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Isolation and Characterization of Oleaginous Yeast

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A B S T R A C T

Oleaginous yeast are known to synthesize and accumulate large amount of intracellular triacylglycerol. Hence, they are considered to be one of the most promising alternate sources for oil production. In this regard, the present study was undertaken to isolate, characterize and screen oleaginous yeast for biomass and lipid production. Eighty yeast isolates were obtained from 19 soil samples collected from different districts of Karnataka. Out of 80 isolates, ten isolates were confirmed to be oleaginous based on Sudan Black B staining. These isolates were further analyzed for biomass and lipid production. The reference strain *Debaryomyces hansenii* var. *hansenii* was also used in the study. The highest biomass and lipid production of 806.03 mg/100ml and 123.51 mg/100ml respectively was recorded by the isolate OYB-1 followed by OYH-2 with biomass production of 790.59 mg/100ml and lipid production of 120.50 mg/100ml. Hence the isolate OYB-1 can be exploited for biodiesel production.

Introduction

Rapid increase in the price of crude oil as well as decrease in oil supplies and environmental consequences of fossil fuels has drawn the attention of researchers towards biofuel production in recent years. To overcome decline in the whole world energy supply and energy security, other alternative energy sources like renewable biofuels are receiving considerable attention. The most favourable renewable energy resources are biodiesel, which can be produced from biomass by transesterification process (Meng *et al.,* 2009). In various parts of the world, different renewable lipids have been used for biodiesel production, including animal fat, waste oils and vegetable oils (Aggelis *et al.,* 1995).

The use of vegetable oils as feed stock for biodiesel production would compete with edible oils leading to increase in the food price. The use of animal fat and waste oil as feed stock can effectively reduce the price of biodiesel but these are limited and cannot meet the demands (Zhu *et al.,* 2008). Hence, alternative source for biodiesel production is currently the interesting area of research.

Microorganisms have often been considered for the production of fats and oils as an alternative to

animal and agricultural sources. Oleaginous microorganisms represent an alternative source for lipid production as they have special feature to accumulate more than 20% lipid per gram dry biomass as carbon storage with similar fatty acid composition as plant oils (Li *et al.,* 2008).

All kinds of microorganisms such as bacteria, microalgae, fungi and yeast produce lipids, but not all of them are available for production of biodiesel. Several species of yeasts are considered as oleaginous species that can accumulate lipid more than 20% of their biomass (Li *et al.,* 2008). They are able to synthesize and accumulate large amounts (up to 70%) of intracellular triacylglycerols of their biomass weight. Hence, they can be used as alternate source for biodiesel production.

Compared to other oleaginous microorganisms, such as filamentous fungi, bacteria and algae, yeasts have a higher growth rate resulting in higher cell density (Li *et al.,* 2007). Lipids produced by yeasts have similar fatty acid profiles compared to those of vegetable oils and are therefore considered as an alternative strategy for the production of second-generation fuels, including biodiesel (Li *et al.,* 2008; Papanikolaou *et al.,* 2003; Papanikolaou and Aggelis, 2011).

Hence the present study was done to isolate characterize oleaginous yeast and analyze biomass and lipid production from these isolates.

Materials and Methods

The experiment was carried out at the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad. The materials used and the methodology adopted are furnished here.

Collection of soil samples and isolation of oleaginous yeast

Soil samples were collected from different districts of Karnataka (Shimoga, Chitradurg, Davangere, Haveri, Bellary, Dharwad and Chikkamagaluru) especially from traditional oil extraction units and these samples were used for the isolation of yeast. Ten gram of soil sample was weighed and added to 100 ml of glycerol enrichment medium (Glycerol 100ml/L, $(NH_4)_2$ SO_4 1g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L and yeast extract 0.2 g/L). It was incubated under shaking condition for 24 h to enhance the cell growth. From this enriched medium, culture dilutions were made and plated on to Yeast extract Malt extract(YM) agar (Glucose at 10 g/L, peptone at 5 g/L, yeast extract at 3 g/L, malt extract at 3 g/L, agar at 20 g/L and pH 7) plates supplemented with ampicillin (10 mg/L). Colonies obtained were purified and maintained on YM agar slants.

Characterization of oleaginous yeast

All the isolates were subjected for microscopic observation and observed for budding characteristic to confirm as yeast. The confirmation of the yeast isolates as oleaginous was done by Sudan Black B staining procedure by following the protocol of Thakur *et al.,* (1988). The isolates were inoculated in to 100 ml Lipid producing medium (Glucose at 50g/L, $(NH_4)_2SO_4$ at 0.2g/L, KH_2PO_4at 0.4 g/L, $MgSO_4$. $7H_2O$ at 1.5g/L, $ZnSO_4$ at 4.4mg/L, $CaCl_2$ at 25mg/L, $MnCl_2$at 0.5mg/L, $CuSO_4$ at 0.3mg/L and yeast extract at 0.75mg/L) in 250 ml Erlenmeyer flask and incubated for 144 h under shaking condition. Two drops of cell suspension from the lipid producing medium was taken on a clean glass slide and smeared on it. Slide was air dried and smear was heat fixed by passing the slide over the flame. Sudan Black B stain (0.3%) was flooded on the smear and kept for 20 min. Then the slide was gently washed with 70% alcohol to remove excess stain. Later, the slide was counter stained with safranin (1%) and kept for 1 min. Finally, the slide was washed gently with distilled water, air dried and observed for lipid globules inside the cells as grey to black colour with pink coloured vegetative cells under microscope at 100X magnification.

Biomass and lipid production from yeast isolates

One ml of overnight grown culture was transferred

to 250 ml Erlenmeyer flask containing 100 ml Lipid producing medium and incubated for 144 h under shaking condition. This culture broth was used for estimation of biomass and lipid content separately.

The biomass of each isolate was estimated by gravimetric method by following the procedure given by Pan *et al.*, (2009). Hundred ml of culture was taken in centrifuge tube and centrifuged at 10,000 rpm for 10 min. The pellet obtained was washed with distilled water and dried in an oven at a temperature of 70°C. Weight of the biomass was recorded and expressed in mg/100ml.

The lipid from yeast isolates was extracted, dried and weighed by following advanced Bligh and Dyer method (Bligh and Dyer, 1959).

Hundred ml of culture broth was taken in centrifuge tube and centrifuged at 13,000 rpm for 10 min. The pellet obtained was washed with distilled water and treated with 15 ml of 4M HCl for 60 min. Then the tube was kept in -80°C for 20 min and immediately transferred to boiling water for 10 min. This procedure was repeated thrice to break down the cell wall. Then the tube was added with 30 ml of methanol/chloroform (1:1) and incubated for 3 h under shaking condition.

Later, the tube was centrifuged at 10,000 rpm for 10 min to separate two layers. The aqueous upper layer was removed using Pasteur pipette and lower organic phase was evaporated to dryness at 70°C in an oven and total lipid was estimated gravimetrically. The lipid content was expressed in mg/100ml.

Results and Discussion

Collection of soil samples and isolation of yeast

Soil samples were collected from the traditional oil mills situated in different districts of Karnataka *viz.*, Shimoga, Chitradurg, Davangere, Haveri, Bellary, Dharwad and Chikkamagaluru. Among 19 soil samples, 3, 7, 5, 1, 1, 1 and 1 samples were collected from Shimoga, Chitradurg, Davangere, Haveri, Bellary, Dharwad and Chikkamagaluru respectively. Total of 80 yeast isolates were obtained from 19 soil samples. All the cultures were observed under the microscope for budding characteristics of the yeast. Out of 80 isolates 11, 27, 19, 04, 06, 07 and 06 isolates were obtained from Shimoga, Chitradurg, Davangere, Haveri, Bellary, Dharwad and Chikkamagaluru respectively (Table 1).Intracellular lipid accumulation is one of the properties of oleaginous organisms. Hence, all the isolates were observed for lipid accumulation inside the cell by using Sudan Black B staining. The yeast isolate which accumulate lipid intracellularly takes the stain and show black to grey colour lipid bodies inside the pink colour vegetative body when observed under microscope at 100X magnification. Out of 80 yeast isolates, only 10 isolates were found to be oleaginous. These ten isolates were obtained from Shimoga (1), Chitradurga (2), Davangere (4), Haveri (1) and Bellary (2) districts of Karnataka.In one of the studies, 79 yeasts out of 479 microbial colonies isolated from Himalaya region, were identified to be oleaginous (Patel *et al.*, 2014). Generally, 3–15% of the randomly selected yeast population is found to be oleaginous (Sitepu *et al.*, 2014), which was also the case in present study.

Significant differences were observed among the isolates with respect to biomass content. The highest biomass content of 806.03 mg/100ml was recorded by the isolate OYB-1, which was significantly superior over all other isolates. The next highest biomass content of 790.59 mg/100ml was observed in the isolate OYH-2. Whereas, other isolates *viz.*, OYM-3, OYC-4, OYC-5, OYC-6, OYD-7, OYR-8, OYU-9 and OYU-10 exhibited the dry biomass content of 765.29, 580.91, 710.87, 690.67, 495.54, 680.50, 425.39, 495.14, 230.60 mg/100ml respectively. The reference strain (*Debaryomyces hansenii* var *.hansenii*) showed least biomass content of 230.60 mg/100ml (Table 2).

Table.1 Details of number of isolates obtained from soil samples of oil spilled sites

Sl.No	Place	Type	Total no. Yeast Isolates	No. of oleaginous yeast isolates	Isolate Number
A. Shimoga district					
1	Bhadravathi	Soil	7	1	OYB-1
2	Mangote	Soil	1	0	
3	Shimoga	Soil	3	0	
B. Chitradurga district					
4	Hosadurga	Soil	6	1	OYH-2
5	Chikkajajur	Soil	4	0	-
6	Holalkere	Soil	4	0	
7	Challakere	Soil	2	0	
8	Ramagiri	Soil	2	0	
9	Malladihalli	Soil	5	1	OYM-3
10	Dummi	Soil	4	0	
C. Davangere district					
11	Channagiri	Soil	6	3	OYC-4 OYC-5 OYC-6
12	Davangere	Soil	4	1	OYD-7
13	Tyavanige	Soil	2	0	-
14	Santhebennur	Soil	4	0	-
15	Bada	Soil	3	0	-
D. Haveri district					
16	Ranebennur	Soil	6	1	OYR-8
E. Bellary district					
17	Ujjini	Soil	6	2	OYU-9 OYU-10
F. Dharwad district					
18	Narendra	Soil	7	0	-
G. Chikkamagaluru district					
19	Krishna Raja Pet	Soil	4	0	-

Table.2 Biomass and lipid content of oleaginous yeast isolates

Sl. No.	Isolates	Biomass content (mg/100ml)	Lipid content (mg/100ml)
1	OYB-1	806.03	123.51
2	OYH-2	790.59	120.50
3	OYM-3	765.29	115.50
4	OYC-4	580.91	96.57
5	OYC-5	710.87	112.59
6	OYC-6	690.67	98.12
7	OYD-7	495.54	108.19
8	OYR-8	680.50	103.52
9	OYU-9	425.39	106.68
10	OYU-10	495.14	110.75
11	Reference strain (*D. hanseniivar.hansenii*)	230.60	19.62
S.Em±		0.52	0.39
CD at 1%		1.95	1.38

With respect to lipid production, significantly highest lipid content of 123.51 mg/100ml was recorded in the isolate OYB-1 followed by OYH-2 with lipid content of 120.50mg/100ml (Table 2). Whereas, other isolates OYM-3, OYC-4, OYC-5, OYC-6, OYD-7, OYR-8, OYU-9 and OYU-10 recorded the lipid content of 115.50, 96.57, 112.59, 98.12, 108.19, 103.52, 106.68 and 110.75mg/100ml respectively. Very least lipid content of 19.62 mg/100ml was observed in reference strain (*Debaryomyces hansenii* var. *hansenii*) (Table 2).Present studies are in conformity with the work of Pan *et al.,* (2009) and Laker *et al.,* (2018) who screened yeast isolates based on lipid accumulation and biomass production. Yeast strains isolated from soil, flower and compost samples exhibited lipid content ranging from 10.44 g/L to 13.41 g/L (Singh *et al.,* 2020). Similarly, Poontawee *et al.,* (2017) reported that the oleaginous yeast *Rhodosporidium fluviale* DMKU-SP314 exhibited highest lipid content of 7.9 g/L and dry biomass of 14.3 g/L.

One of the main challenges to produce second generation biodiesel is to improve the use of non-food materials as nutrients for microorganisms converting them into lipids. In this work isolation of oleaginous yeasts has been done from oil spilled soils. A total 80 isolates were obtained and among them ten isolates were found to be oleaginous. Isolates were characterized as oleaginous upon Sudan Black B staining. The positive isolates were screened for maximum biomass and lipid production. The isolates OYB-1 and OYH-2 exhibited higher biomass and lipid content. So the present work demonstrates the possibility to utilize OYB-1 and OYH-2 for biodiesel production.

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