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Screening of oleaginous yeast isolates of dairy environmental samples for lipid accumulation

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Abstract
A total of 14 oleaginous isolates obtained from dairy environmental samples like dung, feed, fodder, sewage, silage and soil samples were subjected for secondary screening of cells for Sudan black. Once intracellular lipid accumulation was confirmed, through smear, the isolates were phenotyped by subjecting to preliminary and specific tests and based on which the isolates belonged to species of *Candida* (6), *Kluveromyces* (3), *Rhodotrula* (1), *Saccharomyces* (2) and *Yarrowia* (2). Species of *Candida* predominated among the phenotype accounting for 42.86% followed by 21.43% of *Kluveromyces,* 14.29 each of *Saccharomyces* and *Yarrowia* and finally 7.14% of *Rhodotorul*a. pH of yeast cultured MGYP broth showed values in the range of pH 4.6 to 4.3 from initial pH of 6.4 with DMC of 8.00 to 8.80 $\log_{10}$/ml. Soil yeast isolates Y10, phenotyped as *Yarrowia* sp. and Y14 as *Candida* sp. had DMC of 8.80 and 8.60 $\log_{10}$/ml respectively. Dry cell mass of 14 yeast isolates ranged from 10.30 to 57.00 g/l and yield of lipid was 2.80 to 29.00 g/l with per cent lipid of 17.86 to 66.25 per cent. The highest producer of lipid was Y10, *Yarrowia* sp., with 66.25 per cent while Y14, *Candida* sp., had 58.18 per cent of lipid.

Keywords: Oleaginous yeast, malt extract agar, dairy environment, Sudan black, intracellular lipid, *Yarrowia* spp.

Introduction
Oleaginous yeasts have a unique physiology that makes them the best suited hosts for the production of lipids, oleochemicals, and diesel-like fuels. Their high lipogenesis, capability of growing on many different carbon sources (including lignocellulosic sugars), easy large-scale cultivation, and an increasing number of genetic tools are some of the advantages that have encouraged their use to develop sustainable processes. Oleaginous yeasts store above 20% of their dry cell weight as intracellular triacylglycerols. These yeasts are considered as cell factories amenable for the commercial production of biodiesel, polyunsaturated fatty acids, and exotic fats like cocoa butter. Oleaginous yeasts predominantly belong to the genera *Rhodosporidium, Cryptococcus, Rhodotorula, Yarrowia, Lipomyces,* and *Trichosporon* (Adrio, 2017; Athenaki *et al.*, 2018 and Blomqvist *et al*., 2018) [1, 2, 3]. Yeasts are well-suited for commercial production of microbial lipids over other oleaginous microorganisms like algae and filamentous fungi because of its ability to reach high cell densities in a short time having similar composition of fatty acid as those of plant-based oils and capability of producing a variety of metabolic co-products including high-value carotenoids, proteins and polysaccharide rich streams of deoiled biomass, and various organic acids (Sitepu *et al.*,2014; Deeba *et al*.,2016; Parsons *et al.*,2020) [4, 5, 6].
Recent screening and molecular characterization studies have led to the identification of some novel oleaginous yeast strains which include *Cystobasidium oligophagum* JRC1 when grown on a wide range of substrates including carboxymethylcellulose (CMC) and accumulated 36.46% (w/w) lipid on the medium with CMC as sole carbon source (Vyas *et al.*, 2016) [7], *Meyerozyma guilliermondii* BI281A, the lipid production was optimized obtaining 108 mg/L of neutral lipids using pure glycerol as carbon source and the strain proved capable of accumulating oil using raw glycerol from a biodiesel refinery (Ramírez-Castrillón *et al.*, 2017) [8].
Gientka *et al.* (2017) [9] isolated oleaginous yeasts from several fermented foods including Kefir and other dairy products like cheese, milk, and yoghurt. Yeast isolates identified were *Candida inconspicua, Debaryomyces hansenii, Kluyveromyces marxianus, Kazachstania unispora,* and *Zygotorulaspora florentina*. They showed that deproteinated potato wastewater, a starch processing industry waste, supplemented with various carbon sources, including

lactose and glycerol, is a suitable medium for the growth of yeast, which allows an accumulation of over 20% of lipid substances in its cells. According to Patel *et al.* (2019) [10], out of the 1600 described yeast species, only 70 are known to store lipids above 20% of their biomass. Therefore, screening studies are crucial for the discovery of new oleaginous yeast species.

2 Materials and Methods

2.1 Isolation of oleaginous yeast from Dairy environmental samples

The selected black colonies of yeast appeared on malt extract agar incorporated with 3.75 ml of 2% Sudan black were transferred to YEPD broth and incubated at 30 ºC for 48 h. After the growth, observed for turbidity in broth, confirmed the morphology of yeast by simple staining method and further purified thrice by streaking. The yeast isolates were maintained on sterile MEA slants and subcultured once in a month.

2.2 Phenotypic Identification

The purified yeast isolates were subjected to phenotypic identification through preliminary tests like simple staining and Sudan black staining and specific biochemical tests such as oxidative & fermentative test; fermentation or oxidation of lactose & sucrose; production of urease and nitrate reduction to place the yeast isolates to genus level. The medium used and procedure adopted as mentioned by Harrigan (1998) [11].

2.3 Screening of yeast isolates for lipid accumulation

All the phenotyped yeast isolates were subjected to screening for lipid accumulation by growing in malt extract peptone malt broth and carrying out DMC and determining the lipid yield by incubating at 30 °C /5 days.

2.4 Extraction and determination of total Lipids (Bligh and Dyer, 1959) [12].

The optimized cultured broth were centrifuged at 6000 rpm for 10 min for the separation of biomass. The supernatants was discarded and the cell pellets were washed with distilled water, filtered and dried at 60 °C to gain a constant dry mass. By which is considered as fast and rapid technique. The cell mass was suspended in 10 mL of 4M HCl and then incubated at 60°C for 2 h for complete hydrolysis. The acid hydrolyzed mass was then been stirred in 20 mL of chloroform and methanol mixture (1:1) at room temperature for 2 h. The suspension will thoroughly mixed by vortex followed by centrifugation at 1000 rpm for 5 min. The aqueous upper phase and organic lower phase with residing cell debris at interphase will be separated. The lower organic layers and the chloroform was then be allowed to evaporate to obtain the oil content of the yeasts. The oil yield was be estimated by weight.

3. Results and Discussion

3.1 Phenotypic identity of oleaginous yeast isolates:

Oleaginous yeast colonies on Sudan black MEA showed black colonies. The smear of isolates of yeast revealed circular to ovoid cells in simple staining while sudan black staining, exhibited yellow spots inside the cell indicating lipid accumulation. Based on preliminary and specific phenotypic tests conducted for 14 of oleaginous yeast isolates, species of Candida predominated among the phenotype accounting for 42.86% followed by 21.43% of *Kluveromyces*, 14.29 each of *Saccharomyces* and *Yarrowia* and finally 7.14% of *Rhodotorula*. Soil had more yeast isolates while fodder, less of oleaginous yeast (Table 1 and Table 2). On the contrary Pan *et al.* (2009) [13] reported predomination of *Rhodotorula* for 35% where as candida was 20%. While Khobragade *et al.* (2020) [14].

Table 1: Phenotypic identification of yeast isolates obtained from dairy environmental sample

Code of yeast isolates (numbers)	Preliminary identification		Specific identification tests						Identity
	Colony morphology on MEA	Cell morphology	OF Test		Lactose Ferm/Assi	Sucrose Ferm/Assi	Urease Production	Nitrate reduction	
			Oxidative	Fermen Tative					
Y1, Y2, Y5, Y11, Y13, Y14 (6)	White, round, glistening	Oval,budding, asporogenous with pseudomucelia	+	+	Fermenter	Not a fermenter or assimilator	+	+	*Candida* spp.
Y3, Y6, Y4 (3)	Brownish tinged, round	Oval with budding, ascosporogenous	+	+	Fermenter	Not a fermenter or assimilator	-	-	*Kluveromyces* spp.
Y7 (1)	Orange, round, glistening	Oval with budding, asporogenous	+	-	Not a fermenter or assimilator	Assimilator	-	-	*Rhodotorula* sp.
Y8, Y12 (2)	Cream tinged, round Moist, dull	Oval with budding, ascosporogenous	+	+	Not a fermenter of assimilator	Fermenter	-	-	*Saccharomyces* spp.
Y9, Y10 (2)	Whitish, dull, round	Oval,budding, asporogenous with pseudomucelia	+	-	Not a fermenter of assimilator	Assimilator	-	-	*Yarrowia* spp.

Table 2: Sources of phenotyped yeast isolates from dairy environmental samples

Isolated code	Source	Identity (nos & per cent)
Y1, Y2	Dung	*Candida* sp (6 Nos. – 42.86)
Y11, Y13, Y14	Soil	
Y5	Fodder	
Y3, Y4	Feed	*Kluyveromyces* sp (3 nos. – 21.43)
Y6	Sewage	
Y7	Silage	*Rhodotorula* sp (1 no. – 7.14)

Y8	Silage	*Saccharomyces* sp (2 Nos. – 14.29)
Y12	Soil	
Y9, Y10	Soil	*Yarrowia* sp (2 Nos. 14.29)

also found *Schizosaccharomyces* spp. as oleaginous yeast that occurred at a per cent of 27.27 and did not find *Candida* species soil of milk collection centre of Nanded. Even Bardhan *et al.* (2020) [15] found predominant oleaginous yeast species in traditional fermented foods of Manipur as *Rhodotorula* (42.85%) followed by *Pichia* (14.29%), *Saccharomyces* (14.29%) and 7.14% each of *Candida*, *Saturnispora*, *Wickerhamomyces* and *Zygoascus*. In agreement with the present study, Vincent *et al.* (2018) [16] also found predominant yeast as *Candida* spp. accounting for 42.85% followed by species of *Pichia* (33.33%), *Kluveromyces* (19.05) and *Clavispora* (4.76%) from soil samples of Kuching, Malaysia.

3.2 Screening of yeast isolates for lipid accumulation:

All the 14 yeast isolates cultured in malt extract glucose yeast extract peptone broth for 5 days at 30 ºC showed DMC of 8.00 to 8.80 $\log_{10}$/ml (Table 3). Soil yeast isolates Y10, phenotyped as *Yarrowia* sp. and Y14 as

Table 3: Per cent of lipid in oleaginous yeast isolates of dairy environmental samples

Source	Name of the yeast species with code	DMC ($\log_{10}$/ml)	Dry cell weight (g/l)	Lipid content (g/l)	Lipid yield (%)
Dung	*Candida* sp.Y1	8.30	28.00	9.00	32.14[a]
	Candida sp.Y2	8.15	28.00	5.00	17.86 [a]
Feed	*Kluyveromyces* sp.Y3	8.00	57.00	29.00	50.88 [a]
	Kluyveromyces sp. Y4	8.30	13.40	7.00	52.24 [a]
Fodder	*Candida* sp.Y5	8.18	26.00	13.00	50.00 [aa]
Sewage	*Kluyveromyces* sp.Y6	8.48	10.30	3.40	33.01 [a]
Silage	*Rhodotorula* sp. Y 7	8.10	25.00	13.00	52.00 [a]
	Saccharomyces sp. Y 8	8.25	27.00	9.00	33.33 [a]
Soil	*Yarrowia* sp Y9	8.08	14.20	7.10	50.001[a]
	Yarrowia spY10	8.88	24.00	15.90	66.25[a]
	Candida sp. Y11	8.40	27.00	14.00	51.85[a]
	Saccharomyces sp. Y12	8.32	10.50	2.80	26.67[a]
	Candida sp. Y13	8.42	11.50	4.80	41.74[a]
	Candida sp. Y14	8.60	22.00	12.80	58.18[a]
CD (*P*=.05)					41.61

Note

- Medium used: Malt extract glucose yeast extract peptone broth (MGYP) with incubation at 30 ºC for 5 days
- Lipid extraction by using Bligh and Dyer (2013)
- CD – Critical Difference

Candida sp. had DMC of 8.80 and 8.60 $\log_{10}$/ml respectively. *Candida* spp. of 6 numbers found to possess lipid yield of 17.86 to 58.18%, while 3 *Kluveromyces* spp. of 33.01 to 52.24%; 1 of *Rhodotorula* sp. accounting for 52%; 2 of *Saccharomyces* spp. of 26.67 to 33.33% and 2 of *Yarrowia* spp. of 50 – 66.25% feed isolate. *Kluveromyces* sp. Y3 though showed more dry cell mass but lipid content was 29 with per cent yield of 50.88% where as *Candida* sp. Y2 from dung sample had dry mass of 26 with lipid content of only 5 g/l and yield of 17.86. The highest producer of lipid was Y10, *Yarrowia* sp., with 66.25 per cent (dry mass – 24.00 and lipid content – 15.90 g/l) while Y14, *Candida* sp., had 58.18 per cent of lipid (dry mass – 22.00 and lipid content – 12.80 g/l). Though some of yeast isolates produced more biomass which did not indicate high lipid content. The range of lipid production in 14 yeast isolates was 17.86 to 66.25% in yeast malt extract broth at 30 C for 5 days (Table 3).

The lipid accumulated by oleaginous yeast studied by other authors in synthetic and semisynthetic medium have been considered for comparison.On the contrary to the present study, where *Yarrowia* spp. produced lipid of 50 to 66.25% while candida had 17.86 to 58.18 with highest producer of 58.18% but as per Enshaeieh *et al.* (2013) [17], *Yarrowia lipolytica* DSM 8218 used for lipid production as a standard strain possessed 4.21 g/l, 13.28 g/l, 31.7% of lipid quantity, dry biomass and lipid productivity while *Candida albidus* had lipid quantity, dry biomass and lipid productivity of 11.81 19.65 g/l and 60.1%, respectively, in shaking flask cultivation at 150 rpm and 25 °C in nitrogen-limited medium containing per liter 75 g glucose with pH adjusted to 6.5.

A study by Ayadi *et al.* (2019) [18] in fed-batch fermentation using synthetic medium having pH 6.0 with glucose at the rate of 40 g/L, *Rhodotorula mucilagenosa*, Y-MG1 strain produced cell dry weight (DW) of 6.64 ± 0.55 g/L with 2.13 g/L of lipids corresponding to 32.7% of lipid content with incubation at 30 °C for 144 h, which was less compered to present study. In contrast to present study, *S. cerevisiae* 0A03 isolated from rice beer of Assam in yeast malt extract broth (yeast extract -3g/l, malt extract – 3g/l, peptone – 5g/l, glucose -10g/l, pH 6.6) produced lipid content of 12.8% of DW at 28 °C /48 h (Phukan *et al*., 2019) [19].

Conclusion

Candida predominated among the phenotype accounting for 42.86%. The highest producer of lipid was Y10, *Yarrowia* sp., with 66.25 per cent (dry mass – 24.00 and lipid content – 15.90 g/l) while Y14, *Candida* sp., had 58.18 per cent of lipid (dry mass – 22.00 and lipid content – 12.80 g/l) and both were isolated from soil of dairy farm.

Conflict of Interest: None.

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