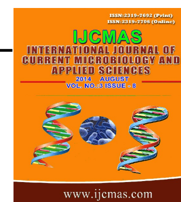

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Original Research Article

Modified Sudan Black B staining method for rapid screening of oleaginous marine yeasts

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A B S T R A C T

A quick and efficient method of screening potential oleaginous marine yeasts and molds is described. Fat globules of marine yeast could not be efficiently stained by Burdon's method of lipid staining. Burdon's method was observed to have limitations to demonstrate fat globules present in marine oleaginous yeasts, marine algae. The staining of LBs in marine yeasts improved as a result of the chemical pretreatment of the dried, heat fixed smears. Out of several combinations tested pretreatment of dried and fixed smear with 1:1 combination of pet ether and absolute ethanol for 2.5 mins at room temperature helped penetration of Sudan black B through the cell membrane and efficiently stained the fat globules in the cytoplasm of marine yeast and of filamentous forms.

Introduction

The interest in screening new sources of fats and oils is continuously growing. Single cell oils (SCOs) accumulated by oleaginous marine yeasts and fungi have emerged as potential alternative feed stocks for biodiesel production. In addition to plants, microbial sources have gained importance as potential source of TAG and poly-unsaturated fatty acids (PUFA) in past few decades. Microbial lipids produced by marine microorganisms are more promising and have been reported to produce medicinally and nutritionally important fats. Oleaginous 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.

The typical oily yeast genera include *Lipomyces, Cryptococcus, Yarrowia, Rhodotorula* and *Candida.* These yeasts vary in the lipid content and can accumulate lipids up to 25%–70% of the biomass, also the lipid profile of these yeasts differ between the species (Athanasios *et al.,* 2011). It would be important to isolate novel strains of microorganisms from environment and screen them for enhanced lipid production capacities. As a first step a simple and rapid technique is required in understanding and demonstrating the potential to accumulate fats by marine yeasts and fungi.

Sudan black B was introduced as a specific

fat stain for the detection of lipids in tissue sections by Lison (1934). Burdon (1946) modified the procedure for demonstrating the intracellular fatty materials in bacteria by preparing dried fixed films for lipid staining with Sudan black B. This method has been applied to films from cultures of all the chief species of bacteria (Kenneth, 1946), many of fungi and dinoflagelletes (Muscatine *et al.,* 1994). Evans *et al.,* (1985) applied replica printing technique in combination with Sudan black B staining as a tool for rapid screening of oleaginous yeasts. Neema and Kumari (2013) reported use of Sudan black B for screening of novel lipid producing isolates from secondary sludge and soil. However this, method has limitations to demonstrate fat globules present in marine oleaginous yeasts, also marine algae. To best of our knowledge, Sudan black B method of wet or dried preparations is not reported as a technique for oleaginous marine yeasts. In this study we have modified Sudan black B staining technique for easy and rapid screening of marine oleaginous yeasts.

Materials and Methods

Isolation of marine yeasts: For isolation of marine yeasts sea water samples were collected at the depth of 150m from different coasts of Konkan, India. The yeasts were isolated on the basal agar media supplemented with sea salt solution, 5% dextrose, 1gm % yeast extract and 5μg/ml of Streptomycin. Isolates were characterized and deposited to Gene Bank.

Optimization of staining conditions and Screening of oleaginous isolates using modified Sudan black B staining: The marine yeast isolates (*Candida* KC966726, *Candida tropicalis* KC966722, *Rhodotorula muciliginosa* KF020690) were stained using original Sudan black B staining protocol and the modified protocol. *Yerrowia lipolytica* obtained as heat fixed dried smear from IBB department of University of Pune was used as reference marine yeast. Variations in Sudan Black B stain preparation were tried so as to improve the penetration of the stain through the cell membrane. Combinations of Sudan black B concentration ranging from 0.3% to 1.5% were dissolved in 80%, 90% and absolute ethanol and were tested for lipid staining. Further, chemical fixation of the dry, heat fixed smear with various chemical agents like diluted acids, and organic solvents was tried. Among organic solvents, combinations of absolute alcohol and pet ether in various proportions (1:1, 1:5, 1:10 and only alcohol or pet ether) were used in pretreatment of the dried, heat fixed smears, varying time of exposure. Out of the various trials the following treatment (step 2) was found to be efficient. The modified Sudan black B staining method includes following steps:

1. Prepare smear using the marine yeasts cells cultivated on MGYP with 3.5% salt (Basal media), grown till early stationary phase, air dry and heat fix the smear.
2. Flood the smear with Reagent A (1:1 ethylalcohol and Pet ether) for 2.5mins, drain off excess reagent.
3. Flood the smear with Sudan black B stain (0.3% in 70% alcohol), keep for 14–15mins till the stain turns greenish blue.
4. Wash the slide to remove the stain.
5. Counter stain with 0.5% saffranin for 30 seconds, wash, dry and observe.

Nile red fluorescence Microscopy:

Accumulation of fat globules was confirmed by the Nile red staining procedure. Nile red was dissolved in 0.1mg/ml with acetone, of which 10 μl was mixed with 100 μl of cell suspension in an Ependorff tube and

observed immediately under a fluorescent microscope using 450-490 nm excitation filter to visualize the fluorescent yellow-gold lipid droplets.

Determination of Lipid content: At the early stationary growth phase cultured yeast cells were harvested, biomass was washed with distilled water and then dried at 60^0C to constant mass. Total cellular lipids were extracted by Soxhlet extraction method (45^oC) with n-Hexane. Solvent was removed under Nitrogen pressure and lipid was determined gravimetrically. All the trials were performed at least in triplicate.

Results and Discussion

Currently, the production of PUFA by marine microorganisms is the subject of intense research and increasing commercial attention. Microbes can be considered as preferred and potential PUFA producers, due to their renewability, fast growth rate and low cost. Considering the safety, performance aspects and percentage of PUFAs, oleaginous marine microbes are the interesting alternative sources to fish oils. Microorganisms particularly microalgae and fungi thought to be the primary producers of Omega-3 PUFAs in marine food chain (Ackman *et al.,* 1964).

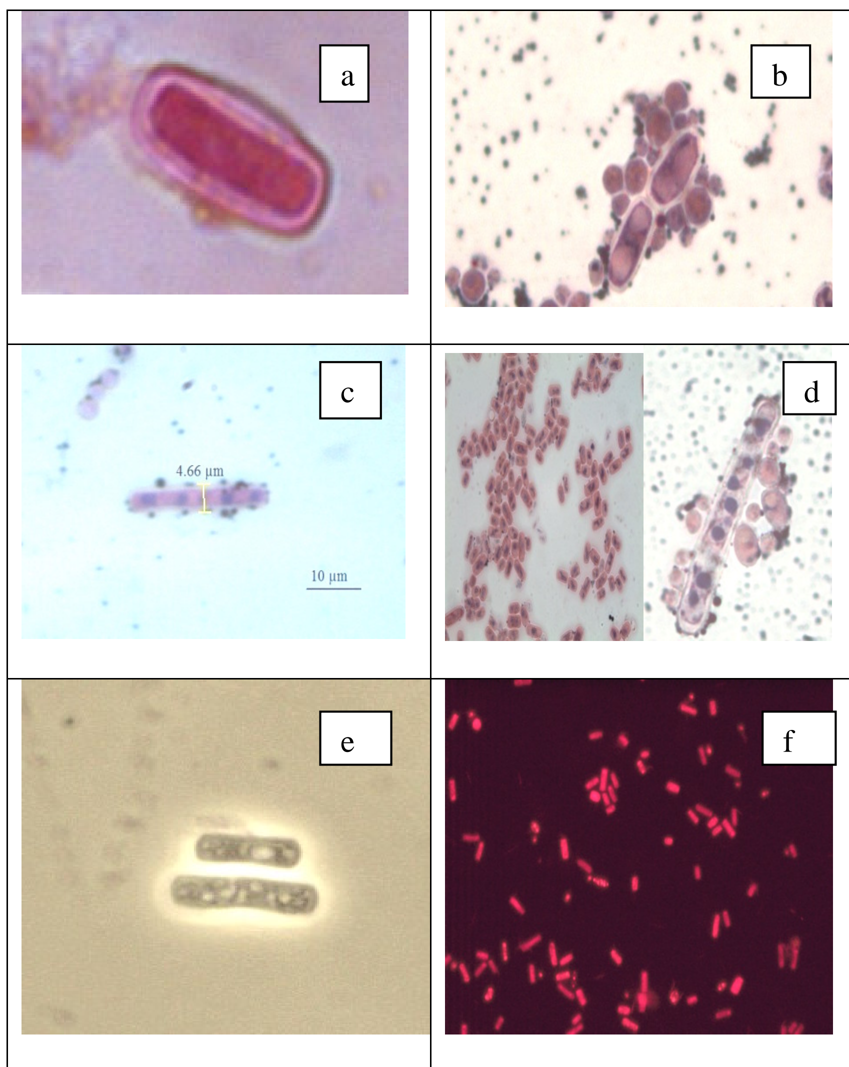
Lipid accumulation in oleaginous yeasts and fungi has been studied mainly with the aim to produce high-value PUFAs and as biodiesel precursors. In general, yeasts and molds can accumulate more lipids over bacteria and microalgae mainly due to rapid growth rate and therefore, have potential as commercial sources of oils (Ratledge, 2004). Screening of potential oleaginous marine yeasts and molds using quick and efficient methods is essential. The accumulated lipids in yeasts get deposited as intracellular lipid bodies (LBs). Microscopic methods reported to detect oil globules particularly in marine yeasts includes staining with fluorescent dye, Nile red (Katre *et al.,* 2012, Beopoulos *et al*., 2008; Mlickova *et al.,* 2004; Kimura *et al.,* 2004; Mahesh *et al.,* 2012). Gupta (2012) used Differential Interference Contrast Nomarski microscopy to determine lipid accumulation ability of marine yeasts. All the above reported methods need specialized instrumentation and thus are expensive. Original Sudan black B staining method is simpler, quick and easy requiring no more efforts than Gram staining (Burdon, 1946). However, Burdon’s fat staining method with Sudan black B has limitations as the oil globules in the cytoplasm of marine yeasts didn’t take Sudan black B (fig.1b) perhaps due to specific membrane chemistry that prevents the penetration of the stain.

The Sudan black B stained only the lipidic matter surrounding the cytoplasmic membrane (fig.1a) while the yeast cells appear pink due to saffranin used as counter stain. The modification in the staining solution by increasing Sudan black B concentration from 0.3% to 1.5% in absolute ethanol that improved penetration of the stain. However, the modified dye solution caused damage to the cell wall as the cells appeared distorted under the microscope. Out of several combinations, pretreatment of dried and heat fixed smear with 1:1 combination of pet ether and absolute ethanol for 2.5mins at RT helped penetration of Sudan black B through the cell membrane and efficiently stained the fat globules in the cytoplasm of marine yeast and filamentous forms (fig.1c and 1d). The modified Sudan black B method can be applied for primary screening of oleaginous marine yeasts and fungi and algae. The current method deviates from Burdon’s method in chemical fixation of the smears.

Table.1 The total lipid content of selected marine yeasts cultivated on basal media

Colony	Biomass Wt.in gms/100ml	Fat droplet no/cell	Lipid yield g/g of biomass
Candida spp.	2.08	15-17	0.246
Candida tropicalis	2.80	3-9	0.168
Rhodotorula muciliginosa	2.76	3-8	0.182

Fig.1 a**:** Cells stained by original Sudan Black B technique stained lipid matter surrounding the cytoplasmic membrane, b: Cells stained by original Sudan Black B technique, the intracellular fat globules remained unstained, c: lipid globules in marine filamentous form (marine *C. tropicalis*) stained with modified method, d: lipid globules in different marine yeasts stained with modified method, e: Wet mount marine *Candida* spp. (Leica DM 2500,100x oil immersion lenses), f: fat globules in marine yeast cell stained with Nile Red and observed with fluorescence microscope



Fixation with combination of pet ether and absolute alcohol leads increased permeability of the stain by solublizing the fatty components of cell membrane of the marine yeasts. The staining of LBs in marine yeasts improved as a result of the pretreatment. We further validated our results with fluorescence microscopy and gravimetric estimation of lipids.

Nile red stained yeasts cells exhibit numerous small discrete fluorescent bodies distributed throughout the cytoplasm. The characteristic of marine yeasts when observed under a fluorescent microscope showed a yellow fluorescent lipid in the yeast cells (fig.1f), while they were red without fluorescence. Overall good correlation was observed between the percentage of LB area to cell surface area as observed in LBs stained with Sudan black B and unstained wet mount observed using Leica DM2500 microscope (fig.1e). The isolates were characterized for biomass, lipid yield and lipid coefficient.

Significance and impact of the study

Screening of marine oleaginous yeasts and fungi has attracted considerable interest mainly with the aim to produce high-value PUFAs and as biodiesel precursors. Screening of potential oleaginous marine yeasts and molds using quick and efficient methods is essential. In this study, the method describes a simple modification of Burdon's Sudan black B staining procedure of lipid staining that could be efficiently demonstrate the lipid bodies present in the marine yeasts and fungi. The modified Sudan black B staining method is of great importance as an efficient, quick screening technique that could be applied for the selection of potential oleaginous marine yeasts, filamentous fungi and algae.

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