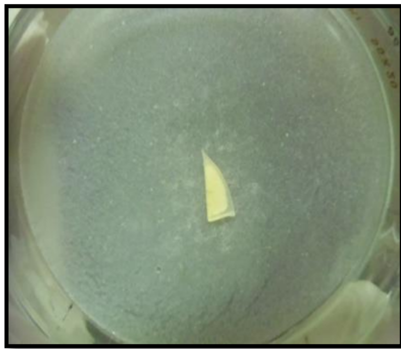
ANNEXURE II

Fusarium oxysporum growth response on carbon substrates

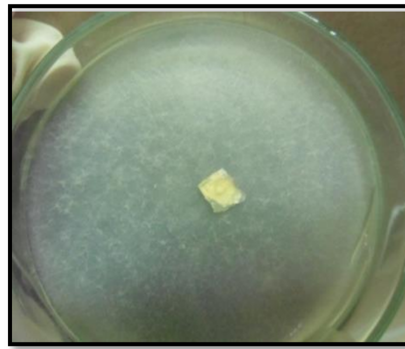


PDA

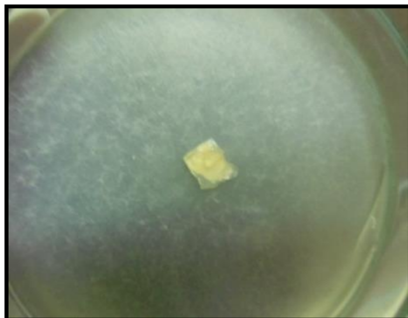
SDA



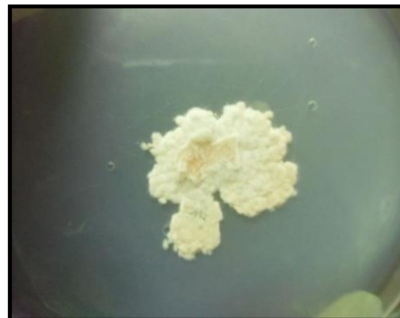
CGW- Cellulose-SA



CMC-A



CGW- CMC-SA



GSA