Optimization of Culture Conditions for the Highest Lipid Production from some Oleaginous Fungi for Biodiesel Preparation

Thanaa H. Ali*, Dina H. El-Ghonemy
Department of Microbial Chemistry,
National Research Centre, Dokki, Cairo, Egypt

ABSTRACT---- Ten different filamentous fungi were screened for their abilities to produce lipid. An oleaginous fungus strain *Trichoderma viride* NRC 314 was found to be the highest lipid producer (>20%) among the tested filamentous fungi. Optimization of culture conditions for maximum lipid production was investigated, and the results reported clearly indicated that, the potato dextrose (PD) liquid medium, at a concentration of 50 g/l for dextrose, was the most suitable medium for lipid production with initial pH 5.0, incubation temperature 28°C, after five days of incubation in a static condition. In addition, neither yeast extract nor sugar cane molasses supplement exhibits any significant effects on lipid accumulation. The GC/Ms analysis indicated that, the n-hexane lipid fraction of *T. viride* 314 was mainly composed of 30.01% Palmitoleic acid (C16:1), 23% Linoleic Acid (C18:2), 13% Linolenic acid (C18:3) and about 8% Erucic acid (C22:1). Furthermore, the total saturated fatty acids represented 8.3%, while that of the unsaturated was 81.74%. Therefore, our study suggests that, SCOs of oleaginous fungi could be used as a potential feedstock for biodiesel production with *Trichoderma viride* NRC 314 as a promising candidate.

Keywords---- Biodiesel; Lipid; Oleaginous fungi; *Trichoderma viride* NRC 314, optimization.

1. INTRODUCTION

Biomass-based biofuel production represents a pivotal approach to face high energy prices and potential depletion of crude oils reservoirs, to reduce greenhouse gas emissions, and to enhance a sustainable economy. Microbial lipids can represent a valuable alternative feedstock for biodiesel production, and a potential solution for a biobased economy (Zinoviev et al., 2010).

The production of biodiesel is based mostly on plant oils, even though animal fats and algal oils can also be used. In particular, soybean, rapeseed, and palm oils are adopted as the major feedstock for biodiesel production. They are produced on agricultural land, opening the debate on the impact of the expansion of bioenergy crop cultures, which displace land from food production. Furthermore, their price restricts the large-scale development of biodiesel to some extent. Recently, the development of processes to produce single cell oils (SCOs) by using heterotrophic oleaginous microorganisms has triggered significant attention (Azocar et al., 2010). These organisms accumulate lipids, mostly consisting of triacylglycerols (TAG) that form the storage fraction of the cell. The occurrence of TAG as reserve compounds is widespread among all eukaryotic organisms such as fungi, plants and animals, whereas it has been rarely described in bacteria (Meng et al., 2009).

Several species of yeasts and filamentous fungi are regarded as oleaginous, since they have the capability to synthesize and accumulate high amounts of TAG within their cells, up to 70% of their biomass weight. These lipids have similar composition and energy value to plant and animal oils, but their production do not compete for food resources, in particular, if it is based on inexpensive carbon sources, such as raw materials, by-products, and surplus. Furthermore, fungal SCOs have short life cycles, display rapid growth rates, unaffected by space, light or climatic variations, easier to scale up and have the ability to utilize a wide range of inexpensive renewable carbon sources such as lingo-cellulosic biomass and agro-industrial residues and wastewater (Ramanaiah et al., 2007; Pant et al., 2009; Pant and Adholeya, 2010; Yousuf et al., 2010; Chen et al., 2012; and Khot et al., 2012).

Several studies reporting lipid accumulation by oleaginous yeasts and filamentous fungi on different renewable substrates such as glycerol, sewage water, whey and molasses have also been reviewed (Subramaniam et al., 2010). The biodiesel quality depends upon the fatty acid composition of the oil feedstock. For an oleaginous microbe to be considered as a suitable feedstock for biodiesel, the total lipid content (>20%) and the type of fatty acids (long chain saturated and/or monounsaturated fatty acids) are important criteria (Ramos et al., 2009).

Lipid content and fatty acid composition of SCOs varies in response to environmental factors such as type of carbon source, pH, temperature and according to the nature of microorganism i.e., it is species and strain-specific (Subramaniam et al., 2010; Venkata and Venkata, 2011). Thus, this manuscript focuses on exploring the potential of fungal strain *Trichoderma viride* NRC 314 to accumulate these lipids. Therefore, the aim of the present study was

optimization of culture conditions to enhance oil biosynthesis by the selected oleaginous fungus strain, *Trichoderma virde* NRC 314 using a low cost cultivation medium containing PD nutrient.

2. MATERIALS AND METHODS

2.1 Chemicals

Potato dextrose medium was purchased from Liofilchem. s.r.l. Bacteriology products, Italy. Chemicals and organic solvents used were of analytical grade and high purity. Olive oil was obtained from a local hypermarket, Cairo, Egypt.

2.2 Microorganism

Ten different filamentous fungi were obtained from Culture Collection of the Microbial Chemistry Department, National Research Centre, Cairo, Egypt. The stock cultures were maintained routinely on slants of Potato dextrose agar (PDA) medium containing (g/l): Potato, 300; dextrose, 20.0; agar; 20.0; distilled water, 1000 ml, adjusted at pH 6.0 before autoclaving (at 121°C for 20 min). The cultures were grown for 7 days at 28°C, and then maintained at 4°C. The slants were subcultured routinely every 4–5 weeks interval.

2.3 *Cultural conditions*

Different filamentous fungi were grown on PDA agar slants for 7 days at 28°C. Conidia were scraped and 5.0 ml of sterile distilled water was added to each slant. One ml aliquot (v/v) of inoculum size (10^7 - 10^8 spores/ml) was used to inoculate fifty milliliters of sterile PD liquid medium in 250 ml Erlenmeyer flasks. The inoculated flasks were incubated for 7 days at 28°C in a static condition.

2.4 Determination of cell dry weight

After the fermentation period, cultures were harvested by filtration (Whatman No.1) and the mycelial mats (in triplicate) were rinsed thoroughly with sterile distilled water to remove unwanted constituents media present on the cell surface. The biomass was kept for drying in a hot air oven at 60°C till a constant weight was achieved. The cell dry weight was determined gravimetrically according to Devi et al. (2009).

2.5 Optimization of culture conditions for the highest lipid production

2. 5. 1. Effect of different media on lipid production

Five different media were used in the present study for biomass and lipid production evaluation, namely as follows: 1) Potato-Dextrose liquid medium, 2) Czapek Dox's medium (Difco, Manual 1972). 3) Potato-Dextrose yeast extract medium containing (g/l): Potatoes, 300; dextrose, 20; yeast extract, 5.0. 4) Potatoes, 300; dextrose, 20; wheat bran, 10. 5) Potatoes, 300; dextrose, 20; sugar cane molasses, 100. The media were adjusted at pH 6.0 before autoclaving (at 121°C for 20 min).

2. 5. 2. Comparison between shaking and static conditions on lipid production

Lipid accumulation in both static and shaking conditions (150 rpm) was evaluated after five days of incubation at 28ºC.

2. 5. 3. Effect of incubation period on lipid production

T. viride NRC 314 was inoculated in the PD liquid production medium adjusted at pH 6.0 and incubated in a static condition at 28ºC for different periods of time i.e., 24, 48, 72, 96 and 120 hrs. During the fermentation, the flasks were taken at regular intervals of 24 hrs.

2. 5. 4. Effect of different carbon sources on lipid production

Equimolar amount of ten different carbon sources namely dextrose, sucrose, xylose, maltose, fructose, raffinose, ribose, arabinose, mannose and lactose were individually added in the production medium. The fungus was inoculated and incubated for 5 days at 28°C. The culture biomass was then collected and used for total lipid production estimation.

2. 5. 5. Effect of initial medium pH on lipid production

This experiment was carried out to evaluate the effect of initial pH medium on the biomass dry weight and lipid production. The pH of the culture medium was adjusted before autoclaving at different pH values ranging from pH 3.0 – pH 8.0 with 1 N HCl or 1 N NaOH.

2. 5. 6. Effect of incubation temperature on lipid production

T. viride NRC 314 was incubated at different incubation temperatures ranging from 20-45ºC for five days in a static condition. After incubation period, lipid production and dry weight of the fungus were evaluated.

2.6 Lipid extraction method

The extraction method for lipid and fatty acid of fungal cultures was carried out according to the method presented by Somashekar et al. (2001). The extraction methods were as follows: chloroform/methanol (2:1), hexane/isopropanol (3:2) and Soxhlet extraction by using hexane.

2.7 Lipid determination

Lipid was estimated according to Mishra et al. (2014). Sulfo-phospho-vanillin reagent (SPV), which was prepared by initially dissolving 0.6 g vanillin in 10 ml absolute ethanol; 90 ml deionized water and stirred continuously. Subsequently, 400 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in a dark bottle. To ensure high activity, freshly prepared phospho-vanillin reagent was recommended.

For SPV reaction of the fungal culture for lipid quantification, a known amount of biomass in 100µl water, which are either suspended in a known volume of liquid culture or harvested via centrifugation at 4000 rpm for 5 min, was used. For routine assay, 2.0 ml of concentrated sulfuric acid (98%) was added to the biomass sample and heated for 10 min at 100°C, then cooled for 5 min in an ice bath. 5.0 ml of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 min at 37°C in an incubator shaker (200 rpm), and the absorbance was measured at 530 nm in order to quantify the lipid concentration within the sample.

2. 8 Fatty acid determination

The fatty acid profile of the oil sample was determined by converting the fatty acids in oil to fatty acid methyl esters (FAMEs) according to the method recommended by Christie and Han (2010). The lipid sample (up to 5 mg) is dissolved in toluene (1 ml) in a test tube fitted with a condenser, and 1% sulfuric acid in methanol (2.0 ml) is added, before the mixture is refluxed for 2 hrs (or alternatively the mixture can be left overnight in a stoppered tube at 50°C). Water (5 ml) containing sodium chloride (5%) is added and the required esters are extracted with hexane (2 x 5 ml), using Pasteur pipettes to separate the layers. The hexane layer is washed with water (4.0 ml) containing potassium bicarbonate (2%) and dried over anhydrous sodium sulfate. The solution is filtered to remove the drying agent, and the solvent is removed under reduced pressure in a rotary film evaporator or in a stream.

2.9 Gas chromatographic conditions

The GC-MS analysis for fatty acid methyl esters (FAMEs) was performed using Agilent Technologies 6890 N (Net Work GC system) USA. Oven was held at initial temperature 50ºC and maintained for 2 min, at rate 10, 8, 5, 6ºC/ min, raised to 70, 170, 200 and 240ºC, at the rate of 2, 9, 5, 10 min and run time 55 min. Injector temp was held at 250ºC splitless. A capillary column HP-5MS (5% phenyl methylsiloxane) has dimension of (length, 30 m, diameter 320 µm, film thickness 0.25 µm). The flame ionization detector temperature was 280°C and flow rate was 1.5 ml/min. The carrier gas was nitrogen, with a flow rate of 30 ml/min. hydrogen flow rate was 30 ml/min and air flow rate 300 ml/min.

3. RESULTS AND DISCUSSION

3. 1 Screening of different fungal strains for their abilities to produce lipid

Results in Table 1, clearly demonstrated that, the majority of tested filamentous fungal strains are oleaginous fungi and have the ability to accumulate lipids in different amounts ranging from 20 - 27% of their dry weight. However, *Trichoderma viride* NRC 314 cells gave the highest amount of lipid accumulation followed by *Penicillium brevicompactum, P. funiculosum, Trichoderma* F635, *Aspergillus oryzae* NRRL 447 and *T. reesei*. While other strains such as *A. niger*, *P. politans* NRC 510 and *A. oryzae* NRRL 3484 gave lipid about 17, 14, 14% to their biomass weight, respectively, while a limited amount of lipid is accumulated by *A. fumigatus.*

Table 1. Screening of different fungal strains for their abilities to accumulate lipid.

Microorganisms	**Biomass (g/l)**	**Lipid (g/l)**	**% lipid/mass**
Aspergillus oryzae NRRL 447	10.0	2.0	20.0
Aspergillus oryzae NRRL 3484	13.4	1.9	14.0
Aspergillus fumigatus DSM 819	13.6	1.25	9.2
***Trichoderma viride* NRC 314**	**13.4**	**3.63**	**27.1**
Trichoderma F6 35	13.8	2.9	20.8
Aspergillus niger	14.0	2.0	17.9
Penicillium funiculosum NRC 258	18.0	3.75	20.8
Penicillium politans NRC 510	13.4	2.0	14.9
Penicillium brevicompactum NRC 829	15.5	3.25	21.0
Trichoderma reesei	14.0	2.9	20.5

Exploitation of oleaginous filamentous fungi for biodiesel production has a more recent history, which, with few exceptions, derives from studies focused to poly-unsaturated fatty acid production (PUFA), such as arachidonic acid and σ-linolenic acid (Li et al., 2011). Lipid accumulation in *M. circinelloides* has been extensively studied (Wynn et al.,

2001), and also it has been used for the first Biodiesel – Feedstock and 74 processing technologies commercial production of microbial lipids (Ratledge, 2004). To our knowledge, limited are the attempts to get lipids with *Aspergillus oryzae* that, conversely, is extensively studied as lipase producer to carry out transesterification of TAG (Adachi et al., 2011).

3.2 Effect of different media compositions on lipid accumulation

A comparative study was carried out to evaluate the effect of different media constituents in enhancing growth and lipid formation by *Trichoderma viride* NRC 314. Data in Table 2, clearly indicated that, the fungus *T. viride* NRC 314 exhibits variation in biomass and lipid yields in the five media tested, however, we can conclude that, the potato-dextrose liquid medium was the most suitable medium for maximum growth and lipid accumulation by *T. viride* NRC 314. On the other hand, the addition of yeast extract or molasses or wheat bran to growth medium were poor in supporting growth with respect to the level produced by organism in PD liquid medium.

Table 2. Growth and lipid (SCOs) yields by *Trichoderma viride* NRC 314 in different physiological conditions.

Experimental conditions	**Weight (g/l)**	
	Biomass	**Lipid**
Types of media		
PD liquid medium	16	4.7
Czapek Dox's medium	12	1.5
PD medium + yeast extract	9.2	1.0
PD medium +wheat bran	13	2.0
PD medium + molasses	10	0.99
Incubation temperature		
20°C	13	2.0
28°C	16	4.66
35°C	13.8	2.2
40°C	9.0	.0.34
45°C	0.0	0.0
Growth condition		
Static	16	4.5
Shaking	14.4	2.8

3.3 Influence of different incubation periods on the fungal biomass formation and lipid accumulation

In the present study, the highest biomass growth and lipid accumulation was noticed on the fifth day of growth (Fig. 1). The time of incubation also showed influence on the fungal biomass formation and lipid accumulation along with the incubation temperature which can lead to efficient utilization of available carbon source in the growth medium leading itself to a higher accumulation of lipid content. Shorter incubation time influenced the substrate utilization by the *Trichoderma viride* leading to less biomass formation and lipid accumulation. Our results are in line with that investigated by Kumar and Banerjee (2013) for fatty acids production by *Aspergillus sp*. They reported that maximum lipid production was found after 5 days of incubation.

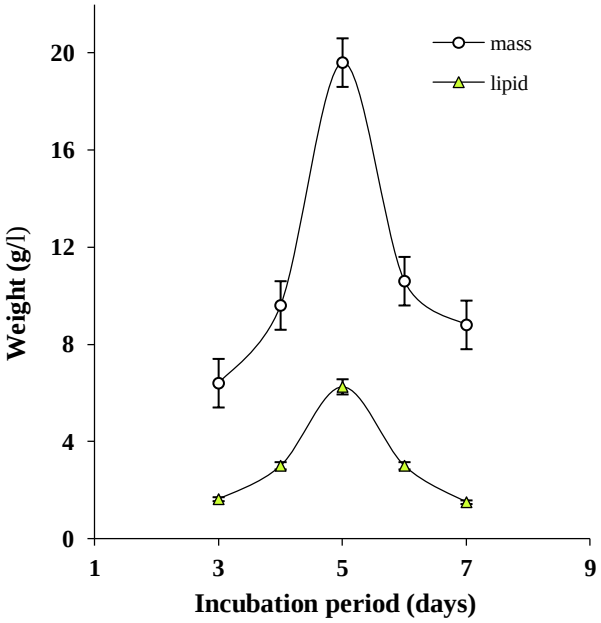

Fig. 1. Effect of different incubation periods on biomass and oil production by *T. viride* NRC 314

3.4 Effect of different carbon sources on lipid production

Among various carbon sources tested, dextrose promoted highest biomass growth and lipid accumulation by *Trichoderma viride* NRC 314 compared to others (Fig. 2), followed by fructose and ribose. In addition, our results revealed that, arabinose and mannose induced the least amount of lipid production among the carbon sources tested as well as the biomass. In contrast, sucrose acts as the better carbon source followed by glucose and dextrose for the growth and lipid accumulation of fungi, but the addition of carbon sources would further increase the cost of oil production in an industrial application (Economou et al., 2011).

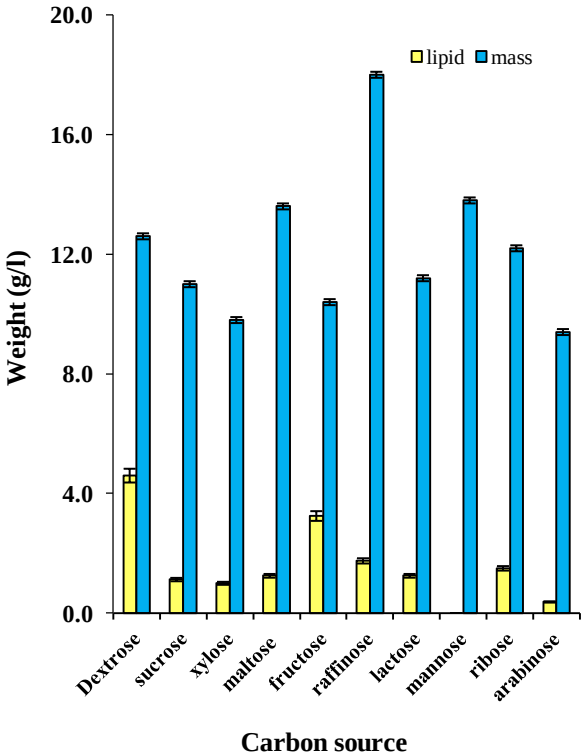

Fig. 2. Effect of different carbon sources on biomass and oil production by *Trichoderma viride* NRC 314

3.5 Effect of different concentrations of dextrose on lipid production

Optimal dextrose concentration was studied by varying its concentration in PD liquid medium from 1.0 - 12%, at the end of incubation period, biomass and oil yield were measured and graphically illustrated in Fig. 3, from which it was obvious that, the most suitable dextrose concentration for maximum production of lipid was 5 - 6% (50-60 g/l). Lipid production was directly proportional to the dextrose concentration up to a certain limit (Chtzifragkou et al., 2010; Chatzifragkou et al., 2011; Peng et al., 2013, Venkata and venkata, 2014). The synthesis of TAG in fungi was influenced by the supply of carbon in the growth medium. Initially, fungi utilize the available carbon source in the medium for growth and cell maintenance, and then produce lipid-free biomasses including functional lipids, and finally, if there is still C-source available, it used to accumulate storage lipids. If a sufficient carbon source is available, the lipid production rate and accumulation will increase up to a maximum level. If the carbon is limited in the medium, or when the carbon supply gets exhausted from extracellular sources, the stored intracellular lipid is mobilized and utilized to sustain generations of cells and production of lipid-free biomass (Park et al., 1990).

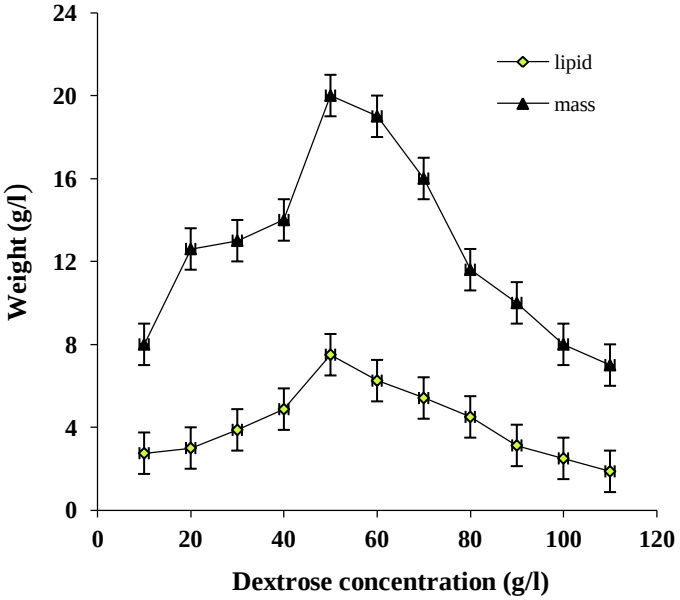

Fig. 3. Effect of different concentrations of dextrose on biomass and oil production by *T. viride* NRC 314

3.6 Effect of initial medium pH on biomass and lipid production

Medium pH was found to be an important factor for biomass formation and lipid accumulation. In the present study, highest biomass and lipid accumulation by *T. viride* NRC 314 was reported in the medium with initial pH 5.0 (Fig. 4). Moreover, there was a drastic decrease in the lipid yields when the organism was grown in a medium either below or above the optimum pH (5.0). This observance was supported by the study reported by Venkata and Venkata (2014). They indicated that pH 5.5 was the suitable pH for *Aspergillus awamori* growth and lipid accumulation. In this concern, Lilly and Barnett (1951) reported that, the hydrogen ion concentration (pH) in the medium was an influencing factor for growth and other life processes like sporulation. It was known that, the function of plasma membrane was to regulate what comes in and goes out of the cells.

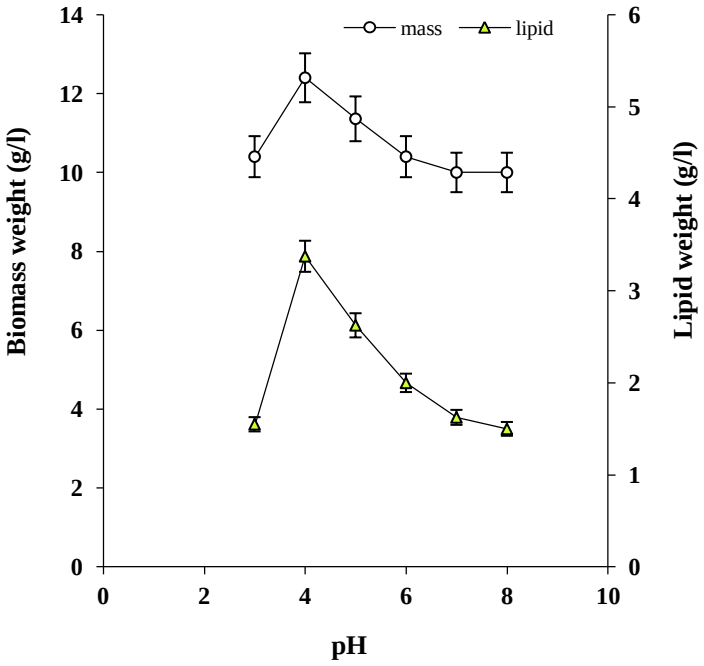

Fig. 4. Effect of initial medium pH on biomass and oil production by *Trichoderma viride* NRC 314

External pH of the culture medium can determine the complex physiological parameters such as membrane permeability and cell morphology. Therefore, the change in the broth pH will affect membrane osmosis to absorb or move certain ions from the surrounding medium. Previous studies reported the influence of broth pH value on the growth kinetics of microorganisms and concluded that the pH of the medium was an important environmental factor affecting cell growth and products formation (Amanullah et al., 2001). In general, fungal strains are more tolerant to acidic than alkaline pH, whereas, pH values 5 - 6 were found to be the suitable pH for most fungal growth as reported by Lilly and Barnett (1951).

3.7 Effect of different incubation temperatures on the lipid production

In the present study, maximum lipid accumulation by *T. viride* NRC 314 was reported at 28ºC followed by 30ºC (Table 2). However, lipid production was drastically reduced gradually when the fungus was incubated at higher temperature (35 - 40ºC). Moreover, neither biomass nor lipid was produced at 45ºC. These results are incongruent with that reported for *Aspergillus awamori* (Venkata and Venkata 2014).

Temperature at 30ºC is optimum for getting the maximum fungal biomass, because in natural environments fungi are exposed to a wide range of temperatures with daily and seasonal variations. All the fugal enzymes show higher activity at this temperature range. At higher temperatures, an increase in nutritional requirements is sometimes observed as reported or *Saccharomyces* sp. (Carlile et al., 2001).

3.8 Comparison between shaking and static condition for lipid production

Data presented in Table 2, clearly indicated that, the lipid production was higher in the static condition than that produced in the shaking condition. Incongruent with our results, Kirrolia et al. (2013) reported that, the static cultures were found to be higher in lipid accumulation (18.35 %) than that found in shaking conditions (13.89 %).

3.9 Lipid extraction methods

The efficacy of three extraction methods for determining the lipid and fatty acid composition of fungal culture was studied. From Table 2, the extraction method: chloroform/methanol (2:1) was found to be the most suitable for lipid and fatty acids extraction from *T. viride* NRC 314 biomass followed by hexane/isopropanol (3:2) and Soxhlet extraction by using hexane. These results are similar to that reported for lipid extraction from *M. circinelloides* by Vicente et al. (2009). They found that the mixtures of chloroform and methanol lead to the highest quantity of extracted lipids.

3. 10 Fatty acid profiles of fungal SCOs

The fatty acid composition of *Trichoderma viride* NRC 314 lipid is transesterified to fatty acid methyl esters with the profiles which are cited in Table 3. The fungal SCOs in the present study, were found to contain a high fraction of mono and polyunsaturated fatty acids mainly 30% C16:1(Palmitoleic), 23% C18:2 (linoleic acid) and 12% C18:3

(linolenic acid) and limited percentage of saturated fatty acids mainly of C12, C17, C 21 and C23 (Fig. 5). This composition is similar to the fatty acid composition oil from microalgae which composed of a mixture of unsaturated fatty acids, such as palmitoleic (16:1), oleic (18:1), linoleic (18:2) and linolenic acid (18:3) with small extent of saturated fatty acids, such as palmitic (16:0) and stearic (18:0) (Halim et al., 2012). This composition of *T. viride* NRC 314 lipid fatty acids is similar about 60% to the types of fatty acids identified in *A. awamori* but differs in the ratio of saturated to unsaturated fatty acids (Venkata and Venkata, 2011).

In contrast to our results, the fatty acid profiles of oleaginous algae and *Yanobacteria*, showed a dominance of C14 and C16 fatty acids with *Chlorella* sp. being rich in C18 (Hu et al., 2008). Our result was also found to be differing from most vegetable oils in being rich in PUFAs, and hence, it is mainly exploited for PUFA production. However, PUFAs with more than 4 double bonds are not desirable for good quality biodiesel. *Trichoderma viride* oil likes *M. circinelloides* oil in differing from most vegetable oils in being quite rich in polyunsaturated fatty acids (Chisti, 2007).

Table 3. Lipid content and fatty acid concentrations of oleaginous fungus *Trichoderma viride* NRC 314

Peak number	RT	Common name	Carbon number	Fatty acid (%)
3	11.373	Caprylic acid	C8:0	0.251
6	15.837	Capric acid	C10:0	0.310
8	18. 746	Lauric acid	C12:0	1.412
9	19.533	Tridecylic acid	C13:0	0.699
11	22.099	Myristoleic acid	C14:1	0.7244
12	25.175	Pentadecenoic acid	C15:1	0.5045
13	**30.428**	**Palmitoleic acid**	**C16:1**	**30.009***
15	32.1	Margaric acid	C17:0	0.7922
16	32.738	Heptadecenoic acid	C17:1	0.951
17	**35.633**	**Linoleic Acid**	**C18:2**	**22.640***
18	**36.515**	**α-Linolenic acid**	**C18:3**	**12.167***
21	40.523	Arachidic acid	C20:0	0.7088
23	41.139	Mead acid	C20:3	1.049
24	42.361	Arachidonic acid	C20:4	1.1
26	44.089	Eicosapentaenoic acid	C20:5	4.181
27	44.589	Heneicosylic	C21:0	3.546
29	**46.242**	**Erucic acid**	**C22:1**	**7.914***
30	48.182	Tricosylic acid	C23:0	0.625
31	51.861	Nervonic acid	C24:1	0.717
Saturated				8.30
Unsaturated				81.70
Others				10.0

The high degree of unsaturation inherent to the FAMEs derived from these fatty acids would evidence lower oxidative stability, but excellent fuel properties at low temperatures, which is an advantage in winter operation (Vicente, et al., 2004). Exploitation of oleaginous filamentous fungi for biodiesel production has a more recent history, which, with few exceptions, derives from studies focused to poly-unsaturated fatty acid production (PUFA), such as arachidonic acid and α-linolenic acid. The most relevant example of this biotechnological application is represented by exploitation of *Mortierella alpina* to produce oils containing n-1, n-3, n-4, n-6, n-7, and n-9 PUFAs (Sakuradani et al., 2009).

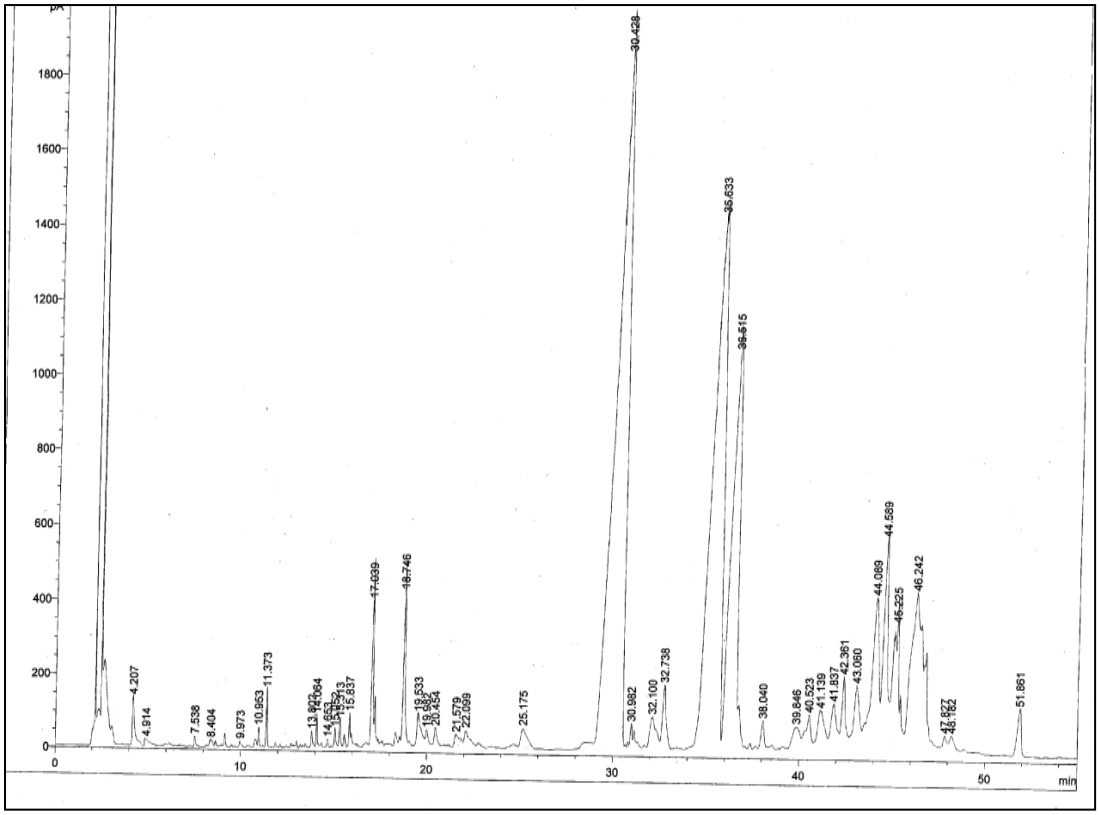

Fig.5. Fatty acids composition of *Trichoderma viridie* NRC 314 biomass by GC Analysis

4. CONCLUSION

Trichoderma viridie NRC 314 was the highest for lipid accumulation (>20%) among the tested filamentous fungi. Optimal conditions for maximum lipid production were evaluated. Lipid content of 43% was achieved on the fifth day of growth, in PD liquid medium at dextrose concentration of 5% (w/v), with initial pH 5.0, and inoculum size 1ml (10^7 - 10^8 spores/m). The optimal incubation temperature for lipid accumulation was reported at 28°C in a static condition. GC-MS study revealed that, the fatty acids palmitoleic, linoleic and linolenic acid were predominant in the lipid sample. Therefore, it can be concluded that production and optimization of lipid enriched biomass from oleaginous filamentous fungi (*T. viridie* NRC 314), could be useful for biodiesel production.

5. REFERENCES

- Adachi D, Hama S, Numata T, Nakashima K, Ogino C, Fukuda H, Kondo A. 2011. Development of an *Aspergillus oryzae* whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production. Bioresource Technology. **102**(12)**:** 6723-6729.
- Amanullah A, McFarlane CM, Emery AN, Nienow AW. 2001. Scale-down model to simulate spatial pH variations in large-scale bioreactors. Biotechnol Bioeng.**73:** 390–399.
- Azocar L, Ciudad G, Heipieper HJ, Navia R. 2010. Biotechnological processes for biodiesel production using alternative oils. Applied Microbiology and Biotechnology. **88(**3**):** 621-636.
- Carlile MJ, Watkinson SC, Graham WG. 2001. The fungi. 2nd ed. San Diego (California): Academic Press.
- Chatzifragkou A, Fakas S, Galiotou-Panayotou M, Aggelis G, Papanicolaou S. 2011. Commercial sugars as substrates for lipid accumulation in *Cunninghamella echinulata* and *Mortierella isabellina* fungi. Eur J Lipid Sci Technol. **112**(9)**:** 1048-1057.
- Chen XF, Huang C, Xiong L, Chen X, Chen Y, Maa LL. 2012. Oil production on waste waters after butanol fermentation by oleaginous yeast *Trichosporon coremiiforme*. Bioresour Technol. **118:**594–597.
- Chisti Y. 2007. Biodiesel from microalgae, Biotechnol. Adv. **25:**294–306.
- Christie WW, Han X. 2010. Lipid Analysis - Isolation, Separation, Identification and Lipidomic Analysis (4th edition), 446 pages (Oily Press, Bridgwater, U.K. and Woodhead Publishing Ltd, Cambridge, U.K.)
- Chtzifragkou S, Fakas M, Galiotou-Panayotou M, Komaitis G, Aggelis, Papanikolaou S. 2010. Commercial sugars as substrates for lipid accumulation by *Cunninghamella* echinulata and *Mortierella isabellina* fungi. Eur J Lipid Sci Technol. 1**12:**1048–1057.
- Devi P, D'souza L, Kamat T, Rodrigues C, Naik CG. 2009. Batch culture fermentation of *Penicillium chrysogenum* and a report on the isolation, purification, identification and antibiotic activity of *citrinin.* Indian J Mar Sci. **38**(1)**:** 38.

- Difco, Manual. (1972). "Difco Manual of dehydrated culture, media and reagents" 9th ED.:245. Difco Laboratories, Detroit, Michigan, USA.
- Economou CN, Aggelis G, Pavlou S, Vayenas DV. 2011. Single cell oil production from rice hulls hydrolysate. Biores Technol. **102:**9737–9742.
- Halim R, Danquah MK, Webley PA. 2012. Extraction of oil from microalgaen for biodiesel production: areview. Biotechnology Advances. **30:**709–732.
- Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A. 2008. Microalgal triacylglycerols as feedstock for biofuel production: perspectives and advances. Plant J. **54:** 621–639.
- Khot M, Kamat S, Zinjarde S, Pant A, Chopade B, RaviKumar A. 2012. Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. Microbial Cell Factories. **11:**1-13.
- Kirrolia A, Bishnoi NR, Sing R. 2012. Effect of shaking, incubation temperature, salinity and media composition on growth traits of green microalgae *Chlorococcum* sp. J. Algal Biomass Utln **. 3** (3)**:**46–53.
- Kumar SP, Banerjee R. 2013. Optimization of lipid enriched biomass production from oleaginous fungus using response surface methodology Indian Journal of Experimental Biology. **51:** 979-983.
- Li CH, Cervantes M, Springer DJ, Boekhout T, Ruiz-Vazquez RM, Torres-Martinez SR, Heitman J, Lee SC. 2011. Sporangiospore size dimorphism is linked to virulence of *Mucor circinelloides*. PLoS Pathogen. **7**(6)**:**e1002086.
- Lilly VG, Barnett HL. 1951. Physiology of fungi. New York: McGraw Hill Book Company Inc. 464.
- Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M. 2009. Biodiesel production from oleaginous microorganisms. Renewable Energy. **34** (1)**:**1-5.
- Mishra S K, Suh WI, Farooq W, Moon M, Shrivastav A, Park M. S. Ji-Won Yang .2014. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresource Technology. **155:**330–333.
- Pant D, Adholeya A. 2009. Nitrogen removal from biomethanated spentwash using hydroponic treatment followed by fungal decolorization. Env Eng Sci. **26:**559–65.
- Pant D, Adholeya A. 2010. Development of a novel fungal consortium for the treatment of molasses distillery wastewater. Environmentalist. **30:**178–182.
- Park WS, Murphy PA, Glatz BA. 1990. Lipid metabolism and cell composition of the oleaginous yeast *Apiotrichum curvatum* grown at different carbon to nitrogen ratios. Can J Microbiol. **36:**318–26.
- Peng W, Huang C, Chen X, Xiong L, Chen X, Chen Y. MaL. 2013. Microbial conversion of waste water from butanol fermentation to microbial oil by oleaginous yeast *Trichosporon dermatis*. Ren Energy. **55:**31–34.
- Ramanaiah SV, Venkata Mohan S, Sarma PN. 2007. Adsorptive removal of fluoride from aqueous phase using waste fungus (Pleurotus ostreatus 1804) biosorbent: kinetics evaluation. Ecol Eng. **31:**47–56.
- Ramos MJ, Fernández CM, Casas A, Rodríguez L, Perez Á. 2009. Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour Technol. **100:**261–268.
- Ratledge C. 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie. **86**(11**):**807–815.
- Sakuradani E, Ando A, Ogawa J, Shimizu S. 2009. Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding. Appl Microbiol Biotechnol. **84**(1)**:**1-10.
- Somashekar D, Venkateshwaran G, Srividya C, Krishnanand, Sambaiah K, Lok esh BR. 2001. Efficacy of extraction methods for lipid and fatty acid composition from fungal cultures. World J Microbiol Biotechnol . **17:**317–320.
- Subramaniam R, Dufreche S, Zappi M, Bajpai R. 2010. Microbial lipids from renewable resources: production and characterization. J Ind Microbiol Biotechnol. **37:**1271–1287.
- Venkata Subhash G, Venkata Mohan S. 2011. Biodiesel production from isolated oleaginous fungi *Aspergillus* sp. using corncob waste liquor as a substrate. Bioresour Technol. **102:**9286–9290.
- Venkata Subhash G, Venkata Mohan S. 2014. Lipid accumulation for biodiesel production by oleaginous fungus *Aspergillus awamori*: Influence of critical factors. Fuel **116:** 509–515.
- Vicente G, Bautista LF, Rodriguez R, Gutierrez FJ, Sadaba I, Ruiz-Vazquez RM, Torres-Martinez S, Garre V. 2009. Biodiesel production from biomass of an oleaginous fungus. Biochem Eng J. **48:**22–27.
- Vicente GM, Martínez J, Aracil A. 2004. Integrated biodiesel production: a comparison of different homogeneous catalysts systems, Bioresour. Technol. **92:**297–305.
- Wynn JP, Hamid AA, Li Y, Ratledge C. 2001. Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*. Microbiology. **147(**10)**:**2857-2864.
- Yousuf A, Sannino F, Addorisio V, Pirozzi D. 2010. Microbial conversion of olive oil mill waste waters into lipids suitable for biodiesel production. J Agri Food Chem. **58:** 8630–8635.
- Zinoviev S, Müller-Langer F, Das P, Bertero N, Fornasiero P, Kaltschmitt M, Centi G, Miertus S. 2010. Next-generation biofuels: Survey of emerging technologies and sustainability issues. Chemistry and sustainability, energy and materials. **3**(10)**:**1106-1133.