PRODUCTION OF FATTY ACIDS BY *YARROWIA LIPOLYTICA* USING AGRO-INDUSTRIAL WASTE AT LOW TEMPERATURE

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ABSTRACT

Single-cell oil has long been considered an alternative to conventional oil sources. The oil produced can also be used as a feedstock for biodiesel production. In this research, Morphological, microscopical, Biochemical tests and VITEK 2 YST ID card are used to identify most lipid screening yeast isolate named Y4. Many parameters (different Carbone sources, different nitrogen sources, PH, Incubation days, shaking, and statistic incubation) were studied using the optimization of one variable at time (OVAT).

On YED agar media, the isolated yeast *Yarrowia lipolytica*Y4 gave the maximum biomass of 4.15±0.04 g/l, lipid yield of 0.86±0.03 g/l and lipid content of 20.67±0.62 %, respectively. Molasses was utilized as a carbon source and it was supporting both yeast growth and lipid production with two-fold increase biomass of 9.36±0.06 g/l, lipid yield of 1.80±0.18 g/l and lipid content of 19.31±1.81 %. Also, the oil profile by gas chromatography was contain 36.17%, 56.54% and 7.26% of saturated, monounsaturated and polyunsaturated fatty acids after 7 days' incubation period at refrigerator and, contain 64.21%, 28.40%, and 7.33% of saturated, monounsaturated and polyunsaturated fatty acids after 30 days' incubation at refrigerator.

Consequently, this work study new insights for the economical oil production with valuable profile using cheap agro-industrial waste molasses as a substrate for growth of marine *Yarrowia lipolytica*Y4 yeast at low temperature 4°C for one week and one month.

Keywords: *Yarrwia lipolytica* -Single cell oils -molasses -psychrophilic marine yeast

1-Introduction

The lipids produced by microorganisms are called microbial lipids or single-cell oils (SCOs) **(Qin *et al.,* 2017)**. The production of microbial oil has many advantages compared with vegetable oils as: the cultivation of microorganisms does not require huge environmental factors **(Amaretti *et al.,* 2010; Thiru *et al.,* 2011)**. Moreover, depending on the fatty acid composition, the oil produced can be exploited for human consumption and in certain valuable industrial applications (paints and coatings, detergents, cleaning products and cosmetics) and as a potential candidate for biodiesel production because its fatty acid profile has a great similarity to vegetable oils **(Xu and Liu, 2017).** A small percentage of microorganisms can synthesize and accumulate 20-87% of their total biomass as intracellular lipids, those organisms are defined as oleaginous microorganisms **(Calvey *et al.,* 2016)**.

Some oleaginous yeast strains, such as *Rhodosporidium sp., Rhodotorula sp., Lipomyces sp.* can accumulate intracellular lipids to levels exceeding 70% of their biomass under nutrient limitation conditions. The yeast oils are now believed as a promising potential feedstock for biodiesel production due to their similar composition of fatty acids to that of vegetable oils. It has been reported that such yeast oils can be used as oil feed stocks for biodiesel production with the catalysis either by lipase or chemical catalyst **(Li *et al.,* 2008).**

As a result of this objective, the prospect of large-scale development of microbial lipids, which constitute a source of valuable fatty acids, is currently a high priority in the medical-dietary sector. The processing of microbial lipids (in most cases fungal or algal oils) containing linolenic acid (LA), eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA) is of particular interest, because these polyunsaturated fatty acids are useful as functional foods and have human health benefits **(Bellou *et al.,* 2016; Ledesma-Amaro *et al.,* 2016).** GLA or the lithium salts of microbial lipids containing this fatty acid present important anticancer characteristics. ARA, EPA and DHA aid fetal brain development, the sight function of the eye, hormonal balance and the cardiovascular system **(Ledesma-Amaro *et al.,* 2016).** The added value of these lipids is of great importance in both the food and healthcare industry **(Sun *et al.,* 2017).**

The composition of fatty acids is influenced by environmental factors., The primary factor is temperature. The most noticeable feature observed in microorganisms upon decreasing the growth temperature is the rapid synthesis of increased proportions of unsaturated fatty acids, particularly hexadecenoic and octadecenoic acids at the expense of their respective saturated acids **(Bhakoo and Herbert, 1979).** One of these wastes is molasses, which is a by-product of sugar industry, consisting of up to 50% (w/w) total sugars, minerals, vitamins and other components **(Najafpour and Shan, 2003).** It has been developed as a cheap carbon source for production of numerous industrially important chemicals, such as lactic acid, citric acid, polysaccharide, microalgal oils, and astaxanthin **(Kotzamanidis *et al.,* 2002; Liu *et al.,* 2015).**

Therefore, according to the above-mentioned findings, the objectives of the present study were:(i) isolation and identification of the marine yeast isolate capable of

yielding high amount of valuable storage lipids, (ii) characterization and comparative analysis of fatty acid profiles of novel isolated yeasts, and (iii) testing the ability of the selected isolate to utilize molasses as a cheap carbon source. Then, the high lipid accumulating oleaginous yeast will be screened using glucose as a carbon source and the growth and lipid production of selected oleaginous yeasts will be grower using cane molasses as a carbon source.

2-Materials and methods

2.1. Sample collection, Isolation and Purification of Yeast

About 26 marine samples were collected from Mediterranean Sea and Red Sea in Egypt according to **Zaky et al. (2014).** Two marine samples were collected at 5-15 cm depth below the surface of sea water for each site. All samples were immediately put in sterile polyethylene bags and stored at 4°C until use.

Yeast isolation was performed according to the methods of **(Pan *et al.*, 2009).** One ml of each marine sample was individually suspended in one ml sterile distilled water and plated on YED agar medium (glucose monohydrate 10 g/l ,yeast extract 10 g/l and agar 18 g/l ,complete to 1 liter of seawater then add 0.05 g of Rose Bengal as antibacterial agent at PH 6.0 ,followed by autoclaving at 121°C .Then The plates were incubated at 4°C for 5 days until the pure colonies were obtained. Several yeast colonies were obtained from different plates and it was purified by single colony transferred repeatedly to a new YED agar plate until pure cultures were confirmed.

2.2. Screening of oleaginous marine Yeast

All isolated marine yeast strains were screened for lipid accumulation using Nile Red Staining assay **(kimura *et al.*, 2004).**

Yeast biomass were picked and incubated in dark with 0.5 ml of 0.1 mM phosphate buffer Solution (PBS) pH 7.4, and 0.05 ml Nile-Red solution (25 µg/ml in acetone).After 30 min in dark, a thin film was prepared on a slide glass and drying by air. Finally, examination all samples were performed under the fluorescence microscope (BX 40, Olympus, Tokyo,Japan) equipped with a CCD camera(U-CMT,Olympus, Tokyo,Japan). To obtained oleaginous yeast must be examined for the presence of blue or greyish colored fat globules withine the cell (**Patnayak and Sree, 2005)**.

2.3. Selection of lipid producing Yeast

All selected oleaginous yeast strains with the highest fluorescence signals were grown on 50 ml YED broth medium in 250 ml triplicate flasks for each yeast before autoclaving at 121°C for 20 min. After autoclaving and cooling, each flask was inoculated at 4°C for 10 days under static conditions with rotator. After incubation time the cells was harvested and the dry biomass, lipid yield and lipid content were determined gravimetrically **(Pan *et al.*, 2009).** The isolate with the highest lipid content were selected for next studies.

2.4. Determination of dry biomass, lipid yield and lipid content.

To determine yeast biomass, the incubation time of each isolate (in triplicate) of culture broth was harvested from the media, then centrifugation and washing three times with distilled water, finally drying at 60^0C till constant weight. Dry biomass weight was determined gravimetrically, expressed in g/l according to **Devi et al. (2009)** and was crushed into fine powder and preserved in desiccator until use.

Lipid yield was determined using phosphovanillin method according to **Helal et al. (2006).** Phosphovanillin reagent was prepared by initially dissolving 0.6 g vanillin in 10 ml absolute ethanol and then in 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 the dark until use. To ensure high activity; fresh phosphovanillin reagent was prepared shortly before every experiment run.Take 0.1g of dry biomass and grinded it with chloroform: methanol (2:1) then put 0.8 ml distilled water and shaking for 30 min. Then centrifugation at 4000 rpm for 5 minutes was carried out for separation to three layers, the upper layer that water contain, the second layer having yeast biomass and the lower layer containing lipid. The lower phase(lipid) was mixed with 200µ saline solution 0.1% NaCl (0.1g NaCl in 100 ml distilled water). Then centrifugation at 4000 g for 10 minutes was carried out for separation of the lower phase and aqueous upper phase. Then the lower phase was evaporated for recovery of lipid. The lipid was transferred to a new tube and added 200µ chloroform, then 0.5 mL of concentrated (98%) sulfuric acid was added to the sample and was heated for 10 min at 100 ºC, and was cooled for 5 min in ice bath or at room temperature. 3 mL of freshly prepared phosphovanillin reagent was added, and the sample was incubated for 15 min at 37 ºC in shaker at 200 rpm. Absorbance reading at 530 nm. The standard calibration curve was performed using canola oil as a standard. Total lipid yield (the amount of lipid extracted from the biomass per liter of fermentation medium) and lipid content (the percentage of lipid to dry biomass) were determined as in the following equation **(Gutierrez *et al.*, 2008).**

$$\textbf{Total Lipid Content (\%)} = \frac{\textbf{Lipid yield (g/L)}}{\textbf{Cell dry weight yield (g/L)}} \textbf{*100}$$

2.5. Identification of the selected isolate.

2.5.1. Morphological and Microscopical identification

Morphological and microscopic identification is used to identify isolated yeasts which actively grow overnight or for up to 2 days on the YED agar plate at 25°C. We studied cultures' morphological characteristics such as colony color, shape, size, texture, and diameter, and the yeast's microscopic characteristics such as cell size, appearance of shape budding, filament forming, and others **(Luttrell, 1977)**.

2.5.2. Biochemical identification tests

Physiological properties used for classifying yeasts containing the following: the ability (i) Certain sugars are semi-anaerobically fermented; (ii) to Aerobic growth with different compounds, each as a sole major source of either carbon or nitrogen (assimilation tests); (iii) to grow without of some vitamins; (iv) to grow in the presence of D-glucose 50% or 60% or NaCl 10% (w/v) + D-glucose 5% (w/v); (v) to grow at many different temperature; (vi) to grow in the presence of cyclohexamide; (vii) to destruction lipids; (viii) to produce starch-like polysaccharide; (ix) to produce urease enzyme ; (x) to acid formation , such characteristics are used for differentiating between yeast species **(Kraepelin *et al.*, 1984)**.

2.5.3. Physiological identification by VITEK

The isolates were subculture twice before testing with a Vitek 2 YST ID card, culture was done on Sabouraud dextrose agar. Identification with using the latest software update of the Vitek 2 identification system. Pure subcultures of the yeast isolates were suspended in aqueous 0.45% (wt/vol) NaCl to achieve a turbidity equivalent to that of a McFarland 2.0 standard (range, 1.80 to 2.20), as measured by turbidity meter. The VITEK 2 instrument automatically filled, sealed, and incubated the individual test cards with the prepared culture suspension. Cards were held at 35.5°C for 18 h, with optical readings taken automatically every 15 min. Based on these readings, an identification profile was established and interpreted according to a specific algorithm. Final profile results were compared to the database, generating identification of the unknown organism **(Hata *et al.*, 2007).**

2.6. Optimization of culture condition and effect of some parameter for biomass production and lipid accumulation.

Isolated yeasts were first cultivated on the basic YED medium (in g/L: glucose10, yeast extract10) and complete to one litre of sea water containing much salts and minerals, and autoclaved for 20 min at 121°C.

2.6.1. Optimization of one variable at time.

The influence of pH (4.5, 5.5 and 6.5), carbon source (glucose, lactose, sucrose, starch and glycerol) at 10 g/l in the medium, nitrogen source (yeast extract, glutamic acid , peptone , ammonium chloride and sodium nitrate) were added at 2 g/l separately in the medium and static and shaking conditions (150 rpm) were studied. After autoclaving and cooling each flask was inoculated with a disk from the margin of 5 days aged yeast cells on agar YED solidified medium in petri dishes and incubated at 4ºC for 7 days under static conditions except for the experiment of shaking at 150 rpm.

2.6.2. Effect of some parameter for biomass production and lipid accumulation.

Also, the effect of incubation period on yeast growth (3, 5, 7 and 10) days, compare between glucose and molasses as a carbon source and different concentrations

of molasses in g/l (20, 40, 60 and 80) were studied. After autoclaving and cooling each flask was inoculated with a disk from the margin of 5 days aged yeast cells on agar YED solidified medium in petri dishes and incubated at 4ºC for 7 days. Aliquot of 50 mL medium in 250 mL Erlenmeyer flasks was prepared in triplicate for each experiment. In all experiments, dry biomass, lipid yield and lipid content were determined as described above except of the test for determine the best days of incubation period for yeast growth, we are depending on optical density.

2.7. Extraction and Transesterification of lipid

Lipid extraction was done according to the method of **Bligh and Dyer, (1959).** Firstly, the dry biomass was grounded with mixture of chloroform: methanol (2:1) then agitated for 20 min at 200 rpm at room temperature. Centrifuge at 6000 rpm for 10 min to recover solvent phase. The same process was repeated two times. The solvent was evaporated, and samples were dried under vacuum, and then the amount of oil is recorded using gravimetric method.

The yeast lipid obtained was mixed vigorously with 20 ml of methanol and 2ml of concentrated sulphuric acid for 2 hours at 70ºC. After the completion of reaction, the mixture could cool at room temperature and then transferred to the separating funnel for obtaining two layers containing upper methyl ester layer and lower glycerol layer. The methyl ester was collected and analyzed using Gas chromatography–mass spectrometry (GC-MS). The chemical composition of the samples was performed using Trace GC-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50°C and then increased by 5°C /min to250 °C hold for 2 min. increased to the final temperature 300°C by 30°C /min and hold for 2 min. The injector and MS transfer line temperatures were kept at 270, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min and diluted samples of 1 µl were injected automatically using Auto sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral database **(El-Shall *et al.*, 2018).**

2.8. Statistical Analysis

All the experiments were performed in triplicates, and statistical analysis was carried out using SPSS (IBM, v. 20). The values are given as mean ±D (standard deviations). Levels of significance were considered at $p \leq 0.05$. Statistical analysis is investigated by ANOVA (one-way analysis of variance) Tukey method for the obtained results.

3. Results and discussion

3.1. Isolation and Screening of Oleaginous Marine Yeast.

In this preliminary study, about 26 yeast isolates designed as WOY1 to WOY26 were isolated from marine samples obtained from different beaches of Mediterranean Sea and Red sea, Egypt. these isolates were tested for growth on YED agar medium at low temperature (4C°) for five days to recognize the pest growth for each isolate , so the pure culture were grown on YED slants and stored at 4C° or preserved with glycerol in freezer until use. These isolates were screened for lipid accumulation by Nile red staining assay. This assay was employed simply to recognize oleaginous lipid species from non-oleaginous as; Nile red reacts only with storage lipid droplets and gives positive red fluorescence signals, which can be detected by fluorescence spectroscopy. Out of screening assay, one isolate was exhibited with the strongest red fluorescence under fluorescent microscope, so these isolates were selected for further study (Figure 1c).

3.2. Identification of the yeast

3.2.1. Morphological and Microscopical identification of the yeast

Based on the colony growth of isolate Y4 (Figure 1a) on YED agar was observed as white to cream, typical smooth, round yeast colonies and fuzzy in appearance **(Barnet, 2002).** Microscopic analysis of the cell was carried out at 40x resolution, they were typical budding and round (Figure 1b). Based on these characters, the oil producing isolate Y4 was identified *as Yarrowia sp.*

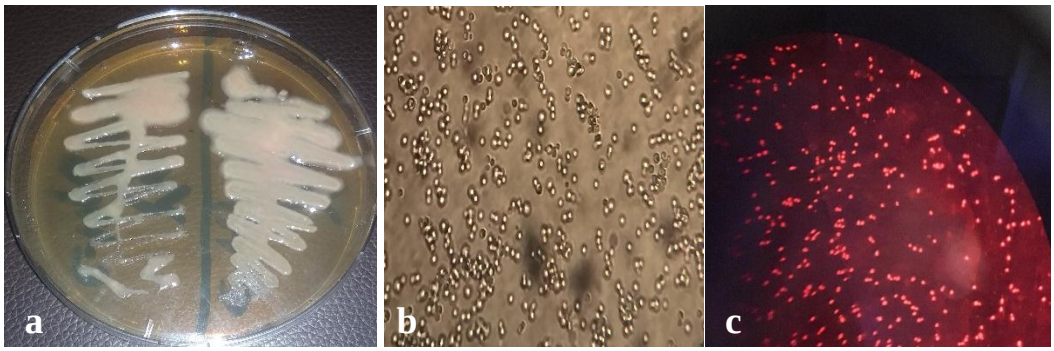

Figure 1. Colony characteristics (a), microscopic features (b), and positive red fluorescence signals for isolate Y4.

3.2.2. Biochemical identification tests

According to **Barnett et al. (2002)** we performed biochemical tests for yeast isolates (Table 1).

Table 1. list of Biochemical tests for Y4 isolate.

	Code	Test name	Y4
Anaerobic (fermentation) tests			
1	F1	D-glucose	(+)
2	F8	Lactose	(-)
3	F13	Starch	(-)
4	F14	D-Xylose	(-)
Aerobic (assimilation) tests			
-Growth tests with carbon source			
	C1	D-glucose	(+)
	C6	D-Xylose	(+)
	C18	Lactose	(-)
	C22	Starch	(-)
	C26	Xylitol	(+)
	C38	DL-Lactate	(+)
	C39	Succinate	(+)
	C41	Methanol	(-)
	C42	Ethanol	(+)
	C43	Propane1,2diol	(+)
	C44	Butane2,3diol	(-)
	C45	Quinic acid	(+)
-Growth tests with nitrogen source			
	N1	Nitrate	(-)
-Growth at different temperatures			
	T0	4 C°	(+)
	T1	25 C°	(+)
	T2	30 C°	(+)
	T3	35 C°	(+)
	T4	37 C°	(-)
	T5	40 C°	(-)
	T6	42 C°	(-)
	T7	45 C°	(-)
-Growth at high osmotic pressures			
	O3	1% Acetic acid	(+)
	O4	50% D-Glucose	(+)
	O5	60% D-Glucose	(-)
	O6	10% NaCl	(-)
	O7	16% NaCl	(-)
-Others			
	M1	Starch formation	(-)
	M2	Urea hydrolysis	(+)

(+); positive growth, (-); negative growth

3.2.3. Physiological identification by VITEK

Physiological identification tests by VITEK are done at Mabarret El Asafra Laboratories – Alexandria city, the isolate Y4 were tested by Vitek 2 YST card and successfully identified as *Yarrowia lipolytica* (Table 2).

Table 2. VITEK 2 YST card report for WOY4 isolate.

Identification information			Analysis time		17.98 hours			
			Status		final			
Selected Organism			99% Probability		*Yarrowia lipolytica*			
			Bionumber		4010000000000020			
Biochemical Details								
3	LysA	(-)	4	IML Ta	(-)	5	LeuA	(+)
7	ARG	(-)	10	ERYa	(-)	12	GLYLa	(-)
13	Tyra	(+)	14	BNAG	(-)	15	ARBa	(-)
18	AMYa	(-)	19	dGALa	(-)	20	GENa	(-)
21	dGLUa	(+)	23	LACa	(-)	24	MAdGa	(-)
26	dCELa	(-)	27	GGT	(-)	28	dMALa	(-)
29	dRAFa	(-)	30	NAGAl	(-)	32	dMNEa	(-)
33	dMELa	(-)	34	dMLZa	(-)	38	lSBEa	(-)
39	lRHAa	(-)	40	XLTa	(-)	42	dSORa	(-)
44	SACa	(-)	45	URE	(-)	46	AGLU	(-)
47	dTURa	(-)	48	dTREa	(-)	49	NO3a	(-)
51	lARAa	(-)	52	dGATa	(-)	53	ESC	(-)
54	lGLTa	(-)	55	dXYLa	(-)	56	LATa	(-)
58	ACEa	(-)	59	CITa	(-)	60	GRTas	(-)
61	IPROa	(-)	62	2KGa	(+)	63	NAGa	(-)
64	dGNTa	(-)						

Note: other well numbers between 1 and 64 not designated in this table are empty.

3.4. Optimization of one variable at time (OVAT) for *Yarrowia lipolytica*.

3.4.1. Choosing between shaking and static incubation

A comparison between incubations of shaking and static culture showed that biomass, lipid yield and lipid content were higher in static conditions than in shaking conditions. Similar findings were reported by **Kirrolia et al. (2012) ; Ali and El-Ghonemy, (2014) ; Ali et al. (2017).** They noticed that static condition gave higher lipid accumulation than the one shaking. This finding may be due to the degree of aeration, which has a great impact on the growth of the yeast and its ability to accumulate lipids (Table 3).

3.4.2. Effect of different carbon sources

One of the most important basic elements needed by living organisms is carbon, The use of different carbon sources depends primarily on the fungal enzyme system and its ability to use such simpler forms or its ability to transform complex carbon compounds into simpler ones, which can be easily used. All tested carbon sources have been found to support both yeast growth and lipid accumulation at different levels. As shown in table (3), the highest biomass (18.84±0.14 g/l), lipid yield (4.57±0.24 g/l) and lipid content (24.24±1.12 %) were recorded when *Yarrowia sp.* was grow in the presence of glucose. On the other hand, glycerol had the lowest biomass (9.62±0.087 g/l), lipid yield (0.71±0.19 g/l) and lipid content (7.39±1.92 %). According to these results glucose was the most better carbon source for lipid production by *Yarrowia sp.* ***(*Ramjegathesh and Ebenezar, 2012).**

3.4.3. Effect of different nitrogen sources

Different levels of cell biomass and lipids were obtained by using different organic and inorganic nitrogen sources as represented in table (3). Results showed that Glutamic acid was a poor nitrogen source with lowest lipid yield (1.14±0.07 g/l) and lipid content (5.66±0.47[c] %). Yeast extract was found to be the most suitable nitrogen source with lipid yield (2.49±0.14 g/l). Yeast extract is the best source of nitrogen for different microorganisms to accumulate lipids because it involves all the metal ions and micronutrients required **(Dyal *et al.*, 2005).**

3.4.4. Effect of initial PH of the medium

Effects of initial pH on cell growth and lipid accumulation of *Yarrowia sp.* were investigated with pH ranging 4.5, 5.5 and 6.5. As shown in Table 3, maximum dry biomass of (11.28±0.19 g/L) and lipid yield (1.68±0.19 g/L) with lipid content of (14.87±1.5 %), were achieved at pH 6.5 (Table 3).

Table 3. The effect of some nutritional and incubational parameters on biomass, lipid yield, and lipid content of *Yarrowia sp.*

Parameter	Dry Biomass (g/L)	Lipid yield (g/L)	Lipid content (%)
Static or shaking			
Shaking	17.82±0.18	4.22±0.18	23.66±0.78
Static	9.85±0.12	1.73±0.23	17.54±2.07
Carbon Source			
Glucose	18.84±0.14[a]	4.57±0.24[a]	24.24±1.12[a]
Lactose	10.64±0.47[b]	1.44±0.21[b]	13.56±1.89[b]
Starch	10.23±0.09[c]	1.09±0.11[b]	10.71±0.97[b]
Glycerol	9.62±0.087[d]	0.71±0.19[c]	7.39±1.92[c]
Nitrogen source			
Yeast extract	18.18±0.21[b]	2.49±0.14[a]	13.68±0.59[b]
Glutamic acid	20.46±0.28[a]	1.14±0.07[b]	5.66±0.47[c]
Peptone	17.72±0.20[b]	1.18±0.11[b]	6.68±0.56[c]

Ammonium chloride	10.31±0.27[c]	2.15±0.15[a]	20.84±1.06[a]
Sodium nitrate	17.76±0.23[b]	2.27±0.19[a]	12.74±0.96[b]
	PH value		
PH 4.5	9.48±0.29[c]	1.18±0.97[b]	12.40±0.63[b]
PH 5.5	10.43±0.19[b]	1.19±0.11[b]	11.43±0.89[b]
PH 6.5	11.28±0.19[a]	1.68±0.19[a]	14.87±1.5[a]

3.5. Effect of some parameter on lipid accumulation of *Yarrwia lipolytica*

3.5.1. Effect of incubation period on yeast growth.

It has been reported that different incubation periods vary influence on yeast growth. therefore, effects of different incubation periods on growth of isolates Y4 were tested. Effects of (3, 5, 7 and 10) days as incubation periods on growth after inoculated each isolate on YED broth (10g glucose monohydrate ,10g yeast extract complete with sea water to 1 liter) and molasses broth(10g molasses ,10g yeast extract complete with sea water to 1 liter) at 4 C° on shaker 150 rpm are presented in Figure (2). The two isolate Y4 exhibited best growth on Molasses more than YED after measured the optical density at 600 nm were 1.166±0.036, are presented in Table (4).

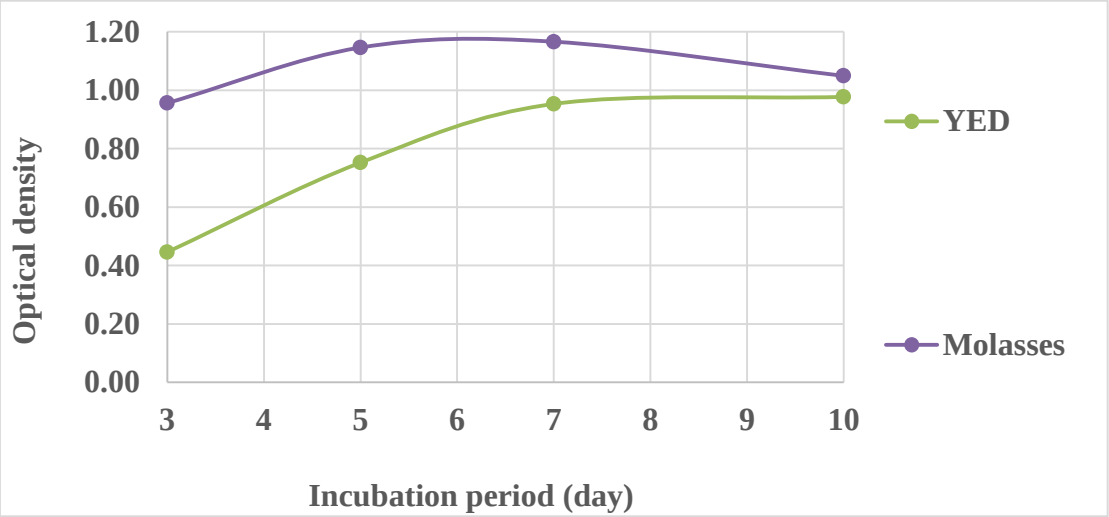

Fig. 2. Effect of incubation period on yeast growth.

Table 4. Effect of incubation periods on yeast growth.

Incubation period	Optical density YED	Molasses
3	0.446±0.038[c]	0.956±0.032[c]
5	0.752±0.073[b]	1.146±0.097[a, b]

7	0.953±0.044[a]	1.166±0.036[a]	
10		0.977±0.023[a]	1.049±0.034[b]

3.5.2. Compare between ordinary medium and molasses with the highest lipid content.

Out of the ten isolates which have positive signs with lipid screening test under microscope, selection of the best one was based on their high Intensity of emitted red fluorescence and lipid content. As presented in (Table 5), isolate Y4 that exhibited lipid yield (0.86±0.03 g/l), (1.80±0.18 g/l), respectively on YED agar medium and molasses that appear the growth on molasses exhibit two and half fold increase more than YED agar after seven days as incubation time on shaker (Figure 3).

Table 5. Biomass, lipid yield and lipid content of the selected isolate which gave the strongest red fluorescence signals on YED and molasses medium.

Isolated Code	Dry biomass (g/l)	Lipid yield (g/l)	Lipid content (%)
$Y4_{YED}$	4.15±0.04	0.86±0.03	20.67±0.62
$Y4_{Molasse}$	9.36±0.06	1.80±0.18	19.31±1.81

$Y4_{YED}$= the isolate on YED medium, $Y4_{Molasse}$= the isolate on Molasses.

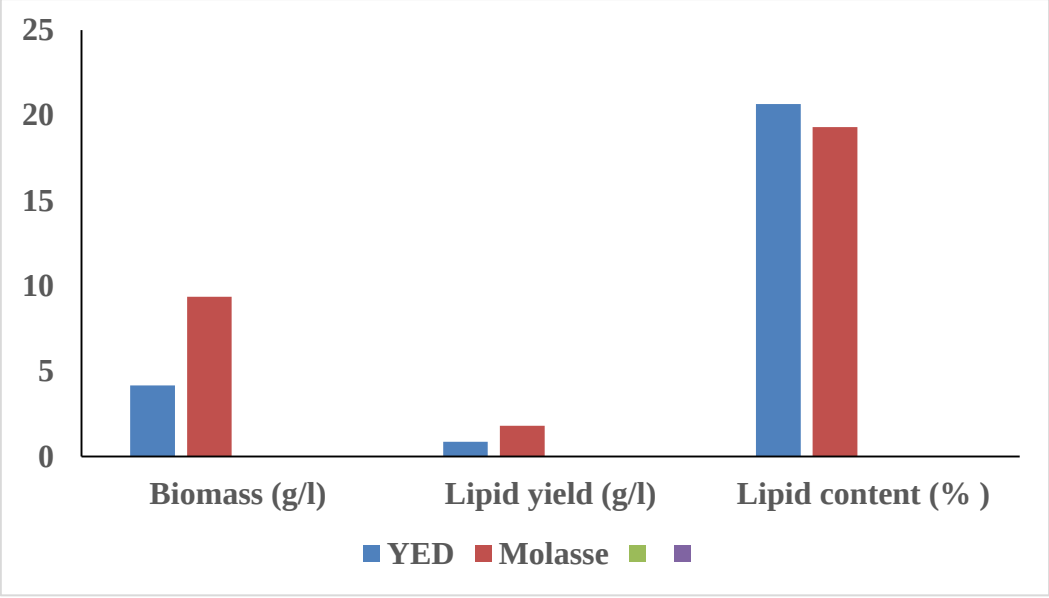

Fig. 3. Effect of ordinary medium and molasses on biomass, lipid yield and lipid content of yeast isolates.

3.5.3. Effect of different concentration of molasses on biomass and lipid yield.

To develop an improved cultivation technique for lipid accumulation by Y4, cultures were investigated to determine the suitable molasses concentration of the initial medium. Therefore, to study molasses concentration on biomass and lipid yield, the concentration of molasses at 20,40,60 and 80 g/L were investigated.

As shown in Table 6, the first isolate Y4 exhibit the best biomass of 5.94±0.29 g/L and lipid yield of 3.06±0.11 g/L was obtained at 60 g/L molasses.

Table 6. Effect of different molasses concentrations on cell growth and lipid accumulation.

Yeast isolates	Molasses concentration (g/L)	Dry biomass (g/L)	Lipid yield (g/L)	Lipid content (%)
Y4	20	4.61±0.22	1.90±0.09	41.24±0.11
	40	4.94±0.18	2.84±0.14	57.50±0.88
	60	5.94±0.29	3.06±0.11	51.54±1.13
	80	2.88±0.11	2.30±0.18	79.99±3.42

3.6. Lipid production using molasses as a carbon source

Many by-products were produced from different industries, which are regarded as waste materials with little value; these materials still contain substances, which are economically valuable, like complex and simple sugars, nitrogen substances, and inorganic salts. All of these components are important for growth of different microorganisms, and therefore, there is a strong potential for using these by-products as substrates in biotechnological production **(Gajdoš *et al.*, 2015).**

Molasses is an industrial by-product, which used in many biotechnological applications (Arshad et al., 2014; Xia et al., 2014; He et al., 2014; Ortiz et al., 2012). Molasses-based cultivation media are indefinite media, which containing different saccharides, nitrogen compounds, and many other substances affecting the growth at different rates depending on molasses batches **(Olbrich, 2006).**

According to Table 7, the growth and lipid production of oleaginous yeast *Yarrowia sp.* on molasses were studied. It was found that molasses support both the growth and lipid production with biomass (4.69±0.03 g/l),(3.46±0.09 g/l) and lipid yield (2.33±0.19 g/l), (2.16±0.16 g/l) respectively after 7 and 30 days incubation , respectively.

Table 7. Effect of two different incubation periods (7 days and 30 days) on biomass, lipid yield and lipid content of *Yarrwia sp.* grown on molasses.

Yeast isolate	Incubation Period (days)	Dry biomass (g/L)	Lipid yield (g/L)	Lipid content (%)
Y4	7	4.69±0.03	2.33±0.19	49.62±3.73
	30	3.46±0.09	2.16±0.16	62.43±2.93

3.7. Fatty acids profile analysis

Fatty acids, both free and as part of complex lipids, are essential components of all vital membranes **(Hassan *et al.*, 2018).** The type of species and growth conditions, i.e., temperature, pH, type of substrate, variation in C/N ratio and oxygen not only influence the efficiency of lipid accumulation, but also the fatty acid profiles of the cellular lipids according to **Jacob (1993).**

According to Table (8), when the *Yarrowia lipolytica*Y4 was grown on molasses after 7 days of incubation period, the yeast oil was found to contain saturated 36.17%, monounsaturated 56.54% and polyunsaturated 7.26% fatty acids with dominant Heptanoic acid(C13) 43.20%, followed by Palmitic acid methyl ester (C17) 27..99% then Nonadecylic acid (C19) (8.18%), respectively ,and after 30 days of incubation period, the yeast oil was found to contain saturated 35.73%, monounsaturated 28.40% and polyunsaturated 7.33%, fatty acids with dominant Palmitic acid methyl ester (C17) 42.78% followed by Heptanoic acid(C13) 19.73% and Nonadecylic acid (C19) (15.99%) , respectively.

Interestingly, significant differences were observed in the fatty acid composition for the yeast isolates at different incubation period. The *Yarrowia lipolytica*Y4 on molasses at incubation period 7 days has a content of omega-3 PUFA (2.82%) and omega-9 MUFA (10.88%) while, at incubation period 30 days has a content of omega-6 PUFA (2.66%), omega-7 PUFA (2.74%) and omega-9 MUFA (5.68%), respectively.

All significant PUFAs are valuable fatty acids and are important to human health, suggesting that the PUFA-rich oil produced by *Yarrowia lipolytica.* Would have high potential in food and therapeutically applications.

Table 8. Fatty acids composition and concentration of extracted total lipids *Yarrowia lipolytica*Y4 and *Candida parapsilosis*Y8 after 7 days and 30 days incubation period using molasses as a carbon source by GC/MS at 4 C°.

Common name	Systematic name	Type	Percentage of fatty acids	
			7 days	30 days
Undecylenic acid	C11	MUSFA	-	1.11
Lauroleic acid (ω -3)	C12:1	PUSFA	2.82	-
Heptanoic acid	C13	MUSFA	43.20	19.73
Nonanoic acid	C15	PUSFA	1.05	-
Pentadecynoic acid	C15	PUSFA	3.39	1.93
Pentadecylic acid	C15:0	SFA	-	1.09
Cis-6,9,12,15-Hexadecatetraenoic acid	C16	MUSAF	2.46	-
Palmitic acid	C16:0	SFA	-	2.66
cis-10-Heptadecenoic acid	C17:2	MUSFA	-	0.51
Palmitic acid methyl ester	C17:0	SFA	27.99	42.78
Oleic acid (ω-9)	C18:1	MUSFA	4.23	1.37
Elaidic acid (ω-9)	C18:1	MUSFA	0.79	-
Stearic acid	C18:0	SFA	-	1.69
Linoleic acid methyl ester(ω-6)	C19:2	PUSFA	-	2.66
Oleic acid methyl ester(ω-9)	C19:1	MUSFA	-	3.02
Nonadecylic acid	C19:0	SFA	8.18	15.99
Cis8,11Eicosadienoic acid(ω-9)	C20:2	MUSFA	5.86	2.66
Paullinic acid (ω-7)	C20:1	PUSFA	-	2.74
SFAs (saturated fatty acids)			36.17	64.21
USFA (unsaturated fatty acid)			63.80	35.73
MUFAs (monounsaturated fatty acids)			56.54	28.40
PUFAs (polyunsaturated fatty acids)			7.26	7.33

4. Conclusions

In the present study, several yeast isolates were screened for its lipid accumulation. one of them Y4 is identified as promising lipid producers a productivity reach to 63%. the identification of isolates is done by several ways such as morphological, microscopical, physiological and vitek 2 YST Card. in this investigation we study the production of fatty acid by yeasts *Yarrowia lipolytica* at low temperature,

because the saturated and polyunsaturated fatty acid are increase according to temperature decrease. The effect of different factors on yeast dry biomass, lipid yield and lipid content were studied. Sugar cane molasses was proved as a suitable carbon source for both yeast growth and lipid production. The oil produced by *Yarrowia lipolytica*Y4 contain saturated, mono-unsaturated and poly-unsaturated fatty acids.

Thus, *Yarrowia lipolytica* represent a promising candidate for production of single cell oils at low temperature (4C°) and can used sugar cane molasses as a carbon source substrate for a long time.

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انتاج الأحماض الدهنية بواسطة Yarrowia lipolytica باستخدام النفايات الصناعية الزراعية في درجات حرارة منخفضة

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يعتبر الزيت الحيوى بديلا عن الزيوت التقليدية وبالتالى فان الديزل والوقود الذى شهد تناقصا كبيرا فى السنوات الاخيرة على مستوى العالم يمكن استبدالة بالوقود الحيوى

يتم تعريف الكائنات المنتجة للدهون على انها ميكروبات لها القدرة على تخزين الدهون بنسبة اعلى من ٢٠ فى المائة من كتلتها الجافة، وجد بالبحث ان الفطريات اكثر قدرة على انتاج الاحماض الدهنية من الميكروبات احادية الخلية مثل البكتريا وتعتبر الخمائر المنتجة للدهون هى ميكروبات قادرة على تحويل الكربوهيدرات الى دهون والتى استخدمت فى القرن الماضى على نطاق واسع فى العديد من الصناعات مثل النبيذ والايثانول الحيوى والخبز والانتاج الدوائى ،تتمتع الخمائر المنتجة للدهون بمعدلات نمو وانتاج دهون عالية و كما تعتبر نفايات قصب السكر من المنتجات الثانوية الرئيسية الناتجة عن عملية إنتاج قصب السكر،ويعتبر الوقود الحيوي هو أحد المنتجات ذات القيمة المضافة والتى يتم انتاجها من نفايات قصب السكر.

في هذه الدراسة ، قمنا بدراسة إنتاج الأحماض الدهنية (الزيوت) من الخمائر البحرية المعزولة من البحار المصري عند درجة حرارة منخفضة والعوامل الموثرة على انتاج الاحماض الدهنية وايضا دراسة جدوى استخدام دبس السكر كمصدر رخيص للكربون لإنتاج الدهون

على وسط YED أجار أعطت الخميرة أقصى كتلة حيوية ٤.١٥ جم / لتر وحاصل دهني ٠.٨٦ جم / لتر ومحتوى دهني ٢٠.٦٧٪ على التوالي وتم استخدام دبس السكر كمصدر للكربون وكان يدعم كلاً من نمو الخميرة وإنتاج الدهون بكتلة حيوية قدرها ٩.٣٦ جم / لتر ، وحاصل دهنى ١.٨٠ جم / لتر ومحتوى دهنى بنسبة ١٩.٣١٪. احتوى ملف الزيت بواسطة جهاز كروماتوجرافي الغاز على ٣٦.١٧٪ و ٥٦.٥٤٪ و ٧.٢٦٪ من الأحماض الدهنية المشبعة ، الأحادية غير المشبعة والمتعددة غير المشبعة على التوالى بعد فترة حضانة لمدة ٧ أيام في الثلاجة. وأيضًا ، احتوى ملف الزيت بواسطة جهاز كروماتوغرافي الغاز على ٦٤.٢١٪ و ٢٨.٤٠٪ و ٧.٣٣٪ من الأحماض الدهنية المشبعة و الأحادية غير المشبعة والمتعددة غير المشبعة على التوالى بعد فترة حضانة لمدة ٣٠ يوم فى الثلاجة

وبناءً على ذلك ، فإن هذه الدراسة تقدم رؤى جديدة لإنتاج الزيت الاقتصادي من الخمائر البحرية *Yarrowia lipolytica* باستخدام النفايات الزراعية الصناعية الرخيصة مثل دبس السكر كمصدر للكربون والنامية عند درجة حرارة منخفضة ٤ درجة مئوية لمدة أسبوع اوشهر

الكليمات المفتاحية : الخمائل البحرية ، الاحماض الدهنية ، دبس السكر