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EFFECT OF ALTERATIONS IN CONVENTIONAL MEDIUM ON LIPID ACCUMULATION AND FATTY ACID CONTENT IN OLEAGINOUS YEASTS

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ABSTRACT

Yeast oils have several biotechnological applications such as fatty acid bioconversion, substrate valorization, polyunsaturated fatty acids production, oil polluted water bioremediation, biodiesel etc. Effects of physical and chemical parameters on lipid content in yeasts, therefore, deserve special consideration. In view of this, two oleaginous yeasts viz. *Yarrowia lipolytica* (NCIM 3589) and *Lipomyces lipofera* (NCIM 3252) were studied for their lipid producing capacities. We observed 1.6 times increase in lipids (8.0 %) in medium A1 for *Y. lipolytica* and 1.9 times increase in lipids (2.4 %) in medium A2 for *L. lipofera* as compared to MGYP. In order to make the medium cost effective, glucose and peptone in MGYP were replaced with local commercial table sugar (LCTS) and urea respectively. Thereby 1.7 fold increase using medium B1 for *Y. lipolytica* and 2.0 fold increase using medium B2 for *L. lipofera* as compared to MGYP was achieved. In media B1 and B2, increase in fatty acids (monounsaturated and linoleic acid in both the yeasts and saturated in *L. lipofera*) was observed as compared to MGYP.

KEYWORDS: **Cost effective media, Fatty acids, Lipids, *Lipomyces lipofera,* MGYP, *Yarrowia lipolytica***

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INTRODUCTION

All microorganisms are able to synthesize lipids for essential functioning of their membranous structures. Some of these organisms, so called oleaginous, store lipids in oil vacuoles in the form of triacylglycerols. Some of these microbial oils have a great potential value since they are composed of commercially important polyunsaturated fatty acids (PUFA)[1,2]. We have earlier reported PUFA producing bacterial isolates from the Antarctic as well as from the Indian Ocean[3,4]. With few exceptions, oleaginous microorganisms are eukaryotes and representative species include algae, yeasts and molds. Yeasts have received the most attention[5] and have shown to be the most promising through the years as source of single cell oil[6]. Generally yeasts have been preferred over molds because their single cell mode of growth has been easier to handle than the mycelial growth of molds and they tend to convert substrate to lipid more efficiently[7]. In the recent years, microbial lipids have also gained importance in biodiesel production. This is mainly due to high energy prices, energy and environment security and concerns about petroleum supplies[8,9]. Thus, oleaginous microorganisms may prove to be a novel substitute for conventional oil in biodiesel production. Therefore, studies on effect of media composition on lipid yields of oleaginous yeasts have become essential prior to their large scale production.

Another noteworthy feature of yeasts is their use as host organism in biotechnology, where the aim may not be to study a gene, but to use cloning to control or improve synthesis of an important metabolic product such as hormone, nutraceutical etc. Therefore, genetically engineered oleaginous yeasts producing higher lipids under optimum conditions could prove to be an excellent source for increased production of desirable oils (ω– 3 and ω– 6 fatty acids). Also over the past several years, extensive research has
been done for microbial production of PUFA as well as their beneficial health effects[10,11]. Among PUFA, linoleic acid (LA), an 18-carbon dienoic fatty acid (18:2 omega-6), is the primary dietary omega-6 PUFA. It is a most abundantly required in mammals and must be acquired from the diet. It is used in the biosynthesis of arachidonic acid (AA) which leads to synthesis of prostaglandins necessary for the repair and growth of skeletal muscle tissue. A diet deficient in linoleate causes mild skin scaling, hair loss etc. [12,13]. In view of this, our studies focus on lipid production using oleaginous yeasts. *Yarrowia lipolytica* and *Lipomyces lipofera* with several important characteristics have induced researchers to study various basic biological and biotechnological applications in detail[14,15,16]. These applications along with non – toxicity of lipids, ease to genetically manipulate and suitability for animal and human use[17,18,19] prompted us to select these cultures.

The present work aims at understanding the effects of altered media composition on lipid accumulation and fatty acid profile in *Y. lipolytica* and *L. lipofera*.

MATERIALS AND METHODS

(i) Chemicals

All the media components were of fermentation grade and procured from Titan Biotech (Delhi, India). Chemicals were purchased from Hi – Media and Merck (Mumbai, India) and were of analytical grade (AG). Local commercial table sugar (LCTS) was purchased from local market.

(ii) Yeast cultures

The cultures of *Yarrowia lipolytica* NCIM 3589 and *Lipomyces lipofera* NCIM 3252 were procured from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India.

(iii) Cultivation media and maintenance conditions

Cultivation medium (MGYP) composed of (in g/l): malt extract 3.0, yeast extract 3.0, glucose 10.0 and peptone 5.0; with pH adjusted to 6.4 –

6.8. The cultures were grown at 30 °C and maintained on MGYP slants. 10 % (by volume) inoculum (10^8 CFU/ ml) was used for all experiments. Lipids and biomass were estimated as described below. All further experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml medium with shaking at 200 rpm at pH 6.5. The conditions of time and temperature were optimized as given below.

(iv) Effect of temperature and incubation time

The yeast cultures were incubated in MGYP at different temperatures (20, 30 and 37 °C). At each temperature, the incubation time was varied as 24, 48 and 72 h. The conditions which yielded maximum lipids were used further to study effect of altered media composition.

(v) Effect of different sugars and nitrates

Glucose in MGYP was replaced individually by equimolar concentration of different sugars viz. galactose, xylose, ribose, fructose, lactose, maltose and sucrose. The conditions of time and temperature optimized earlier were used for both the cultures respectively. Concentration of the sugar yielding maximum lipids was further optimized over a range of 0.05 M to 1.0 M to determine the best suitable concentration. Peptone in the medium was replaced individually with ammonium nitrate and sodium nitrate. Concentration of nitrate in the medium was varied in the range 0.1 to 1.25 %. The medium yielding maximum lipids was named as A1 for *Y. lipolytica* and A2 for *L. lipofera.*

(vi) Effect of 'LCTS' and 'urea'

In another set of experiments glucose and peptone in MGYP were replaced with LCTS and urea respectively to make the medium cost effective. Firstly glucose was replaced by LCTS and its concentration varied in the range of 1- 5 %. Further peptone in the medium was replaced by urea (0.1 to 1.25 %). The medium yielding maximum lipids was named as B1 for *Y. lipolytica* and B2 for *L. lipofera.*

(vii) Determination of yeast biomass and lipid content

To determine the lipid content in yeast cells, lipids were extracted, dried and weighed based on the method of Bligh and Dyer[20] with modifications. Briefly, a 50 ml sample was centrifuged at 5000×*g* for 5 min, after which the cells were washed twice with 50 ml of distilled water, then added into 10 ml of 4 M HCl, 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 phases. After centrifugation the lower phase containing lipids was recovered with a Pasteur pipette and evaporated to dryness under reduced pressure of nitrogen for 10 min. The dry lipids were weighed and estimated using phosphovanillin reagent[21].

(viii) Preparation of fatty acid methyl esters (FAMES)

Y. lipolytica and *L. lipofera* were grown in B1 and B2 repectively, since these media yielded maximum lipids. Cells were harvested by centrifugation (Plastocraft, India) at 10,000 rpm for 12 min. Following centrifugation, the supernatant was discarded, the cell pellet resuspended in 1.0 % NaCl (w/v) and recentrifuged. Each culture tube was capped and stored at 4°C. Cells were reweighed, to which a fresh solution of the transesterification reaction mix (methanolic HCl (0.6 N) 4 ml) was added in the tubes[22,23]. The tubes were capped tightly and the solutions were vortexed for 5–10 s and heated in a 80°C± 2°C water bath for 2 h. The tubes were then cooled quickly in ice. The resultant FAMES were extracted twice by adding 2 volumes of hexane and then 1 volume of hexane by centrifugation at 5,000 rpm for 15 min. The upper phase of hexane layer was separated and stored for gas chromatography analysis.

(ix) Gas chromatography analysis

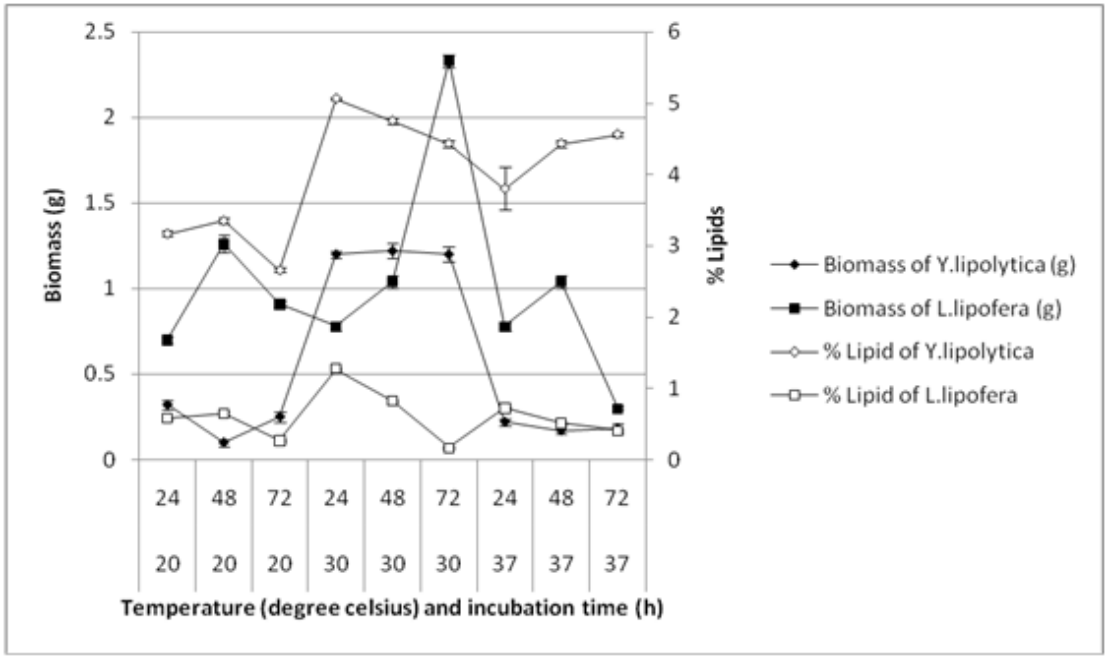
Analyses of the FAMES were performed with a Chemito GC 1000 equipped with a 50 m x 0.25 mm internal diameter cross-linked methyl silicone fused-silica CP-SIL 88 capillary column and flame ionization detector. Samples were injected in the split mode at 100°C for 5 min. Then the oven was programmed from 100 to 198°C at the rate of 1.5°C min^{-1} and held for 9 min. Nitrogen was used as a carrier gas, and the injector and detector were maintained at 225 and 250°C, respectively. Peak areas were quantified using chromatography software (IRIS 32, India).

(x) Statistical analysis

All the experiments were carried out in triplicate, the values presented in the graphs are those of the mean of three independent experiments and the error bars indicate standard deviation. For statistical analysis, the mean values and standard deviation (mean ± SD) were calculated and tested. Statistical analysis of variance (ANOVA) was performed on all values and tested for $p < 0.05$ for significance.

RESULTS

Effect of temperature and incubation period on biomass and total lipids of Y. lipolytica and L.lipofera



Maximum lipid content of 5.1 % and 1.3 % were obtained for Y. lipolytica and L. lipofera respectively at 30 °C after 24 h (Fig. 1).

Figure 1
Cultures were grown in MGYP at pH 6.5

1. Effect of different sugars

In *Y. lipolytica* i) maximum biomass was obtained using glucose in the medium ii) addition of maltose in the medium instead of glucose yielded maximum lipid content of 5.6 % (Fig. 2 A). Maltose concentration in the medium was varied in the range of 0.05- 1.0 M. Maximum lipid content of 6.0 % was obtained at 0.6 M maltose (Fig. 2 B). In *L. lipofera* addition of sucrose in the medium yielded maximum lipid content of 1.4 % (Fig. 2 A). Therefore, sucrose concentration in the medium was varied in the

range of 0.05- 0.6 M. Biomass was maximum at 0.05 M sucrose. However, maximum lipid content of 1.6 % was obtained at 0.4 M sucrose (Fig. 2 C).

Effect of different sugars on biomass and total lipids of Y. lipolytica and L.lipofera

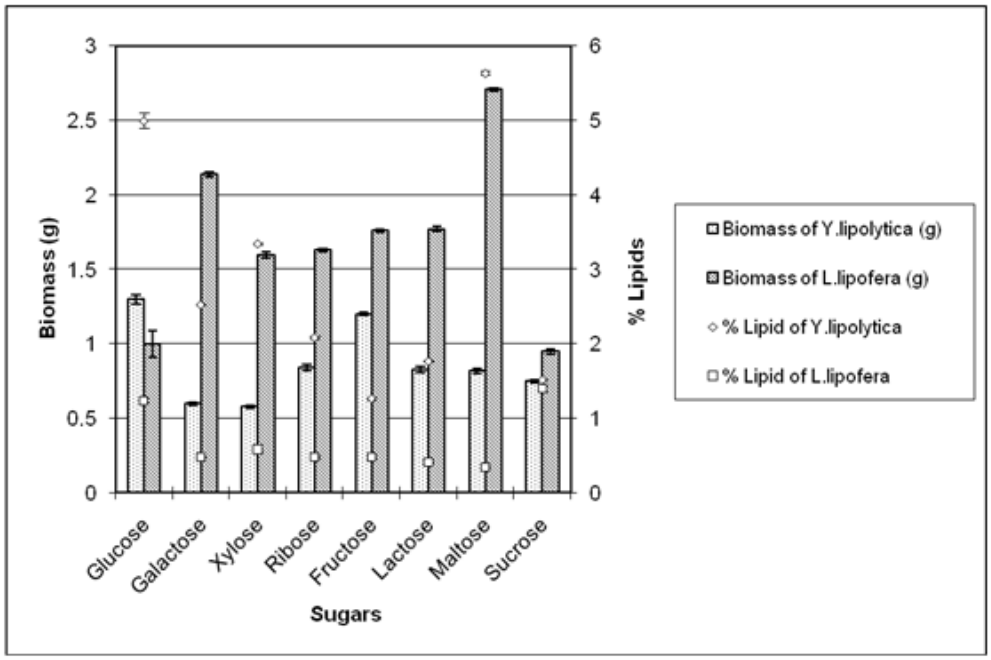


Figure 2A

Glucose in MGYP was replaced with different sugars. Cultures were grown at 30° C for 24 hr at pH 6.5.

Effect of various concentrations of maltose on biomass and total lipids of Y. lipolytica

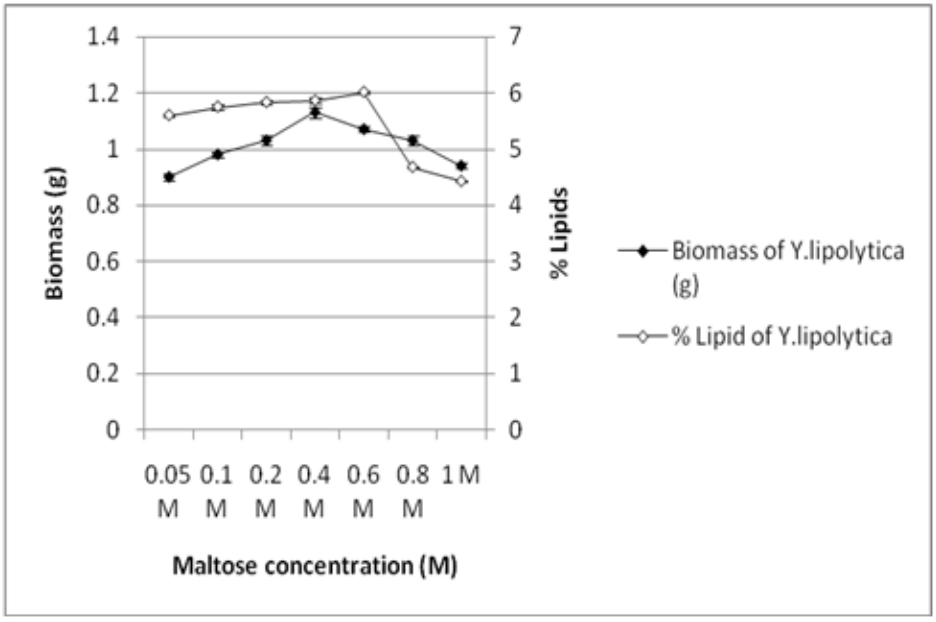


Figure 2B

Cultures were grown at 30° C for 24 h at pH 6.5.

Effect of various concentrations of sucrose on biomass and total lipids of L.lipofera

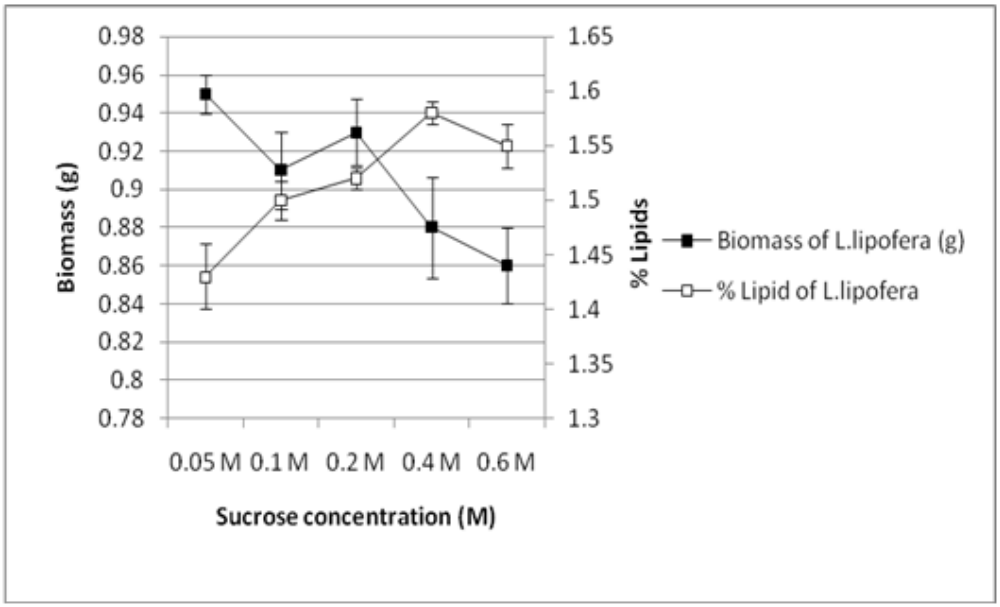


Figure 2C
Cultures were grown at 30° C for 24 h at pH 6.5.

2. Effect of nitrates

Peptone was substituted with nitrates to examine the effect on lipid yields. In *Y. lipolytica* i) highest lipid yield of 6.1 % was obtained using sodium nitrate (Fig. 3 A). ii) Its concentration was varied in the range of 0.1 – 1.25 % with highest yield of 8.0 % at 1.0 % sodium nitrate (Fig. 3 B). In the case of *L. lipofera*, lipid yields were 2 % in presence of ammonium nitrate (Fig. 3 A), with maximum of 2.4 % at 0.25 % ammonium nitrate (Fig. 3 C).

Effect of different nitrogen sources on biomass and total lipids of Y. lipolytica (in presence of 0.6 M maltose) and L.lipofera (in presence of 0.4 M sucrose)

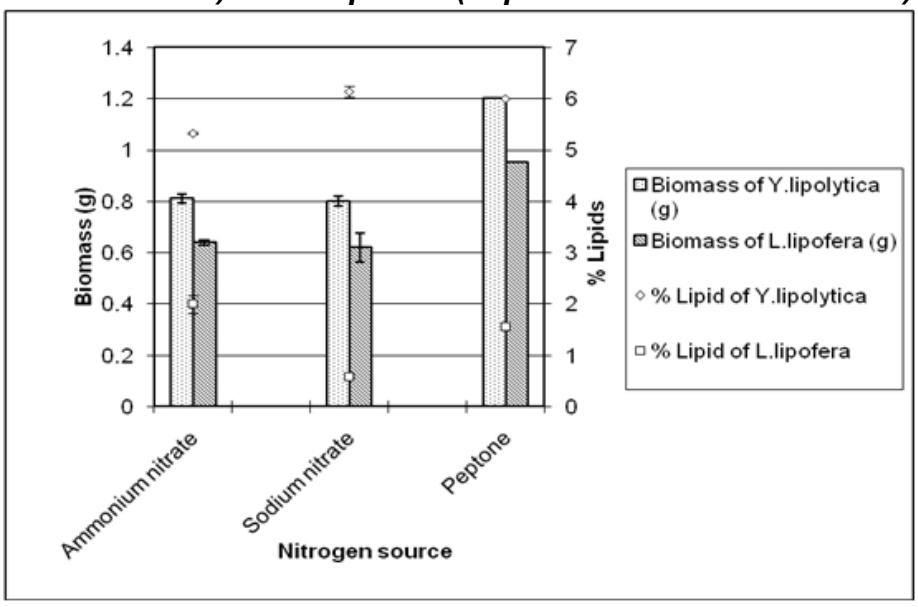


Figure 3A
Peptone in the medium was replaced with different nitrogen sources. Cultures were grown at 30° C for 24 h at pH 6.5.

Effect of various concentrations of sodium nitrate on biomass and total lipids of Y. lipolytica

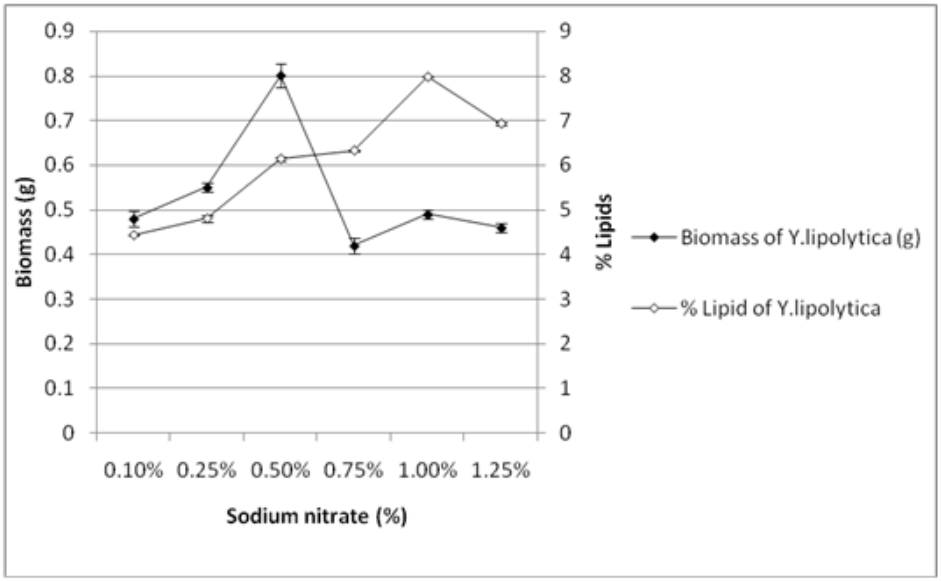


Figure 3B
Cultures were grown at 30° C for 24 h at pH 6.5.

Effect of various concentrations of ammonium nitrate on biomass and total lipids of L.lipofera

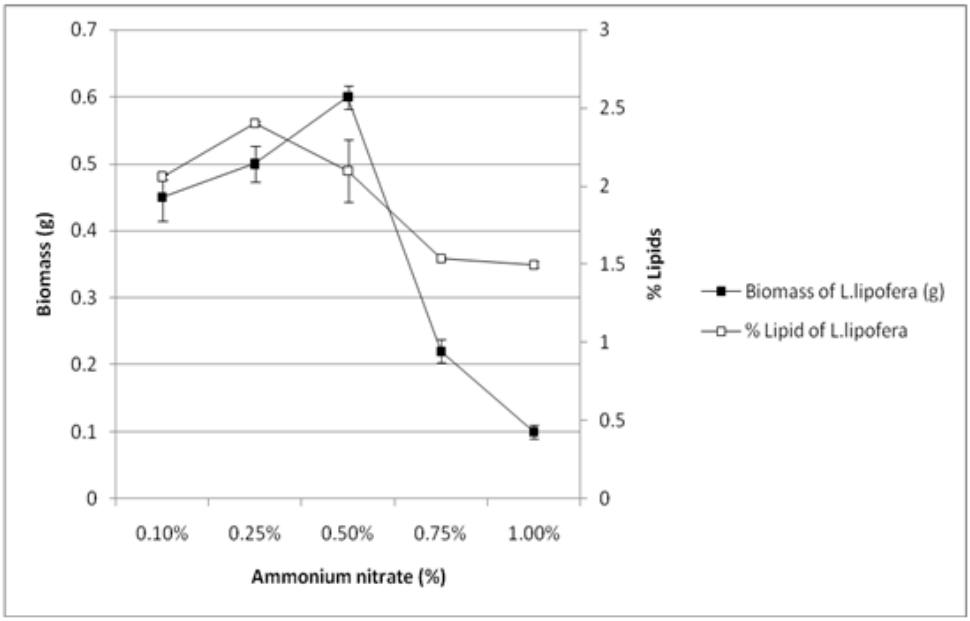


Figure 3C
Cultures were grown at 30° C for 24 h at pH 6.5.

Y. lipolytica and *L. lipofera* exhibited 1.6 and 1.9 times more lipids in medium A1 (0.3 % malt extract, 0.6 M maltose, 0.3 % yeast extract and 1.0 % sodium nitrate) and A2 (0.3 % malt extract, 0.4 M sucrose, 0.3 % yeast extract and 0.25 % ammonium nitrate) respectively as compared to MGYP.

3. Effect of 'LCTS' and 'urea'

In another set of experiments, LCTS was used in the range of 1 % to 5 % as a substitute for glucose in MGYP. In *Y. lipolytica* maximum lipids i.e. 5.8 % were obtained at 2 % LCTS and in *L. lipofera,* it was 1.4 % at 1 % LCTS (Fig. 4 A).

Effect of various concentrations of LCTS on biomass and total lipids of Y. lipolytica and L. lipofera

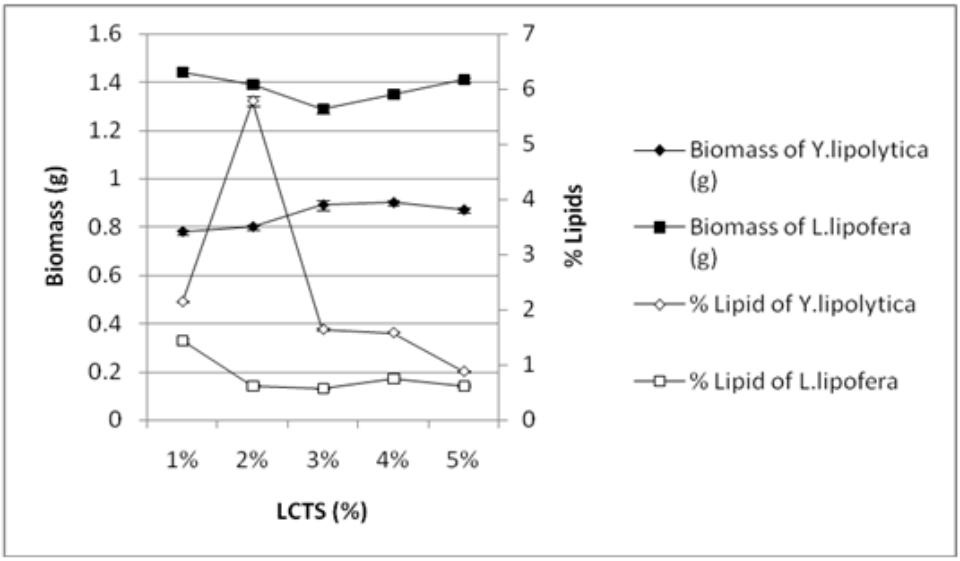


Figure 4A

Glucose in MGYP was replaced with various concentrations of LCTS. Cultures were grown at 30° C for 24 h at pH 6.5.

To examine effect of urea, in LCTS containing medium, peptone was replaced with urea (0.1 % to 1.25 %). In *Y. lipolytica*, maximum lipid of 8.5 % was obtained at 0.25 % urea (Fig. 4 B), while, in *L. lipofera,* it was 2.6 % at 1.0 % urea (Fig. 4 B).

Effect of various concentrations of urea on biomass and total lipids of Y. lipolytica (2 % LCTS) and L.lipofera (1 % LCTS)

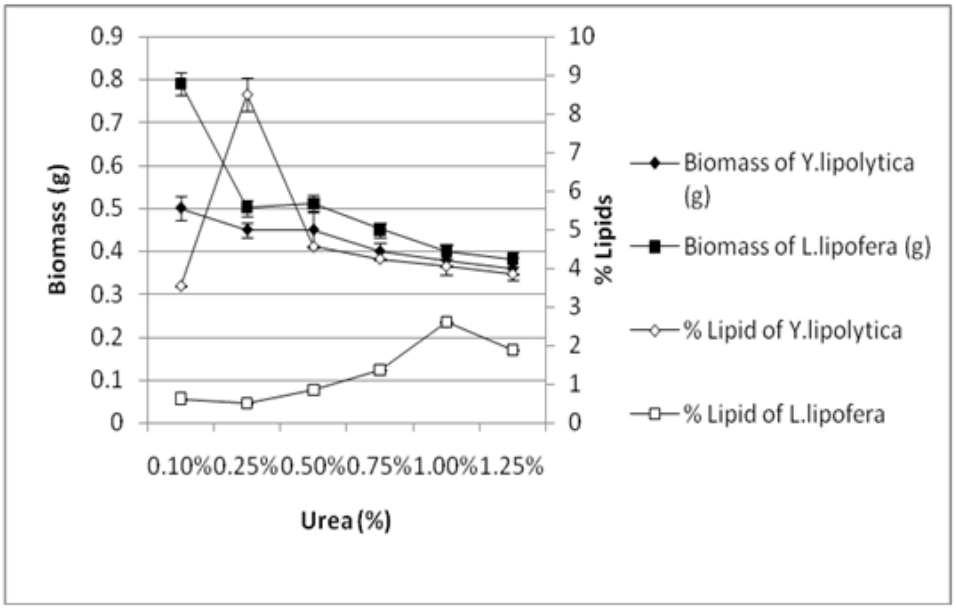


Figure 4B

Peptone in MGYP was replaced with different concentrations of urea. Cultures were grown at 30° C for 24 h at pH 6.5.

Y. lipolytica and *L. lipofera* showed maximum lipid accumulation in medium B1 (0.3 % malt extract, 2.0 % LCTS, 0.3 % yeast extract and 0.25 % urea) and B2 (0.3 % malt extract, 1.0 % LCTS, 0.3 % yeast extract and 1.0 % urea) respectively. It was 1.7 and 2.0 fold increase as compared to MGYP and 6.1 and 7.7 % increase in comparison to A1 and A2 respectively.

4. Fatty acid analysis:

The media containing LCTS and urea (B1 and B2) were found to be not only cost effective but also yielded highest lipid contents as compared to that with commercial chemicals (A1 and A2). Therefore, fatty acid profiles of both the cultures in MGYP were compared with that in medium B1 and B2 respectively (Table 1). Notably in B1 and B2 we observed i) 2.6 fold increase in saturated fatty acids in *L. lipofera* ii) 1.4 and 6.0 times increase in monounsaturated fatty acids in *Y. lipolytica* and *L. lipofera* respectively and iii) 1.2 and 2.1 times increase in linoleic acid content in *Y. lipolytica and L. lipofera* respectively.

Table 1

Percentage fatty acid composition of Y. lipolytica and L.lipofera. Cultures were grown in MGYP as well as in altered media (B1 and B2).

	Y. lipolytica		*L.lipofera*	
Fatty acids	**MGYP**	**B1**	**MGYP**	**B2**
C (14:0) Myristic acid	1.4±0.28	1.1 ±0.14	2.5±0.42	8.6±0.14
C (16:0) Palmitic acid	13.5±0.28	13±0.7	10.9±0.28	23.2±0.42
C (18:0) Stearic acid	2.2±0.14	2.8±0	5±0.28	16.5±0.56
C (16:1) Palmitoleic acid	9.7±0.28	22.9±0.28	N.D.	N.D.
C (17:1) cis-10-Heptadecanoic acid	N.D.	1±0.14	N.D.	11.2±0.28
C (18:1 n9c) Oleic acid	23.8±2.55	21.8±0.99	5.8±0.28	24.6±0.56
C (18:2 n6c) Linoleic acid	32.1±0.28	37.4±0.7	7.4±0.28	15.9±0.42

Values represent mean ± standard deviation of two observations and they were tested for statistical significance at P< 0.05.

N.D. Not detected

DISCUSSION

Lipid – producing (oleaginous) microorganisms have been known for many years, and their potential as alternative sources of plant oils has been periodically assessed[24]. These lipids can be used in different industrial processes as mentioned earlier. Yeasts are considered to be major source of single cell oil that can be used as alternatives to vegetable oils. These uses are somewhat dependent on an inexpensive and abundant yeast feedstock. Therefore, cultivation of yeasts at large scale using cost effective raw material is need of the hour. Keeping this in mind, we have examined effects of alterations in MGYP on lipid accumulation in oleaginous yeasts. Earlier reports on lipid accumulation during primary anabolic growth in *Y. lipolytica* indicated that this was influenced by the medium pH, incubation temperature, aeration rate and an endogenous supply of easily bioconvertible substrates[25]. We observed maximum lipid yields at the end of 24 hrs in both the cultures.

This is ideal since shorter incubation periods are always preferred while considering potential industrial processes for lipid production[26]. The total lipid content of yeast cells is reported to increase as the culture ages until the stationary phase of growth is reached. Thereafter, following the exhaustion of glucose in the medium, there is a rapid decrease in the lipid content of the cells indicating the dynamic state of storage lipids in these organisms[26]. Our analysis of residual reducing sugar in the medium (data not shown) showed that sugar was not completely utilized by the yeasts when the lipid contents were high. Thus, before the depletion of 'C' source in the culture medium, oleaginous microorganisms are not able to consume their own lipid reserves[27,28,29].

Further, experiments of change in media composition by replacing glucose and peptone with different sugars and nitrates resulted in increase in lipid yields. We observed significant increase of 1.6 and 1.9 times in lipids using media A1 and A2 in *Y. lipolytica* and *L. lipofera* respectively. Inorganic nitrogen sources have also been reported to increase lipid synthesis in some fungi[30,31]. The key to lipid accumulation lies in allowing the amount of nitrogen supplied to the culture to become exhausted within about 24 - 48 h. Exhaustion of nutrients other than nitrogen can also lead to the onset of lipid accumulation[32] but in practice, cell proliferation is most easily effected by using a limiting amount of nitrogen (usually NH_4^+ or urea) in the medium. The excess carbon which is available to the culture after nitrogen exhaustion continues to be assimilated by the cells and is converted directly into lipid. Our results of high lipid accumulation at low nitrogen concentration in *L. lipofera* are in line with these observations. Fundamental differences in nitrogen metabolism between different species of oleaginous yeasts may account for the ability of various nitrogen compounds to stimulate lipid accumulation[33]. Considerable decrease in lipid contents observed in *Y. lipolytica* in presence of ammonium nitrate may attribute to dual effect of NH_4+ simultaneously repressing both
transport and the catabolic systems for dissimilation of the amino acids[34]. Further, we have attempted to economize the media using LCTS and urea. The results were significant as increase in lipid yields were observed for both the cultures in B1 and B2 in comparison to MGYP. Urea is widely utilized by yeasts[35]. Significant increase in lipid percentage in presence of urea compared to that of peptone may be due to the extent of NH_4^+ release and accumulation during metabolism[36]. Increase in lipid content was also reported in *Rhodosporidiurn toruloides* CBS 14 with urea as a principle nitrogen source[33]. Tsiege et al. (2012)[37] studied lipid yield of *Y. lipolytica* Po1g and its suitability for biodiesel production. The authors also concluded that different strains of *Y. lipolytica* have different lipid yields in different media.

The fatty acid profile of the yeasts was observed to differ in presence of MGYP and B1/ B2 which was found to be dependent on 'C' and 'N' source since other parameters were kept constant. In presence of urea, amount of linoleic acid increased at a loss of palmitic and oleic acid in *Y. lipolytica*. Similar result is noted in *Cryptococcus curvatus*[38]. Presence of urea as a nitrogen source was also found to drastically change intracellular fatty acid content in *Chlorella*[39]. We observed higher contents of oleic acid in *Y. lipolytica* as compared to that reported for *Y. lipolytica* grown on agro- industrial residues[40]. In case of *L. lipofera*, lipids with a high content of stearic acid (C18:0) and oleic acid (C18:1) were observed which can be of high prospects for biodiesel production, due to their properties of higher value oils, oxidative stability and their potential for adaptability in industrial production of biodiesels[41]. Thus, in totality unsaturated fatty acids (particularly C_{16} and C_{18}) were increased in both the yeasts when grown in altered medium. Papanikolaou et al. (2009)[42] have also reported increase in unsaturated C_{16} and C_{18} fatty acids in *Yarrowia lipolytica* strains cultivated on glucose.

CONCLUSION

In conclusion, B1 and B2 appeared to be the cost effective media for oleaginous yeasts for production of higher lipids as well as omega- 6 fatty acids.

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