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SESAME OIL INCORPORATED MEDIUM FOR ISOLATION AND ENUMERATION OF LIPOPHILIC YEASTS

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ABSTRACT: This communication reports on an easy and cost effective mycological medium, developed for primary isolation and culture of lipophilic yeasts. The medium has been modified and adjusted specifically to promote the growth of lipophilic yeasts and simultaneously suppress the growth of other fungi and bacteria. The traditional medium of Sabouraud's dextrose agar (SDA) with olive oil overlay was modified by incorporating different oils (coconut, palm, corn, olive and sesame) in the medium. The oil incorporated SDA medium was compared with other routinely used media such as Modified Dixon's, IMU – Mf and Modified Leeming – Notman, for the culture of lipophilic yeasts. From the results obtained, it was observed that the medium with sesame oil exhibited the highest viable count within 48 hours. Thus, this medium can be used in the conventional microbial procedures such as isolation of pure culture by streaking; enumeration (viable count) using spread plate technique, and anti-microbial sensitivity testing, for lipophilic yeasts.

INTRODUCTION: *Malassezia* species are lipophilic yeasts that are found to be a part of normal animal and human cutaneous commensal microflora [1]. Being lipid dependent, they are normally found in areas that are rich in sebaceous glands [2]. Ghahfarokhi and Abyaneh [1] have suggested that these microorganisms have been considered as medically important yeasts because of their involvement in the etiology of some important skin disorders including pityriasis versicolor, foliculitis, seborrhoeic dermatitis and dandruff. They could also be possibly associated with other important human diseases which are not known presently, due to insufficient data available in this field, compounded by the lack of convenient culture medium to study those [3].

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This work is an effort to develop a suitable medium for the cultivation of the lipophilic yeasts so as to isolate and study them in diseased conditions.

MATERIALS AND METHODS:

Culture Media: The different culture media and media supplements used in the study are listed in **Table 1.**

Formulation of culture medium: The basal formulation of the medium contained Sabouraud's dextrose agar that consists of nutrients and trace elements required for the growth of most yeasts and fungi [4]. The basal medium was supplemented with nutrients that would promote the growth of lipophilic yeasts [4]. Examples of these nutrients used: olive oil, coconut oil, palm oil, corn oil and sesame oil. Different polysorbates were tested for efficient mixing of these oils in the medium. Tetracylline selective supplements were used to inhibit bacterial overgrowth. Standard strain of *Malassezia furfur (MTCC 1374)* was used to test

the ability of the culture medium to support effective colony isolation and growth of lipophilic yeasts [5]. All the inoculated plates were incubated at 37°C and examined twice daily for evidence of growth of microorganisms up to 7 days. The number of days taken for the yeast colonies to be visible and the progressive size of the colonies for each day were recorded for both primary culture and subculture[3]. Further, the composition of the medium was modified in terms of the type and quantity of oil and tween, so as to obtain the most effective growth medium for lipophilic yeasts. Each formulation was tested twice for the isolation and growth efficiency of lipophilic yeasts using a standard strain of *M. furfur*, so as to ensure reproducibility of results. Nine most effective compositions were selected to carry out viable count of the standard strain of *M. furfur (MTCC 1374).* Amongst these nine media, the best medium was identified on the basis of colony count obtained and compared with some known media available for lipophilic yeasts.

TABLE 1: LIST OF CULTURE MEDIA AND MEDIA SUPPLEMENTS USED IN THE STUDY

Media used	Oils used	Tween used
1. Sabouraud's agar + Antibiotic + 1% Tween + 2 % Sterile Oil (incorporated)----- Enumeration	Olive oil Coconut oil	Tween 20 Tween 40
2. Sabouraud's agar + Antibiotic + Tween.	Palm oil	Tween 60
3. Modified Dixon's Agar medium.	Corn oil	Tween 80
4. IMU – MF Agar medium.	Sesame oil	
5. Modified Leeming – Notman Agar medium.		
6. Sabouraud's agar + sterile oil (overlay) ----- Isolation		

Comparative study for primary isolation and enumeration: Modified Leeming-Notman agar [3, 6], Modified Dixon's agar [7], Sabouraud's dextrose agar (SDA) with olive oil overlay [8], and IMU-Mf agar [9] were prepared and inoculated according to specifications described in literature. The growth observed on these known media was compared with the growth observed on the new medium under investigation. The growth was confirmed microscopically using Gram – stained preparations.

METHODOLOGY:

Isolation: Hexagonal streaking [10] was performed on five Sabouraud's agar plates and overlaid with olive oil, sesame oil, palm oil, corn oil and coconut oil respectively. Simultaneously hexagonal streaking was carried out on oil and tween (20, 40, 60, and 80) incorporated Sabouraud's agar plates. Plates were incubated at 37°C up to 7 days. Each plate was observed for appearance of isolated colonies in each zone.

Enumeration: A saline suspension of 48 - 72 hour old culture of a standard strain of *M. furfur (MTCC – 1374)* having an approximate density of 10^7- 10^8 cfu/ml adjusted as per microscopic haemocytometer count, was prepared aseptically in sterile suspension tubes.

Viable counts were performed on the oil incorporated agar plates as well as on the known media plates using spread plate technique [10]. Incubation was carried out at 37°C up to 7 days. Isolates were identified on the basis of colony morphology and microscopic examination.

Statistical analysis:

Isolation: The growth obtained in each zone was observed and the number of days required for visible colonies to appear in each zone in individual medium was noted. The growth pattern in oil overlay and oil incorporated medium was compared visually on the basis of the number of zones showing growth, the density of growth in each zone and the growth time required by the organism. The data obtained was tabulated and a clustered column graph was plotted.

Enumeration: The data derived in the form of colony count as CFU/ml x 10^7 was tabulated and evaluated. The Mean +/- SD values were calculated and a clustered column graph was plotted. The data obtained for the new medium was compared with the data obtained for Leeming Notman agar medium using an unpaired student's t test, considering a probability (p) value of 0.05 or less as the level of significant association.

RESULTS: The oil incorporated composition was developed after a number of modifications to obtain the culture medium that provided optimal growth of a standard strain of *Malassezia furfur* (MTCC – 1374). The final formulation for the solid medium contained Sabouraud's dextrose agar Medium, Tween 80 and Sesame oil. In this study olive oil and corn oil supported good growth of lipophilic yeasts. The growth efficacy was found to be minimal with coconut and palm oil. The best growth was obtained with sesame oil. Amongst the polysorbates, Tween 80 was found to be the best emulsifying agent which itself allows good growth of lipophilic yeasts.

Isolation: The results showing incubation time required for *M. furfur* colonies to appear on each medium with different oils has been represented in **Table 2**. Also a comparison between the growth pattern obtained on oil incorporation medium and oil overlay medium is shown in **Table 2.**

TABLE 2: INCUBATION TIME AND GROWTH PATTERN

Oil	Growth On Culture Media					
	Oil Incorporation Medium			Oil Overlay Medium		
	24 Hrs.	48 Hrs.	72 Hrs.	24 Hrs.	48 Hrs.	72 Hrs.
Olive	No Growth	Zone 1& 2	Zone 1, 2, 3, & 4	No Growth	No Growth	Zone 1, 2 & 3
Sesame	No Growth	Zone 1&2	Zone 1, 2, 3, & 4	No Growth	Zone 1	Zone 1, 2, 3, 4, 5, & 6
Palm	No Growth	Zone 1& 2	Zone 1, 2, 3, & 4	No Growth	Zone 1	Zone 1, 2, 3, 4, & 5
Corn	No Growth	Zone 1	Zone 1, 2,& 3	No Growth	No Growth	Zone 1 & 2
Coconut	No Growth	Zone 1& 2	Zone 1, 2, 3, & 4	No Growth	No Growth	Zone 1, 2, 3, & 4

It was observed that, individual colony formation could not be obtained on SDA plate with oil overlay while excellent isolation was obtained on oil incorporation medium. Though all these oils supported growth of the yeast, the colony morphology was observed to be the best in sesame oil media. The growth pattern observed on different media is shown in **Fig. 1.**

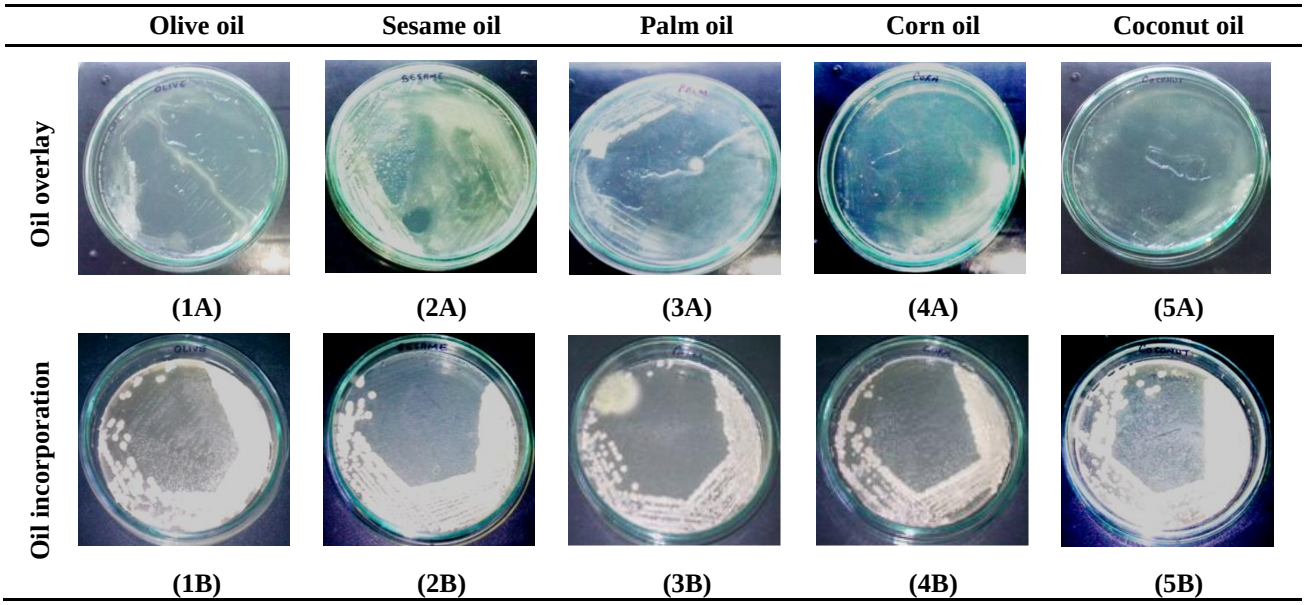


FIG. 1: COMPARISON OF GROWTH PATTERN OF *MALASSEZIA FURFUR* ON OIL OVERLAY AND OIL INCORPORATION MEDIA

As observed in **Fig. 2** the growth time on oil overlay medium was more than oil incorporated medium. All the oil incorporated media gave good growth within 48 hours, while amongst the oil overlay media growth within 48 hours was observed only in sesame oil and palm oil.

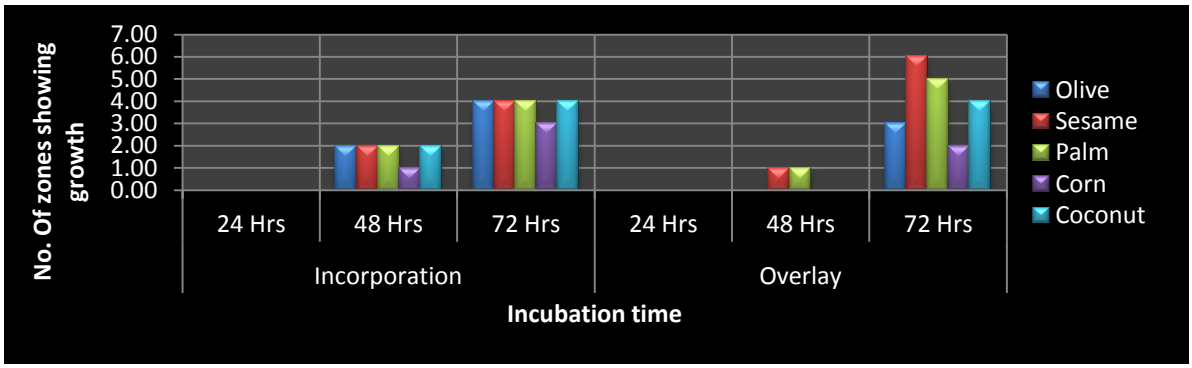


FIG. 2: DIFFERENCE IN GROWTH TIME ON VARIOUS MEDIA

Thus, it can be noted that, the growth efficiency is more in oil incorporated medium than in the oil overlay medium.

Enumeration: The oil incorporated medium was further analyzed for its efficiency in the enumeration of lipophilic yeast and the growth pattern was compared with that on SDA + polysorbate medium. The results obtained for growth on polysorbate and oil incorporated medium are given in **Table 3** in the form of colony counts.

The data derived in the form of colony count as CFU/ml x 10^7 was evaluated by calculating the Mean +/- SD values.

Table 4 shows the Mean +/-SD values obtained for all the oil incorporated media.

TABLE3: AVERAGE COLONY COUNT OF *MALASSEZIA FURFUR* ON POLYSORBATE AND OIL INCORPORATED CULTURE MEDIUM

Oil	Average colony count (CFU/ml) x 10^7							
	Polysorbate							
	Tween 20		Tween 40		Tween 60		Tween 80	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
Olive	2.17	3.28	6.28	5.913	1.505	1.465	16.195	17.53
Sesame	5.575	3.835	6.1	8.42	1.6115	2.7	19.33	19.595
Palm	5.53	5.17	5.415	5.361	1.205	1	10.22	10.92
Corn	5	5.37	4.4	5.437	2.03	1.25	17	13.42
Coconut	3.24	5.335	2.58	5.636	1.1955	1.125	10.25	10.815
Only Tween	3.045	3.525	0.595	0.505	1.191	2.87	15.75	11.83

(Y1 – average colony count of set 1; Y2 – average colony count of set 2)

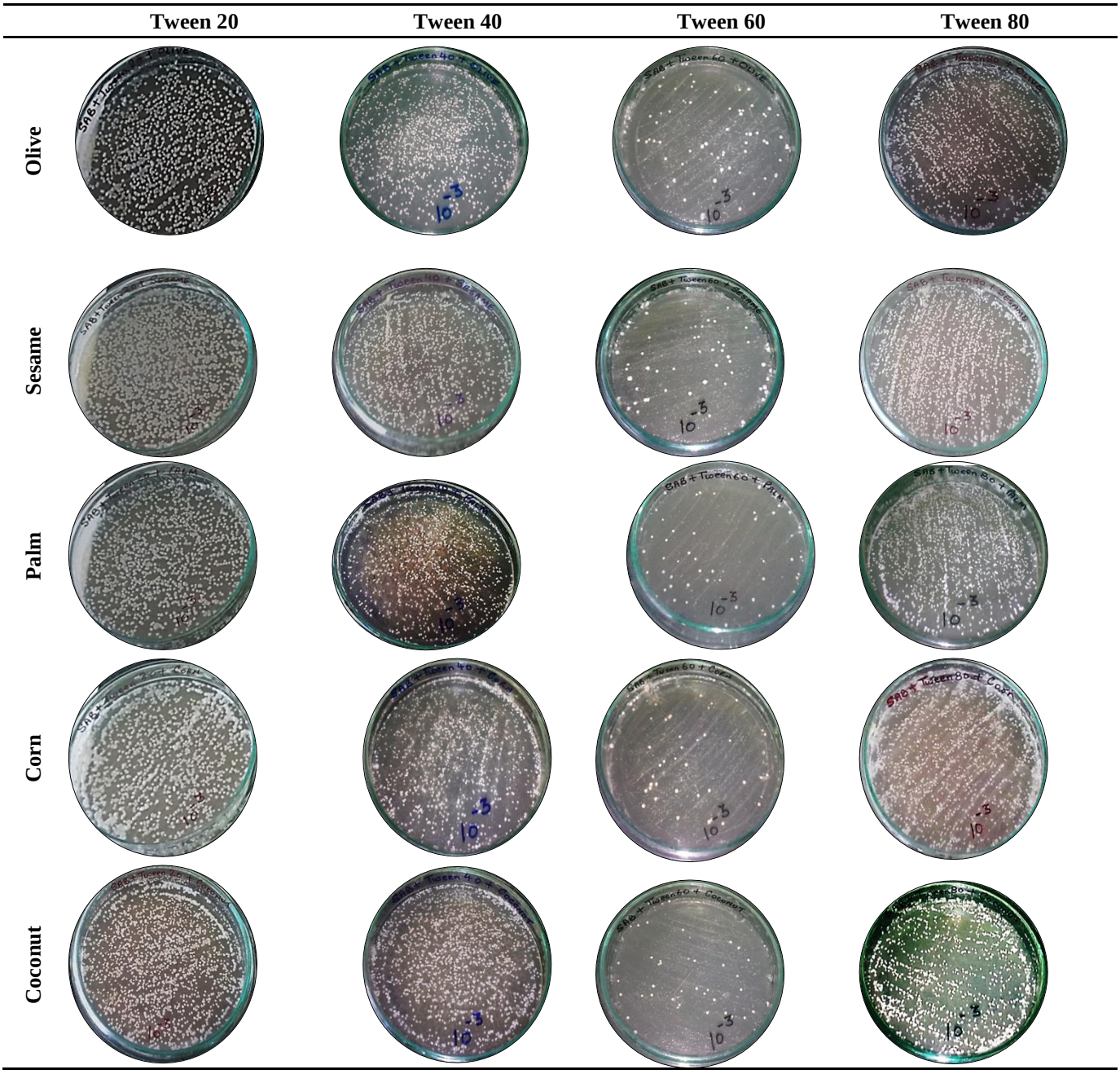
TABLE 4: THE CALCULATED MEAN +/- SD OF THE COLONY COUNTS

Oils	Mean +/- SD of colony count (x 10^7)							
	Polysorbates							
	Tween 20		Tween 40		Tween 60		Tween 80	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Olive	2.725	0.785	6.097	0.260	1.485	0.028	16.863	0.944
Sesame	4.705	1.230	7.260	1.640	2.156	0.770	19.463	0.187
Palm	5.350	0.255	5.388	0.038	1.103	0.145	10.570	0.495
Corn	5.185	0.262	4.919	0.733	1.640	0.552	15.210	2.531
Coconut	4.288	1.481	4.108	2.161	1.160	0.050	10.533	0.400

SD – Standard Deviation

On comparison of the colony counts amongst the oils, it was found that except with polysorbate 60, sesame oil gave the maximum growth with all the polysorbates, the best growth being with polysorbateate 80. The extent of growth obtained in different oil incorporated media can be observed in **Fig. 3**

FIG. 3: EXTENT OF GROWTH OBTAINED IN 10 $^{-3}$ DILUTION AFTER 72 HOURS OF INCUBATION



The oil incorporated medium was also compared with the standard culture media available, i.e. Modified Leeming - Notman agar medium, Modified Dixon's agar medium and IMU-Mf medium. The data obtained has been represented in **Fig. 4.** The growth obtained in Modified Leeming-Notman agar medium was comparable with the SDA + Olive oil + Tween 40 medium ($p = 0.059$), but less than all other oil incorporated media. The statistical data obtained confirms that the medium with sesame oil and polysorbate 80 shows better growth ($p = 0.008$) on comparison with the standard medium i.e. Modified Leeming-Notman agar.

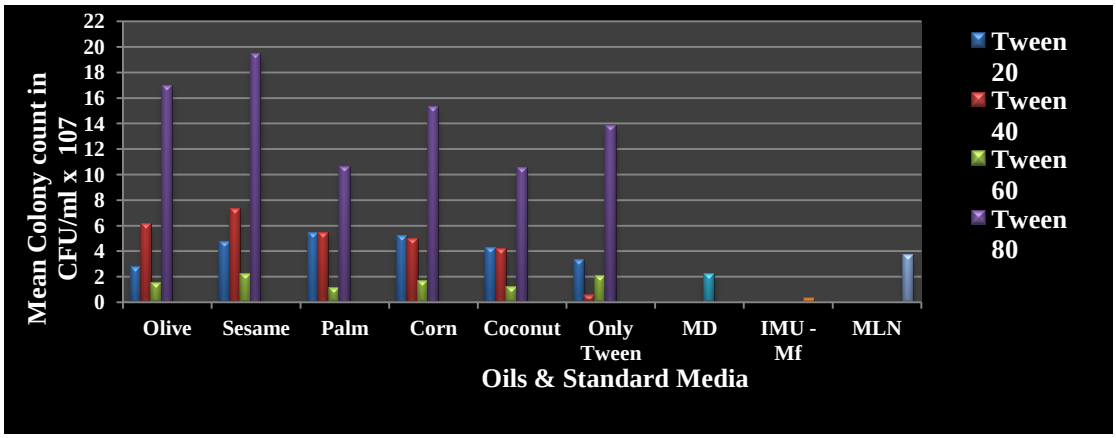


FIG. 4: GRAPHICAL REPRESENTATION OF THE COLONY COUNT (CFU/ML) X 10 [7] OBTAINED ON DIFFERENT MEDIA. MD - Modified Dixon's; MLN - Modified Leeming – Notman

DISCUSSION: As mentioned in literature, the genus *Malassezia*, comprises lipophilic and lipid dependent yeasts that require long chain fatty acid (C12 up to C24) supplementation for growth and survival.[11] It is also known that studies on the role of *M. furfur* and other lipophilic yeasts in human diseases have been severely hampered by the lack of a simple and convenient culture medium, especially for primary isolation from either normal or diseased tissues.

Traditionally, Sabouraud's dextrose agar (SDA) with olive oil overlay was used for primary isolation and subculture of these lipophilic yeasts. Although this classical medium has served its useful purpose, it has a number of drawbacks [3]. One of the major problems with SDA and olive oil overlay medium is the inability to obtain individual isolated colonies upon streaking out the culture on the agar.

Various media have been developed by early researchers (Panja, Shifrine and Marr), but these media were disappointing because growth on these media was inconstant and resulted in rapid loss of cultures [11, 12]. Development of modified Dixon agar and Leeming and Notman agar allowed excellent growth, isolation and maintenance of these nutrient dependent microorganisms [3, 6, 7]. These complex media contain Ox bile, polysorbates and oil supplements as major components. But, the drawback of these media is their complex and expensive nature [13]. The main purpose of this study was to develop an easy and cost effective medium to obtain well isolated colonies of lipophilic yeasts.

Malassezia was used to test the ability of the culture medium to support effective colony formation, isolation and growth of lipophilic yeasts.

Since lipophilic organisms require oil as a major component for growth, it was incorporated into a solid medium instead of an overlay. The oils to be incorporated into the medium were determined by reviewing the oils mentioned in literature.

Further selection of oils was based on the ease of availability and its cost effectiveness. In all, five different oils were selected and studied for their utilization in the growth of the lipophilic yeasts. The oils used were olive oil, coconut oil, palm oil, corn oil and sesame oil.

On the basis of the lipid composition of the oils used in the study, different esters/polysorbates were selected for the emulsification of these oils into the medium. Based upon the varying fatty acid composition there are 4 major polysorbates: Polysorbate 20 (monolaurate), Polysorbate 40 (monopalmitate), Polysorbate 60 (monostearate), and Polysorbate 80 (monooleate) [14].

According to the fatty acid composition of the selected oils it is known that olive oil has the maximum that is 71 % of oleic acid, coconut oil has the maximum that is 47 % of lauric acid, palm oil has the maximum that is 45 % of palmitic acid, corn oil has the maximum that is 58 % of linoleic acid and sesame oil has the maximum that is 40 % of oleic acid [15, 16].

Thus, it can be known that olive oil will be best emulsified by monooleate that is polysorbate 80, coconut oil will be best emulsified by monolaurate that is polysorbate 20, palm oil will be best emulsified by monopalmitate that is polysorbate 40, corn oil will not be emulsified very well in any of the polysorbates and sesame oil will be best emulsified by monooleate that is polysorbate 80. Although the best polysorbate for emulsification of the oils is known, the oils were emulsified with each polysorbate separately and studied.

Isolation: A comparison of the oil overlay medium and oil incorporated medium was made to find out the best medium for obtaining individual isolated colonies of these lipophilic yeasts. Hexagonal streaking was performed as mentioned in the methodology. Upon incubation different media showed varied growth patterns of the organism. In the oil overlay plates, maximum growth was observed in plate with sesame oil, followed by palm oil, coconut oil, olive oil and corn oil. Growth observed was confirmed on the basis of colony morphology and microscopic examination using Gram staining technique. Although the medium supported excellent growth of the yeast, isolated, individual colonies were not obtained. Yeast cells were dispersed throughout the medium in the oil making it difficult to pick up a yeast colony by wire loop for staining or preparation of saline suspension as the colonies were mixed with oil.

In the oil incorporation medium except corn oil, all the other oils i.e. olive oil, sesame oil, palm oil and coconut oil showed good growth of the organism. Though all these oils supported growth of the yeast, the colony morphology was observed to be the best in sesame oil media. Organism confirmation was carried out morphologically and microscopically using Gram staining technique. Well isolated, individual colonies were obtained in this media which were easy to pick up using a wire loop and could be used for staining and saline suspension preparation.

Upon comparison of the growth time on oil overlay and oil incorporated media, it was found that, all the oil incorporated media gave good growth within 48 hours, while oil overlay medium gave good growth with all the oils only after 72 hours. On comparison of the two types of media, it was found that sesame oil is the best oil supporting growth of the lipophilic yeast in both the media types.

Enumeration: Enumeration of the lipophilic yeast was carried on the oil incorporated medium in order to determine the efficiency of this medium. Also the growth pattern of the lipophilic yeast was studied using each polysorbate separately in Sabouraud's dextrose agar. It was found that the organism did show a minimum amount of growth in SDA + polysorbate medium, but not as good as with oil in the medium. Among the polysorbates, the best growth was observed in polysorbate 80, followed by polysorbate 20, 60 and the least growth was observed in polysorbate 40.

The colony counts of the oil incorporated media revealed that, sesame oil showed maximum growth with all the polysorbates, except polysorbate 60. Sesame is followed by olive oil, corn oil, palm oil and coconut oil. Along with polysorbate 20, olive oil has given the best result.

The oil incorporated medium was also compared with a few other culture media already available for lipophilic yeasts. Amongst the standard culture media available, the best growth was obtained in Modified Leeming - Notman agar medium followed by Modified Dixon's agar medium and then IMU-Mf medium. On comparison of the standard media and the oil incorporated media it was found that the sesame oil incorporated medium and the Modified Leeming-Notman agar medium performed equally well in terms of the duration taken by the sub cultured colonies of *M. furfur to* become visible (after 24 hours), and the size of the individual colony after 4 days. Both media performed better than Modified Dixon's agar medium and IMU-Mf medium. The viable count results reveal that the colony count in the sesame oil incorporated medium was much higher as compared to that in Modified Leeming-Notman agar medium.

Previous studies by Shifrine *et al*, Kaneko *et al* and Nazzaro Porro *et al* have suggested the importance of oleic acid as a fatty acid requirement for *Malassezia* species [12, 6, 17].

The results obtained in this study for isolation and enumeration using the oil incorporated medium; also reveal that the presence of oleic acid is essential for the growth and proliferation of *Malassezia* species *in vitro*. Thus, we conclude that the medium with sesame oil and polysorbate 80 can be used as a simple, easy and cost effective medium for the primary isolation and enumeration of lipophilic yeasts. It can also serve as a useful medium for the revival and maintenance of standard strains.

The medium can further be explored for its ability to isolate lipophilic yeasts from clinical samples, thus adding new avenues for research in the field of lipophilic yeast biology. Further, the medium can also be used for performing antifungal susceptibility test against *Malassezia* so as to develop efficient treatment strategies.

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