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Biodiesel Production from Oleaginous Yeast: A Review

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ABSTRACT

This study explored a strategy to convert agricultural residues into microbial lipid, which could be further transformed into biodiesel. Demand of alternative fuels is increasing day by day due to the present crisis of petroleum based fuels. Biodiesel is one of the most demanding alternative fuels. One probable solution is to use microorganisms; especially oleaginous species, because of their higher lipid content and almost similar composition as plant/animal lipid. For this experiment, yeast was selected because of several reasons like easy availability, rapid growth rate, and higher lipid accumulation capacity, capable to grow on a variety of media etc. Among the 250 yeast strains screened for xylose assimilating capacity, eight oleaginous yeasts were selected by Sudan Black B test. The lipid content of these 8 strains was determined by soxhlet extraction method. The optimal fermentation conditions were obtained as follows: glucose as carbon source 100 g/l; yeast extract and peptone as nitrogen sources at, respectively, 8 and 3 g/l; initial p^H of 5.0; Inoculation volume of 5%; temperature at 28°C, shaking speed of 180 r/min, cultivated for 96 h. More encouraging results were observed for the lipid production with alternative carbon sources. In this experiment, yeast was adapted to accumulate maximum quantity of lipids by providing metabolic stress condition, which was later converted into biodiesel by acid transesterification. Elimination of lipid extraction step has made the process much faster and easier as compared to traditional methods. Presence of fatty acid methyl esters were confirmed by high performance thin layer chromatography (HPTLC) and gas chromatography (GC). Fatty acids composition was determined by gas chromatography (GC) by comparing with standards.

Keywords: *Oleaginous yeast, Economic production of biodiesel, Yeast biomass, Single step transesterification method*

INTRODUCTION

Biodiesel can be an interesting alternative for energy resource and may be used as substitute for petroleum-based diesel. Increasing in its production, biodiesel has attracted a broad public interest. The conventional method for biodiesel production is to transesterify plant oil with methanol (Krawczyk, 1996; Ma and Hanna, 1999). However, the cost of biodiesel is currently more expensive than that of conventional diesel due to high cost share (70 - 85%) of the raw material. Increasing interest is generated to explore ways to reduce the high cost of biodiesel, especially the cost of the raw materials (Wu and Miao, 2006). If biodiesel can be produced from agricultural and forestry residues, the environmental benefits can be much more significant than the economic benefits (Hill, *et al*., 2006).

Biodiesel, a non-petroleum based diesel fuel could be defined as alkyl esters of long chain fatty acids. In the principle process of biodiesel production which is also known as transesterification, lipids are converted into fatty acid methyl/ethyl esters in the presence of suitable catalyst and alcohols. These catalysts may be either chemical or enzymatic. (Ataya, *et al*., 2007; Bajpai and Tyagi, 2006; Demirbas, 2008; Lotero, *et*

al., 2005; Vasudevan and Briggs, 2008). Biodiesel production is based on either oleaginous yeast sources. (Vasudevan and Briggs, 2008; Canakci and Sanli, 2008; Bhattacharyya, 1999; Pinto, *et al*., 2005). Problem with these kinds of sources are their limited availability which becomes a major limiting factor in biodiesel production. Now a days, scientists have also focused on algae (micro and macro) and genetically modified microorganisms as an alternative oleaginous yeast due to limited availability of other sources. (Kalscheuer, *et al*., 2006; Aresta, *et al*., 2005; Chisti, 2007). It is assumed that, as this biodiesel is produced from biological method it is renewable, eco-friendly and biodegradable. Reference have also shown that this kind of biodiesel contains less sulfur as well as CO and it also lacks polyaromatic hydrocarbons as present in petroleum diesel (Bajpai and Tyagi, 2006).

As mentioned before, major obstacles with oleaginous yeast based sources are their higher cost and lesser availability. Scientists have focused on the oleaginous microorganisms, as it is possible to produce sufficient biomass by using fermentors and ultimately production of higher quantity of lipids in the form of stored lipids within the cell. (Li, Du and Liu, 2008). Other advantages also include smaller area for production as easy and rapid oil extraction methods and ability to grow on a wide variety of media. Yeast, fungi and certain microalgae are examples for such microorganisms. (Li, Du and Liu, 2008; Antoni, *et al*., 2007). Major lipid components found in yeast and fungi are triglycerides having C16 and C18 long chain fatty acids, which are very similar to the vegetable oil. (Blagovi, *et al*., 2001; Gill, *et al*., 1977). Under normal growth conditions, microorganisms do not accumulate higher quantity of lipid, but their ability could be enhanced by providing metabolic stress conditions. (Gill, *et al*., 1977; Zhao, *et al*., 2008). One of the most common and widely used methods is to provide limited supply of nitrogen with excess carbon source. Under nitrogen limiting condition, replication of yeast is inhibited after certain growth cycles and excess sugar is accumulated in the form of triglycerides within cell as reserved energy source. (Gill, *et al*.,1977; Mulliner and Daum, 2004).

Yeasts

Yeasts are defined as ascomycetous or basidiomycetous fungi that are capable of reproducing by budding or fission and form spores which are not enclosed in a fruiting body (Boekhout and Kurtzman,1996). They are first classified based on its sexuality (Ascomycotina or Basidiomycotina) or the lack of sexual phase in the life cycle (Deuteromycotina). The lower taxonomic subdivisions (families, subfamilies, genera, species and strain) are determined by its morphological, physiological and genetic characteristics including sexual reproduction (Kurtzman and Fell, 1998).

Yeasts Diversity

The number of discovered yeasts has been increasing from year to year. More than 2500 yeast species were published by 2005. It is assumed that only 1% of yeast species is currently known which represents approximately 1500 species. The total numbers of yeast species on earth are expected to reach 150,000 (Barriga, *et al*., 2011). The diversity of yeast species in particular niches is determined by its capability of utilizing different carbon source and its nutritional selectivity as it exhibits great specialization for habitat (Phaff and Starmer, 1987). Yeasts can be isolated from the terrestrial, aquatic and aerial environment. Plant is the preferred habitat of yeasts community. A few species are found to have commensalism or parasitic relationships with animals. Extreme environments like low water potential (high sugar or salt concentration) and low temperature may be inhabited by yeasts (Walker, 1998; Walker, 2009).

There are a broad diversity of yeast cells including its size, shape and colour. Cell sizes of yeasts are influenced by its species and growth condition. The length of some yeast cells are only 2–3 µm while the other species may reach the length of 20–50 µm (Phaff and Starmer, 1987). Most yeasts have a width in the range of 1–10 µm. Generally, the sizes of brewing strains of *S. cerevisiae* are larger than laboratory strains (Hough, *et al*.,1987). Many yeast species including *Saccharomyces spp*. are ellipsoidal or ovoid in shape and have creamy colour colonies (Walker, 1998; Walker, 2009).

In the present study, yeast was isolated and adapted for accumulation of lipids by providing metabolic stress conditions. Yeast biomass was directly used for acid transesterification without prior lipid extraction; Presence of fatty acid methyl esters was determined by HPTLC and GC.

Immobilization

Immobilized cell technology is commonly applied in fermentation process. The benefits of immobilized cells over free cells include higher cell density per volume of reactor, easier separation from the reaction medium, higher substrate conversion, less inhibition by products, shorter reaction time and control of cell replication (Duarte, *et al.,*2013). The immobilization of yeast cells and its productivity are influenced by several factors such as the surface characteristics of the carrier, pore size, water content, hydrophilicity and magnetism (Vucurovic, *et al.,* 2009). Immobilization should be performed under mild condition to maintain the activity of the cells (Calinescu, *et al.,* 2012).

Immobilization of yeast cells

Cells can be immobilized by different types of methods like adsorption, crosslinking, encapsulation and entrapment. Entrapment is carried out by the polymerization of an aqueous solution of acrylamide monomers in which microorganisms are suspended. It is commonly used to overcome the problems of degradation and limitation of mass transfer. It avoids the release of cells while allowing diffusion of substrates and products(Chibata and Wingard,1983). This method allows high biomass loading which results in high ethanol productivity. Entrapment method is widely used due to its simplicity, non-toxic, less expensive, reversible and good mechanical properties. Entrapment can be operated at extremely high dilution rates without causing washout of cells. Most of the researches involving the immobilization of microbial cells were focused on gel entrapment. The most commonly used gels are in the form of spherical beads with diameters in the range of 0.3–5 mm. However, gel has limited mechanical stability which can be easily damaged by the growth of the microbial cells and carbon dioxide production. Moreover, the presence of phosphates causes the weakening of calcium alginate gels (Margaritis and Merchant, 1987).

Adsorption is a very popular way of cell immobilization due to its simple, cheap and fast method. Cells are attached to the surface of the material by electrostatic force such as Van der Waals forces, ionic bonds, hydrogen bridges or covalent interactions. Ionic attraction is used to immobilize yeast cells. The supporting material used must have a high affinity in order for the yeast strain to withstand the environmental conditions present within the bioreactor. In most cases of continuous ethanol production, adsorption is carried out by circulating a concentrated suspension of yeast cells through the bioreactor for several hours. Adsorption technique does not require the use of toxic chemical and the yeast cells can be maintained in a viable state. Theabsorbed-cell system is limited by lower biomass loading and lower feed flow rates compared to entrapped-cell system. This is because the number of yeast cells that can be absorbed on the carrier is limited by the surface area of the carrier (Margaritis and Merchant, 1987).

The other commonly used method for cells immobilization is encapsulation which encloses cells within a thin semi-permeable membrane. The cells are free to move in the inner liquid core inside the capsule. However, the space is limited by the outer membrane (Ylitervo, *et al.,* 2011). In fermentation, the molecular dimensions of the microcapsules limit the growth of cells and the size of both nutrients and products. The rate of substrate transfer into the capsules will determine the rate of reaction. Encapsulation method gives several advantages such as mechanical and chemical stability of the membrane system, possibility of high loading and regulation of the fermentation reaction by selective diffusion of substrate and products (Phillips and Poon, 1988).

There are many types of supporting materials that have been used in yeast cells immobilization such as calcium alginate, sugarcane bagasse, delignified cellulosic materials, orange peel, spent grains, corn cobs, kcarrageenan, wood blocks, porous cellulose, zeolite, loofa sponges and sorghum bagasse (Yu, *et al.,* 2007). The support used in immobilization must be conducive to cell viability and have proper permeability for the diffusion of oxygen, essential nutrients, metabolic waste and secretory products across the polymer network. There are two types of polymers that are used as carrier in yeasts immobilization which are natural and synthetic polymers. The benefits of using natural polymers are low price and no impurities produced from chemical reaction. Synthetic polymers exhibit high chemical and biological stability, mechanical resistance to abrasion, permeable to reagents, and have large surface, capacity and porosity (Stolarzewicz, *et al.,* 2011).

METHODS

Cell biomass determination for fungi

The fungal biomass of screened isolates, mycelia were harvested from the incubated flasks by suction filtration through Whatman No 1 filter paper and thoroughly washed with distilled water. Then filtered mycelia dried at 60° C in an oven for 15 hrs. The weight of the dried sample was taken and dry biomass was expressed in gl-1.

Cultural Condition

50 ml of this broth were dispensed in 250 ml conical flasks, sterilized and incubated at 30°C, 200 rpm for 7 days in an incubator shaker. The fungal mycelium was filtered through Whatman No.1 filter paper. Liquids and biomass content of filtered fungal mycelium were analyzed. Based on their earlier study, glucose was found to be the best source for lipid production. Hence the glucose was taken at different concentration to find out the optimum level for maximum lipids production.

Oil production of selected oleaginous fungi in flask cultivation experiments

Identified fungi were cultivated in 250 ml conical flasks containing 50 ml of the oil production medium in the incubator shaker at 30°C and a shaking speed of 200 rpm. In order to select the optimal conditions, effects of carbon sources (glucose, fructose, sucrose and lactose), nitrogen sources (ammonium chloride – NH_4Cl, ammonium sulphate – $(NH_4)_2SO_4$, and yeas extract), temperature (20, 25, 30, 35 and 40°C) and initial p^H levels (5.5, 6.5, 7.5 and 8.5) on oil production of the selected fungal species were investigated. The carbon utilization pattern and lipid production by the selected fungal species were studied under different concentrations of glucose and nitrogen. The lipid content in biomass and the biomass growth was measured every day. All the above experiments were conducted with three replications and culturing was performed in an incubator shaker at 30°C, 200 rpm for 7 days.

Extraction of fungal lipids

Fungal lipids were extracted from the dried mycelia as described by Bligh and Tyer method (Bligh and Dyer, 1959). The influence of different nutritional (carbon and nitrogen source) and growth conditions (p^H and temperature). The lipids and biomass production by fungal isolate with different carbon sources were also studied with following composition Glucose 30.0 (0.16 M), Yeast extract 5.0 g/l, Distilled water 1000 ml, p^H 5.4, Glucose in the broth was replaced individually with different carbon sources. (Ahmed, *et al*., 2006).Different carbon sources like glucose, fructose, sucrose and lactose were used in the concentration of 0.16 (M) in one gram of fungal isolate as mycelial suspension.

They present great diversity. They grow exponentially fast as they divide once every 20 to 90 min, they are able to utilize cheap substrates, they can be cultivated at large scale, spontaneous mutants can be easily isolated and strains can be genetically manipulated. Among microorganisms, yeast seem to be the most adapted microorganisms for biotechnological applications as bacteria store excess carbon as polysaccharides, and lipids
mostly in form polyhydroxyalkanoates or wax ester, while yeasts accumulate carbon as glycogen and lipids mostly in the form of TAG.

Fewer than 30 of the 600 known species of yeasts are found to be oleaginous. The best known oleaginous yeasts are typically found, but not exclusively, in genera such as *Candida, Cryptococcus, Rhodotorula, Rhizopus, Trichosporon, Lipomyces* and *Yarrowia* (Ratlege*et al.,* 1990). On average, these yeasts accumulate lipids to levels corresponding to 40% of their biomass. However, under conditions of nutrient limitation, these levels may exceed 70% of their cell dry weight (CDW). The lipid contents and fatty acid profiles of some representative oleaginous yeast are presented in table 1. We observe that lipid content and profile differs between species, however it can be acknowledged that the main FA produced by oleaginous yeast are similar to those produced by plants and are mainly consisted by: myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linoleic acid (C18:3).

Incidentally, this is the type of oil composition that has been recommended for biodiesel production (Steen et al., 2010). Actually, the most common feedstock used for its production is rapeseed and soybean oil, having FA profile similar to yeasts. This profile is crucial for the utility of biodiesel as defines the physical and chemical properties of the lipid mixture, i.e viscosity, energetic density and melting point. The above specifications refer to the use of

biodiesel in standard diesel engines, alone or blended with regular diesel fuel. Otherwise, biodiesel can also be used as a low carbon alternative to heating oil.

Furthermore, the development of genetic tools for yeast engineering coupled with culture strategy give us the ability to control and modulate the fatty acid profile produced. This can broaden the utility of the FA mixture produced and meet the specifications of aviation fuels or the needs of the oleo chemical industry. Another advantage is that estimation of quantitative and qualitative productivity of the yeast process is superior to plant cultivation as fermentation is not subjected to climate changes, microbial or insect infections. This could lead to the definition of an industrial process with identical performances around the world. However, a low cost starting material is essential for the economic viability of the process.

To this end, we should acknowledge the yeasts ability to convert renewable carbohydrates or industrial by-products such as glycerol or lactose into fatty acids. The major byproduct in conventional biodiesel production is glycerol: for every tone of biodiesel manufactured, 100 kg of glycerol is produced. As a consequence, the increase in global biodiesel production resulted in a crash to glycerol's market price (Thurmond, 2008). It would be thus a great opportunity for yeasts to use this cheap by-product as starting material to regenerate fatty acids for biodiesel production in a recycling nature process. In order to better understand the potential of yeasts as oil producers, we would try to briefly overview the mechanisms of the cell leading to lipid accumulation. We would also try to provide information about the advances made in order to increase the amount of lipids produced and to modify the FA profile.

Table 1: Lipid accumulation and fatty acid profiles of selected oleaginous yeast

Species	Lipid content (% CDW)	Major fatty acid residues (% w/w)					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Cryptococcus curvatus	58	25	Trace	10	57	7	0
Cryptococcus albidus	65	12	1	3	73	12	0
Candida sp	42	44	5	8	31	9	1
Lipomycesstarkeyi	63	34	6	5	51	3	0
Rhodotorulaglutinis	72	37	1	3	47	8	0
Rhodotorulagraminis	36	30	2	12	36	15	4
Rhizopusarrhizus	57	18	0	6	22	10	12
Trichosporonpullulans	65	15	0	2	57	24	1
YarrowiaLipolytica	36	11	6	1	28	51	1

Extraction of Lipid

Lipid was extracted only for comparative analysis in HPTLC and to calculate percentage conversion of total lipids into biodiesel. After 5 days of incubation on a rotary shaker at 200 rpm at 25°C, produced biomass was collected by centrifugation at 8500 rpm for 20mins. The pellet was washed twice with distilled water and used for lipid extraction. Blight and dyer method with some modifications was used for lipid extraction (Blight and Dyer 1959). In the process, cell suspension was prepared by suspending known quantity of biomass in known volume of sterile distilled water. To this mixture of chloroform: methanol (1:2) (3.75 ml/ml) of suspension) was added and vortex at least for 15mins. Then 1.5ml of chloroform/ml of suspension was added and mixed for 2 minutes. In the next step, 1.5ml of distilled water/ml was added and mixed. After centrifugation at 8000 rpm for 10mins, lower solvent phase was collected and dried at room temperature. The residue was suspended in known volume of chloroform: methanol (2:1) and stored at 4 -8 °C.

Biomass Production

To screen and select the highest biodiesel producer among purified fungal isolates, they were cultured in basal medium (in g/l: yeast extract 0.5, $MgSO_4.7H_2O$ 0.4, KH_2PO_4 2.0, $CaC1_2$ 0.5, $CuSO_4$ $5H_2O$ 0.05 and 5% glucose (w/v), with initial p^H 6). Flasks were removed every 24 hours for seven days and microbial cells were harvested from the media by centrifugation and washed with distilled water three times, then

freeze dried at -50°C. Exacted weight was taken, and then total lipids were extracted from the dried biomass with chloroform: methanol, volume ratio of 2:1. Ultrasonication to favor cell membrane disruption during extraction was done. The mixture containing extracted lipids was separated from residual biomass by centrifugation and the solvent fraction was transferred to a new tube. Then the residual of solvent was removed in a rotary evaporator followed by lyophilization to determine the ratio of extracted lipids in compare to the cell dry weight.

Screening for oleaginous fungi

After reaching to the pure culture, fungi were allowed to grow for 3 days. So, the amount of oleaginous microorganisms and the content of lipid accumulated could reach a certain level. Subsequently, the isolates that appeared earlier and grew the fastest were picked to visualize the intracellular lipids inside fungal cells. Fungal biomass was stored in a dark with 0.5 ml PBS solution and 0.05 ml Nile-red solution (Nile red 25 µg Nile-red/ acetone 1000 ml) for 30 min (Lim, *et al*., 2003). Then, stained lipid bodies were photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan).

Direct Transesterification

Among the various methods of transesterification, acid transesterification was chosen for production of FAMEs. Acid transesterification was selected because studies (Demirbas, 2008; Fallon *et al.,* 2007). have shown that, it is possible to convert variety of lipids into their respective esters by acid transesterification without prior extraction. In this modified process, dried yeast biomass was mixed with 20 volume of methanol containing concentrated sulfuric acid to a final concentration of 0.02 mol/l. Reaction was carried out in a tightly closed glass bottle at 70° C with constant stirring for 24 hrs. At the end of reaction, suspension was cooled and filtered. To the filtrate, hexane was added to recover the FAMEs. Upper hexane layer containing FAMEs was collected and used for various analyses. Similar procedure was used to produce FAMEs from yeast grown under normal condition to compare FAMEs composition produced by yeast grown under nitrogen limiting media.

Screening of oleaginous yeast

The isolated 250 yeast strains were further screened using a rapid and sensitive technique. The cells were stained with Sudan Black B so that the absorbance measured at 580 nm gave lipid concentration in the fermentation broth using unstained cells as control (Thakur, 1989). The total lipid concentration of the selected 8 strains was determined by Soxhlet extraction method (Dai et al., 2005). The 8 oleaginous yeast strains were precultured in inoculum medium, and then 24 h old preculture was inoculated on nitrogen-limited medium for 3 days at 28°C with shaking at 140 r/min in 2000 ml Erlenmeyer flasks containing 500 ml media.

Analytical methods

Cell biomass was determined after harvesting mycelia from the cultures by filtration through Whatman No.1 filter paper. The harvested mycelia were thoroughly washed with sterile distilled water and then dried at 60°C in an oven for 15 h. Residual glucose in the culture filtrate was determined in accordance with the method used by (Somogyi, 1952). Fungal oils were extracted from the dried mycelia using the solvent choloroform: methanol (2:1) using a soxhelt extractor (Bligh and Dyer, 1959) and then the solvents were evaporated in a rotary evaporator. The amount of oils was measured using gravimetric method.

The fatty acids present in the fungal oils were determined after conversion into fatty acid methyl esters (FAMEs). The method was used by Morison and Smith (1964). After addition of 2 ml of 0.5 M KOH in methanol to the oils along with two standards (pentadeconoic acid and ribitol), the mixture was boiled in a water bath at 90°C for 30 min. After cooling down to ambient temperature, 2 ml of 14% BF_3 in methanol was added and the mixture was boiled at 90°C for 30 min. After cooling down, 2 ml of water and 1 ml of hexane was added and the mixture was centrifuged at 5000 rpm at 28°C. The top liquid layer was filtered through 0.22 µm cellulose acetate membrane filter paper and then analyzed with gas chromatography (GC) (ASTM American standard for testing of materials). The GC conditions were 70 ev (m/Z) 50-550, source temperature at 230°C and quadruple temperature at 150°C in the EI mode with an HP-5ms capillary column (30 m x 0.25 mm i.d., 0.25 mm film thickness; J&W scientific, USA). Helium was the carrier gas at a flowrate of 1.0 ml/min. The inlet temperature was 300°C and the

oven temperature was programmed at 150°C for 2 min, increase at 4°C /min up to 300°C and then at 300°C for 20 min. Samples were injected at 1µl with a split ratio of 50:1. The extracted crude oil samples were subjected to the analysis of the physical and chemical properties, including kinetic viscosity, specific gravity, flash point, fire point, cloud point, pour point, calorific value, carbon residue, free fatty acids, acid value and ash content) according to the procedures of American Standards for Testing of Material.

Molecular genetics of yeasts

The production of biodiesel is founded on the ability of yeasts to catabolize six-carbon molecules such as glucose into two carbon components diesel, without proceeding to the final oxidation product which is CO_2. Crabtree positive yeasts such as *S. cerevisiae* accumulate diesel in the presence of oxygen, however *Candia albicans* which is a crabtree-negative yeast catabolizes sugars into CO_2 in the presence of oxygen (De Deken, 1966). The presence of six carbon carbohydrates represses the oxidative respiration pathway in Crabtree positive yeasts and energy for growth is generated via glycolysis. Upon depletion of six carbon molecules, the catabolism shifts to oxidation of two carbon molecules into CO_2(Postma, *et al.,*1989). This phenomenon is termed at the 'diauxic shift'. The process of biodiesel production via fermentative metabolism and the diauxic shift is dependent upon the enzyme Alcohol Dehydrogenase which is encoded on the ADH1locus. ADH1 catalyzes the reduction of acetaldehyde to diesel during the fermentation of glucose; it can also catalyze the reverse reaction which is the conversion of diesel into acetaldehyde, albeit with a lower catalystic efficiency (Bennetzen and Hall, 1982).

Molecular characterization of oleaginous yeasts

Genomic DNA was extracted from cultures grown on 40 g/L glucose, 5 g/L peptone, 5 g/L yeast autolysate, and 20 g/L agar (GPYA) medium for three days using the FastDNA kit (BIO101, Carlsbad, CA, U.S.A.) with the "FastPrep" Instrument (Q-Biogene). Primers V9G (de Hoog and Gerrits van den Ende 1998) and LR5 (Vilgalys and Hester 1990) were used to amplify the region of the rRNA gene operon that includes the 3′ end of the smallsubunitrRNA gene, the ITS regions (ITS 1, ITS 2 and the intervening 5.8S rRNA gene), and the D1/D2 domains of the 26S rRNA gene of the large subunit, as described by Knutsen et al. (2007).

The PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8 % (w/v) agarose gel containing 0.1 µg/mL ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.85) and examined under UV-light. The amplicons were sequenced in both directions using the primers LR0R (Vilgalys and Hester 1990) and LR5 for the D1/D2 domain, while the primers V9G and ITS4 (White et al. 1990) were used for the ITS domain (ITS 1, ITS 2 and the intervening 5.8S rRNA gene). The BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) was used according to the manufacturer's recommendations and the products were analyzed on an ABI Prism 3730XL DNA Sequencer (Perkin-Elmer). A consensus sequence was computed from the forward and reverse sequences with SeqMan version 8 from the Lasergene package (DNASTAR). All sequences of the studied strains were blasted against sequences in GenBank and the CBS yeast database in order to identify the oleaginous yeasts. Sequences of theD1/D2 of the 26S rRNA obtained during this study and related sequences from the Gen- Bank (NCBI) database were aligned and phylogenetic analyses were done using MEGA 7 version (Kumar *et al.,* 2016). The phylogenetic relationship of these yeast strains is displayed in a distance based Neighbor-Joining tree.

HPTLC Analysis

HPTLC was carried out by using CAMAG V instrument (Germany). Silica Gel G 254 plates were used as stationary phase, on which a known quantity of previously extracted yeast lipids and esterified product were applied. The plate was developed in hexane:diethyl ether(9:1). Plate was stained with phosphomolybdic acid (5% in ethanol) and heated at 105°C until the spots were visualized. Stained plates were scanned using CAMAG V scanner at 534 nm.)

Biodiesel production and analysis by gas chromatography

Microbial oil was extracted as mentioned above before transesterfication. The transesterfication reactions were carried out using sulfuric acid as catalyst in flasks at following conditions: 30:1 molar ratio of methanol to oil, 160 rpm, 5 h of reaction time, temperature at 55°C and 80% catalyst amount based on oil weight (Liu, *et al*., 2004; Wu and Miao, 2006). The reaction mixture was cooled and undisturbed until two layers were formed in a

separating funnel. The upper layer (biodiesel) was separated with petroleum ether and the final biodiesel product was obtained by evaporating the ether from the solution. The fatty acid methyl esters of biodiesel were analyzed' by GC/MS. It was performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50-550; source at 230 °C and quadruple at 150 °C) in the El mode with an HP-5ms capillary column (30 m ' 0.25 mm i.d., 0.25 mm film thickness; J & W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 ml/min. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The injection volume was 1 ml, with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

CONCLUSION

In this study, a simple method was used to isolate oleaginous yeast from soil and used for biodiesel production. It was shown that several strains produced lipids in high yields when fed with carbohydrates. This is an efficient way of producing lipids from carbohydrates. Therefore, potentially it could be used to produce a large amount of lipids form biodiesel.

Engineered organisms using *S. cerevisiae* platforms are an interesting option, but are still full of challenges if industrial application and large-scale production are intended. Biodiesel are a commodity that needs to be competitive with the oil industry products, and therefore, the operating cost is a major concern. A bio-based economy using engineered microorganisms may be promising, but multiples aspects need to be dealt with before this option can become a reality. Sterile condition, pre-treatments, raw material costs and contamination by different microflora are aspects still needing a solution.

The costs of microbial lipids are still too high in order to compete with plant oils for biodiesel manufacturing. Cheap carbon sources have necessarily to be used as carbon sources for the cultivation of these microorganisms and the performance of the bioprocess has to be further improved in terms of both the yield and the productivity. The exploration of the natural biodiversity is a promising strategy to identify novel oleaginous species that assimilate and get fat on agro-industrial residues, particularly the lingo-cellulosic biomass and crude glycerol from biodiesel industry.

REFERENCES

1) Ahmed. S, Sudheer. P, Ashok. K, Sanjit. B, Prasad. N, Effects of various process parameters on the production of linolenic acid in submerged fermentation. *Food, Technol. Biotechnol,* 44, 283-287, 2006.

2) Ahmed. S.u, sudheer. K.S, ashok. P, sanlit. K and Prasad. B.N, Effects of various process parameters on the production of linolenic acid in submerged fermentation. Food technol. *Blotechnol,* 44(2), 283-287, 2006.

3) Antoni. D, Zverlov. V. V, and Schwarz. W. H, "Biofuels from microbes," *Applied Microbiology and Biotechnology,*77, 23-35, 2007.

4) Aresta. M, Dibenedetto. A, Carone. M, Colonna. T, and Fragale. C, "Production of biodiesel from macroalgae by supercritical CO 2 extraction and thermochemical liquefaction," *Environmental Chemistry Letters*,3, 136-139, 2005.

5) Ataya. F, Dube .M. A, and Ternen. M, "Acid¬CatalyzedTransesterification of Canola Oil to Biodiesel under Single- and Two-Phase Reaction Conditions," *Energy and Fuels,* 21, 2450-2459, 2007.

6) Bajpai. D and Tyagi. V. K, "Biodiesel: Source, Production, Composition, Properties and Its Benefits," *Journal of Oleo Science*, 55(10), 487-502, 2006.

7) Barnett JA, Payne RW, Yarrow D, Yeast - Characteristics and Identification. Cambridge, *Cambridge University Press,*18-226, 1990.

8) Barriga. E.J.C,Libkind. D, Briones. A.I, Yeasts biodiversity and its significance: case studies in natural and human-related environments, ex situ preservation, applications and challenges, in: Grillo. O (Ed.), *Changing Diversity in Changing Environment, InTech, Europe,* 55–86, 2011.

9) Bennetzen. J.L, Hall. B.D, The primary structure of the Saccharomyces cerevisiae gene for alcohol dehydrogenase, *J. Biol. Chem*, 257, 3018–3025, 1982.

10) Bhattacharyya. D. K, "Biodiesel from minor vegetable oils like karanja oil and nahor oil," *Fett/Lipid*, 101(10), 404-406, 1999.

11) Blagovi. B, Rup. J, Mesari. M, Georgi. K, and Mari. V, "Lipid Composition of Brewer's Yeast," *Food Technology and Biotechnology*, 39(3), 175-181, 2001.

12) Bligh E.G. and Dyer. NJ, A rapid method of total lipid extraction and purification. *Can J.Biochem. Physiol,* 37, 911-917, 1959.

13) Blight. E and Dyer. W, "A rapid method of total lipid extraction and purification," *Canadian Journal of Biochemistry and Physiology,* 37, 911-917, 1959.

14) Boekhout. T, Kurtzman. C.P, Principles and methods used in yeast classification and an overview of currently accepted yeast genera, in: K. Wolf (Ed.), Nonconventional Yeasts in Biotechnology, *Springer-Verlag, Berlin*, 1–99, 1996.

15) Botes A, Todorov SD, Dicks Leon MT, Identification of lactic acid bacteria and yeast from boza. *Process Biochem,* 42(2), 267-270, 2006.

16) Calinescu. I, Chipurici. P, Trifan. A, Badoiu. C,Immobilisation of *Saccharomyces cerevisiae* for the production of bioethanol, *Ser. B: Chem. Mater. Sci,* 74, 33–40, 2012.

17) Canakci. M and Sanli. M, "Biodiesel production from various feed stocks and their effects on the fuel properties," *Journal of Industrial Microbiology and Biotechnology,* 35, 431-441, 2008.

18) Cerik. M, Andrani. P and Sajbidor. J, Effect of extraction methods on lipid yield and fatty acid composition of lipid classes containing A-linolenic acid extracted from fungi. *JAOCS,* 73(3), 357-365, 1996.

19) Chibata. I, Wingard. L.B, Immobilized Microbial Cells: Applied Biochemistry and Bioengineering, 4th ed., *Academic Press, New York,* 203–215, 1983.

20) Chisti. Y, "Biodiesel from microalgae," *Biotechnology Advances*, 25, 294-306, 2007.

21) Dai. CC, Shi. Y, Wang. AQ, Yuan. ZL, Studies on Inoculation Types of y-linolenic Acid Fermentation by a High-producing Filamentous Fungus Strain. *Chin. Food Sci,* 26(4), 55-58, 2005.

22) Dai. YJ, Yuan. S, Ge. F, Chen. T, Microbial hydroxylation of imidacloprid for the synthesis of highly insecticidal olefin imidacloprid. *Appl. Microb. Cell Physiol,* 71,927-934, 2006.

23) De Deken. R.H, The Crabtree effect: a regulatory system in yeast, *Microbiol,* 44, 149–156, 1966.

24) deHoog. G.S,Gerrits van den Ende AHG. Molecular diagnostics of clinical strains of filamentous basidiomycetes. *Mycoses.* 41,183–9, 1998.

25) Demirbas. A, "Comparison of transesterification methods for production of biodiesel from vegetable oils and fats," *Energy Conversion and Management*, 49, 125-130, 2008.

26) Duarte. J.C, Rodrigues. J.A.R, Moran. P.J.S, Effect of immobilized cells in calcium alginate beads in alcoholic fermentation, *AMB Express,* 3, 31, 2013.

27) Frances Duncan, Applied Microbiology Laboratory Manual by Frances Duncan with contributions from Valerie Walker and Neil Clark Fourth Edition, 2005.

28) Gill. C.O, Hall. M. J, and Ratledge. C, "Lipid Accumulation in an Oleaginous Yeast (Candida 107) Growing on Glucose in Single-Stage Continuous Culture," *Applied and Environmental Microbiology,*33(2), 231-239, 1977.

29) Hall. M. J and Ratledge. C, "Lipid Accumulation in an Oleaginous Yeast (Candida 107) Growing on Glucose under Various Conditions in a One- and Two-Stage Continuous Culture," *Applied and Environmental Microbiology*, 33(3), 577-584, 1977.

30) Hill Nelson. E, Tilman. D, Polasky S, Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *PNAS*.103, 11206-11210, 2006.

31) Hough. J.S, Briggs. D.E, Stevens. R, Young. T.W, Malting and Brewing Science. *Hopped Wort and Beer 2 Chapman and Hall, London,* 527–536, 1982.

32) Kalscheuer. R, Stolting. T, and Steinbu. A, "Microdiesel : Escherichia coli engineered for fuel production," *Microbiology,* 152, 2529-2536, 2006.

33) Krawczyk T, Biodiesel-Alternative fuel makes inroads but hurdles remain. *Inform,* 7, 801-829, 1996.

34) Knutsen. A.K, Robert. V, Poot. G.A, Epping. W, Figge. M, Holst-Jensen. A, Skaar. I, Smith. M.T,Polyphasic re-examination of Yarrowialipolytica strains and the description of three novel Candida species: *Candida osloensisssp*nov, *Candida alimentariasp*nov and *Candida hollandicasp*nov.*Int J SystEvolMicrobiol*, 157, 2426–35, 2007.

35) Kumar. S, Stecher. G, Tamura. K, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *MolBiolEvol,* 33, 1870–74, 2016.

36) Kurtzman. C.P, Fell. J.W, Definition, classification and nomenclature of the yeasts, in: Kurtzman. C.P. Fell. J.W. (Eds.), The Yeasts: A Taxonomic Study, *Elsevier Science BV, Amsterdam* , 3–5, 1998.

37) Li. Q, Du. W, and Liu. D, "Perspectives of microbial oils for biodiesel production," *Applied Microbiology and Biotechnology,*80, 749-756, 2008.

38) Lim. H, Ming. H, Park. Y, Choi S, Food Technol. *Biotechnol,* 41(2), 137-144, 2003.

39) Liu. G, Yuan. S, Dai. C,*Food Microbiol,* 21(3), 299-304, 2004.

40) Lopandic K, Zelger S, Banszky LK, Identification of yeasts associated with milk products using traditional and molecular techniques. *Food Microbiol,* 23(4), 341-350, 2006.

41) Lotero. E, Liu. Y, Lopez. D. E, Suwannakarn. K, Bruce. D. A, and Goodwin. J. G, "Synthesis of Biodiesel via Acid Catalysis," *Industrial Engineering Chemistry Research,* 44, 5353-5363, 2005.

42) Ma F, Hanna MA, Biodiesel production: a review. *Bioresour. Technol,* 70,1-15, 1999.

43) Margaritis. A, Merchant. F.J.A, The technology of anaerobic yeast growth, in: D.R. Berry, B.I. Russell, G.G. Stewart (Eds.), *Yeast Biotechnology, 84 Unwin Hyman Ltd, London*, 37–53, 1987.

44) Mulliner. H and Daum. G, "Dynamics of neutral lipid storage in yeast*.," *ActaBiochimicaPolonica,* 51(2), 323-347, 2004.

45) Morison. WR, Smith. LM, Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lip. Res*, 53, 600-608, 1964.

46) Phaff. H.J, Starmer. W.T, Yeasts associated with plants, insects and soil, in: Rose. A.H, Harrison. J.S (Eds.), *The Yeasts Biology, Academic Press, London*, 123–180, 1987.

47) Phillips. C.R, Poon. Y.C, Immobilization of Cells, 1st ed., *Springer-Verlag, Berlin*, 48–49, 1988.

48) Pinto.A. C, Guarieiro. L. L. N, Rezende. M. J. C, Ribeiro. N. M, and Ednildo. A, "Biodiesel : An Overview," *Journal of Brazilean Chemical Society*, 16(6), 1313-1330, 2005.

49) Postma. E, Verduyn. C, Scheffers. W.A, Van Dijken. J.P,Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae, Appl. Environ. Microbiol,*55, 468–477, 1989.

50) Ratlege. C, Tan. K, Oils and fats: production, degradation and utilization by yeasts. *Yeast biotechnology and biocatalysis,* 223-54, 1990.

51) Smith. VH, Sturm BSM, DeNoyelles FJ, Billings SA, The ecology of algal biodiesel production. *Trends Ecol. Evol*, 25, 301-309, 2009.

52) Somogyi. M, Notes on sugar determination. *J. Biol. Chem,* 200, 245-247, 1952.

53) Steen. EJ, Kang. Y, Bokinsky. G, Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature*, 463, 559-62, 2010.

54) Stolarzewicz. I, Bialecka-Florjanczyk. E, Majewska. E, Krzyczkowska. J, Immobilization of yeast on polymeric supports, *Chem. Biochem. Eng,* 25, 135–144, 2011.

55) Thakur MS, Prapulla SG, Karanthsuadans NG, Estimation of intracellular lipids by the measurement of absorbance of yeast cells stained with Sudan Black. *Enzyme Microb. Technol,* 11(4), 252-254, 1989.

56) Thurmond. W, Biodiesel 2020: A global market survey. *E.M. Online, 2008*.

57) Tucker, C. M, Taxonomy of the genusPhytopthora de Bary. Res. Bull. *Missouri Agric. Exp.stn,*153, 1-208, 1931.

58) Vasudevan. P. T and Briggs. M, "Biodiesel production - current state of the art and challenges," *Journal of Industrial Microbiology and Biotechnology,* 2008.

59) Vucurovic. V, Razmovski. R, Popov. S, Ethanol production using *Saccharomyces cerevisiae* cells immobilised on corn stem ground tissue, *Zb. Matic-. Srp.Prir. Nauk,* 116, 315–322, 2009.

60) Walker. G.M, Yeast Physiology and Biotechnology, 1st ed., *John Wiley & Sons Ltd, Chichester,* 1–13, 1998.

61) Walker. G.M, Yeasts Fungi BiolAppl, in: M. Schaechter (Ed.), *The Desk Ecyclopedia of Microbiology*, *Elsevier, Oxford*, 1174–1187, 2009.

62) Watanabe. T,Zygospore induction in Mortierellachlamydospora by the soaking- plain-water-agar-culture method. *Mycologia,* 82, 278¬282, 1990.

63) Wu. Q, Miao. X,*bioresour.Technol,* 97, 841-846, 2006.

64) Wu Q Y, Miao XL, Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol,* 97, 841-846, 2006.

65) Ylitervo. P, Franzen. C.J, Taherzadeh. M.J, Ethanol production at elevated temperatures using encapsulation of yeast, *J. Biotechnol,* 156, 22–29, 2011.

66) Yu. J, Zhang. X, Tan. T, A novel immobilization method of *Saccharomyces cerevisiae* to sorghum bagasse for ethanol production, *J. Biotechnol,* 129, 415–420, 2007.

67) Zhao. X, Kong. X, Hua. Y,Feng. B, and Zhao. Z. K, "Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomycesstarkeyi," *European Journal of Lipid Science and Technology,*110, 405-412, 2008.