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Yeasts as a Promising Tool for Microbial Oil Production

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ABSTRACT

In the present study, thirty three of oleaginous yeast isolates were obtained from different location from Egyptian soil by using enrichment technique on YPD medium. These isolates were screened on its ability to accumulate lipid within cells on nitrogen limiting medium. Estimation of biomass, lipid weight and lipid content were done for all isolates. Five isolates exhibited high values of lipid content (more than 10%). One isolate was found to produce lipid content up to 14.96% and it was identified as *Rhodosporidium toruloides*. The optimal conditions were studied in different nitrogen limited media to produce high amount of lipids compared to *Lipomyces starkeyi* as a reference strain. The highest lipid content obtained when both strains were grown on pre-production medium (1) and nitrogen limited medium 2 (NLM 2) at 25 ^{o}C, at 100 rpm for 5 days. Chloroform: methanol (2:1) was the suitable solvent system to extract high amount of lipids from yeast cells. Different carbon and nitrogen sources were also studied. Sucrose and glycerol were the best carbon sources to accumulate high amount of lipid content for *R. toruloides* and *L. starkeyi*, respectively. Whereas, the mixture of peptone and ammonium sulphate (1:1) and sodium nitrate were the best nitrogen sources for producing high quantity of lipids by the tested strains reached to 41.28% lipid content for *R. toruloides* and 32.52% by *L. starkeyi*. So, it can be use *R. toruloides* in the economic production of lipids under the tested conditions.

Key words: Oleaginous yeast, *Rhodosporidium toruloides*., optimization, carbon sources, nitrogen sources, nitrogen limited medium.

Introduction

Oleaginous microorganisms such as bacteria, yeast, fungi and microalgae can accumulate high amounts of lipids under appropriate cultivation conditions, greater than 15-20% of dry biomass (Li *et al*., 2008 and Amaretti *el al*., 2010).

Different species from yeasts including *Cryptococcus curvatus, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis* and *Rhodotorula graminis* have been investigated for lipid production by many researchers (Angerbauer *et al*., 2008 and Matsunaga *et al*., 2009). Oleaginous yeast species have the capability to accumulate over 70% of its dry cell biomass and grow to high densities with biomass yields of 10 to 100 g/L reported over 3-7 days (Liu *et al*., 2008).

The major lipid components of oleaginous yeasts is triacylglycerides which mostly composed of C16 and C18 series long chain fatty acids, which are quite similar to those of vegetable oils such as rapeseed oil and soybean oil (Ratledge and Wynn, 2002). The utilization of oleaginous yeast for lipid production could prove more advantage than microalgae or vegetable oils. Cultivation of yeasts is not affected by environmental conditions, seasonal production or geographic location as in the case of vegetable oil (Zhu *et al*., 2008). They grow much faster than microalgae, short life cycle and resistance against climatic and seasonal changes (Li *et al*., 2008).

Oleaginous microorganisms are known to accumulate lipids intracellular when a nutrient in the medium (e.g. the nitrogen or the phosphorus source) becomes limited and the carbon source is present in excess. Nitrogen limitation is the most efficient condition for inducing lipogenesis (Ratledge and Wynn, 2002). Using of various media by oleaginous yeast can be achieved besides traditional laboratory media with glucose, glycerol or fructose as carbon source, also other media, which accumulate in industrial processes (Papanikolaou *et al*., 2002). Inorganic nitrogen sources were suitable for cell growth but not suitable for oil production, while organic nitrogen sources such as peptone was suitable for oil production but not good for cell growth (Huang *et al*., 1998 and Liu *et al*., 2000).

The aim of this study was isolation of oleaginous yeasts from different location of Egyptian soil and evaluates their ability to produce high amount of lipid by applying some processes.

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Material and Methods

Isolation of oleaginous yeasts

Samples from different plants rhizosphere were collected from different locations, *i.e*. Alexandria, Damietta, Fayoum, Giza, Ismailia, Kafr-El-sheikh, Qalubia, South of Sinai, Sohag, and Sharqya governorates, Egypt by an enrichment technique using yeast peptone dextrose medium (YPD) Seki et al., (1985). The flasks containing 50 mL liquid medium were inoculated with one gram from each samples and incubated on a rotary shaker (100 rpm) at 25 °C for 2 days. Enriched cultures were then streaked on (YPD) agar plates medium and incubated at 25 °C until yeast colonies appeared. The developing colonies on the plates were picked, purified and maintained on YPD agar medium at 4 °C for further studies.

Lipomyces starkeyi NRRL 11557 used throughout the current study as a reference strain was provided by Microbiology Department, Soils, Water and Environment Research Institute, Agriculture Research Center (ARC), Giza, Egypt.

Cultivation and enrichment media used

Different media were used in this study for isolation, propagation and production of lipid from yeast (Table, 1).

Table 1: Composition of different media used (g/l)

Components	YPD	Preproduction medium 1	Preproduction medium 2	NLM 1	NLM 2	NLM 3	NLM 4	NLM 5
Glucose	20.0	40.0	2.0	100.0	70.0	60.6	80.0	100.0
Peptone	20.0	5.0	-	3.0	-	-	-	-
Yeast extract	10.0	15.0	-	8.0	0.75	10.0	1.0	1.0
Na2HPO4. H2O	-	-	6.0	-	-	-	-	-
KH2PO4	-	-	3.0	-	0.4	1.0	0.8	0.5
K2HPO4	-	-	-	-	-	-	0.2	-
NaCl	-	-	0.5	-	-	-	0.2	-
NH4Cl	-	-	10.0	-	-	-	-	2.0
(NH4)2SO4	-	-	-	-	0.2	10.0	3.0	-
MgSO4.7H2O	-	-	-	-	1.5	1.0	0.5	1.0
CaCl2.2H2O	-	-	-	-	-	-	-	-
ZnSO4	-	-	-	-	4.4mg	-	-	-
CaCl2	-	-	-	-	25.0mg	-	-	-
MnCl2	-	-	-	-	0.5mg	-	-	-
CaCO3	-	-	-	-	0.3mg	-	-	4.0
Agar	20.0	-	-	-	-	-	-	-
pH	5.6	6	5	5	6	6	6	6

NLM: nitrogen- limited medium

Screening

The obtained isolates were screened on the basis of total lipid concentration. Isolates were pre-cultured in YPD medium, by inoculation of 250 mL conical flasks containing 100 mL of YPD broth medium with loop of tested isolates. The inoculated flasks were incubated on a rotary shaker (100 rpm) for 48 h. at 25 °C. After that, Five mL of pre-culture were added to 250 mL flasks containing 100 mL of NLM 1 and incubated at 25 °C for 5 days with shaking speed at 100 rpm. The cells were harvested for lipid extraction.

Identification of oleaginous yeast strain

The selected oleaginous yeast strains were identified based on its 26S rDNA sequence. The 26S rDNA was amplified by PCR using various universal primer sets and sequenced. The obtained sequences were BLAST searched against National Center for Biotechnology Information (NCBI) database.

Optimization of culture conditions for lipid production

Different solvent systems

Different mixtures of solvents were tested to detect the suitable extraction method which gives the highest lipid content. These mixtures were chloroform: methanol 1:1 (Pedersen, 1962), chloroform: methanol 2:1 as control (modified Bligh and Dyer, 1959), hexan: isopropanol 3:2 (Ratledge and Wilkinson, 1988), and benzen: methanol 1:2 (Jacob, 1992). All parameters of yeast growth, lipid weight and lipid content were detected. The selected mixture was apply in the following experiments.

The selection of pre-production media for lipid accumulation

Lipomyces starkeyi and *Rhodosporidium toruloides* were cultivated on pre-production media before transferring to nitrogen limiting medium. So that, three different types of preproduction media were tested, these media were YPD medium as control (Seki *et al*., 1985), preproduction medium 1 (Dai *et al.*, 2007), and preproduction medium 2 (Van Overbeek *et al*., 1995). These media were inoculated with the two yeast strains and incubated at 25 ºC with rotary shaker at a rate of 100 rpm for 2 days. Then, 5 mL from each flask was transferred to NLM 1 and incubated at 25 ºC with shaking (100 rpm), for 5 days. Cells were harvested for lipid extraction and determination of the cells dry weight.

Selection of suitable medium for Lipid production

The two yeast strains were separately grown on five recommended as lipid production media (nitrogen limited medium NLM). These media were, NLM 1 as control (Xiao, 2006), NLM 2 (Ratanaporn and Karraphan, 2011), NLM 3 (Guojie *et al.,* 2012), NLM 4(Gao-Qiang *et al.,* 2010) and NLM 5 (Tanaka *et al.*, 1971). These media were prepared and inoculated with 5 mL of suitable pre-culture medium. Lipid content and cells dry weight of yeast strains were determined after 5 days of incubation at 25 ºC with shaking (100 rpm). Most suitable medium achieved high amount of lipid content was selected.

Carbon sources

This experiment was carried out to study the effect of different carbon sources on growth and lipid content by selected yeast strains. Therefore, glucose as control in suitable production medium was replaced with different sources of carbon in equal amount of carbon content to that present in original medium (28.0 carbon g/L). The carbon sources used were glucose, fructose, sucrose, raffinose, xylose, glycerol, sugarcane molasses, and acid or alkali hydrolyzate of sugarcane bagasse. The condition of lipid production as mentioned before. The growth and lipid content were determined.

Nitrogen sources

Different nitrogen sources of suitable production medium were replaced by equivalent nitrogen amount (0.084 g/L), these sources were (ammonium sulfate as control, ammonium acetate, ammonium citrate, potassium nitrate, sodium nitrate, beef extract peptone, yeast extract, and combined mixture between peptone and ammonium sulfate (1:1). The propagation was carried out as mentioned before and the growth and lipid content were determined.

Analytical methods

Preparation of beet molasses

Preparation of beet molasses was carried out according to method described by Pandey and Agarual (1993).

Preparation of bagasse sugar cane

The acid hydrolyzate of bagasse was done by using method of Candido *et al*. (2012).

Determination of yeast cells dry weight (Granger et al., 1993)

Five mL of cultures were harvested and centrifuged at 5000×g for 5 min. Harvested biomass was washed twice with 5 mL of distilled water and then dried at 60 °C to constant weeight. The biomass was determined gravimetrically.

Determination of lipid content

To determine the lipid content in yeast cells, lipids were extracted, dried and weighed, based on the method of Bligh and Dyer (1959) with modifications. This is a fast procedure allowing complete lipid extraction. Briefly, a 50 mL sample was centrifuged at 5000×g for 5 min after which the yeast was washed twice with 50 mL of distilled water, then added into 10 mL of 4M HCl, and incubated at 60 °C for 2 h. Then the acid-hydrolyzate was stirred with 20 mL of chloroform/methanol mixture (2:1) at room temperature 3 h, followed by centrifugation at 2000×g for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. Then, the lower phase containing lipids was recovered with pasteur pipette, and the solvents were evaporated. The dry lipids were weighed.

$$\text{Lipid content} = \frac{\text{Single cell oil weight (g L}^{-}\text{)}}{\text{Cell dry weight (g L}^{-}\text{)}} \times 100$$

The significance of various treatments was evaluated by Duncan's multiple range at P value 0.05 (Duncan, 1955). Statistical analysis was made using a software Package "Costat", a product of Cohort soft wear INC., Berkley, California.

Results and Discussion

Thirty-three isolates were obtained from different locations as shown in Table (2). The quantitative assessments (cell dry wt., lipid wt. and lipid content) of all tested isolates were done after growing on N-limited medium. It was observed that five isolates exhibited high values of lipid content (more than 10%) whereas, twenty-eight from these isolates recovered less than 10% of lipid content.

Table 2: Isolation and quantitative assessment of lipid content for yeast isolates grown on N- limited medium.

No. sample	Location	Lipid weight (g/L)	Cell dry weight (g/L)	Lipid content %
1	Alexandria	0.1966	10.1692	1.93
2	Alexandria	0.3932	12.6942	3.1
3	Alexandria	0.1318	4.3359	3.04
4	Alexandria	0.656	16.3729	4.01
5	Damietta	0.2598	7.5788	3.43
6	Damietta	0.3789	4.3518	8.7
7	Fayoum	0.3286	8.3719	3.92
8	Fayoum	0.561	14.3732	3.9
9	Fayoum	0.094	2.3338	4.03
10	Fayoum	0.4312	8.694	4.96
11	Giza	0.119	3.7248	3.19
12	Giza	0.1475	4.3123	3.42
13	Giza	0.4842	13.1687	3.68
14	Giza	1.3761	9.2006	14.96
15	Ismailia	0.1973	7.215	2.73
16	Ismailia	0.3204	8.3214	3.85
17	Ismailia	1.0563	10.7388	9.84
18	kafr-ElSheikh	0.1124	5.347	2.1
19	kafr-ElSheikh	0.4243	11.6989	3.63
20	kafr-ElSheikh	0.3933	7.3273	5.37
21	Qalubia	0.2017	3.1751	6.35
22	Qalubia	0.861	8.1809	10.53
23	Qalubia	0.695	6.2972	11.04
24	Sharqya	0.1985	6.1594	3.22
25	Sharqya	0.3727	7.3593	5.06
26	Sharqya	0.6506	9.3742	6.94
27	Sharqya	1.652	16.3881	10.08
28	Sharqya	0.8539	7.7124	11.06
29	Sohag	0.0482	2.0884	2.31
30	Sohag	0.562	17.2729	3.25
31	Sohag	0.3174	9.1843	3.46
32	South Sinai	0.241	6.3036	3.82
33	South Sinai	0.2276	4.7086	4.83

The values of lipid content were taken as detector for selection of different oleaginous yeast isolates. These results are agreement with Dyer *et al*. (2002) reported that oleaginous microorganisms are able to accumulate lipids to level greater than 15% of their cellular dry weight. Many investigators reveled that some yeast strains such as *Rhodotorula glutinis*, *Yarrowia lipolitica* and *Lipomyces starkeyi* able to accumulate high percentage of lipid in their cells reached to 70% of their biomass dry weight (Angerbauer *et al.,* 2008).

Identification of the selected oleaginous yeasts

One isolate that showed the most potential ability in lipid content (14.96%) was selected and identified using phenotypic and genotypic techniques. The entire (ITS1)-5.8 rRNA-(ITS2) regions were successfully amplified from yeast template DNA by using one fungus-specific universal primers pair, ITS1 and ITS4. The BLAST search revealed that ITS1-5.8 rRNA-ITS2 region DNA from yeast isolated had sequence similarity exceeded 99% with *Rhodosporidium toruloides* (*R. toruloides*).

Optimization of culture conditions for lipid production

Solvent system

The obtained results (Table, 3) showed that the mixture of chloroform: methanol (2:1) succeeded to obtained high efficiency for extracting lipid from damage cells, of *L. starkeyi* (15.39%) and *R. toruloides* (17.62%)

Table 3: Effect of solvent system on lipid content (dry weight to 10 g cell dry weight from *L. starkeyi* and *R. toruloides*).

Solvent system	*L. starkeyi*		*R. toruloides*	
	Lipid weight (g/L)	Lipid content (%)	Lipid weight (g/L)	Lipid content (%)
Chloroform: Methanol (1:1)	1.34	13.43	1.15	11.58
Chloroform: Methanol (2:1)	1.53	15.39	1.76	17.62
Hexan: Isopropanol (3:2)	0.99	9.92	1.17	11.75
Benzen: Methanol (1:2)	1.21	12.19	1.63	16.32
LSD	0.1653	1.1853	0.0645	0.7502

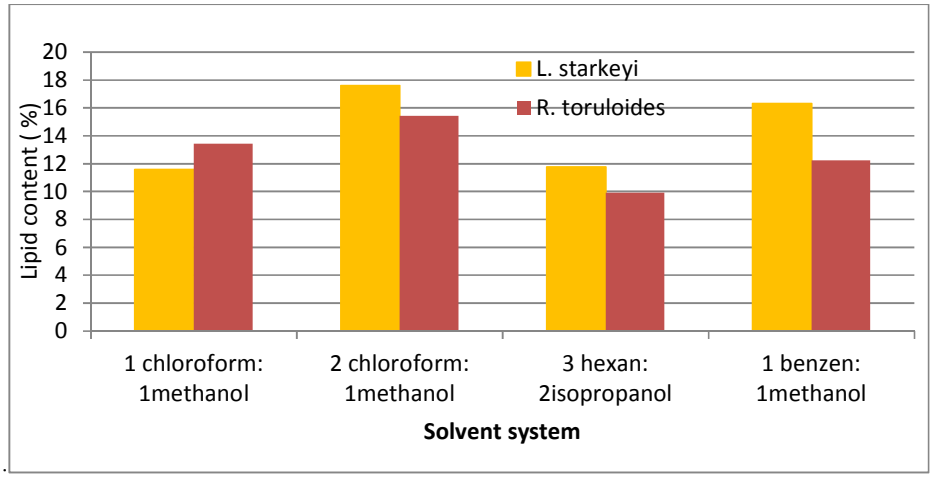

Fig. 1: Lipid content of *L. starkeyi* and *R. toruoloide* under different solvent systems

Selection of pre-production media for lipid accumulation

The growth and lipid content of *L. starkeyi* and *Rh.toruloides* were estimated as shown in Table (4). Results revealed that both strains reached its high value of biomass being 6.61 and 10.27 g/l, respectively, when they were grown initially on pre-production medium 1. The corresponding figures for lipid content were 21.77 and 23.24%, respectively.

Table 4: Effect of different pre-production media on biomass (g/L), lipid weight and lipid content of *L. starkeyi and R. toruloides* grown for 5 days at 25 °C under shaking condition.

	L. starkeyi			*R. toruloides*		
	Dry weight g/L	Lipid weight g/L	Lipid content %	Dry weight g/L	Lipid weight g/L	Lipid content %
preproduction medium 1	6.61	1.44	21.77	10.27	2.4	23.34
YPD medium	6.04	1.06	17.61	9.68	1.49	15.42
preproduction medium 2	1.69	0.14	8.41	1.66	0.08	5.03
LSD	0.2554	0.0334	0.9875	0.7889	0.1431	0.9097

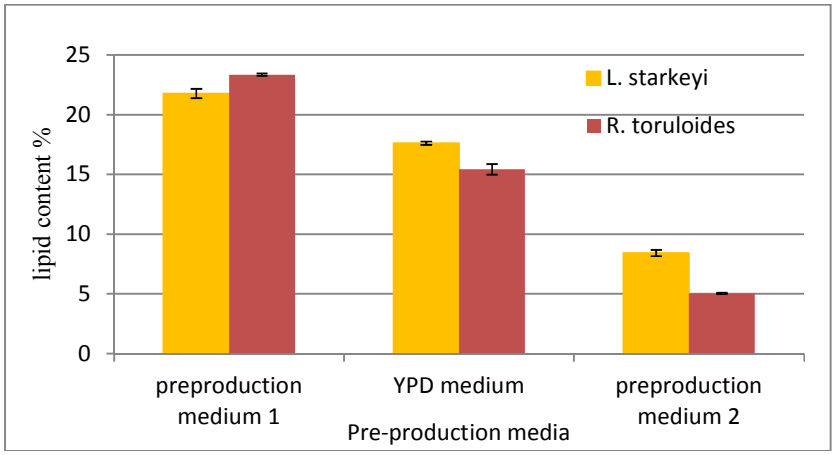

Fig. 2: Lipid content of *L. starkeyi and R. toruloides* at different pre-production media.

Selection of suitable medium for lipid production

Data presented in Table (5) and illustrated by Fig. (3) revealed that N- limited medium (2) achieved the highest growth biomass by *R. toruoloides* and *L. starkeyi* being 10.43, and 6.88 g/L, respectively. In addition, the lipid weight was high significance with *R. toruoloides* and *L. starkeyi* reach to 3.50 and 1.74 g/L, respectively. Consequently, lipid content reached 33.50 for *R. toruoloides* and 25.30% for *L. starkeyi,* when both strains were grown NLM 2. So that, these results could be concluded the nitrogen limited medium no 2 was the preferable medium for lipid production by *R. toruoloides* and *L. starkeyi.*

Table 5: Effect of different N- limited media on cell dry weight, lipid weight and lipid content in *L. starkeyi and R. toruloides* during 5 days under shaking conditions.

Different N-limited media	*L. starkeyi*			*R. toruloides*		
	Dry weight (g/L)	Lipid weight (g/L)	Lipid content (%)	Dry weight (g/L)	Lipid weight (g/L)	Lipid content (%)
NLM 1	6.61	1.44	21.77	10.27	2.40	23.34
NLM 2	6.88	1.74	25.30	10.43	3.50	33.50
NLM 3	5.28	1.10	20.89	5.88	1.67	28.31
NLM 4	4.20	0.82	19.60	4.31	0.64	14.95
NLM 5	2.70	0.39	14.46	5.34	0.67	12.60
LSD	0.135	0.063	0.790	0.131	0.055	0.492

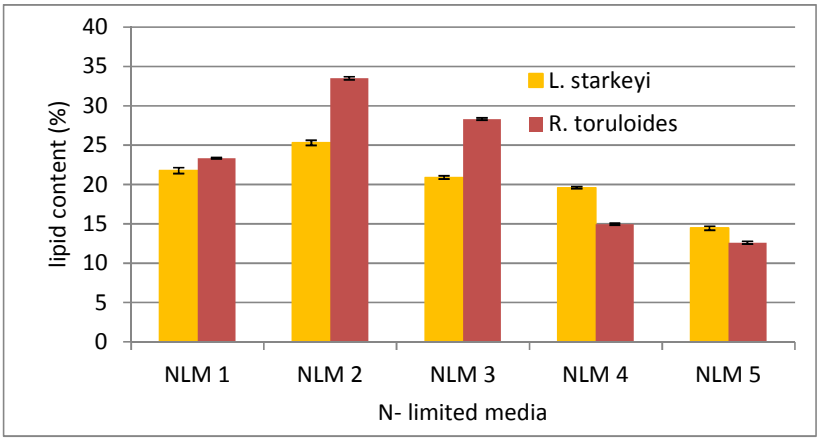

Fig. 3: lipid content (%) of *L. starkeyi and R. toruoloides* under different N-limited media.

Carbon Sources

Different sources of carbon were tested in this study to evaluate the most suitable one for lipid content (Table 6 and Fig. 4). Data obviously determined cell dry weight, lipid weight and lipid content with different carbon sources by two yeast strains under investigation.

Results revealed that molasses attained higher biomass production by *R. toruoloides* and *L. starkeyi* than other carbon sources being 15.28 and 7.21 g/L, respectively.

Table 6: Effect of different carbon sources on biomass production, lipid weight and lipid content in *L. starkeyi and R. toruloides* during 5 days under shaking conditions.

	L. starkeyi			*R. toruloides*		
Carbon sources	Dry weight (g/L)	Lipid weight (g/L)	Lipid content (%)	Dry weight (g/L)	Lipid weight (g/L)	Lipid content (%)
Glucose	5.34	1.26	23.69	12.30	4.18	33.95
Fructose	5.57	1.35	24.17	11.00	3.16	28.75
Sucrose	6.79	1.76	24.87	11.77	4.47	37.98
Raffinose	5.15	1.56	30.27	4.59	1.55	33.83
Xylose	6.19	1.82	29.48	0.98	0.36	36.34
Glycerol	6.65	2.04	30.73	3.84	1.06	27.45
Molasses	7.21	0.66	9.10	15.28	1.76	11.54
Bagasse	1.48	0.25	16.95	2.58	0.18	6.82
LSD	0.5521	0.0992	0.8547	0.5193	0.1804	1.0379

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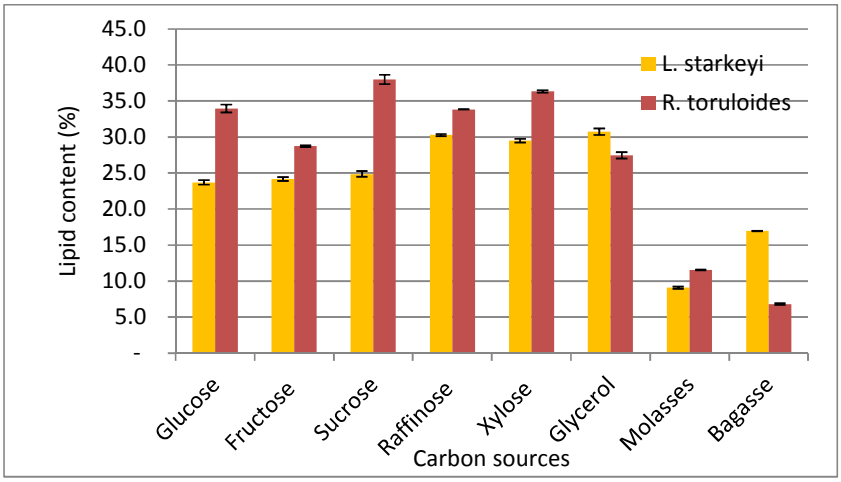

Fig. 4: lipid content for *L. starkeyi* and *R. toruloides* at different carbon sources.

The accumulated lipid which presented by lipid weight was 4.47 g/L, when *R. toruloides* grown on sucrose as a carbon source with achieving lipid content 37.98%, while glycerol was the best carbon source to lipid weight and lipid content for *L. starkeyi* being 2.04 g/L and 30.73%. Molasses and sugar cane bagasse recorded less values of lipid weight and lipid content in both yeast strains.

These results are agreement with Syed *et al*. (2006) who investigated lipid production in different fungal species using various carbon sources such as glucose, sucrose, lactose, soluble starch. Bialy *et al*. (2011), who reported *L. starkeyi* able to utilize industrial glycerol, for the production of lipids.

These data revealed that sucrose and glycerol were the best source of carbon for *R. toruloides* and *L. starkeyi.*

Nitrogen sources

Data presented in Table (7) and illustrated by fig (5) showed that the growth weight and lipid content of *L. starkeyi* and *R. toruloides* cultivated on different organic and inorganic nitrogen sources in NLM 2 at 25 ºC for 5 days. Each tested strain was grown individually after choosing the suitable carbon source.

Amm. sulphate was the most efficient N- source on biomass (cell dry weight) of *L. starkeyi* being 9.62 g/L followed by potassium nitrate (9.25 g/L), whereas the lowest value was recorded by the mixture one. On the other hand, the optimize value of lipid weight was recorded with potassium nitrate (2.59 g/L), while the highest lipid content being 32.52% was recorded with sodium nitrate.

As seen in Table (7) and fig. (5) amm. acetate was the best source of nitrogen for cell dry weight of *R. toruloides* strain (13.16 g/L). Whereas lipid content were recorded the highest value reached to 41.28% with mixture of peptone and ammonium sulfate than other nitrogen sources (1:1). These findings confirmed the results obtained by Pakawat (2009) and Leonidas *et al*. (2014), who revealed that the complex organic nitrogen sources (yeast extract and peptone) were more favorable for the production of lipids. In contrast with Syed *et al*. (2006) showed that the total lipid content produced by *L. starkeyi* from the medium containing yeast extract was higher than that medium containing peptone.

Table 7: Effect of different nitrogen sources on biomass production, lipid weight and lipid content in *L. starkeyi and R. toruloides* during 5 days under shaking conditions.

	L. starkeyi			*R. toruloides*		
	Dry weight g/L	Lipid weight g/L	Lipid content %	Dry weight g/L	Lipid weight g/L	Lipid content %
Ammonium acetate	8.45	2.17	25.71	13.16	3.88	29.52
Ammonium citrate	8.26	2.49	30.13	11.54	2.47	21.43
Ammonium sulfate	9.62	2.30	23.93	12.43	3.52	28.31
Potassium nitrate	9.25	2.59	27.98	11.28	4.37	38.72
Sodium nitrate	6.88	2.24	32.52	11.87	3.78	31.87
Beef extract	7.81	2.23	28.50	12.12	4.79	39.54
Peptone	9.14	2.40	26.27	12.81	4.51	35.18
Yeast extract	7.33	2.23	30.35	10.75	3.75	34.94
Peptone:Amm.sulphate (1:1)	5.51	1.59	28.90	11.59	4.79	41.28
LSD	0.5913	0.2023	1.0293	0.0479	0.0359	0.1854

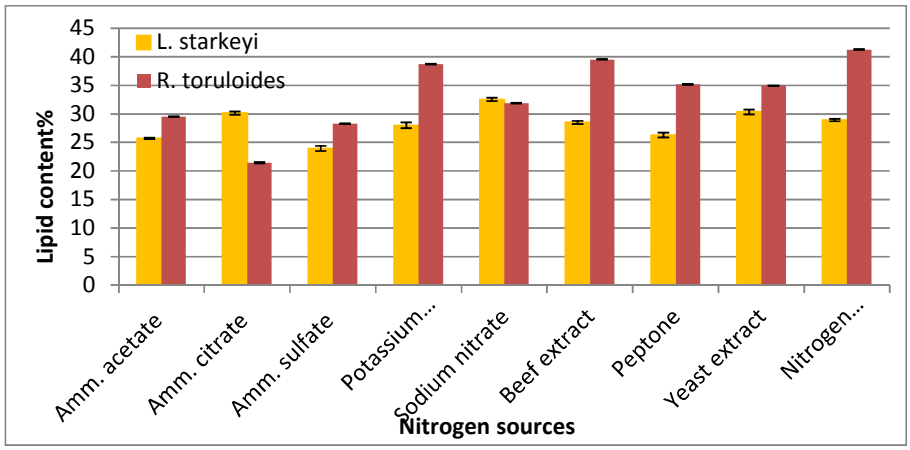

Fig. 5: lipid content for *L. starkeyi* and *R. toruloides* at different nitrogen source.

Conclusion

It could be concluded that one isolate out of 33 yeast isolates was the most efficient yeast strain to accumulate a considerable amount of lipids in nitrogen limited medium. This strain was identified *Rodosporidium toruloides* by using PCR technique. This strain and *Lipomyces starkeyi* (as reference strain) produced 41.28 and 32.52% lipid content in nitrogen limited medium 2 using shaking flasks (100 rpm) at 25 ºC for 5 days. Sucrose and glycerol as a source of carbon, moreover, mixture of peptone and ammonium sulphate, (1:1) and sodium nitrate as nitrogen sources, were the best sources for lipid production by *R. toruloides* and *L. starkeyi* strains, respectively, and using of solvent mixture chloroform : methanol (2:1) was suitable for extraction system for both strains.So, it is recommended to use this yeast strain for production of high amount of lipids.

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