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# A modified mycological medium for isolation and culture of *Malassezia furfur*

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*Abstract*

A mycological medium was developed for primary isolation and culture of lipophilic yeasts. It was initially based on published information of nutrients and trace components that would promote the growth of these yeasts. It was subsequently modified and adjusted to specifically promote the growth of lipophilic yeasts and simultaneously avoid the luxurious growth of other fungi and bacteria. With this medium, the conventional bacteriological procedures such as microbial streaking for pure culture and anti-microbial sensitivity testing could be carried out for these lipophilic yeasts.

*Key words:* medium, culture, *Malassezia furfur*

## INTRODUCTION

The taxonomy of lipophilic yeasts, presently classified under the genus *Malassezia*, had undergone many changes since its first detection and implication as the cause of pityriasis versicolor by Eichstedt in 1846.[1] In 1953, Robin named the yeast as *Microsporum furfur* which he thought was closely related to the dermatophyte *Microsporum audouinii*.[1] In 1874, Malassez described this yeast isolated from the scalp lesion of a patient as "spores". Following which, various names had been used to describe this yeast-like organism such as *Pityrosporum (P) ovale*, *Saccharomyces ovale*, *P. orbiculare*.[1] In 1889, Billion created the new genus *Malassezia* to accommodate this microorganism.[1] The lipophilic nature of the *Malassezia* organisms was first described in 1939 by Benham.[2] The great micromorphological polymorphism and the lack of suitable methods for isolation in the early period were the main reasons that made the study and classification of this yeast difficult.[3] The genus has recently been revised based on the morphology, ultrastructure, physiology and molecular biology of these yeasts and presently expanded to comprise seven species: *M. pachydermatis*, *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtuse*, *M. restricta*, and *M. slooffiae*.[4,5] With the exception of *M pachydermatis*, the remaining six species are lipid-dependent yeasts that required long chain fatty acids for in-vitro growth.[6-8]

These yeasts are considered as the normal cutaneous microbiota of humans and some warm-blooded animals.[9-13] However, under certain circumstances, they are associated with a variety of diseases in both humans and animals.[14-18] They were implicated as the aetiological agents of systemic infection especially in patients on long term intralipid therapy, as well as the cause of a number of skin disorders in healthy people such as pityriasis versicolor, seborrheic dermatitis, dandruff and Malassezia folliculitis.[14-21] They could also have possibly associated with other important human diseases which are not known presently due to insufficient interest in further scientific research in this field compounded by the lack of convenient culture medium to study them. This report describes the development of a modified relatively simple mycological medium, IMU-Mf (International Medical University-Malassezia furfur) medium, for direct isolation of these lipophilic yeasts from clinical specimens and subsequent subculturing of these yeasts for anti-fungal assay and other studies.

## MATERIALS AND METHODS

*Literature search*

An extensive stepwise search was made for published information on the type and quantity of nutrients required for growth of lipophilic yeasts as well as any trace element, mineral or vitamin that could possibly promote or inhibit their growth. Information on the structural composition, in particular, lipids of these yeasts was included in the search. Published papers on a number of existent culture media for lipophilic yeasts were reviewed.[1-9, 22-38] Information on

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nutrients that would promote the growth of bacteria and filamentous fungi was also taken into consideration in the formulation of the culture media.

*Formulation of culture medium*

The initial formulation of IMU-Mf medium contained a combination of all the known nutrients and trace elements required for and promoted the growth of lipophilic yeasts at slightly higher than the specified concentration. Examples of these nutrients and trace components used were: yeast extract, peptone, tryptone, dextrose, cow milk, olive oil, lanolin, Tween (40, 60, 80), asparagines, glycine, desiccated ox-bile, thioglycolate, and glycerol. Bacto-agar was used in a concentration of 1.5% as the solidifying base. Chloramphenicol (50 µg/ml) and cycloheximide (200 µg/ml) were used to inhibit bacterial overgrowth in this study. Clinical specimens from patients with tinea versicolor collected on an adhesive cellophane tape and an earlier isolate of *Malassezia furfur*, obtained from a patient with similar illness using the conventional Sabouraud dextrose agar with olive oil overlay (Fig. 1a), were used to test the ability of the culture medium to support effective isolation and growth of lipophilic yeasts. 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. The same growth assay was repeated twice to ensure reproducibility of the results.

Subsequently, each component of the nutrients sequentially underwent stepwise reduction, even to the stage of omission for some components, if each revised medium did not impair the isolation and growth of the lipophilic yeast in comparison with the initial formulation. For each revised formulation, the isolation and growth efficiency of lipophilic yeasts from both clinical specimens and an earlier *M furfur* isolate were tested thrice to ensure consistency of the formulation before moving into the next.

Concurrently, tests were carried out to identify the types of oil that would promote the optimal growth of an earlier isolate of *M. furfur* using Sabouraud agar as the basal medium. The types of oil included in the tests were: olive oil (Brand: Basso, LughliO, Fragata, Bulerias), coconut oil, palm oil used for cooking (Brand: Olina, Eagle, Knife, Neptune, Labour), butter oil, and lard oil.

*Preparation procedure*

For each revised formulation, a hundred milliliter of the medium was prepared in a 250-millilitre Schott screw-cap bottle. The required quantity of heat-stable nutrients and trace components were weighed and added into the bottle containing 100 ml of double-distilled water. After adjusting the pH to 6.2 by adding 1M HCl dropwise, the medium was heated in a microwave oven to near-boiling temperature. The required quantity of lipid compound was added into the mixture and mixed by gently swirling the bottle. The resultant mixture was autoclaved for 15 minutes at 121°C and allowed to cool to approximately 50°C in a water-bath. Subsequently, the heat labile nutrients and trace components with the necessary anti-microbials (chloramphenicol and cycloheximide) that were disinfected earlier using a 0.2 micron filter were added. After a good mix by gentle swirling of the bottle to avoid bubble formation, about 15 ml of the medium was immediately poured into each sterile Petri dish inside a biosafety class II cabinet and allowed to cool and solidify.

*Comparative study for primary isolation with other culture media*

Leeming-Notman agar,[7] Dixon's agar,[8] Sabouraud dextrose agar (SDA), and IMU-Mf agar were prepared according to specifications described by manufacturers. The same concentration of chloramphenicol (50 µg/ml) and cycloheximide (200 µg/ml) was added to all preparations. Adhesive cellophane tapes containing clinical specimens obtained from patients with pityriasis versicolor were cut into rectangular stripes of 1X0.5 cm. Each strip was carefully laid on the surface of a respective culture agar plate and for the SDA plate, the cellophane tape was overlaid with olive oil. All the inoculated plates were incubated at $35^0$C and examined twice daily for evidence of growth of microorganisms up to 7 days. The presence of *Malassezia* yeasts in the culture was identified by micromorphology in Gram-stained preparations. Other microbial growths in the culture plates were identified as filamentous fungi by their gross morphological growth on culture plates, bacteria or yeasts by Gram stain. Final species identification of *Malassezia* species was by polymerase chain reaction amplification followed by sequencing of the internal transcribed space 1 ribosomal DNA sequences.[39]

*Statistical analysis*

The data derived was tabulated and evaluated by Chi-square test, using Epi Info 6 (Center for Disease Control and Prevention, Atlanta) free computer program for any statistically significant association. A probability (p) value of 0.05 or less was taken as the level of significant association for each ordinal variable with the relevant adjusted variables.

## RESULTS

The IMU-Mf medium was developed after more than 100 modifications to optimize the culture medium that provided optimal growth of *M. furfur* from both clinical specimens and subcultures of isolates (Fig. 1b and 1d). The final formulation for the solid medium contained the following nutrients and trace components per liter: Bacto-agar 12 gram, dextrose 10 gram, yeast extract 10 gram, peptone 3 gram, sodium chloride (NaCl) 2 gram, desiccated ox-bile 2 gram, thioglycolate 2 gram, L-asparagine 2 gram, palm oil 10 ml, and Tween 80 10 ml. The liquid medium contained all the components mentioned above except the Bacto-agar. All the components could be increased 2 to 4 folds without obvious effect on the growth and primary isolation of M. furfur-like yeasts except for the desiccated ox-bile, which impaired the growth when its concentration was increased 2-fold or more above the specified concentration.

In this study, coconut oil did not support good growth of *M. furfur* yeasts. It failed to isolate lipophilic yeast from clinical specimens using Sabouraud dextrose agar as the basal culture medium. A few brands of palm oil (Olina, Eagle, Knife, Neptune, Labour) used for normal cooking in Malaysia supported the growth of lipophilic yeasts equally well from both primary clinical specimens and subcultures of isolates. The growth efficacy was found to be as good as the commercially available olive oil (Fig. 1f).

In comparison of IMU-Mf medium with a few other culture media for lipophilic yeasts, IMU-Mf agar medium performed as well as Leeming-Notman agar in the duration taken by the subcultured colonies of *M. furfur to* become visible (after 24 hours), and the size of the individual colony after 4 days is shown in Fig. 1d. Both media performed better than Dixon's agar. Individual colony formation could not be obtained in SDA plate with oil overlay (Fig. 1c). In terms of culture performance for primary isolation from clinical specimens of patients with pityriasis versicolor, IMU-Mf medium managed to obtain isolation of M. furfur-like yeasts from 24 of the 25 clinical specimens in this small study. Leeming-Notman agar, Dixon's agar and SDA with olive oil overlay were able to obtain positive M. furfur-like yeasts from 23, 21, and 21 clinical specimens respectively (Table 1). Though IMU-Mf had the highest isolation rate of lipophilic yeasts, there was no significant difference in the isolation rate between IMU-Mf medium in comparison with other media (Fisher exact, $p = 0.349$). Heavy growth of either bacteria or fungi (filamentous or other yeasts) or both was found in all those culture media which failed to culture M. furfur-like yeasts from the clinical specimens despite the presence of anti-microbials. Leeming-Notman agar had a higher rate of bacterial growth in comparison with IMU-Mf medium ($\chi 2 = 4.86$, $p = 0.027$). Sabouraud dextrose agar with olive oil overlay had a significant higher rate of fungal growth in comparison with Leeming-Notman agar ($\chi 2 = 4.5$, $p = 0.034$) but there was no significant difference in comparison with IMU-Mf medium ($\chi 2 = 2.17$, $p = 0.141$) though the number of fungal contaminant growth was higher in SDA than in IMU-Mf medium (Table 1).

## DISCUSSION

Lipophilic yeasts, as the name suggests, are lipid-dependent yeasts that require long chain fatty acids for *in-vitro* growth. Studies of 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 disease tissues. Traditionally, Sabouraud dextrose agar (SDA) with olive oil overlay was used for primary isolation and subculture of these lipophilic yeasts. Although this classical medium served its useful purpose, it has a number of set-backs. One of the major set-backs, as also shown in this small study, is that primary isolation for lipophilic yeasts tends to be hampered by other fungal contamination and overgrowth (Fig. 1a). This is probably due to the composition of the SDA medium that includes a high concentration of glucose to promote the growth of various fungi. The other major problem with SDA and olive oil overlay medium is the inability to "streak" out the culture to obtain individual colonies (Fig. 1c). The findings in this study also demonstrated that when the use of olive oil was in excess, the lipophilic yeasts would incorporate extra lipid into the cell body to the extent that the yeasts remained at the

**TABLE 1: Comparative study of IMU-Mf agar with other agar culture media for isolation of lipophilic yeasts from clinical specimens derived from patients with pityriasis versicolor.**

| No. | IMU-Mf | | | Leeming-Notman | | | Dixon's | | | Sabouraud + oil | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | #Mf | *B | Fungus | #Mf | *B | Fungus | #Mf | *B | Fungus | #Mf | *B | Fungus |
| 1 | + | | | + | + | | + | + | | | | + |
| 2 | + | | | + | | | + | | | + | | |
| 3 | + | | | | + | | | + | | | | + |
| 4 | + | | + | + | + | + | | + | | + | | + |
| 5 | + | | | + | | | + | | | + | | |
| 6 | + | + | | + | + | | + | + | | + | | |
| 7 | + | | + | + | | + | + | | + | + | | + |
| 8 | | + | | | + | | | + | | | + | + |
| 9 | + | | | + | | | + | | | + | | |
| 10 | + | | | + | | | + | | | + | | |
| 11 | + | | + | + | | | + | | | + | | + |
| 12 | + | | | + | + | | + | | | + | | + |
| 13 | + | | | + | + | | + | + | | + | | |
| 14 | + | | | + | | | + | | | + | | |
| 15 | + | | + | + | | + | + | | + | + | | + |
| 16 | + | | | + | + | | + | | | + | | |
| 17 | + | | | + | | | + | | | + | | |
| 18 | + | | + | + | | + | + | | + | | | + |
| 19 | + | | | + | + | | + | + | | + | | |
| 20 | + | | | + | | | + | | | + | | |
| 21 | + | | | + | + | | + | + | | + | | + |
| 22 | + | + | + | + | + | | | + | | + | + | + |
| 23 | + | | | + | | | + | | | + | | + |
| 24 | + | | | + | | | + | | | + | | |
| 25 | + | | | + | | | + | | | + | | |
| Total | 24 | 3 | 6 | 24 | 11 | 4 | 21 | 9 | 3 | 21 | 2 | 12 |

#Mf = Lipophilic yeasts having morphology of *Malassezia furfur* in Gram stain.
*B = Presence of bacterial growth.
Fungus: Inclusive of either filamentous fungi or yeasts other than *Malassezia furfur*.

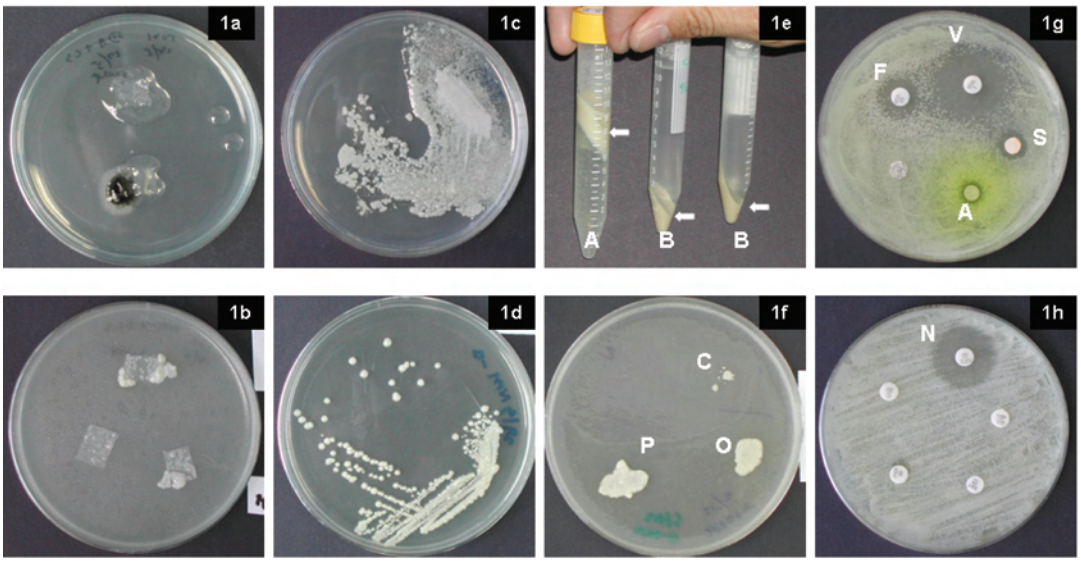


FIG. 1: A composite photograph demonstrating a number of uses of IMU-Mf culture medium in comparison with Sabouraud dextrose agar (SDA). Fig. 1a: Strips of cellophane tape containing clinical specimens from patients with pityriasis versicolor cultured on SDA with olive oil overlay. Fig 1b: Similar specimens cultured on IMU-Mf agar without oil overlay. Fig 1c: A known isolate of *M. furfur* streak-cultured for 4 days on SDA plate with olive oil overlay. Fig. 1d: *M. furfur* cultured on IMU-Mf agar without oil overlay for 4 days. Fig. 1e: *M. furfur* cultured on SDA plate with excessive oil overlay remained in the upper aqueous layer after 10 minutes of centrifugation at 1500 g (A). *M. furfur* cultured in IMU-Mf agar pelleted at the bottom of tube after similar centrifugation (B). Fig. 1f: Growth of a fixed inoculum of *M. furfur* in 25 µl of coconut oil (C), palm oil (P), and olive oil (O) after 4 days of culture on SDA plate. Fig. 1g: Agar disk diffusion drug susceptibility sensitivity testing using anti-fungal discs; variconazole (V), fluconazole (F), selenium sulphite (S), and acriflavine (A). Fig. 1h: Agar disk diffusion drug susceptibility testing using antibiotic discs; netilmicin (N).

upper aqueous layer despite centrifugation at high centrifugal force (Fig. 1e).

There are many advantages of the IMU-Mf medium in comparison with the traditional medium. One of the major advantages of IMU-Mf medium is the incorporation of the lipid components required by the lipophilic yeasts into the aqueous phase of the medium. With this property, the conventional bacterialogical practices of culture purification and anti-microbial sensitivity testing can be applied to these lipophilic yeasts (Fig. 1g). An interesting observation made while using the agar disk diffusion test with antibiotic and anti-fungal disks was the incidental finding that *M. furfur* was sensitive to the antibiotic netilmicin (Fig. 1h) and moderately sensitive to gentamicin.

One of the major challenges in conducting a study on cutaneous microbiota of humans or other animals comes from the ever presence of multiple microflora, which are often resistant to a number of antibiotics. Thus, the challenge in the initial designing of IMU-Mf media was to find ways to reduce (i) the protein content as much as possible to discourage bacterial growth, (ii) the sugar content to discourage growth of fungi but at the same time would not interfere with the growth of lipophilic yeasts. The finding in this limited study with other media showed that IMU-Mf agar significantly reduced the risk of bacterial contamination in comparison with Leeming-Notman agar and fungal growth in comparison with Sabouraud dextrose agar.

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