STUDY ON THE DISTRIBUTION OF OLEAGINOUS YEASTS FROM THE WATER SAMPLES OF BAY OF BENGAL

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Abstract: Oleaginous yeasts are a promising solution for lipid production which is rich in nutritional value and can be used in the production of bio-fuels, value added products and pharmaceutical protein production. This study outlines an efficient and much competitive approach of analysing the marine deep sea water for lipid production and identified accumulation of lipids. In this study oleaginous yeasts from deep-sea water were isolated and extracted lipids through gravimetric method. The marine water was collected from Bay of Bengal at different depths of 2m, 50m, 500m, 1500m, 2000m and 2500m from three different stations. Out of 65 yeasts, three were identified to accumulate more than 30% of lipids and selected for further studies.

Keywords: lipids, oleaginous yeasts, sea water, Bay of Bengal.

INTRODUCTION

Microorganisms are widely distributed in the marine environment but the exploration of microbes is very limited. The activity and the characteristic features of marine microbes are different from the terrestrial sources **(Carr *et al*., 2003).** Their number and distribution depend on organic materials and other environmental factors. Specifically marine yeasts are not only found in sea water, sediments and plants of marine habitats but also in digestive tract of marine animals and sea birds. Marine yeasts are unique in nature when compared to the terrestrial by their unique properties like high osmotic tolerance and industrial enzyme productivity **(Kutty & Philip *et al*., 2008).** Nowadays yeasts are widely applied for the production of commercial products like bio-ethanol, lipids, fatty acids etc, **(Evan *et al*., 1984).**

Lipids are produced by all microorganisms usually in the range of 6 to 8% of their dry biomass principally as components for the cell membrane. Microorganisms, including yeasts, bacteria, filamentous fungi and microalgae can synthesize and accumulate more than 20% lipids in their biomass. These microorganisms are called oleaginous organisms (**Patel *et al.*, 2020**). Yeasts are advantageous for lipid production over other sources, as they can accumulate oil up to 80% of their dry weight and their cultures can be easily scaled up.

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 **(Robak & Balcerek, 2018).** The oleaginous yeasts, when compared to other oleaginous organisms can accumulate 40% of lipid and require small area for their cultivation. The main objective of this study is to isolate potential oleaginous yeasts that can accumulate more than 40% of lipid with the basal medium.

The accumulation of lipid in microorganisms especially in yeasts is done through

(i) *Denovo* biosynthesis: Hydrophilic substrates like carbohydrates are fermented and converted into lipids; and
(ii) (ii). *Exnovo* biosynthesis: Hydrophobic substrates like oil and alkanes are converted into lipids (**Papanikolaou & Aggelis, 2011; Huang *et al*., 2017).**

Accumulation of lipid is a complex process that involves many metabolic reactions spanning multiple cellular organelles in eukaryotic cells. The major intermediates in lipid biosynthesis are fatty acids, and are primarily found in lipid bodies for storage. Yeasts are industrially important microorganisms widely used in several industries including biofuel production, agricultural, biocontrol and other biotechnological applications **(Passoth & Schnurer, 2003; Hill *et al.*, 2006;** Matsushika et al., 2008). Particularly marine yeasts are found in sea water, sediments and also in the digestive tracts of marine creatures (**Van uden & Brance, 1963; Taysi & Vanuden, 1964; Kawakita & Vanuden, 1965; 3 Fell, 1967; Vogel *et al.*, 2007; Kutty & Philip, 2008).**Yeasts are isolated from both non- living environmental sources like sea water, marine sediments and living organisms like marine plants and animals. The abundance of yeast decreases with increase in sea- depth. The common ascomyces yeasts found in shallow marine environment are Candida, Debaryomyces, Kluyveromyces, Pichia and Saccharomyces. The basidiomycetes yeasts (Cryptococcus, Rhodosporidium, Rhodotorula and Sporobolomyces) are found in deep waters (**Kohlmeyer *et al.*, 1979**). In nature, there are only a few microorganisms have the ability to convert carbohydrates into oils and store them under the right conditions. Microorganisms that can accumulate lipids have been recognized for a long time and their potential as alternative plant oil sources has been evaluated on a regular basis **(Birch *et al.*, 1976; Woodbine, 1959**). Yeasts are largely utilized in the food industry to produce alcohol and carbon dioxide, which are critical in the brewing, distillation and baking sectors **(Yanagida *et al.*, 2002).** Oleaginous microorganisms have a noteworthy applicability in the production of lipids that can be utilized as a substitute for coca butter. Some oleaginous yeast has a unique ability to produce lipid profiles with high stearic acid content and little palmitic and oleic acid content similar to cocoa butter **(Papanikolaou *et al.*, 2011; Muniglia *et al*., 2003).**

MATERIALS AND METHODS

Collection of Samples and Isolation of Oleaginous Yeasts

Sea water samples were collected at the depth of 2m, 50m, 500m, 1500m, 2000m and 2500m from three different stations (MS1 Lat: 17º52.492'N Long: 88º48.018'E; MS2 Lat: 16º52.650'N Long: 86º42.102'E; MS3 Lat: 13º59.9726'N Long: 85º00.0316'E) in Bay of Bengal using Sagar Nidhi. Sabouraud's Chloromphenicol Agar, (**Loureiro *et al.*, 2005**), Yeast Extract, Peptone and Dextrose Agar (YEPD), Minimal Salt Agar with 2% Dextrose **Ykema *et al.*, (1989**) were used for the isolation of oleaginous yeasts from the samples. All the media were prepared with 1:1 dilution of filtered sea water and distilled water and autoclaved at 121º for 15-20 minutes. The water samples were serially diluted and plated. The plates were kept at 28±1ºC for 7 days.

Isolation and Colony Characterization

The mucoid colonies were selected from the inoculated plates and sub-cultured in glycerol and glucose agar media **(Ykema *et al.*, 1989).** The colony morphology of the selected strains was characterized. All the selected cultures were stocked in glycerol for further use.

Primary Screening of Oleaginous Yeasts

Preliminary screening of oleaginous yeasts was done using sudan black staining.

Sudan Black Staining: Sudan black stain 0.3 % is prepared in 70% ethanol and used directly for the staining. Sudan black staining was done according to the method by (**Kitcha & Cheirsilp (2011).** After drying, the samples were visualized under microscope.

Secondary Screening by Lipid Extraction

Lipid extraction was performed according to the method by **Bligh and Dyer** (1959). The isolated yeast cells were inoculated in glucose medium. After 48 hours incubation, the medium was centrifuged at 10000 rpm for 10 minutes to recover the yeast cell pellets. The collected pellets were washed with sterile distilled water. The cell pellets were kept at -80ºC overnight and then subjected to lyophilization. After lyophilization dried cells were weighed around 0.1g and suspended in 5 ml PBS buffer. The cell suspension was kept for autoclaving at 121ºC for 15 mins. After that cells were allowed to cool and to that equal volume of Chloroform: Methanol (2:1) was added and kept in a shaker at constant rpm for 1 h. The suspension was centrifuged at 10000 rpm for 5 mins. The upper aqueous phase containing lipid was collected and evaporated in rotary vacuum evaporator. The lipid weight was measured. The percentage of lipid accumulation was calculated according to the formula: Lipid accumulation (%) = Total lipid (g) / Total biomass (g).

RESULTS

Isolation and Colony Characterization

More than 300 colonies were identified in 10 ³ dilutions. The cells were identified based on differences in morphology and total colonies were counted. In YEPD agar, more number of mucoid colonies was observed when compared to other media. The mucoid colonies from all three media were noted and those colonies were again sub-cultured in glycerol and glucose media. Overall 67 isolates were selected based on the mucoid characteristics. The viscous sticky growth of selected yeasts was confirmed in both glucose and glycerol media. The isolates grew well in the glycerol medium when compared to glucose medium as shown in the Figure 1.

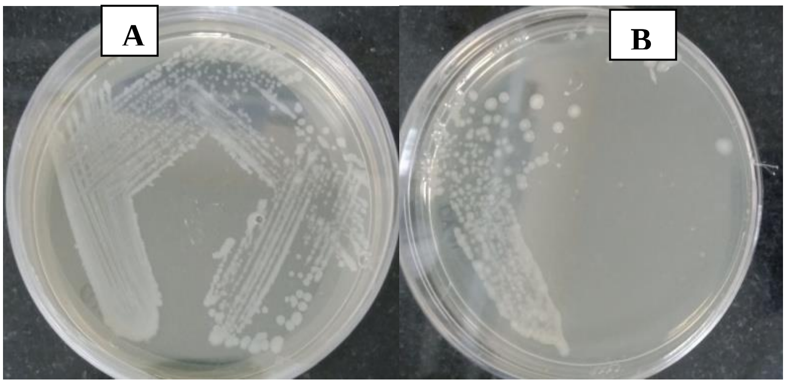
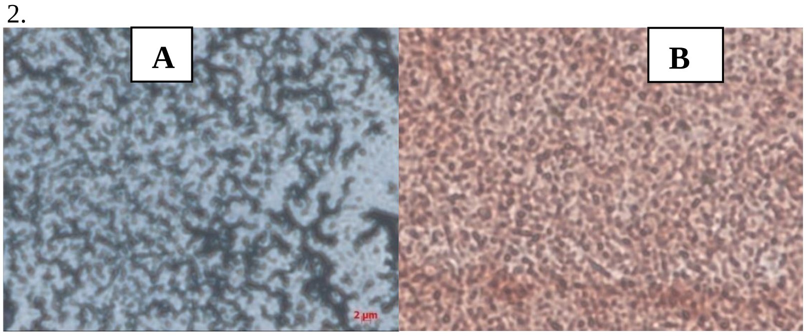


Figure 1: Yeast cells in A. Glycerol Medium; B. Glucose Medium

Primary Screening of Oleaginous Yeasts Sudan Black Staining:

The selected isolates were visualized for lipid accumulation using sudan black staining. Sudan black B stain only the lipid globules and the counter stain saffranin stained the yeast cells as shown in the figure (Figure 2). The oleaginous cells show lipid bodies stained yellow **(with lighter cytoplasm). The non-oleaginous cells were stained completely pink with no** observation of yellow stain as shown in the figure 2.



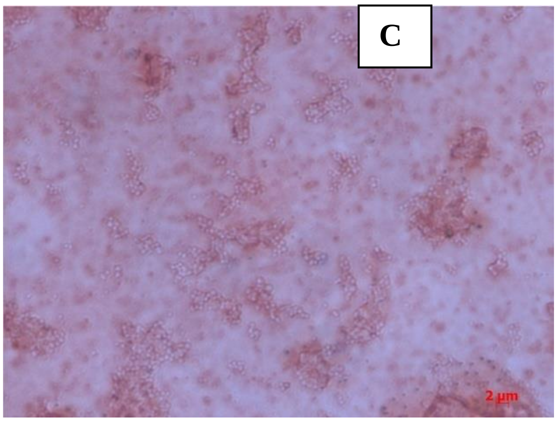


Figure 2: Microscopic Image of Isolated Yeast Strains.
A & B. Lipid Bodies Stained Yellow (presence of lipid accumulation)
C. No Observation of Yellow Stain (absence of lipid accumulation)

Totally 67 strains were screened and only 4 strains showed accumulation of lipid above 30% whereas 7 strains showed 20% accumulation (Table 1). Forty strains showed 10-20% accumulation and 16 strains exhibited accumulation less than 10%. The screening results of isolated yeast samples were shown in Table 1. The extracted lipid was viscous as shown in the Figure 3.

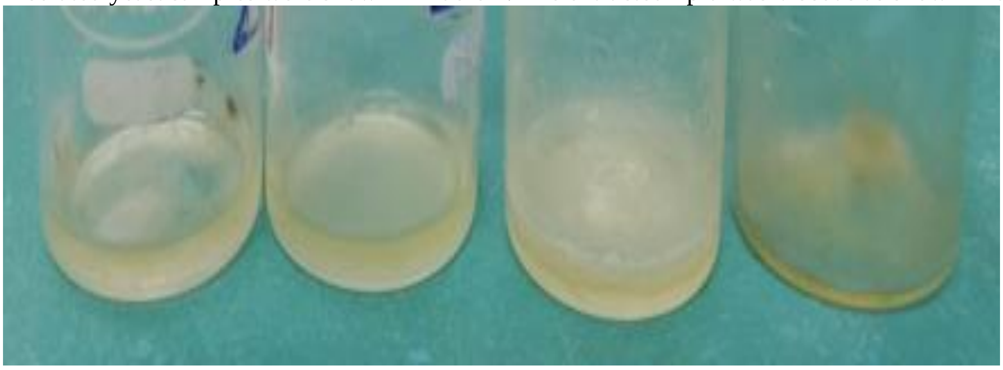
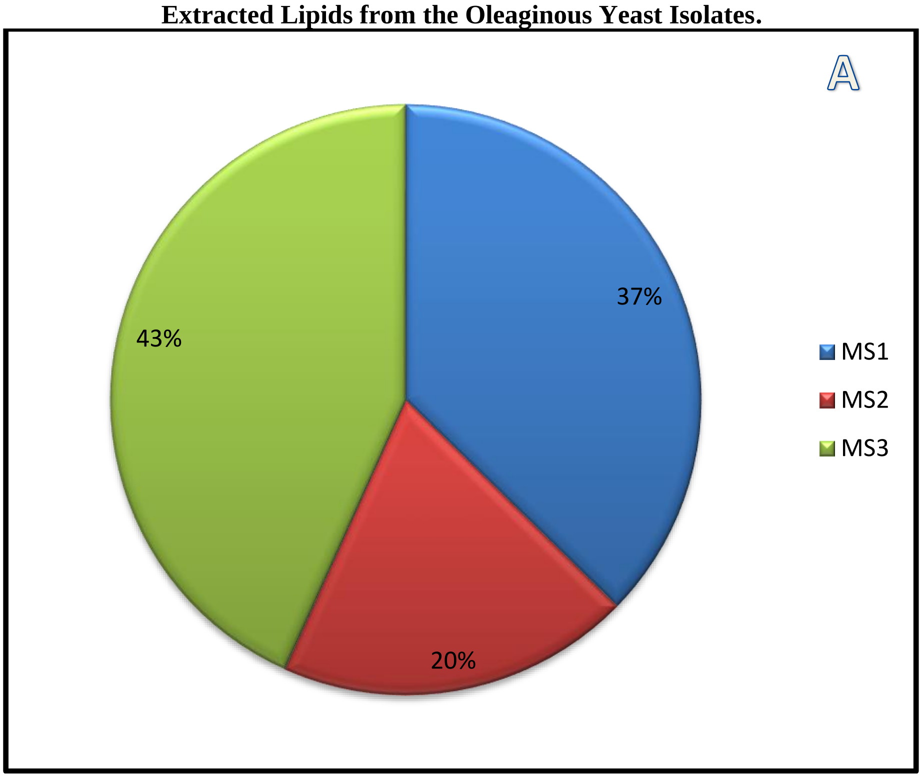


Figure 3:
Extracted Lipids from the Oleaginous Yeast Isolates.



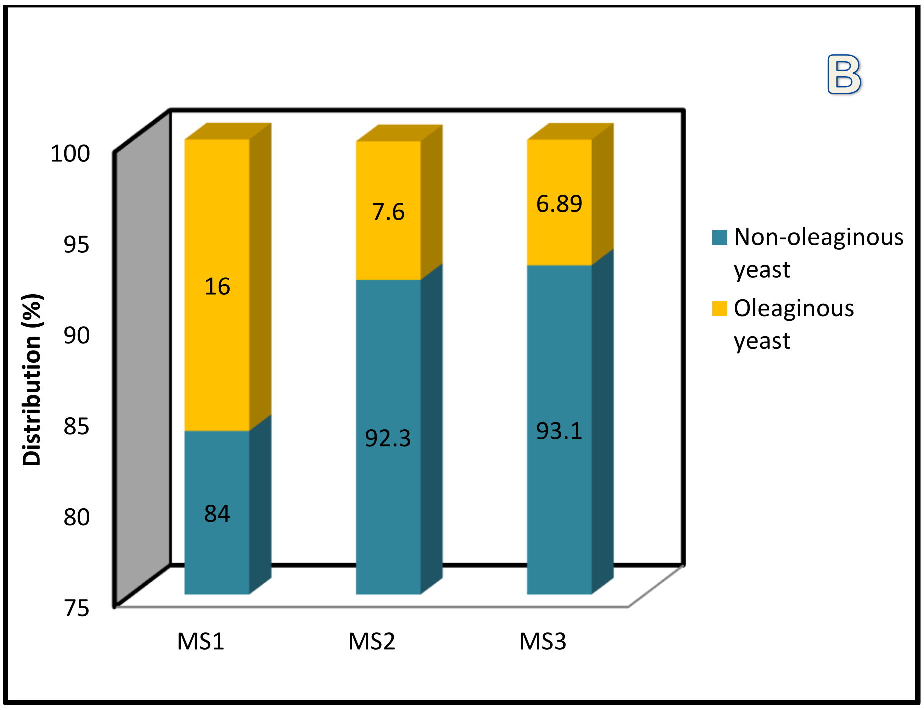


Figure 4: A. Distribution of Yeasts in Different Stations of Bay of Bengal
B. Percentage Distribution of Oleaginous Yeast

Among the Samples from Different Stations of Bay of Bengal It is observed that the distribution of yeasts was higher in the station MS3 than MS1 and MS2. The distribution of yeasts is very minimal in MS2. The detailed distribution of yeasts in different stations of Bay of Bengal is depicted in the Pie Chart (Figure 4). Even though the distribution of yeasts was higher in MS3, the presence of oleaginous yeast is very less. The presence of oleaginous yeast in MS2 station is 7.6% which is 1% higher than the MS3 station. MS1 has the maximum percentage of around 16% compared to other two stations. This is the first report on the distribution of oleaginous yeasts in the Bay of Bengal. Further research is needed on the exploration of oleaginous yeasts for commercial applications.

Table 1: Isolation and Characteristic Features of Oleaginous Yeasts from Three Different Stations of Bay of Bengal.

Culture Code	Station Details	Depth (m)	Lipid (%)	Sudan Black Staining
OYA1	MS2 Lat:16°15.650'N Long:86°42.102'E	2	20	Negative
OYA2		2000	6.6	Negative
OYA3		2000	12.5	Negative
OYA4		500	20	Positive
OYA9		2000	14.2	Negative
OYA11		5	20.5	Positive
OYA15		2500	12.5	Negative
OYA16		2500	8.3	Positive
OY57		1000	17.49	Negative
OY58		1000	17.53	Positive
OY60		1000	17.48	Positive
OY62		500	17.21	Positive
OY63		500	17.31	Negative
OYA5	MS1 Lat : 17°52.492'N Long:88°48.018'E	50	30.3	Positive
OYA6		50	4.5	Negative
OYA7		50	6.4	Negative
OYA17		1500	20	Negative
OYA18		1500	16.6	Positive
OYA10		2	6.4	Positive
OY1		2	8.3	Negative
OY2		2	9.2	Negative
OY3		50	7.45	Negative
OY4		1500	25	Positive
OY14		2000	16.6	Positive
OY16		2000	16.6	Positive
OY17		2000	18.56	Negative
OY35		1000	16.6	Positive

OY45		1000	12.5	Positive
OY46		1000	13.2	Negative
OY47		1000	18.3	Negative
OY48		1000	12.47	Negative
OY49		1000	14.2	Negative
OY50		1000	8.53	Negative
OY51		1000	7.27	Negative
OY54		500	6.29	Positive
OY55		500	7.28	Positive
OY56		500	33.27	Positive
OY65		200	32.28	Positive
OYA19	MS3 Lat : 13º59.9726'N Long:85º00.0316'E	3000	16.6	Negative
OYA20		3000	12.5	Positive
OYA22		1000	13.59	Negative
OYA23		1000	15	Negative
OY6		3000	14.33	Negative
OY7		3000	10.78	Negative
OY10		2	15.37	Positive
OY12		5	12.5	Positive
OY19		5	16.6	Negative
OY20		5	12.5	Negative
OY21		1000	16.39	Positive
OY24		50	16.34	Positive
OY25		50	15.78	Negative
OY26		50	13.75	Positive
OY27		1000	7.6	Negative
OY28		1000	14.33	Negative
OY29		1000	19.6	Negative
OY30		1000	17.48	Negative
OY31		1000	20	Negative
OY32		500	14.88	Positive
OY33		500	14.2	Positive
OY37		2000	8.3	Negative
OY38		2000	6.25	Negative
OY39		2000	23.46	Positive
OY41		2000	17.32	Negative
OY42		3000	16.45	Negative
OY43		3000	7.6	Negative
OY52		2000	15.38	Negative
OY53		2000	32.5	Negative

CONCLUSION

Studies on the distribution patterns of yeasts isolated from the three different stations (MS1, MS2 and MS3) of Bay of Bengal showed that the distribution of yeasts was higher in the station MS3 as compared to stations MS1 and MS2 whereas the distribution of oleaginous yeasts was higher in the MS2 station as compared to stations MS1 and MS3 .

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