

Original Research Article

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Biodiesel Production by *Pichia occidentalis* MH879824.1

Laila Ramadan, Yasser Fathy Abdelaliem* and Salem Abdelfattah Mahfoz

Department of Agricultural Microbiology, Faculty of Agricultural, Fayoum University, Egypt

*Corresponding author

ABSTRACT

Keywords

Honey Oleaginous yeasts, Biodiesel, lipid extraction, Fatty acids analysis, 26S rDNA gene, Nitrogen-limited medium

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A lipid producing isolate (LPI 65) was identified by a molecular genetics technique as *Pichia occidentalis* MH879824.1 was investigated for its ability to produce biodiesel. The isolate was screened for its ability to accumulate lipids within the cells by culturing on nitrogen-limited medium (NLM) and using Sudan black B staining technique. Estimation of lipid content % was performed. The effect of different glucose concentrations in NLM on dry biomass and lipid weight were conducted and results showed that the best concentration of glucose was 70 g/L. The possibility of using different wastes such as sugar cane molasses, waste food oil and shortening, whey permeate, and waste motor oil as carbon sources for lipid production were determined, and the results showed that the best waste for lipid production was waste motor oil. Optimization of fermentation conditions such as effect of NLM composition, NLM pH, incubation temperature, and incubation period were also investigated and results of these tests showed that the best lipid accumulation when NLM5, at pH 5.5 and incubated at 28°C for 120 h. Fatty acids profile analysis achieved that the lipids extracted from the isolate mainly contained the principal fatty acids (triacylglycerol's, TAGs) which are similar to that of vegetable oils mainly oleic, linoleic and palmitic acid.

Introduction

In the last few decades, oil prices have been experiencing an increasing trend. The main sources used in the energy and transport sector are still petroleum based. However, the gap is closing as a result in reducing production costs of bio-based materials (Martínez *et al.*, 2015). In the same way, bioethanol and biodiesel are still the main biofuels used in transport applications, with biogas also presenting a few large-scale applications in some localized European areas. The extensive use of liquid biofuels

was initially controversial due to the impacts on food prices. Advanced biofuels, although it is a term that is not yet well defined, are rapidly becoming a reality. These fuels may be defined based on the conversion technologies used, which are still in the research and development pilot or demonstration phases, and are commonly referred to as second- or third-generation biofuels (Balan *et al.*, 2013). In 1993, Ratledge stated that lipids accumulating microorganisms called oleaginous microorganisms are defined as microbes with the capacity to accumulate a lipid content of

greater than 20%. Lipids produced from these types of microorganisms are known as single cell oils (SCO) to clearly identify their origin from microbial sources. The oil produced has the same triacylglycerol (TAGs) structure as plant oils. While Zhang *et al.*, (2011) found that as a major component of cell membranes, fatty acids are synthesized in high flux and converted into phospholipids in all organisms. The long hydrocarbon chain is energy-rich, which makes it an ideal precursor for biofuels.

The advantages of utilizing biodiesel replacing conventional fuel are that biodiesel in engines are non-toxic, biodegradable, renewable and less pollutant in emissions. Engine life can be prolonged by reducing the frequency of engine part replacement and by increasing the lubricity. Due to the high cost of feedstock used for biodiesel synthesis, it is not yet globally commercialized. The oleaginous yeasts are the best source as biodiesel producer and can be used for future research programs Karthika and Karthika, (2018).

More than 95% of biodiesel production feed stocks come from edible oils, which exert a lot of pressure on the cost of raw materials. Moreover, it is a cause of deforestation in some countries due to the increase in agricultural land required (Leung *et al.*, 2010, and Olkiewicz *et al.*, 2012). The use of waste cooking oil as a feedstock may represent a reasonable alternative that also solves the problem of waste oil disposal (Lin *et al.*, 2011 and Fierro *et al.*, 2014).

The use of low-cost carbon sources for the production of SCO has been extensively studied as a mean of reducing costs, and thus becoming competitive with traditional energy crops for oil production (Helwani *et al.*, 2009, Li and Wang 1997, and Gouda *et al.*, 2008). Alvarez and Rodriguez (1992) stated that

molasses has not been traditionally studied as a suitable substrate due to its high content of organic nitrogen.

The study of Katre *et al.*, 2012 is one of the few reports of the conversion of waste cooking oil and motor oil into valuable lipids. Nigrocincta (Denise *et al.*, 2002). Most microorganisms do not grow in honey because of its low water activity of 0.6 (Molan, 1992). Hydrogen peroxide, methylglyoxal, bee defensin, pH, osmotic effect as well as leptosin were known to be responsible for the antimicrobial effects of honey (Mandal, 2011; Kato *et al.*, 2012).

Materials and Methods

Yeast isolation

Pickle Carrots sample was serially diluted and added to plates, then YEPD medium (Glucose - 2.0%, Yeast Extract - 1.0%, peptone - 2.0% pH-6.0) was poured. Plates were incubated at 28°C for 48 hrs. Selected colonies were further streaked on YEPD plates to obtain pure culture (Pan *et al.*, 2009).

Cultivation medium

Nitrogen-limited medium Marjan *et al.*, (2013) with some modifications) which containing g/L Glucose 70.0, (NH₄)₂SO₄ 2.0, KH₂PO₄ 7.0, MgSO₄·7H₂O 1.5, NaH₂PO₄ 2.0, Yeasts extract 0.75, pH 5.5 was used.

Qualitative assay of lipid production from yeast

The purified yeast culture cultivated on NLM Marjan *et al.*, (2013) and after 5 days of incubation period at 28- 30 °C, the yeast culture was further screened for its cellular lipid by qualitative analysis with Sudan black B staining technique according to (Anuradha *et al.*, 2014) .

Cultivation of yeast for lipid production

Standard inoculum

Standard inoculum of the isolate prepared by inoculation of conical flask (250 ml volume) containing 50 ml of inoculum's medium (Pan *et al.*, 2009) with a loop of the tested yeast. The inoculated flask was incubated on rotary shaker (160 rpm) for 48 hours at 28° C. Under aseptic conditions, five ml of the content of the flask was used as an inoculum for 50 ml of productive medium.

Lipid production

Five ml of propagated culture of yeast isolate was transferred into flasks containing 50 ml of production medium, then incubated at 28 °C in shaking incubator (160 rpm) for 5 days (Pan *et al.*, 2009). Total yeast lipids were extracted according to the procedures described by Bligh and Dyer (1959) with modifications described by (Pan *et al.*, 2009), then lipid content was determined. A 50 ml sample was centrifuged at 5000 rpm for 10 min, after that the yeast cells were washed twice with 50 ml of distilled water, and then 10 ml of 4 M HCl were added into, and incubated at 60 °C for 1 to 2 h. Then the acid-hydrolyzed mass was stirred with 20 ml of chloroform/methanol mixture (1:1) at room temperature for 2 to 3 h, followed by centrifugation at 2000 g for 5 min at room temperature to separate the aqueous upper phase and organic lower phase. After that the lower phase containing lipids was recovered with a Pasteur pipette, and evaporated at room temperature. The dry lipids were weighed.

Effect of carbon sources on lipid production

The influence of carbon sources (Glucose, sugar cane molasses, whey permeate, waste food oil, waste food shortening, and waste

motor oil) on biomass weight and lipid production were investigated.

Effect of glucose concentration

The propagation was carried out as mentioned before and the cultures were inoculated in basal NLM with different glucose concentrations being 40, 70, and 100 g/ liter.

Effect of sugar cane molasses concentration

Sugar cane molasses was obtained from Sugar & Integrated Industries Company in Al-Hawamdeya, Egypt, and kept in refrigerator at 4°C until used. Molasses was diluted with water in an equal volume using the method of Johnson *et al.*, (1995) with some modifications.

The concentration of sugar cane molasses was at 83.2, 145.8, and 208.2 which equalize 40, 70, and 100 g/L glucose respectively, then completed to one liter and the components of NLM except glucose were added.

Effect of whey permeate

Whey permeate sample was obtained from the model dairy unit, Faculty of Agriculture, Fayoum University, and stored at 4 °C for the preparation of the fermentation medium. Whey permeate was sterilized at 121 °C for 15 min for coagulation of protein residues and then filtered through filter paper.

The supernatant was collected and used with the components of NLM g/L glucose 20g - (NH₄)₂ SO₄ 2g - KH₂PO₄ 7g - NaH₂PO₄ 2 g - MgSO₄. 7H₂O 1.5 g - Yeast extract 0.75g and added to a liter of permeate (20g glucose/ L which equal 70g sugar/L). The components of NLM without any carbon source were added to a liter of permeate (control) as described by Ykema *et al.*, (1988).

Effect of waste motor oil, waste food oil, and waste food shortening as carbon sources

Waste food oil, used in the present study was obtained from local market of Fayoum – Egypt, from shop selling fried vegetables (potatoes, green pepper, eggplant, and falafel), and Waste food shortening obtained from local market of Fayoum – Egypt, from shop selling different types of fast food such as, chicken, meat products, waste motor oil was obtained from Fayoum – Egypt also. Using the method of Heba El Bialy *et al.*, (2011) with some modifications, each type of oil (5, 10, and 15 g) were added to tween 80: water (25: 75) and autoclaved separately. Autoclaved oil was aseptically mixed with basal medium at a final content 5, 10, and 15 g/L. Glucose concentration used in this experiment was 10 g/L for waste food oil, and shortening, and waste motor oil supplemented medium as growth starter for yeast isolates.

Optimization of fermentation conditions for lipid production

Effect of NLM composition, NLM pH, incubation temperature, and incubation period were also investigated. Different five nitrogen limited media, NLM 1 Gao-Qiang *et al.*, (2010), NLM 2 Pan, *et al.*, (2009), NLM 3 Gohel *et al.*, (2013), NLM 4 Pirozzi *et al.*, (2014), and NLM 5 Marjan *et al.*, (2013) with some modifications were used, pH was 4.5, 5.0, 5.5, and 6.0, incubation temperature was 25, 28, and 35°C, and incubation period was 96, 120, and 144h.

Fatty acids analysis (GC- MS fraction) of extracted lipids

Fatty acids analysis was confirmed at Oil and Fat Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

Preparation of fatty acids methyl esters

Fatty acids methyl esters prepared from total lipids by using rapid method according to the method of ISO 12966-2 (2011). Where, fatty acids methyl esters were formed by transesterification with methanolic potassium hydroxide as an intermediate stage before saponification take place. Approximately 0.1g of the lipid sample was placed in 5 ml screw-top test tube and 2 ml of isooctane were added to the tube then the tube was shaken. Methanolic potassium hydroxide solution (0.1 ml, 2N) was put on the cap fitted with a Polytetrafluoroethylene (PTFE)- joint, tighten the cap, and shaken vigorously for 30 seconds. The tube was left to stratify until the upper solution became clear and the upper layer containing the methyl ester was decanted. The isooctane solution is suitable for injection into the gas chromatography.

Gas liquid chromatography (GLC) of methyl esters of fatty acids

Fatty acids methyl esters were injected in to (HP 6890 series GC) apparatus provided with a DB- 23 column (60m x 25µm). Gas carrier was N₂ with flow rate 2.2 ml/min, splitting ratio of 1:50. The injector temperature was 250°C and that of flame ionization detector (FID) was 300°C. The temperature setting was as follows: 150 °C to 210 °C at 5°C/min, and then held at 210 °C for 25 min. Peaks were identified by comparing the retention times obtained with standard methyl esters.

Yeast identification

Yeast isolate was identified according to (White *et al.*, 1990). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

Results and Discussion

Effect of carbon sources on lipid production

The effect of glucose concentrations in NLM on lipid production

Data in Figure 1 indicate the effect of different glucose concentrations as a carbon source on the production of dry biomass, and lipid weight. The addition of 70 g glucose gave 26.0 g, and 11.6g for the both parameters, respectively. These results are higher than those obtained by Ratanaporn, and Ratanaporn (2011) which was 8.8 g/L dry biomass and 4.1 g/L lipid production at a concentration of glucose 80 g/L, and in agreement with the results obtained by Gohel *et al.*, (2013) who showed that the best concentration of glucose for lipid production was 70 g/L.

Effect of sugar cane molasses as a carbon source on lipid production

Data in Figure 2 indicate the effect of sugar cane molasses as a carbon source on the production of dry biomass, and lipid weight. Isolate LPI 65 gave 11.8 g for lipid weight when 83.2 g (40 g sugar) were used, while the highest dry biomass 32.7 g/L was obtained when 208.2 g molasses (100 g sugar) were added to the fermentation medium. These results are better than those obtained by Noura El-Naggar *et al.*, (2011) who used 4.2 g sugar cane molasses with *Candida albicans*, where cell weight and lipid weight only reached 6.5 g/L and 1.1 g/L, respectively.

Effect of whey permeate as a carbon source on lipid production

Results in Figure 3 revealed that the addition of 20 g glucose gave the highest dry biomass and lipid production with the tested isolate.

The LPI65 gave 37.8 g dry biomass superior to the control which only gave 8.47. Recording lipid weight, it is clear that the addition of 20 g glucose gave more than three times the control; 10.4 and 3.4 g, respectively.

Effect of using different concentrations of waste motor oil as a carbon source on lipid production

Data in Figure 4 illustrate the effect of using different concentrations of waste motor oil as a carbon source on the production of dry biomass, and lipid weight. The addition of 15 g/L waste motor oil gave 18.09 g, 14.6g for the both parameters, respectively, followed by 10 and 5 g/L.

Effect of using different concentrations of waste food oil as a carbon source on lipid production

Batch cultures were investigated to determine the possibility of using waste food oil as a carbon source for lipid production. Data in Figure 5 show the effect of using different concentrations of waste food oil as a carbon source on the production of dry biomass, and lipid weight. The highest dry biomass and lipid weight 36.6 and 5.2, respectively, were obtained when 15 g/L waste food oil were added. The results in this study are higher than those obtained by Heba El Bialy *et al.*, (2011) who reported that the maximum lipid content was 12.9 %, and dry biomass was 9.12g/L whereas in the present study the maximum lipid content and the maximum dry biomass was 44.0 % and 36.6g/L respectively.

Effect of using of waste food shortening as a carbon source on lipid production

Batch cultures were investigated to determine the possibility of using waste food shortening as a carbon source for lipid production. In Figure 6 data show that the highest dry

biomass and lipid weight 40.1g and 5.2, respectively, were obtained when 15 g/L waste food shortening were added. It's clear that the higher concentration of waste food shortening, the highest biomass and lipid.

Optimization of fermentation conditions for lipid production

The effect of NLM pH on lipid production

The growth and lipid production of yeast were affected by the medium pH as shown in Figure 7. Isolate LPI 65 gave the highest dry biomass 35.2 g when pH was 6.0, but the highest lipid 12.0 g was recorded, when the pH was 5.5. These results are in agreement with those obtained by El – Fadaly *et al.*, (2009) who showed that the best initial pH was 5.5 for the production of cell biomass and lipids.

The effect of incubation temperature on lipid production

Data in Figure 8 indicate the effect of different incubation temperatures on the production of dry biomass, and lipid weight. The highest dry biomass and lipid weight 22.8 g and 9.9 g were obtained at 28°C. These results disagree with the results obtained by Noura El-Nagggar *et al.*, (2011) who showed that the best incubation temperature was 25°C for the production of cell biomass and lipids. When the temperature increased to 28°C the growth rate decreased, while 25 °C gave the highest dry biomass (8.3 g/L), and the highest lipid weight (1.6 g/L). The temperature 25°C, gave the highest dry biomass (12.8 g/L), and the highest lipid weight (7.2 g/L) Marjan *et al.*, (2013).

Table.1 Fatty acids analysis (GC- fraction) of extracted lipids

Fatty acids		Fatty acids %
Name	Symbol	LPI 65
Myristic acid	C14:0	0.68
Myristoleic acid	C14:1	0.18
Pentadecanoic acid	C15:0	0.24
Pentadecenoic acid	C15:1	1.00
Palmitic acid	C16:0	15.10
Palmitoleic acid	C16:1	5.57
Heptadecanoic acid	C17:0	1.08
Heptadecenoic acid	C17:1	1.68
Stearic acid	C18:0	4.79
Oleic acid	C18:1	34.41
Linoleic acid	C18:2	19.96
Gamma-linolenic acid	C18:3n6	0.76
α-Linolenic acid	C18:3n3	11.11
Stearidonic acid	C18:4	0.84
Arachidic acid	C20:0	1.10
Gondoic acid	C20:1	0.28
Eicosadienoic acid	C20:2	0.16
Arachidonic acid	C20:4	0.40
Eicosatrienoic acid methyl ester	C20:3n3	0.35
Behenic acid	C22:0	0.06
Eicosatetraenoic acid	C22:4	0.20

Fig.1 The effect of glucose concentrations in NLM on lipid production

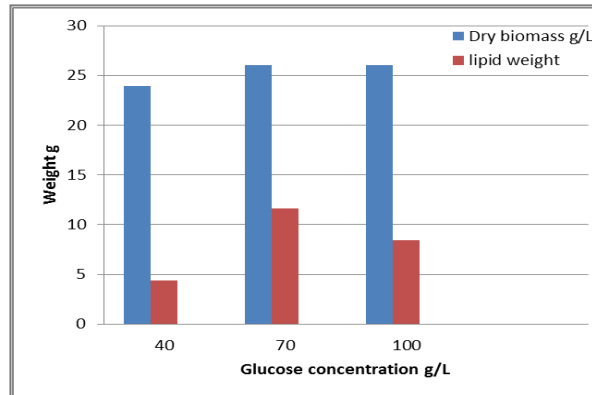


Fig.2 Effect of sugar cane molasses as a carbon source on lipid production

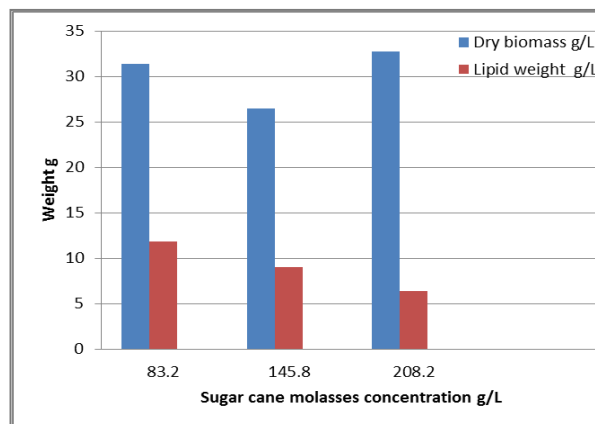


Fig.3 Effect of whey permeate as a carbon source on lipid production

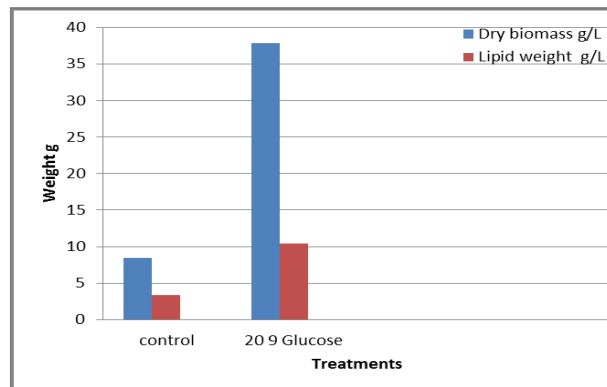


Fig.4 Effect of using waste motor oil as a carbon source on lipid production

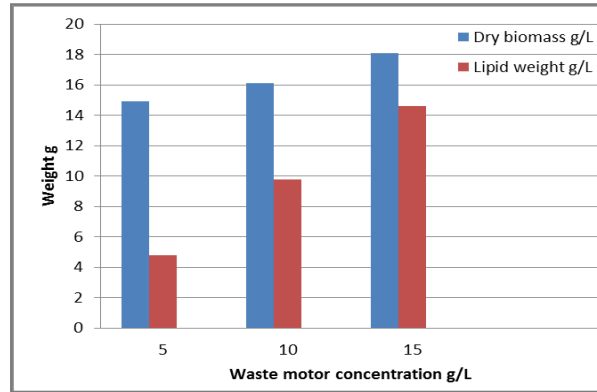


Fig.5 Effect of using different concentrations of waste food oil as a carbon source on lipid production

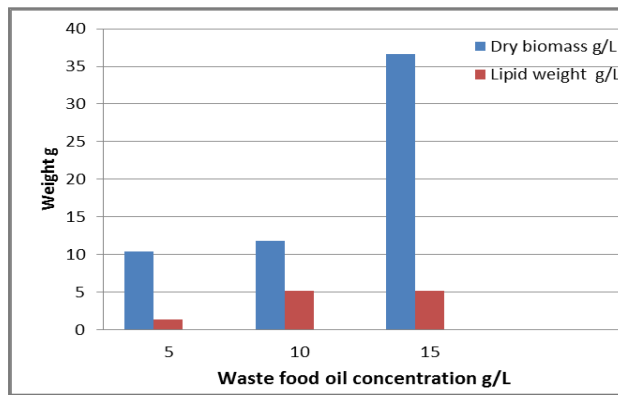


Fig.6 Effect of using waste food shortening as a carbon source on lipid production

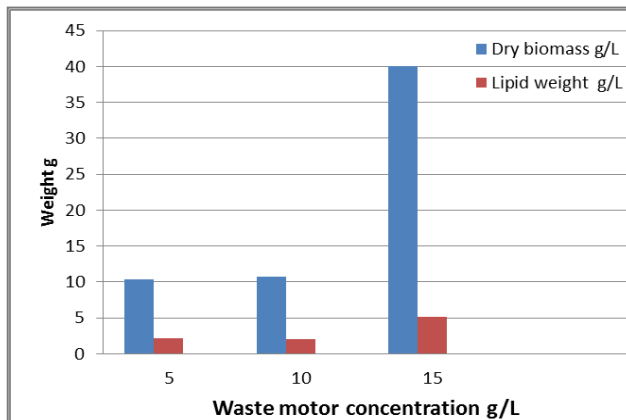


Fig.7 The effect of NLM pH on lipid production

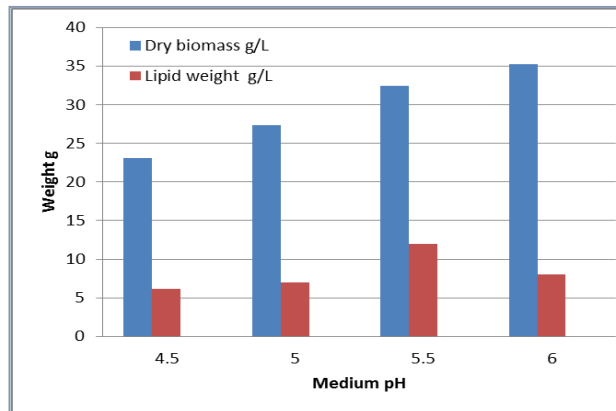


Fig.8 The effect of incubation temperature on lipid production

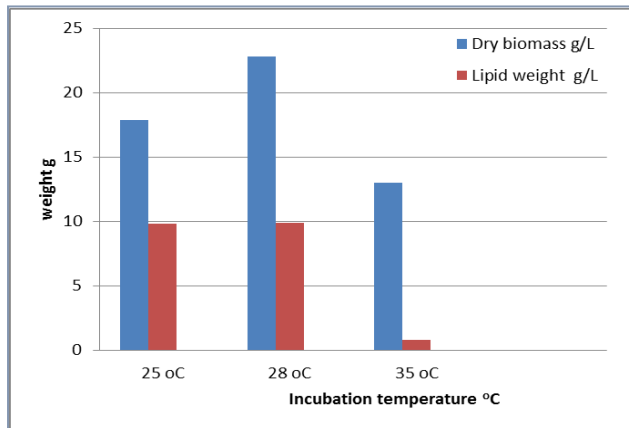


Fig.9 The effect of incubation period on lipid production

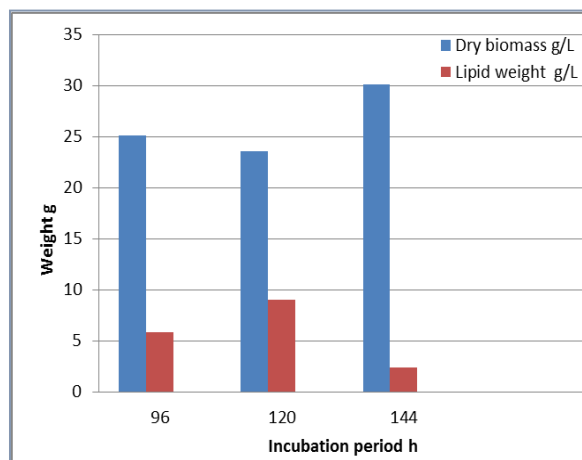


Fig.10 The effect of NLM composition on lipid production

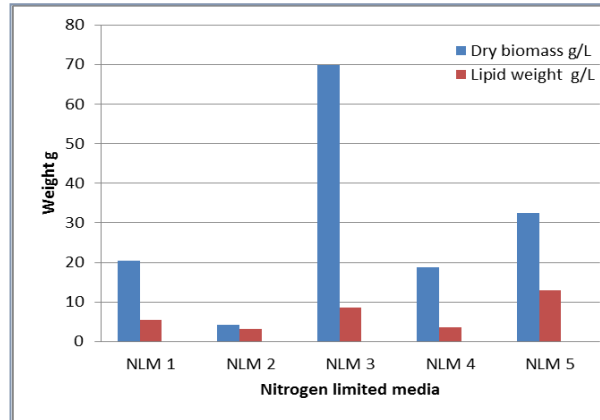


Fig.11 Chromatogram of fatty acids methyl esters of LPI 65

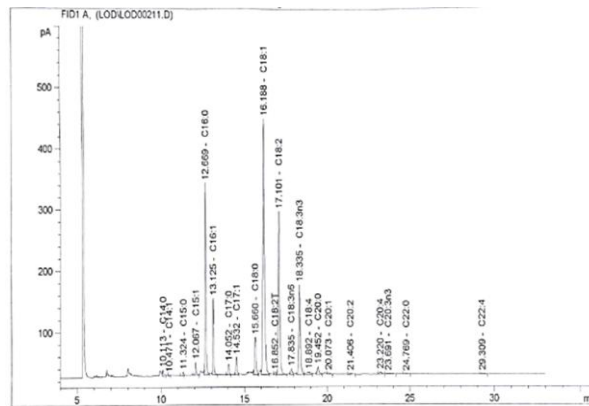
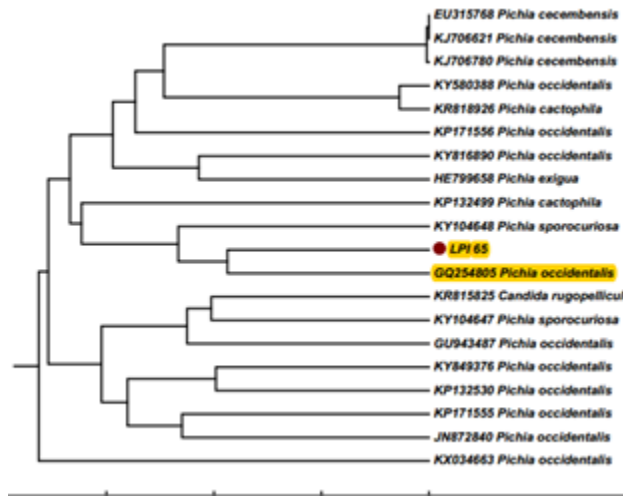


Fig.12 Contiguous of yeast LPI 65

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GGCTAGGATACTCTTACACGTGCGTGAGCGCAAGCAAACACGAAAACTGT
AGTACGAGAGTCAAACAAACCAAAAAACAAACTTTCAACAACGGATCTCTTGG
TTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCC
ATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGG
CATGCCTGTTTGAGCGTCGTTTCCTTCTTGCTTGGGAGCAGAAATGGGGGGCCC
TGGCATTGGGGCCGCTCTGAAAAGAAACGTTGCGGGCGAAGCGAACTATGAGTAG
GACGCTTGGCCGCCGAACCTTAATACATAAGCTCGACCTCAAATCAGGTAGGAATA
CCCGCTGAACCTTAAGCATATCAAAGGCCGGAGGAA
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Fig.13 Phylogenetic tree of ITS sequences of rDNA from yeast samples isolated in the present study LPI 65, aligned with related sequences accessed from the Gen Bank



The effect of incubation period on lipid production

The growth and lipid production of yeasts were affected by fermentation period as shown in Figure 9. Data in the figure show the effect of different incubation periods on the production of dry biomass, and lipid weight. The highest dry biomass 30.1 g was obtained after 144 h, but the highest lipid weight 9.0 g after 120 h. These results disagree with those obtained by Noura El-Naggar *et al.*, (2011), who showed that the best fermentation period was after 72 h, where lipid content reached 20.0 %, where after 24 h lipid content was only 16.9 %. And after 72 h Marjan *et al.*, (2013) reported that lipid content reached 57.5 %, where in the present study the highest lipid content reached 38.1% after 120 h.

The effect of NLM composition on lipid production

Data in Figure 10 indicate the effect of NLM composition on the production of dry biomass, and lipid weight. The highest dry biomass 69.9 g was obtained on NLM 3, and the highest lipid weight 12.9 g was obtained

on NLM 5. These results are higher than those obtained by Abdou, (2015) where NLM 1 gave 4.0 g/L cell dry biomass, and lipid weight 0.82 g/L, less than the results obtained by Pan *et al.*, (2009) NLM 2 gave 22.3 g/L cell dry biomass, and lipid weight 5.6 g/L, and in agreement with those reported by Marjan *et al.*, (2013) who also added that the production of lipids in the medium with glucose as a sole carbon source was more efficient than xylose in NLM 5, where glucose gave the highest cell dry biomass was 17.4 g/L, and the highest lipid weight 7.1 g/L, when the medium composition was modified with increasing glucose concentration to 70 g/L.

Fatty acids analysis (GC - MS fraction) of extracted lipids

The highest dominant fatty acid among the fatty acids profile of isolates LPI 65 was the oleic acid (C18:1) with content 59.48%, followed by linoleic acid (C18:2) with content 19.96%, palmitic acid (C16:0) with content 15.10%, as shown in table 1 and Figure 11. These results are in agreement with those obtained by Dalia Abdel Mawla (2015), who showed that the highest dominant fatty acids

were the oleic acid with content ranging from 27.85 to 55.69%, followed by palmitic acid with content ranging from 16.08 to 31.52%, which nearly the same percentage obtained in the present study.

Yeast identification

The 26SrDNA gene sequences of LPI 65 were compared with sequences in the Gene-Bank database. A phylogenetic tree based on 26SrDNA sequences Figure 12, revealed the affiliation of isolate LPI 65 to the genus *Pichia*, comprising 99% to 100% similarity with related strains of *Pichia occidentalis* MH879824 Figure 13.

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