DISSERTATION

ON

Biodiesel production from waste lignocellulosic materials using in-house oleaginous yeast

Submitted by

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In partial fulfillment for the award of the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

Department of Biotechnology

THAPAR UNIVERSITY, PATIALA

JULY, 2015

DECLARATION

I hereby declare that the thesis entitled "**Biodiesel production from waste lignocellúlosic materials using in-house oleaginous yeast**" being submitted in partial fulfillment of the requirements for the degree of **Master of Science** in **BIOTECHNOLOGY** under Faculty of Biotechnology of Thapar University, Patiala, during the academic year 2015, is a bonafide record of my original work carried out under guidance and supervision of **Dr. A.S. MATHUR, RESEARCH MANAGER, BERC, R&D, INDIAN OIL CORPORATION LIMITED** and has not been presented elsewhere.

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CERTIFICATE

This is to certify that **Ms. Simarpreet Kaur Chawla**, M. Sc. (Biotechnology), a student from **Thapar University, Patiala,Punjab** has undergone Industrial Training and completed her project work at DBT IOC Centre for Advanced Bio-Energy Research, Indian Oil Corporation Limited, R&D Centre, Faridabad during the period **7th Jan, 2015 to 30th June,2015.**

She worked on the project **"Lipid Production from waste lignocelluloesic materials using In-house oleagenous yeast"** in Bio-Energy Research Centre, IOC R&D Centre, Faridabad, Haryana.

She was found sincere and hard working during the training period.

I wish her all the success in her future career.

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CERTIFICATE

This is to certify that the thesis entitled "**Biodiesel production from waste lignocellulosic materials using in-house oleaginous yeast**" by **SIMARPREET KAUR CHAWLA, (301301014)**, submitted in partial fulfillment of the requirements for the degree of **Master of Science** in **BIOTECHNOLOGY** under Faculty of Biotechnology of Thapar University, Patiala, during the academic year 2015, is a bonafide record of work carried out under our guidance and supervision.

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ACKNOWLEDGEMENT

I would like to express my gratitude and appreciation to all those who gave me the possibility to complete this report. I would also like to acknowledge the Head of Department and my course coordinator **Dr. Dinesh Goyal**, and faculty of Biotechnology, who gave the support and confidence to work outside the campus. I express my deep sense of gratitude to **Dr. D.K. Tuli**, Executive Director, Bioenergy Research Centre for trusting me with this project at IOCL, R&D. I would like to express my sincere thanks to **Mr. Ravi Prakash Gupta**, Senior Research Manager and Head of Department, Bioenergy Research Centre, IOCL, R&D for providing me with the best possible facilities for my project. I am in great debt to **Dr. A.S Mathur**, Research Manager, Bioenergy Research Centre, R&D, IOCL, Faridabad for sharing his experience and guiding me throughout the project

A special thanks to my final year project guide **Dr. Dilip Singh**, Research Associate Bioenergy Research Centre, IOCL, R&D whose stimulating suggestions and encouragement helped me to coordinate my project, especially in writing this report. I would like to thank **Dr Preeti Mehta**, Research Associate, Bioenergy Research Centre, IOCL, R&D for her invaluable effort with experiments and her constant support throughout the project. My sincere thanks to my fellow lab mates for their cooperation and help throughout the project.

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ABBREVIATIONS

DCW	Dry cell weight
NDLH	Non- detoxified liquid hydrolysate
LCBs	Lignocellulosic biomass
TFA	Total fatty acid
HMF	Hydoxymethyl furfural
FAME	Fatty acid methyl ester
GC-FID	Gas chromatography-fluid ionization detector

ABSTRACT

Oleaginous yeast strain *Rhodotorula* sp. DBTIOC-ML3, previously isolated from India marine biodiversity, was used in this study. Strain DBTIOC-ML3 can not only tolerate high concentration of inhibitors such as acetic acid, HMF and furfural but also produce high biomass containing significant amount of lipid, which underlines the robustness of this strain. Major aim of this study was to replace costly yeast extract with cost effective nitrogen sources. For which different inorganic and organic nitrogen source were tested. Spent yeast from ethanol fermentation was found viable option to replace yeast extract in the media. Autolysis of the spent yeast released significant amount of protein and other nutrients, which was subsequently used as nitrogen and nutrient source in combination with non-detoxified liquid hydrolysate (NDLH) of different biomass. Ability of this strain to utilize sugars from NDLH of different lignocellulosic biomass and nitrogen source from spent yeast makes it suitable candidate for lipid production, which can be subsequently used for biodiesel production. Fatty acid profile of this strain contains large amount of palmitic and oleic acid, which further underlines the potential of this strain for biodiesel production.

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Chapter 1

Introduction

Depleting conventional energy resources, rising fuel prices, alarming greenhouse gas (GHG) levels in the atmosphere and the effects of global warming are among the many important environmental issues that the global community must grapple with in the coming decades [1]. Combustion of fossil fuels (e.g. coal, oil and gas) is the primary source of energy and currently supplying 80% of the total global energy demand, which will cause a decline of petroleum reserves at a rate of between 2% and 3% per year and expected to vanish completely in next 100 years [2].Researchers are focusing extensively on the development of alternative sources of energy such as nuclear, solar, wind, hydro and biofuel.

Biodiesel is one of the most prominent biofuel and is prepared by transesterification of triacylglycerols. Biodiesel has many advantages, such as inherent lubricity, superior flashpoint, biodegradability, and negligible sulphur content (Moser BR et al 2009.,) It is composed of monoalkyl esters with long-chain fatty acids, which can be derived from oil resources in the form of triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and free fatty acid (FA). Biodiesel contributes no net carbon dioxide or sulphur to the atmosphere and emits less gaseous pollutants than normal diesel. In spite of the favourable impacts that the commercialization of biodiesel could provide, the cost of production of oil raw materials is a significant cost deterrent [3].Based on source of raw material, biodiesel can be classified into 1^{st} gen, 2^{nd} gen and 3^{rd} gen biodiesel.First generation biodiesel is produced directly from edible oil such as soyabean oil, palm oil etc. Large amount of edible oil is being diverted for biodiesel production, resulting into surge in commodity oil prices, which raises the issue of food vs fuel debate. Second generation biofuels overcome the limitations of first generation biofuels. They are produced from non-edible oils such as Jatropha, Karanja oil. Second generation biodiesel don't compete with food crops, although oil materials can be obtained from plants, but energy and acreage are required for sufficient production of oilseed crops, contributing negatively to the overall economics of biodiesel production. Therefore, exploring ways to reduce the high cost of biodiesel is an important research theme, particularly for methods focused on lowering the cost of oil raw material. The use of microbes as feedstock for biodiesel production could be advantageous due to their shorter life cycle, lower land usage, easier scale up and lesser effect of

seasonal variations[4]. Biodiesel from these microbes is called as 3rd gen biodiesel. Oleaginous microorganisms such as algae [5], yeast and fungus have capability to accumulate high concentration of lipids and can be exploited for competitive production of biodiesel.Microbial biofuels, therefore, have the potential to replace petroleum fuels, but their realisation depends on technological and economic advances [6].

Currently biodiesel is more expensive than conventional diesel mainly because of the high cost (70%–85%) for the raw material. If biodiesel could be produced from low-cost biomass growing on agricultural marginal land or even from waste biomass, it could markedly enhance the supply pool of raw materials, realize the environmental benefits much greater than food-based biofuels, and lower the price of biodiesel (Dai et al. 2007; Islas, Manzini, and Masera 2007; Schubert 2006). Thus with the increasing demand for biodiesel production, alternative cheap sources are to be used which would help in the production of good quality biodiesel. Different agro industrial wastes showed remarkable capacity to be used for microbial lipid production i.e molasses, glycerol, whey, plant waste hydrolysates etc. Oleaginous microorganismscould utilize a variety of carbon sources such as the xylose, glucose and thus can accumulate a large amount of intracellular lipid. Furthermore, supplementing the hydrolysates with the additional nitrogen source also enhanced the production of intracellular lipid

Chapter 2

Literature review

2.1 Oil producing microorganisms

Many microorganisms across different kingdoms exhibit the ability to store significant oil content. Heterotrophic microorganisms such as fungi are extremely efficient in utilizing different classes of sugars such as pentose and hexoses, and can be grown for oil production on cheap carbon sources. Most microalgae are photosynthetic but have the ability to switch between different modes of growth, that is, phototrophic, heterotrophic and mixotrophic, depending on the pigment system and carbon source. For example, *Chlorella* and*Scenedesmus* can be grown in these different modes, while other microalgae such as *Schizochytrium* are exclusively heterotrophic. Microorganisms that accumulate more than 20% of their dry weight as lipids are designated as oleaginous microorganisms. Lipid content in many microalgae such as *Schizochytrium, Botryococcus, Nannocloropsis, Dunaleilla*(Table 1)and fungi such as*Rhodotorulla, Mortierella* (Table 1) exceeds more than 50 % of their dry cell weight (DCW, depending on various physiological parameters. Culture conditions and nutritional and environmental factors significantly affect lipid accumulation and composition. Nitrogen stress, salt stress and low aeration are among the well-known physiological factors that enhance lipid accumulation in the cell during fermentation, but at the expense of cell growth. Microorganisms with high lipid content coupled with high biomass productivity are the good candidates for biodiesel production (Table 2.1a and 2.1b). Photosynthetic microalgae like *Chlorella*, when grown under heterotrophic mode, have shown higher biomass and lipid content as compared to growth under their phototrophic modes [7]. The heterotrophic mode offers great flexibility in terms of light independence, easy scale up for industrial use, negligible likelihood of contamination, high degrees of growth control and reduced harvesting costs due to higher cell density.

Table.1 Oil content (>20 % of DCW) of oleaginous yeasts

Microorganisms	Habitat (/F/M)*	Lipid content (% of DCW)
Aspergillus	F/M	57
Candida	F/M	58
Cryptococcus	F/M	58-65
Cunninghamella	F	35
Entomophthora	F/M	43
Fusarium	F/M	34
Humicola	F/M	75
Lipomyces	M	64
Mortierella	F/M	66-86
Pellicularia	F/M	39
Rhodotorula	F/M	72
Torulaspora	M	40-50
Trichosporon	F	45
Umbelopsis	F/M	20-40
Waltomyces	M	64
Yarrowia	F/M	40-60

2.2. Collection and isolation of oil producing microorganisms

Collection and identification of fast growing, hyper lipid containing microorganisms is one of the key milestones in successful commercial biodiesel production. Collection of oleaginous microorganisms is influenced by several physio-chemical factors such as annual rainfall, salinity, dissolved oxygen level, temperature, pH, macronutrient levels, and carbon content of the selected habitat [8, 9]. Since habitat conditions affect the physiology of local microorganisms, physiological conditions of a habitat differ from place to place, offering enormous opportunities for isolation of diverse groups of oil producing microorganisms with high biomass and lipid producing ability. These microorganisms can be collected from various habitats such as fresh water, brackish water, marine, hyper saline water bodies, or mangroves. Hyper saline environments, thermophilic springs and eutrophic marine water bodies are the excellent sites for

collection of oleaginous microorganisms [10, 11]. Various parameters such as pH, altitude, and temperature of the site should be measured during sample collection.

Different techniques are currently being employed for the successful isolation of oleaginous microorganisms, which include conventional methods such as medium enrichment, single-cell isolation by micropipette, serial dilutions, micromanipulation, atomized cell sprays, gravimetric separation, and high throughput techniques like Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) [7, 12, 13]. Serial dilution and single cell isolation are the two most widely used techniques for the isolation, whereby single cells are collected by micropipette and placed into culture media followed by streaking on agar plates having different combination of antibiotics [14]. The combination of antibiotics should be carefully selected based on the targeted microorganisms to be isolated. The two above mentioned techniques are easy to implement and effective only for small sample size. For large scale screening, FACS and MACS [15-17] are most frequently used. In FACS, samples are serially diluted and inoculated in 96 well plate followed by the addition of lipid staining fluorescent dyes such as nile red, BODIPY$^{505/515}$[18, 19]. Based on fluorescence, these cells are sorted into high lipid, moderate and low lipid containing cells. In MACS, samples are serially diluted and labelled with magnetic nanoparticles based on the surface properties of targeted microorganism, these cells are then passed under a magnetic field leading to separation of microorganisms based on their surface magnetic properties.

2.3. Examples of cost-effective nutrients for cultivation

The type of carbon source, optimal quantity and input cost are crucial parameters to be taken in to account in the design of fermentation protocols in biodiesel industry. Cheap carbon sources such as waste glycerol from the biodiesel industry or fish industry, sugar cane juice, molasses, beer residues, soybean cake, coconut water and sweet sorghum juice were investigated for lipid production to make the process commercially viable [20-22]. Oleaginous microbes are reported to grow well in media having these carbon sources, without compromising yield. In fact, some reports have shown higher biomass and lipid production than glucose using coconut water or raw glycerol or beer residue as the carbon source [23, 24]. This increment in biomass and lipids can be attributed to the presence of other necessary growth factors and trace elements in these cheaper carbon sources. Quilodran (2009) reported as much as 2-7 fold increases in biomass and

lipid accumulation when waste liquid residues from beer or potato chips were used as carbon sources. The ability of oleaginous microbes to utilize carbon sources from a broad spectrum of waste materials makes them an attractive feedstock for biodiesel production, although continuous supply of some of these materials at an industrial scale is not sustainable for large scale biodiesel production. Oleaginous yeasts can utilize xylose as a carbon source once glucose is consumed in medium [25] opposite to ethanologenic yeasts, which cant ferment xylose into ethanol.

The availability and type of the nitrogen sources in the medium are key parameters affecting total lipid yield and fatty acid profiles. Nitrogen content dictates the cell division rate since most of the vital processes of cell division such as protein synthesis, and nucleic acid synthesis are under direct control of nitrogen levels in the medium. Generally, nitrogen is consumed in the medium within 48-72 hours. Once nitrogen is consumed, AMP deaminase is up regulated, shifting acetyl Co~A flux towards lipid biosynthesis. Glucose content under nitrogen stress therefore significantly affects lipid production, with higher glucose levels resulting in higher lipid content due to perpetually more glucose being channelled into fatty acid synthesis pathways [26]; typical behaviour of oleaginous microorganisms. Lipid accumulation normally begins in the late log phase and continues until the early stationary phase or until complete exhaustion of the carbon source. Although nitrogen depletion in the medium enhances lipid content by several multiples but at the expense of biomass yield, so appropriate C/N ratios needs to be selected in the medium for optimal biomass and lipid production. Most of the oleaginous microorganisms are reported to grow well on organic carbon sources such as peptone, tryptone, yeast extract, and corn steep liquor or inorganic nitrogen sources such as ammonium sulphate, ammonium acetates, nitrates, and monosodium glutamate [27-29]. Although the quantity of nitrogen sources needed for fermentation is far less than that of carbon sources, availability of cheap nitrogen sources will require a breakthrough to reduce costs; a foremost priority for the biodiesel industry. Inorganic nitrogen sources are significantly cheaper than their organic counterparts, but the question as to their longer term sustainability remains. The ability of oleaginous microbes to utilize a wide range of nitrogen sources provides ample opportunity to take advantage of potential cheap nitrogen sources in fermentation.Researchers have grown oleaginous microbes on various cheap nitrogen sources such as soybean meal, skimmed milk, distillery wastewater. Wastewater effluents from ethanol fermentation, tanneries, slaughter houses, and the milk industry containing nitrogen up to 4-5g/L, represent cheap, abundant

nitrogen sources. While these can be explored as alternative organic nitrogen sources [30], they must be pre-treated before use to remove toxic materials, as these can restrict growth. The balance between treatment cost and productivity must be analysed to establish the suitability of these effluents as nitrogen sources.

2.3.1Lignocellulosic biomass as an important source for the lipid production

Commercial success of biodiesel from oleaginous microorganisms will depend on the long term supply of nutrients, particularly carbon source, and the availability of large quantities of this source at a low cost. Carbon sources including glucose, glycerol and volatile fatty acids (VFAs) are being used in biodiesel production, but have not proven to fully satisfy the necessary conditions for sustainability of the process.

Lignocellulosic materials are available in large quantity and are of interest as cheap carbon sources for biofuel production. About 910 million metric tonnes of wheat straw was produced worldwide in 2011, calculated by multiplying the global wheat production (approximate 701 million metric tonnes, UNFAO 2011) by a factor of 1.3 [31]. Cellulose and hemicellulose are the two major components of wheat straw, ranging from 35-45% and 20-30%, respectively, followed by lignin (8-15%). However, composition varies by location. Glucose is predominantly present in cellulose and xylose in hemicellulose. These sugars are tightly packed in the lignocellulosic matrix, reducing their accessibility to hydrolytic enzyme and the release of sugar. Thus prior to enzymatic hydrolysis, Pre-treatment is necessary to increase the substrate accessibility and subsequent sugar release from the tightly packed lignocellulosic matrix.

Hexose sugars are widely used as a carbon source for ethanol fermentation, applying different strains of *Sacchamycescerevisiae*[32], *Kluyveromycesmarxianus[33] orZymomonasmobilis*[34] and process for hexose to ethanol production is well established. Pentose sugars are the second most abundant sugar present in any lignocellulosic material. However efficiency of pentose sugar fermentation is quite low, as compare to hexose sugar fermentation, due to lack of enzymatic machinery necessary for pentose fermentation in most of the natural strains of S.*cerevisiae*[34, 35]. S.*cerevisiae*strains were genetically modified to overcome this drawback. However those strains showed little improvement in terms of ethanol yield, productivity and tolerance [36]. Pentose sugars can be assimilated into lipid by oleaginous yeasts such as *Rhodotorula*[37], *Lipomyces*[38]and*Trichosporon*[39]. Pentose assimilation to lipid by

oleaginous yeast is efficient as compare to pentose fermentation to ethanol by ethanologenic yeast. Energy density of lipid is higher when compared to ethanol, thus it is more efficient to convert pentose sugars into lipid rather than ethanol.

Hemicellulose is the second most abundant polysaccharide after cellulose in lignocellulosic materials. The conversion of lignocellulose into lipid together with hexose fermentation to produce ethanol may provide an economical route for biofuel production. Efficient conversion of lignocellulosic biomass into biofuel requires effective Pre-treatment before enzymatic hydrolysis. However, this Pre-treatment can result in the formation of inhibitors such as acetic acid, furfural and hydroxymethyl furfural (HMF). These inhibitors have adverse effects on both ethanol fermentation [40, 41] and lipid assimilation [31, 42]. Liquid hydrolysates need to be detoxified to remove inhibitors before fermentation or lipid assimilation. Detoxification processes such as over liming, activated charcoal, neutralization or evaporation have been demonstrated to be effective for inhibitor removal, but also remove some of the sugars and so reduce yield. The detoxification step also enhances the energy footprint of the process, resulting in higher input costs. Therefore it would be beneficial to isolate oleaginous yeast which can convert pentose sugars into lipid in the presence of inhibitors.

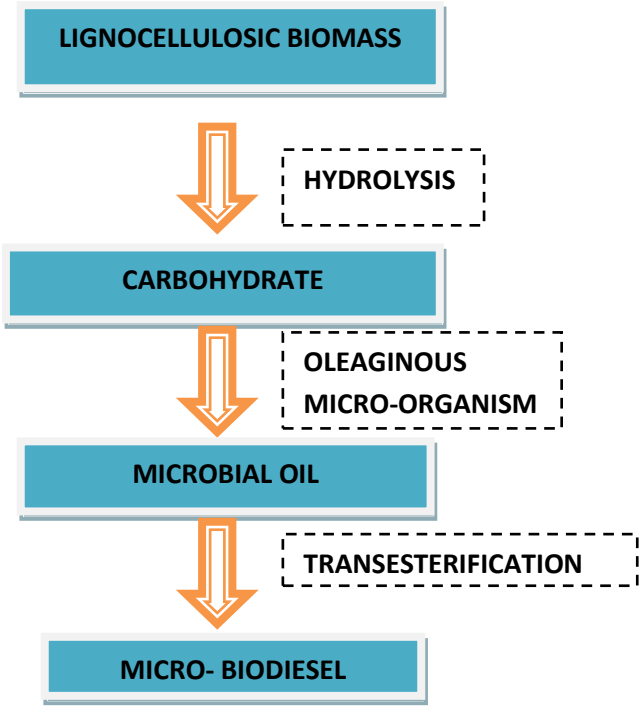

Fig.1: Biodiesel production from lignocellulosic biomass using oleaginous yeast

2.3.2Pre-treatment of biomass with dilute sulphuric acid for the production of microbial oil using oleaginous yeast

The hydrolysate from the dilute sulphuric acid Pre-treatment of lignocellulosic biomass is used for microbial oil production. Wheat straw consists of 35–45% cellulose, 20–30% hemicellulose, and 8–15% lignin (Saha et al., 2005). Although these two carbohydrate components in the biomass can be converted to fermentable sugar monomers for biofuel production, the direct enzymatic hydrolysis is impeded due to the physico-chemical and structural cell wall composition of the biomass. Thus, biomass Pre-treatment prior to enzymatic hydrolysis is essential to enhance the accessibility of cellulase to cellulose. Among various chemical Pre-treatment methods, dilute sulfuric acid is the most commonly applied catalyst (Taherzadeh and Karimi, 2008). At moderate temperatures, most of the hemicellulose is effectively removed and recovered by dilute sulfuric acid as dissolved sugars and acetic acid in the hydrolysate (Wyman et al., 2005). Since hemicellulose is the second most abundant polysaccharide in lignocellulosic biomass, the full utilization of these sugars in the hydrolysate is required for economical biofuel production.

Moreover, in dilute sulfuric acid pretreated wheat straw hydrolysate various degradation products are present, which mainly include acetic acid from acetyl groups in hemicellulose, furfural from pentose, and 5-hydroxymethylfurfural (HMF) from hexose, and these compounds strongly inhibit microorganisms during microbial oil production.

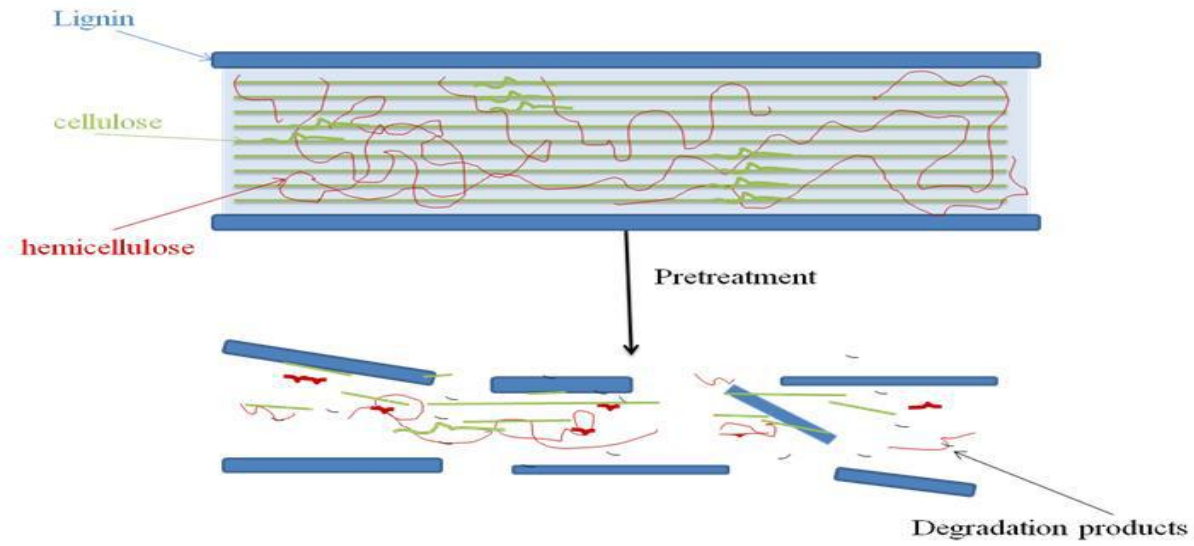

Fig.2: Effect of Pre-treatment on Lignocellulosic biomass

2.3.3. Spent yeast broth from ethanol fermentation as alternative to yeast extract

With the increase in ethanol production, large volume of broth containing spent yeast is expected to be generated. Disposing this broth directly into environment may damage the delicate ecosystem of the locality. However treating this broth before discharging may further add cost to ethanol production. Therefore adding value to this spent broth may helpful to address the broth discharge issues associated with ethanol fermentation. Yeast cells contain a lot of protein, carbohydrates, lipid, vitamins, and minerals. Yeast extract (YE) comprises the water-soluble components of the yeast cell, the composition of which is primarily amino acids, peptides, carbohydrates and salts. Nitrogen components and vitamins are the value of yeast extract because of their nutritional characteristics. The carbohydrates are not such an important nutritional component of YE but it is important to know their recovery kinetics if cell walls are used for carbohydrate isolation (glucans, mannans) after yeast extract production. YE is manufactured by breaking down the cells using endogenous or exogenous enzymes. There have been many reports on the manufacturing processes (K. Breddametal 1991; S.J. Choi etal 1998). Autolysis by endogenous enzymes occurs naturally in yeasts when they complete the cell growth cycle and enter the death phase. Autolysis is a traditional disruption method in YE production but it has some disadvantages: low yield, difficulty in solid–liquid separation due to the high content of residue in the autolysate, poor taste characteristics as a flavour enhancer and risk of deterioration due to microbial contamination.

The yeast biomass recovered from beer production/ethanol production is an interesting residue, since it is abundant and low cost. It contains 45-65% proteins and 8-12% nucleic acids (Halasz and Lastity, 1991). When autolysis is done on this spent yeast biomass, the total protein and nucleic acid of the yeast biomass will be released up in the media. This is high in nutrient content and can be efficiently utilized in the cultivation of oleaginous microorganisms. Oleaginous microorganisms require nitrogen components for its growth and thus in can be supplemented using spent yeast as the nitrogen source for the accumulation of lipid and thus microbial oil production.

In this study, a fast growing oleaginous yeast strain i.e. *Rhodotorula* sp. ML-3, previously isolated from Indian marine biodiversity was studied. Biomass and lipid production potential of this strain was studied in detail under different culture conditions utilizing different pentose rich non-detoxified hydrolysates and various nitrogen sources. Potential of autolyzed spent yeast from ethanol fermentation was also studied in detail. This strain is capable of producing substantial amount of biomass and lipids when grown on medium containing on NDLH and spent yeast broth.

Chapter 3

Materials and methods

3.1 Reagents and Chemicals

All the chemicals and regents used in this study were either analytical or molecular grade. Media constituents for example D-glucose, glycerol, acetic acid, and xylose or yeast extract, peptone, sodium nitrate, potassium nitrate, ammonium chloride, ammonium acetate, ammonium sulphates were procured from Sigma-Aldrich or Merck. Sea salt (Instant Ocean, USA) was added in the medium to mimic marine environment. Antibiotics such as Penicillin G, Streptomycin and Rifampicin and Nystatin were sourced from Sigma-Aldrich. Methanol, chloroform, hexane, toluene (Merck or Fischer), Methyl nonadecanoate, Butylatedhydroxytoluene, Potassium bicarbonate (Sigma-Aldrich) were used in lipid extraction, FAME preparation and FAME analysis.

3.2 Preparation of non-detoxified liquid hydrolysate (NDLH) of different lignocellulosic biomass

Dried lignocellulosic biomass of size 0.25mm was mixed with 100 ml acidified water (2 ml concentrated H_2SO_4 n 98 ml distil water). The mixture was then autoclaved at 121^{o}C for 60 minutes and filtered using Whatman no. 1 filter paper to remove solid particles. Since the filtrate obtained was highly acidic so conditioning of the filtrate had to be done to bring it to desired pH of 7. For obtaining the desired pH of 7, filtrate was taken in Erlenmeyer flask and calcium hydroxide was added to flask with simultaneous vigorous shaking to dissolve the amount of calcium hydroxide added to bring the desired pH. Once desired pH of 7 is achieved, it was centrifuged at 5000 rpm for 15 min in centrifuge bottles where the supernatant is taken and the pellet is discarded upon centrifugation.

3.3 Culture maintenance, biomass production and sugar consumption of oleaginous yeast DBTIOC-ML3 on NDLH of dilute acid pretreated different lignocellulosic biomass (LCBs)

The yeast strain used in this study i.e. *Rhodotorula* sp. ML-3, was previously isolated from Indian mangrove diversity. Culture was maintained at 25 °C on agar plate having glucose 5 gL^{-1}, yeast extract 2 gL^{-1}, peptone 2 gL^{-1}, sea salt 18 gL^{-1} (to make 50 % sea water strength), agar 10 gL^{-1}. Plates were sub cultured each 25 days. For long term storage of the culture, glycerol stocks of this strain were prepared. For glycerol stock preparation, 50 % v/v glycerol solution in water was prepared and autoclaved at -121°C for 20 min. 500 µl of 48 h -72 h old culture was added in 2 ml sterile cryovials containing 500 µl of 50 % sterile glycerol solution and stored at -80°C.

1 µL of culture was taken from plate and inoculated in seed medium containing glucose 30gL^{-1}, yeast extract 10gL^{-1}, peptone 1gL^{-1}, sea salt 18 gL^{-1}. All the media used in this study were sterilized by autoclaving at 121°C for 15 min. 5% v/v of 48 h old inoculum was transferred into production media having dilute acid pretreated non detoxified pentose sugar rich hemicellulose fraction of lignocellulosic biomass of wheat straw, sugarcane bagasse, corn cob, corn stalk and cotton stalk. Cultures were incubated at 25°C, 4 day at 150 rpm for biomass production. 4 day old cultures were harvested and washed with 0.5 M NaOH (to dissolve lignin and other solid particles, which may settle along with biomass during centrifugation thus interfering with biomass calculation) followed by two time washing with distil water. Biomass was freeze dried for 24h – 48 h and stored at -80°C for subsequent studies.

3.4 Effect of different nitrogen sources on biomass production and sugar consumption by oleaginous yeast DBTIOC-ML3 on NDLH of dilute acid pretreated wheat hydrolysate

Different nitrogen sources such as sodium nitrate, potassium nitrate, ammonium chloride, ammonium acetate and yeast extract were added in the production media to study biomass production and sugar consumption by oleaginous yeast DBTIOC-ML3. Production media containing these nitrogen sources (10 gL^{-1}), peptone (1 gL^{-1}), sea salt (18 gL^{-1}) and NDLH of wheat hydrolysates. Production media was autoclaved at 121°C at 15 min followed by

centrifugation at 5000 rpm for 15 min under sterile condition. Pellet was discarded and supernatant was used as production media for further studies. Subsequently 50 ml media was transferred aseptically in 250 ml Erlenmeyer flask and flasks were inoculated with 48 h old 5 % (v/v) culture. Cultures were incubated at 25°C, 4 day at 150 rpm for biomass production. 4 day old cultures were harvested and washed with 0.5 M NaOH (to dissolve lignin and other solid particles, which may settle along with biomass during centrifugation thus interfering with biomass calculation) followed by two time washing with distil water. Biomass was freeze dried for 24h – 48 h and stored at -80°C for subsequent studies.

3.5 Autolysis of spent yeast cells from ethanol fermentation

Spent yeast broth after ethanol fermentation was harvested and centrifuged at 5000 rpm for 15 min at 4°C. Harvested wet yeast biomass was washed twice with distil water to remove traces of media components. 100 g of wet biomass was suspended in 500 ml of 8 % saline solution (in 1000 ml flask) and incubated at 60ºC for 12 h at 150 rpm in rotatory shaker. After 12 h, suspension was centrifuged at 5000 rpm for 15 min at 4°C and supernatant plus pellet were separately stored at 4°C. Protein released in the supernatant and remained in pellet was estimated by using Lowry assay to quantify the degree of cell lysis due to osmotic shock. Lowry assay was performed for both conditions i.e before and after autolysis of biomass.

3.6 Cultivation of oleaginous yeast DBTIOC-ML3 on different media compositions

3.6.1 Media containing different concentration of NDLH of wheat straw and supernatant of autolysed spent yeast

NDLH of acid pretreated wheat straw and supernatant of autolysed spend yeast broth was mixed in different proportions. Peptone (1 gL^{-1}) was also added in the production media. Rest of the culture conditions and harvesting methodology was same as mentioned in section 3.3

Media no	Composition (v/v)
Media-1	100% NDLH
Media-2	75% NDLH+25%spent yeast
Media-3	50% NDLH+50%spent yeast
Media-4	25% NDLH+75%spent yeast
Media-5	100%spent yeast
Media-6	100%NDLH+3.32 g/L Yeast extract

Table.2 Composition of different production media

3.6.2 Media containing NDLH of different lignocellulosic biomass and supernatant of autolysed spent yeast

25 ml of NDLH of different acid pretreatedlignocellulosic biomass (wheat straw, sugarcane bagasse, corn cob, corn stalk and cotton stalk) was mixed with 25 ml supernatant of autolysed spent in 250 ml flask. Peptone (1 gL^{-1}) and sea salt (18 gL^{-}) was added in this media. Rest of the culture conditions and harvesting methodology was same as mentioned in section 3.3

3.6.3 Media containing NDLH of different lignocellulosic biomass and pellet of autolysed spent yeast

10 g of different lignocellulosic substrates such as wheat straw, sugarcane, cotton, corn cob, corn stalk were mixed with 100 ml of 2% (v/v) conc. H_2SO_4 in 250 ml Erlenmeyer flask followed by addition of 15 g of wet pellet of autolysed spent yeast. Flasks were autoclaved at 121°C for 60 min. After autoclaving, hydrolysates were centrifuged at 4500 rpm for 15 min at 25°C. The supernatant was taken while the pellet was discarded. The pH for each hydrolysate was brought to 7 by NaOH pellets followed by centrifugation at 4500 rpm for 20 min to remove precipitates. Peptone (1 gL^{-1}) and sea salt (18 gL^{-}) was added in each NDLH followed by autoclaving at 121°C for 15 min. Rest of the culture conditions and harvesting methodology was same as mentioned in section 3.3

3.6.4 Media containing NDLH of different lignocellulosic biomass and spent yeast broth (after ethanol fermentation)

Two experiments were carried out to study the direct utilization of spent yeast culture (after ethanol fermentation) as nitrogen source for the cultivation of strain DBTIOC-ML3. In first experiment, 50 ml of spent yeast culture (after ethanol fermentation) was mixed with 50 ml of 2% (v/v) conc. H_2SO_4 in 250 ml Erlenmeyer flask. 10 g of different lignocellulosic biomass (wheat straw, sugarcane, cotton stalk, corn cob, corn stalk) were also added in the solution. Flasks were autoclaved at 121°C for 60 min. In second experiment, 50 ml spent yeast culture was added in acidic NDLH of acid pre-treated different lignocellulosic biomass and heated at 100°C for 10 min. Hydrolysates were centrifuged at 4500 rpm for 15 min at 25°C. The supernatant was taken while the pellet was discarded. pH of each hydrolysate was brought to 7 by calcium hydroxide followed by centrifugation at 4500 rpm for 20 min to remove precipitates. Peptone (1 gL^{-1}) and sea salt (18 gL^{-1}) was added in each NDLH followed by autoclaving at 121°C for 15 min. Rest of the culture conditions and harvesting methodology was same as mentioned in section 3.3

3.7 Analytical Methods:

3.7.1 Sugar and inhibitor analysis of NDLH with High performance liquid chromatography (HPLC)

NDLH was neutralized according to the protocol followed by Yu et al 2011[31] with little modifications. Calcium hydroxide was added in NDLH until pH reaches 7 while stirring at 30°C followed by centrifuge at 12000 rpm for 30 min to remove precipitate. 1 ml of NDLH was filtered with syringe filter and injected in HPLC system (Waters, USA) equipped with refractive index detector and BioradAminex HPX-87H column (Bio-Rad, USA) for peak identification and quantification. 0.005 M sulfuric acid was used as Mobile phase with flow rate of 0.6 mL min^{-1}. Peaks were identified and quantified based on retention time and calibration curve of standards respectively. Sugars analysis included glucose, xylose, and arabinose while inhibitors included acetic acid, HMF, Furfural.

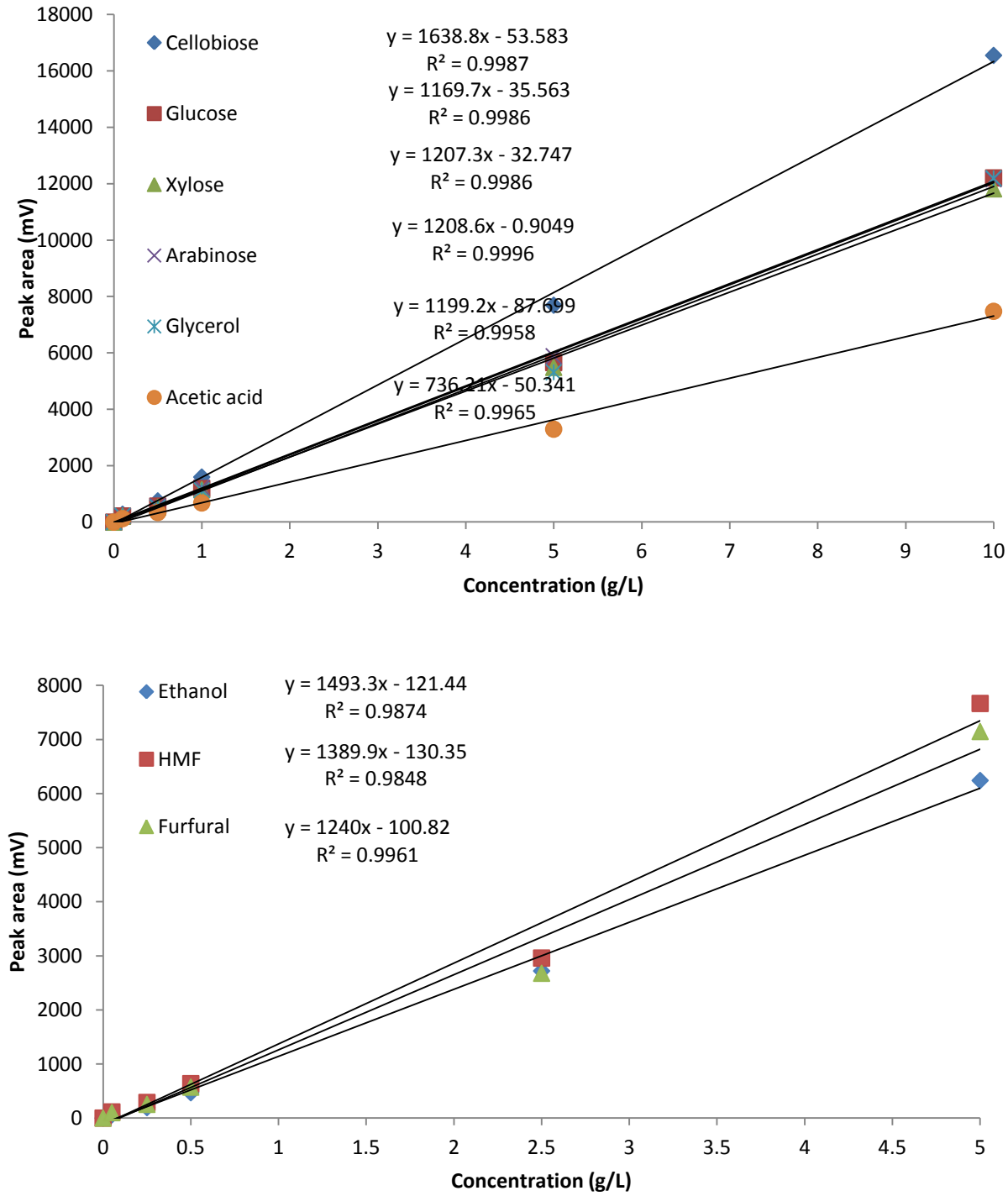

Fig.3 Standard curve for sugar and inhibitors

3.7.2Protein estimation of supernatant and pellet of autolysed spent yeast cells usingLowry method

Protein content of supernatant and pellet after autolysis of spend yeast cells were estimated by Lowry method. For Lowry analysis, 60 µl of supernatant (from autolysed spent yeast) was mixed with 940µl distilled water. Meanwhile pellet of autolysed spent yeast was first washed with distilled water followed by suspending in 1ml distilled water. From the resultant pellet solution, 60 µl was mixed with 940µl distilled water.

Lowry assay

Protein content was estimated using the Folin-Ciocalteau reagent according to Lowry's Method (Lowry *et al*, 1951).

Reagents:

(A) Solution 'A' 2% Sodium carbonate(Na_2Co_3) in 0.1N NaOH

(B) Solution 'B' 1% Sodium potassium tartarate in H_2O, 0.5% $CuSo_4.5H_2O$ in H_2O

(C) Reagent I: 50 ml of solution 'A' + 1ml of 'B'

(D) Reagent II: 1 part folin-phenol[2N] in 1 part of water

Reaction: 100 µL of protein/BSA was mixed with 900 µL of distil water. 5ml of freshly prepared solution "C" was added and incubated at room temperature for 10 minutes. To this, 0.5 ml Folin reagent was added and incubated in dark at room temperature for 30 minutes. The absorbance was measured @ 660nm in UV-1800 SHIMADZU UV-Spectrophotometer.

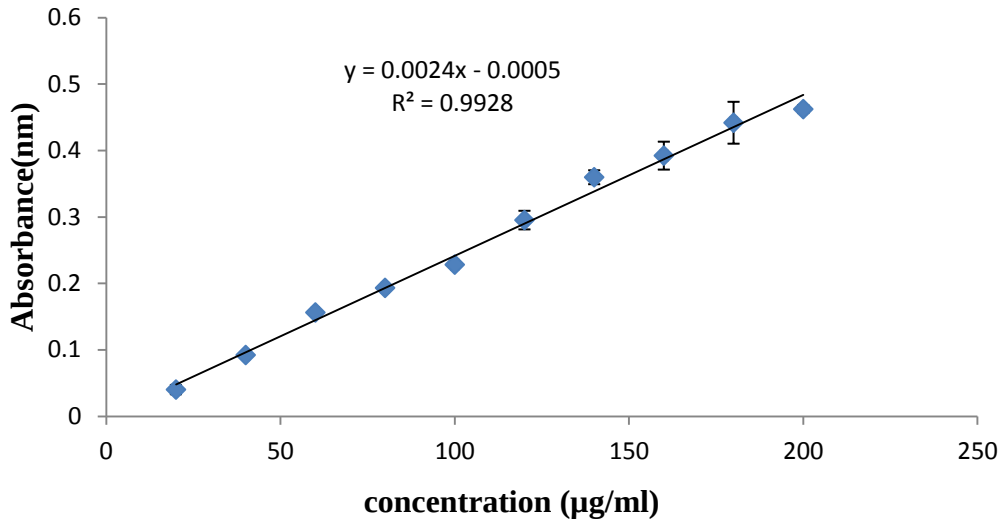

Fig. 4 Lowry standard curve for protein estimation

2.9 Lipid extraction and FAME analysis

For lipid extraction, protocol as reported by Gupta et al (2012), was followed with some modifications [5]. 10 mg of freeze dried biomass was suspended in 600 µl solvent (chloroform: methanol, 2:1) in 2 ml centrifuge tube. Chloroform was added first followed by methanol and vortexed for 3 minutes before centrifuge at 13000 rpm for 15 minutes. This process was repeated 2-3 times for complete extraction. Hexane was added into the lipid extract and upper layer was harvested carefully with Pasteur pipette followed by evaporation under nitrogen. Lipid was measured gravimetrically. For FAME analysis, 500 µl toluene was added in dried lipid extract followed by 100 µl internal standard (methyl tricosanoate C19:0) and 100 µl butylatedhydroxytoluene (BHT). Acidic methanol (400 µl) was added into tube and kept for 10-12 hours at 50 °C. 1 ml of 5 % NaCl was added followed by the addition of 1 ml hexane and upper layer was transferred into fresh tube. FAMEs were washed with 1 ml of 2 % Potassium bicarbonate and moisture was removed by passing it through anhydrous sodium sulphates. FAMEs were concentrated under nitrogen and analyzed with GC-FID system (Perkin Elmer clarus680, US), equipped with fast-GC capillary column (Omegawax100, 15 m x 0.1 mm, 0.1 µm thickness). 1 µl of FAMEs were injected at injector temperature 250 °C. Oven temperature was started increasing from initial 140°C to final 280 °C with ramping rate of 40 °C/sec and hold for 2 min. Detector was set at 260 °C. Hydrogen was used as carrier gas with velocity rate 50 cm sec^{-1}. Fatty acid peaks were identified and quantified with Totalchrome chromatography software (Perkin Elmer, US).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Pre-treatment of different lignocellulosic biomass and sugar/Inhibitor profile

Different lignocellulosic biomass (LCBs) such as wheat straw, sugarcane bagasse, corn cob, corn stalk and cotton stalk were taken for this study to assess the robustness of strain DBTIOC-ML3 to utilize pentose sugar rich non-detoxified liquid hydrolysate of different LCBs, since each LCB has different sugar and inhibitor profile (Fig.5). Xylose was predominant in NDLH (21 g/L to 36 g/L) of all the LCBs, since during pre-treatment condition, hemicellulose is reported to break first, into xylose and arabinose. Along with xylose, some amount of glucose (3 g/L to 10 g/L) was also reported into xylose rich NDLH. Xylose and glucose together constitute major portion of hemicellulose of all the LCBs. Apart from xylose and glucose, little amount of arabinose was also reported in the stream. Xylose units in hemicellulose are connected by acetyl group. Therefore upon Pre-treatment, acetyl group converts into acetic acid and comes in hemicellulosic streams of all NDLH. Maximum amount of acetic acid was reported in corn cob and corn stalk (around 3.28g/L). Furfural and HMF also came in stream due to some breakdown of xylose and glucose respectively.

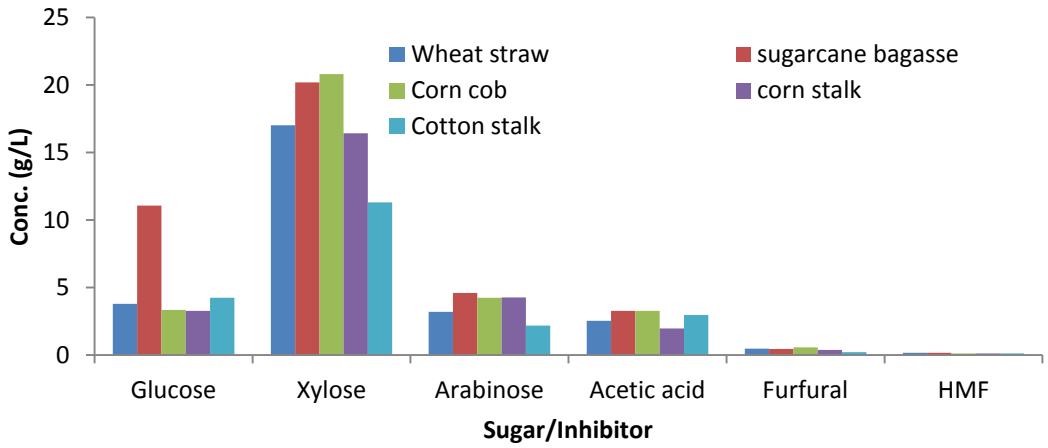

Fig. 5 Sugar and inhibitor profile of different LCBs at same Pre-treatment condition

4.2 Autolysis of spent yeast cells by osmotic shock

Once ethanol fermentation is finished, these spent yeast biomass is discarded. These yeast cells are composed of mainly amino acids, peptides, carbohydrates and salt. Therefore this biomass can be considered as potential sources of nitrogen and vitamins for the cultivation of important oleaginous microorganisms, however whole cell yeast cannot be utilized as such for nitrogen source. Cells need to be disrupted to release the nutrients in the supernatant. However employing cost and energy efficient method for cell disruption remains one of the major challenges. Osmotic shock induced autolysis is one of the most widely used method for yeast cell disruption. In this study 8% saline solution was used to give osmotic shock to the cells, which resulted into release of protein in the supernatant. This released protein content was measured to determine the degree of cell disruption.

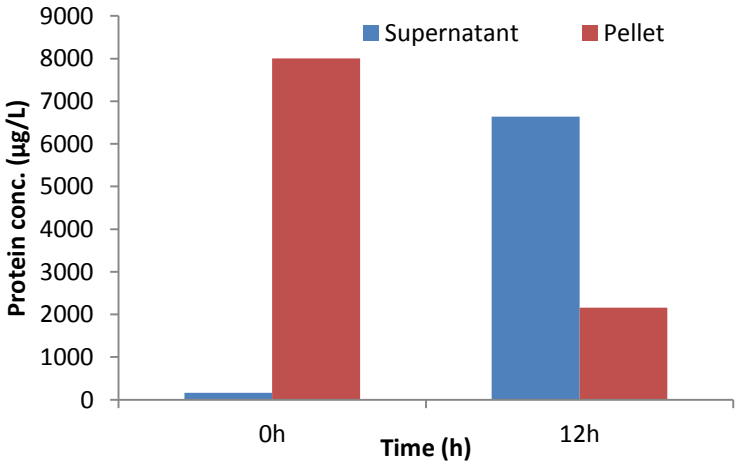

Fig.6 Concentration of the protein in supernatant and pellet upon autolysis

Fig.6 clearly shows the increased protein content in supernatant from 162.5 µg/ml at 0h to 6640 µg/ml at 24h, whereas protein content in pellet (biomass) decreased from 8001 µg/ml at 0h to 2160 µg/ml at 24h. The release of more than 80 % protein from pellet clearly underlines the efficiency of autolysis methodology. In the subsequent studies, this protein rich supernatant was used as nitrogen source for the cultivation of DBTIOC-ML3 strain.

4.3 Cultivation of strain DBTIOC-ML3 using NDLH of different LCBs and yeast extract

Strain DBTIOC-ML3 showed complete utilization of sugars and acetate from media within 4 day of cultivation in spite of presence of inhibitors in the media. Most of the oleaginous microbes can't grow in the presence of the inhibitors such as furfural and HMF. Oleaginous biomass varied from 7.12 g/L in cotton stalk to 14.05 g/L in corn cob. From the table.3, it is clear that biomass production is independent of type of LCBs and inhibitor concentration; however it is depending on total sugar content present in NDLH. This strain can tolerate broad range of inhibitor concentration. Maximum biomass (14.05 g/L) was reported with corn cob whereas lowest biomass (7.12 g/L) was recorded with cotton stalk. Interestingly after 4 d of cultivation, no furfural content was recorded in NDLH of all LCBs. Either furfural was consumed by the strain or it converted into HMF, since HMF concentration in the media went up in NDLH of all the LCBs after 4 d of cultivation.

Hydrolysates	Media	Glucose (g/l)	Xylose (g/l)	Acetate (g/l)	HMF (g/l)	Furfural (g/l)	**Biomass (g/L)**
Cotton stalk	Control (0d)	4.24	11.3	2.96	0.12	0.22	**7.12**
	Residual (4d)	0	0	0	0.33	0	
Corn cob	Control (0d)	3.33	20.80	3.28	0.12	0.57	**14.05**
	Residual (4d)	0	0	0	0.56	0	
Corn stalk	Control (0d)	3.27	16.41	1.97	0.13	0.39	**10.49**
	Residual (4d)	0	0	0	0.25	0	
Sugarcane	Control (0d)	11.07	20.18	3.26	0.17	0.46	**11.34**
	Residual (4d)	0	0	0	0.38	0	
Wheat straw	Control (0d)	3.78	17.02	2.54	0.16	0.47	**13.37**
	Residual (4d)	0	0	0	0.60	0	

Table.3 Sugar consumption and biomass production by DBTIOC-ML3 in different NDLH

4.4 Cultivation of strain DBTIOC-ML3 on different nitrogen source and NDLH of wheat straw

Nitrogen source type and content in the medium influence the biomass and lipid productivity in the fermentation since nitrogen content determines growth rate in initial phase of the culture followed by lipid accumulation phase triggered by nitrogen exhaustion in the medium. Different nitrogen sources are taken and metabolized through separate pathway [43]. In this work different organic and inorganic nitrogen sources such as yeast extract, ammonium chloride, ammonium acetate, potassium nitrate, sodium nitrate and were used as nitrogen source in the medium. Maximum biomass was observed with yeast extract ($12.31 gL^{-1}$) opposite to lowest with ammonium acetate (2.4 g/L) (Table.4). Addition of inorganic nitrogen sources in the media resulted into decreased biomass production. Evans et al (1984) observed the almost similar trend while culturing 17 oleaginous yeast on different organic and inorganic nitrogen sources [44]. Yeast extract is reported to have other nutrients such as vitamins, growth factors along with nitrogen. This can be the reason behind higher biomass production with yeast extract [45].

Nitrogen sources	Media	Glucose (g/l)	Xylose (g/l)	Acetate (g/l)	HMF (g/l)	Furfural (g/l)	**Biomass (g/L)**
Sodium nitrate	Control (0d)	11.64	25.29	3.06	0.48	0.28	
	Residual (4d)	4.84	1.90	0	1.47	0	**5.8**
Potassium Nitrate	Control (0d)	10.86	23.92	2.88	0.47	0.28	
	Residual (4d)	3.63	1.04	0	2.63	0	**6.46**
Ammonium chloride	Control (0d)	12.15	25.27	3.76	0.40	0.22	
	Residual (4d)	0.74	1.14	0	2.16	0	**7.45**
Ammonium acetate	Control (0d)	11.37	23.28	4.76	0.36	0.25	
	Residual (4d)	11.30	22.2	4.57	0.42	0.25	**2.4**
Yeast extract	Control (0d)	12.17	24.58	4.50	0.46	0.25	
	Residual (4d)	0.05	0.10	0	2.3	0	**12.31**

Table.4 Sugar consumption and biomass production by DBTIOC-ML3 on different nitrogensources

4.5 Cultivation of strain DBTIOC-ML3 on media containing different concentration of NDLH of wheat straw and supernatant of autolysed spent yeast cells

Different media compositions were prepared by mixing NDLH of wheat straw and supernatant of autolysed spent yeast cells in different proportions as mentioned in table.2. Most of the sugars was totally consumed within 4 d. Maximum biomass (12.4 g/L, 11.05 g/L) was recorded when NDLH and supernatant of autolysed spent yeast cells were mixed in 1:1 or 1.5:1 ratio respectively. However further increase in proportion of supernatant resulted into significant drop in biomass production. This clearly shows that biomass production is governed by amount of sugar present in medium. When ratio of NDLH and supernatant was decreased from 1:1 to 1:1.5, biomass was dropped from 12.4 g/L to 8.65 g/L respectively. Further drop in ratio resulted into restricted growth of the strain. When 100 % of supernatant of spent yeast cells was used as media, very little biomass was observed.

Media	Day	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)	**Biomass (g/L)**
	Control (0d)	19.06	36.29	5.82	3.70	0.59	0.41	
Medium 1	Residual (4d)	17.97	37.88	0.29	3.18	0.67	0.40	**1.9**
	Control (0d)	14.57	26.82	4.61	2.98	0.42	0.22	
Medium 2	Residual (4d)	0.06	4.28	0.19	0.83	0.73	0	**11.05**
	Control (0d)	10.42	18.18	3.49	2.11	0.29	0.18	
Medium 3	Residual (4d)	0	0.37	0	0	0.35	0	**12.4**
	Control (0d)	5.92	9.06	2.22	1.32	0.16	0	
Medium 4	Residual (4d)	0	0.23	0.01	0.10	0.35	0	**8.65**
	Control (0d)	1.44	0	0	0	0	0	
Medium 5	Residual (4d)	0	0	0	0	0	0	**0.65**
	Control (0d)	18.88	35.37	5.86	3.69	0.54	0.35	
Medium 6	Residual (4d)	1.18	20.93	5.18	0	0.81	0	**11.1**

Table.5 Sugar consumption and biomass production by DBTIOC-ML3 on different media composition

4.6 Cultivation of strain DBTIOC-ML3 on media containing NDLH of different LCBs and supernatant or pellet of autolysed spent yeast cells

Ethanologenic yeasts are widely used in breweries, however with the recent push in bioethanol production from 1st and 2nd generation carbon sources, large amount of spent yeast is expected to be generated. This spent yeast cannot be used back for ethanol fermentation however these cells are rich in nutrients. Therefore these yeasts cells were autolysed with saline solution to release nutrients in supernatant. However cells are not totally autolysed thus leaving behind significant amount of nutrients in the cells (fig.6). Therefore if pellet and nutrient rich supernatant can be utilized as nitrogen source, this will add value to the spent yeast cell leading to value addition to ethanol fermentation. In this study, nutrient rich supernatant and pellet of yeast cells were separately mixed with NDLH of different LCBs. Although significant amount of inhibitors i.e. acetic acid, HMF and furfural are presents in all NDLH, oleaginous yeast strain DBTIOC-ML3 was able to significantly utilize all the carbon source including acetate and furfural from media. This is an important characteristic of this strain, utilizing sugars from NDLH and nitrogen source from spent yeast cell, thus totally utilizing nutrients from waste resources. Ability of this strain to utilize broad range of NDLH from different LCBs also reflects the robustness of this strain despite the presence of different concentration of inhibitors. With nutrient rich supernatant as nitrogen source, maximum biomass was recorded with wheat straw (14.0 g/L) followed by corn stalk (13.18 g/L) (Table 5a). Lowest biomass (10.75 g/L) was recorded with sugarcane bagasse. Similar trend was observed when pellet from autolysed spent yeast cells were used as nitrogen source in the media (table.5b), however overall biomass production and sugar consumption was lower as compare to culture grown in media containing nutrient rich supernatant as nitrogen source. Since nitrogen content in pellet was almost one third of the supernatant. This could be one of the probable reason for low biomass production and sugar consumption in the medium.

LCBs	Day	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)	**Biomass (g/L)**
	Control (0d)	2.34	16.12	3.14	2.22	0.32	1.01	
Cotton	Residual (4d)	0	0	0	0	0.65	0	**11.50**
	Control (0d)	2.19	15.42	2.13	2.18	0.19	1.29	
Corn stalk	Residual (4d)	0	0	0	0	0.24	0	**13.18**
	Control (0d)	5.47	11.0	2.12	2.04	0.24	1.5	
Corn cob	Residual (4d)	0	0	0	0	0.41	0	**12.33**
	Control (0d)	3.92	18.99	2.38	1.97	0.32	0.91	
Sugarcane	Residual (4d)	0	0	0	0	0.51	0	**10.75**
	Control (0d)	4.24	15.43	2.60	1.64	0.29	1.34	
Wheat straw	Residual (4d)	0	0	0	0	0.41	0	**14.0**

LCBs	Day	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)	**Biomass (g/L)**
	Control (0d)	6.94	13.47	1.15	1.71	0.12	0.51	
Cotton	Residual (4d)	0	2.14	0	0	0.18	0	**8.65**
	Control (0d)	5.12	14.82	0	1.12	0.11	1.11	
Corn stalk	Residual (4d)	0	3.15	0	0	0.22	0	**8.91**
	Control (0d)	5.81	21.24	0	1.75	0.22	1.22	
Corn cob	Residual (4d)	0	6.15	0	0	0.32	0	**8.31**
	Control (0d)	11.07	14.87	0	1.67	0.11	0.71	
Sugarcane	Residual (4d)	0	4.15	0	0	0.21	0	**9.45**
	Control (0d)	6.55	18.07	0	1.58	0.19	0.45	
Wheat straw	Residual (4d)	0	7.13	0	0	0.21	0	**7.34**

Table.6 Sugar consumption and biomass production by DBTIOC-ML3 on NDLH of different LCBs and (a) supernatant (b) pellet of spent yeast cells

4.7 Cultivation of ML-3 in the media containing different lignocellulosic substrates and whole cell spent yeast broth.

From section 4.6, it is clear that supernatant and pellet of autolysed spent yeast from ethanol fermentation can be used as nitrogen source for the cultivation of oleaginous yeast DBTIOC-ML3. However autolysis of spent yeast by osmotic shock not only increases cost of the process but also energy footprint of the process, since lot of energy will be required to maintain temperature at 60°C to carry out osmotic shock. Therefore if broth containing yeast cells after ethanol fermentation can be used as such for nitrogen source in the medium, this will save energy and money both. Therefore in these experiments, in one case spent yeast broth was directly added into acidic NDLH. Since acidic pH coupled with high temperature will drive the autolysis of yeast cells during pre-treatment of LCBs. With nutrient rich nitrogen source, maximum biomass was recorded with corn cob (11.35 g/L) followed by wheat straw (10.63 g/L) (Table 6). Lowest biomass (6.54 g/L) was recorded with cotton stalk. Cotton stalk also had lowest sugar. In the second case, whole cell yeast broth was mixed with NDLH of different LCBS in 1:1 ratio and heated at 100 °C for 10 min. Subsequently this combination was used as media for the cultivation. Significant biomass was recorded in this experiment along with total sugar consumption. This approach gives a real advantage over other methods using spent yeast as nitrogen source. This method gives ease of integration with ethanol plant, where heated and acidic NDLH stream from different LCBs is mixed with whole cell spent yeast broth for some time. Heating and acidic pH will drive the autolysis of the cell. The resultant solution can be directly used as media for the cultivation of oleaginous yeast *Rhodotorula* sp. DBTIOC-ML3 for oil production. This is expected to bring down the production cost of oil significantly, since both of media component does not fetch premium prices in the market.

LCBs	Day	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)	**Biomass (g/L)**
	Control (0d)	1.15	8.12	1.12	2.01	0.17	0.48	
Cotton stalk	Residual (4d)	0	0	0	0	0.21	0	**6.54**
	Control (0d)	1.88	8.50	1.77	1.03	0.14	0.43	
Corn stalk	Residual (4d)	0	0	0	0	023	0	**9.63**
	Control (0d)	1.81	13.02	1.16	1.53	0.12	0.51	
Corn cob	Residual (4d)	0	0	0	0	0.32	0	**11.35**
	Control (0d)	0.70	12.12	2.11	1.35	0.11	0.35	
Sugarcane	Residual (4d)	0	0	0	0	0.25	0	**9.50**
	Control (0d)	2.04	12.77	1.11	1.46	0.08	0.31	
Wheat straw	Residual (4d)	0	0	0	0	0.15	0	**10.63**

Table.7 Sugar consumption and biomass production by DBTIOC-ML3 on media containing NDLH of different LCBs and whole cell spent yeast culture

4.8 Fatty acid profile of strain DBTIOC-ML3

Fatty acid analysis of strain DBTIOC-ML3 showed that major fatty acids were palmitic acid (C16:0) and oleic acid (C18:1) comprising 70 % to 90% of the total fatty acids (TFAs), irrespective of culture conditions. This shows the suitability of this strain for biodiesel production. Palmitoleic acid (C16:1) and stearic acid (C18:0) are the other major fatty acids present, followed by traces of linoleic acid and linolenic acid (fig.1). NDLH of different LCBs appear to favour the production of oleic acid (around 43%-50 % of TFA) over palmitic acid (around 28%-41% of TFA). However the opposite trend was observed when glycerol was used as the carbon source, with palmitic acid becoming the major FA (46 % of TFA) with oleic acid at 28% of TFA (fig.1). The FAME profile of oil extracted from biomass grown on yeast extract and ammonium chloride were similar, with the majority of fatty acids being palmitic acid (almost 34% - 41% of TFA) and oleic acid (42% to 45% of TFA). The amount of palmitoleicacid

present was 24% of TFA, which was relatively high compared with that produced in other medium. Maximum amount of palmitic acid was produced when yeast extract was used as nitrogen source, whereas oleic acid levels were high when ammonium chloride was used as the nitrogen source. With yeast extract palmitic acid content was around 30% of TFA however oleic acid remained around 50% of TFA. Other nitrogen sources such as ammonium acetate, potassium nitrate, sodium nitrate resulted in a similar fatty acid profile. Irrespective of which carbon source was used an increase in the carbon source resulted in the combination of palmitic acid (C16:0) and oleic acid (C18:1) constituting almost 90 % of TFA (fig.2). Oleic acid content was higher in cultures fed with xylose (46% of TFA) when comparing with glucose (37% of TFA) irrespective of C/N ratio. Thus, level of palmitic acid and oleic acid in the oil can be tweaked at desirable level by changing culture conditions.

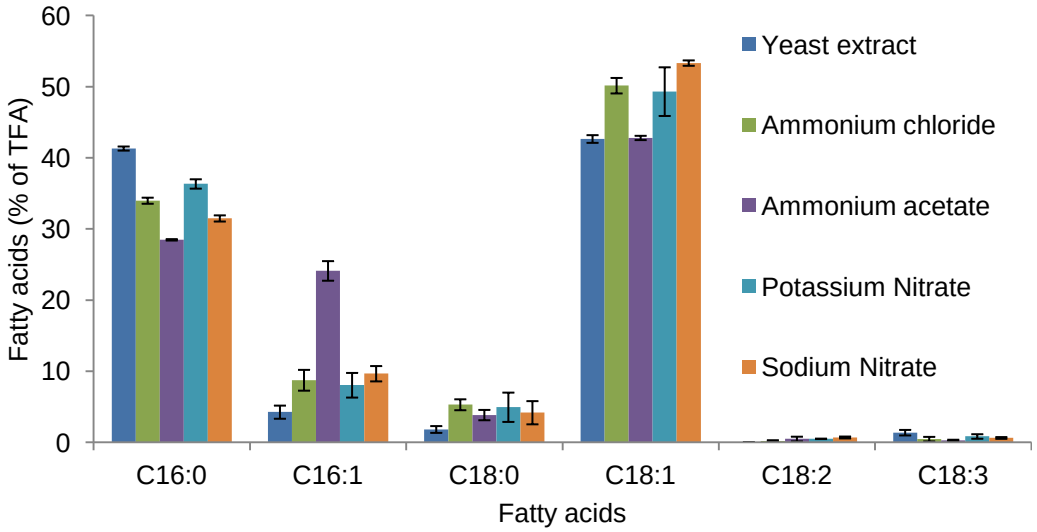

Fig.7 Fatty acid profile of strain *Rhodotorula* sp.DBTIOC-ML3 under different nitrogen sources

CONCLUSION AND FUTURE DIRECTIONS

Oleaginous yeast *Rhodotorula* sp.DBTIOC-ML3 can utilize broad range of NDLH of different LCBs along with different nitrogen sources. Although inorganic nitrogen sources are comparatively cheaper than yeast extract but biomass production and sugar consumption from NDLHS streams was significantly lower than yeast extract. Therefore in this study spent yeast from ethanol fermentation were tried as nitrogen source for the cultivation of this strain. Autolysis of spent yeast resulted into release of protein and other nutrients from cell, which were later used as nitrogen source in the media. This strain can efficiently utilize autolysed spent yeast as nitrogen source. Biomass production and sugar consumption was comparable with yeast extract. Therefore spent yeast is promising and cost effective alternative of yeast extract without compromising biomass and lipid productivity. Fatty acid profile of this strain revealed the suitability of the extracted lipid for biodiesel production, since it contained large amount of oleic acid and palmitic acid along with traces of poly unsaturated fatty acids.

Most of the work in this study was done in shake flasks. However moving from shake flask to bioreactor may further increase biomass production and sugar consumption rate, since all the parameters affecting the productivity can be controlled. These parameters include pH, agitation, mixing, gas flow, dissolved oxygen and mass transfer rate. Lipid content was also around 25 % of dry biomass in shake flask studies. Therefore a fed batch or continuous system can be designed in a way, where nitrogen stress can be induced in the bioreactor to enhance lipid content.

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