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CHARACTERISED IDENTIFICATION AND LOW COST PRESERVATION OF *MALASSEZIA* spp.-ENABLING FUTURE POSSIBILITY FOR CONTROL

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ABSTRACT

In a study, the scalp scales of infected and healthy persons (age between 18-35 years) were observed to contain predominantly the yeast Malassezia. The species were identified as Malassezia furfur (37.93%), Malassezia restrictum (22.41%), Malassezia pachydermatis (17.24%), Malassezia globosa (5%) in proportion and rest was other forms of fungi. Several classical and modern techniques have been employed by workers to identify different Malassezia species and to study its control mechanism. But remarkable success could not be achieved in standardizing the processes due to the changing behavior of the organism during culturing and difficulty in revival of the species after long term preservation. The present study has established novel ways to make simultaneous identification of different Malassezia spp. in culture (10 spp.) and to make easy long-time preservation. Interesting observations were also recorded about Malassezia during the study like, it is a part of rare to mild infected scalp (50% Malassezia spp. of total microorganisms) but not necessarily in the heavily infected dandruff scales (only 14.28% Malassezia spp. of total microorganisms). The long-term preservation of Malassezia in selected broth coated ceramic beads at –20^{0}C was achieved. Similarly, a trial with only water as medium in the vial to store this fungus at –20^{0}C has also worked out with high percentage of revival after six months. The coordinated identification methods and the cost-effective preservation approaches envisaged through this piece of research certainly opened new possibilities to study more of Malassezia spp. and its genuine control over scalp and skin.

KEY WORDS

Malassezia spp., dandruff, tween assimilation, lipophilic yeasts, preservation

INTRODUCTION

Malassezia, an opportunistic yeast pathogen related to the Basidiomycota has been identified with 15 lipophilic species by different authors in their studies, when isolated from healthy and diseased human and animal skin (1). Over 150 years, *Malassezia* has been of interest to medical microbiologists in view of it being a member of the human cutaneous microbiota and etiologic agent of certain skin diseases (2). In the present-day health conscious society, hair loss and hair disorders have become great concern to individuals and to the hair therapists. Various reasons and conditions are assigned to hair problems such as too oily or dry skin conditions of the scalp and severe form of dandruff (seborrheic dermatitis). These conditions are associated with hair loss due to depletion of lipids present in different layers of epidermis and dermis resulting weakening of hair root (3, 4). Hence, isolation, culture and studies of scalp micro-fungal flora are undertaken by researchers that include *Malassezia*. The major difficulty in isolation of *Malassezia* is of its slow growing nature and variation of growing period in the culture. Long time preservation

(more than a month) and successful revival of its culture are other hurdles encountered with this fungal pathogen.

Present era has seen a considerable increase in our understanding of the effects of low temperatures and freezing of living cells. Many classical methods and improved techniques have been applied for enhancing the quality of low temperature preservation and revival of *Malassezia* cultures. Hence, it is necessary to do research and find novel methods in the preservation, storage and revival of *Malassezia* spp. and add new dimensions to the earlier results and understandings. Overcoming freezing temperature from -80^{0}C to lower temperature and alternatively changing the culture medium to achieve viable storage for longer time duration will be significant. These new techniques should also be reliable, cost-effective and simple for making future studies easy and developing preventive measures to the menace of predominant *Malassezia* species causing dandruff and hair disorders. The present research application has attempted to address these issues.

MATERIALS AND METHODS

Study Period and Study Sample:

A research study was conducted from August 2016 to December 2017 as a part of studies related to hair disorder cases, prevalently found among youth between age group 18 – 35 years. Informed written consent was taken from the randomly sampled individuals. The samples were categorized from suspected 78 cases based on the presence of rare to mild, moderate and severely visible scales over the scalp (dandruff).

Collection of Sample:

The scalp of individuals was visually observed with magnifying lens for locating the infection sites. Head scrapings were taken from infected scaly sites by using sterile cotton swab or by sterile scalpel on sterilized butter papers at Dirghayu Ayurveda Chikistyalaya, Latur. Numbered and coded sample packets were transported to the laboratory and further processing were conducted at the Department of Biotechnology, College of Computer Science and Information Technology, Latur.

Sample Processing:

The collected samples were studied for identification of the microorganisms in each coded sample using different standard methods and the critical observations made during the conduct of the study were systematically recorded, statistically analyzed and left for interpretation in light of allied references.

Direct Microscopy:

Skin scrapings, as collected were mounted on 20 % KOH for direct microscopic examination for each sample. These were assessed by observing morphological features of yeast and budding yeast cells or of hyphae, etc. under 40X objective lens. Only KOH positive samples were cultured (5).

Culture:

The evaluated positive samples of the infected scalp/ scaly samples were plated over Glucose peptone agar (GPA) {2% w/v Glucose, 1% w/v peptone, 1%v/v olive oil} and modified Dixon (mDixon) agar (3.6% w/v malt extract,1% w/v peptone, 1% w/v desiccated ox-bile, 1% v/v Tween–40, 0.2% v/v Glycerol, 0.2% v/v olive oil, 2.5% w/v agar in distilled water) containing 0.05% chloramphenicol and 0.05% cyclo-hexamide for isolation of scalp infecting microorganisms (5, 6, 7). The inoculated plates were incubated at 32 ^{0}C and examined on days 3-7 and then at weekly intervals till three weeks for any developing colonies. After three weeks of incubation, the plates without growth were considered negative and discarded. In order to achieve pure cultures, each positive sample was further processed. Entry of observations in a detailed proforma, clinical evaluation and mycological evaluation were performed based on macro and microscopic morphology.

Microscopic and Biochemical Characteristics:

The morphological identification criteria used to identify *Malassezia* spp. are the cell size, shape, colour, margin, elevation, surface, consistency, Gram's staining and the budding pattern (8). The biochemical tests such as Catalase reaction, Urease test, splitting of Esculin (TE slant) were conducted for identification of Lipid-dependent species. Tween assimilation method (i.e. 20, 40, 60, 80 and cremophor), cremophor assimilation (EL slant) tests (8, 9), Sugar fermentation and tryptophan tests, etc. were used as additional tests to differentiate the species.

Gram Staining:

The morphology of the yeast cells was studied by making Gram stained smears of the isolate on slide and being examined under oil immersion (100X) objective lens, looking for unipolar budding yeast that are described as bottle-shaped organisms (10).

Catalase Reaction:

Presence of catalase was determined by using a drop of hydrogen peroxide (6% solution) and production of gas bubbles - considered as a positive reaction (10).

Urease Test:

A loop, full of cells from 4-5 days old cultures were suspended in urea broth / urea agar slant and incubated at 37^{0}C. The reaction was read after 1-4 hrs of incubation and if found doubtful then after 24 hrs. The urease test with a bright pink to violet color considered positive and yellow colour development considered negative results (9).

Splitting of Esculin (ß-glucosidase):

The inoculating needle with fresh *Malassezia* yeast was deeply inoculated in the tube containing esculin agar (Hi Media, India) and tubes were incubated for 5 days at 32^{o}C. The splitting of esculin is indicated by darkening (black) of the medium. The ß- glucosidase activity of different *Malassezia* species was assayed by this test (11).

Tween Assimilation Test:

For each isolate, the ability of the organism to utilize individual Tween was examined by the procedure: Yeast suspension was prepared in 3 ml distilled water and sterile Glucose peptone agar 18 ml, supplemented with 0.05% Chloramphenicol and Cyclohexamide was melted and allowed to cool to approximately 50^{0}C. This mixed suspension and GPA agar were poured into plate. After solidification, five holes were made in the agar base by means of a 2 mm diameter punch and the holes were filled with 10 ul of Tween 20, 40, 60, 80 and cremophor respectively. The plates were incubated at 35^{0}C for a week. Utilization of tween was assessed by the degree of growth of the lipophilic yeasts around individual wells (11, 12).

Assimilation of Cremophor (EL slant):

Cremophor EL which can indicate assimilation of castor oil was composed of 6.5g of SDA and 1 ml of cremophor EL. This EL slants were used to determine the ability of the organism to utilize polyethoxylated castor oil. Observations were noted about appearance of colonies. (12).

Sugar Fermentation:

Sugar fermentation tests for positive / negative results were performed according to the procedure described by Gordon 1979 (13, 14).

Tryptophan Test:

The modified Dixon medium (mDixon) was prepared by replacing peptone with 0.6 % filtered sterilized L-tryptophan. Other ingredients remained constant in the medium. Culture plates were incubated at 32^{o}C.All isolates were evaluated macroscopically after 1–7 days for specific pigment induction (15).

Identification based on Molecular characterization:

The molecular analysis by DNA sequencing of the cultured organisms were conducted at Aavanira Biotech (P) Ltd. Pune, and at National Chemical Laboratory, Pune to confirm the organisms' identifications.

Preservation:

Six different preservation methods for the culture with some variables were studied like; freezing at −20°C a) in mDixon broth, b) plain glucose peptone broth (GPB), c) glucose peptone broth containing olive oil, d) mDixon and GPB coated on ceramic beads, e) in sterile water and f) emmons medium storage under refrigeration and at room temperature. Every month, viability of stored isolates was tested using solid media in triplicate sets.

RESULTS:

The data and results obtained from the present study of scalp contents of healthy and affected individuals as said and described with processes above are represented here under in Tables and Figures.

Table 1: Microscopic Examination of Scalp scraping with KOH (n=103)

Type of Organisms	% Total of KOH Test	% Total Gram Staining Test
Fungal cell and hyphae	24.57	24.57
Only Yeast cells	74	74

Table 2: % of *Malassezia* species from KOH positive test (of total 78 cases)

Culture on	***Malassezia* – Positive**	***Malassezia* – Negative (but positive for other microorganisms)**
mDixon agar	74.35	25.64

Table 3: Morphological characteristics of *Malassezia* species isolates

Sample Code	Size	Shape	Colour	Margin	Elevation	Surface	Texture	Consistency	Emulsi-fiability	Budding	Identi-fication
1, 4, 10, 16, 22, 23, 27, 28, 29, 34, 40, 44, 48, 51, 53, 56, 60, 64, 67, 71, 74, 78	3mm	Globose	Creamish	Entire	Convex	Smooth	Soft	Friable	Emulsifiable	Monopolar	*Malassezia furfur*
3, 9, 43, 46, 47, 49, 50, 55, 58, 61, 65, 73, 77	2mm	Globose	Creamish	Lobate	Raised	Smooth	Dull	Brittle	Emulsifiable	Monopolar	*Malasseziarestrictum*
5, 19, 20, 24, 36, 42, 54, 59, 69, 76	5mm	Cylindrical	Creamish	Entire	Convex	Smooth	Shiny	Butyrious to brittle	Emulsifiable	Monopolar	*Malassezia pachydermatis*
6, 21, 26, 45	4mm	Cylindrical	Pale yellow	Entire	Flat	Rough	Shiny to dull	Butyrious	Non emulsifiable	Monopolar	*Malassezia dermatis*
7, 11, 66	3mm	Cerebriform	Whitish	Wrinkled	Raised	Rough	Shiny	Brittle	Emulsifiable	Monopolar	*Malassezia globosa*
15, 72	5mm	Globose	Pale Cream	Folded	Elevation at center	Smooth	Shiny	Butyrious	Emulsifiable	Monopolar	*Malassezia sympodialis*
12	2mm	Cylindrical	Yellowish crm	Undulate	Flat	Rough	Dull	Butyrious	Less emulsifiable	Monopolar	*Malassezia japonicum*
2	3mm	Ellipsoidal	Pale yellow	Folded	Raised	Rough	Dull	Butyrious	Less emusifiable	Monopolar	*Malassezia sloffiae*
13	2mm	Cylindrical	Whitish	Folded	Slightly Lobate	Smooth	Shiny	Brittle	Emulsifiable	Monopolar	*Malassezia obstula*
8	3mm	Cylindrical	Pale yellow	Entire	Slightly Lobate	Smooth	Shiny	Butyrious	Emulsifiable	Monopolar	*Malassezia equina*

Table 4: Biochemical characteristics of cultured organisms from infected scalp and their identification

Sample Code	Confirmatory Tests			Tween assimilation					Growth on mDA		Growth on			Incubation	Identification
	Cata Lase	Urease	B-gluco sidase	20	40	60	80	Cremo EL	At 37^{0}C	At 40^{0}C	GPA	EL Slant	TE Slant	On mDA At 32^{0}C	
1, 4, 10, 16, 22, 23, 27, 28, 29, 34, 40, 44, 48, 51, 53, 56, 60, 64, 67, 71, 74, 78	+	+	+	+	+	+	+	+	+	+	+	Growth	Growth Black zone	7 days	*Malassezia furfur*
3, 9, 43, 46, 47, 49, 50, 55, 58, 61, 65, 73, 77	-	-	-	-	w	w	-	-	-	-	+	No growth	No growth	7 days	*Malassezia restrictum*
5, 19, 20, 24, 36, 42, 54, 59, 69, 76	-	+	-	-	-	+	+	-	+	+	+	Growth	Growth Black zone	7 days	*Malassezia pachydermatis*
6, 21, 26, 45	-	-	-	+	+	+	W	-	+	-	+	No growth	No growth	7 days	*Malassezia dermatis*
7, 11, 66	+	-	-	w	-	-	-	-	-	-	-	No growth	No growth	7 days	*Malassezia globosa*
15, 72	+	+	+	+	+	+	+	-	+	-	+	No growth	Growth Black zone	7 days	*Malassezia sympodialis*
42	+	+	+	w	w	+	+	W	+	-	+	Growth	Growth Black zone	7 days	*Malassezia japonicum*
2	+	-	-	+	+	+	W	-	-	-	+	No growth	Growth No color change	7 days	*Malassezia slooffiae*
13	+	-	-		-	-	-	-	+	-	-	No growth	No growth Black	12 days	*Malassezia obstula*
8	+	-	-	w	+	+	+	-	+	-	+	No growth	No growth	12 days	*Malassezia equina*

(+) = Positive, (-) = Negative, mDA, = modified Dixon Agar, w =weak, EL = Cremophor EL

Table 5: Sugar fermentation characteristics of *Malassezia* species isolates

Name of Organisms	Fructose	Dextrose	Xylose	Maltose	Mannitol	Rhamnose	Raffinose
Malassezia furfur	_	+	+	+	_	_	_
Malassezia restrictum	+	_	_	+	+	_	_
Malassezia pachydermatis	_	_	_	_	_	_	_
Malassezia dermatis	_	_	_	_	_	_	_
Malassezia globosa	_	+	_	+	+	_	_
Malassezia sympodialis	_	_	_	_	_	_	_
Malassezia japonicum	_	_	_	_	_	_	_
Malassezia slooffiae	_	_	_	_	_	_	_
Malassezia obstula	_	_	_	_	_	_	_
Malassezia equine	_	_	_	_	_	_	_

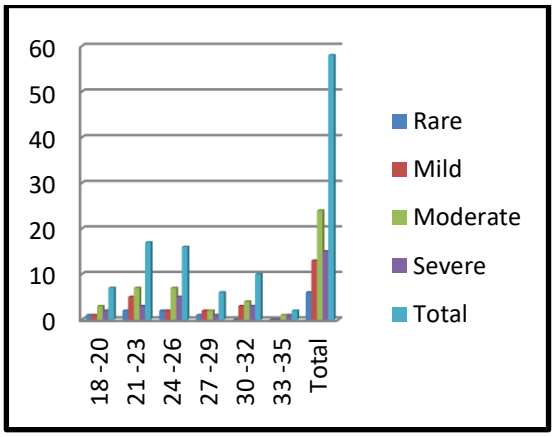

Fig. 1: Frequency of scalp infected individuals based on severity

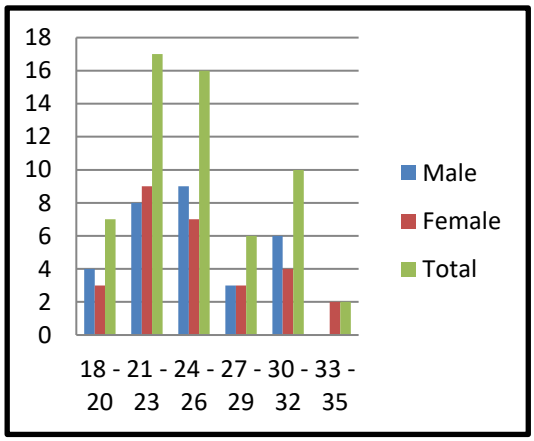

Fig. 2: Distribution of individuals on the basis of age and gender

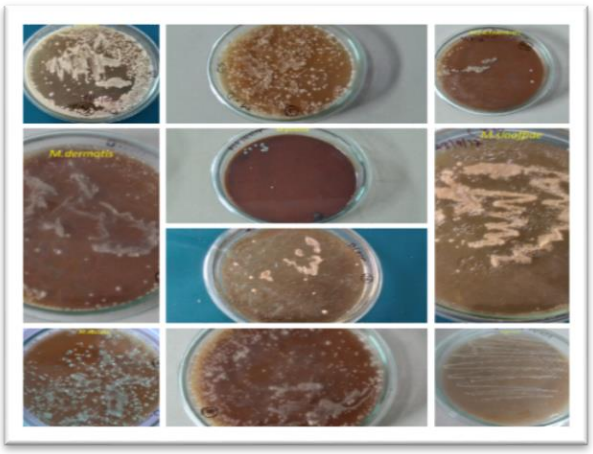

Fig. 3: Colony morphology of *Malassezia*

Fig. 4: Biochemical tests of spp. grown on mdixon agar at 32^{0}c *Malassezia* species

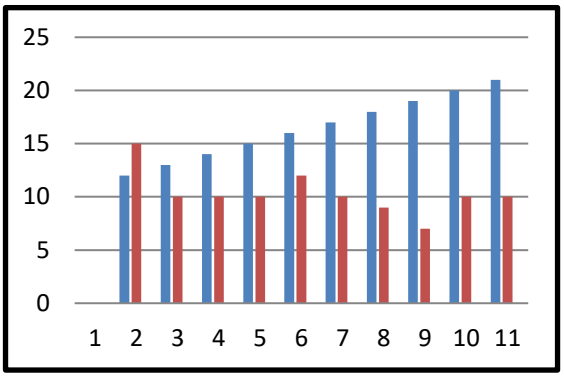

Fig. 5: Distribution of incubation days on

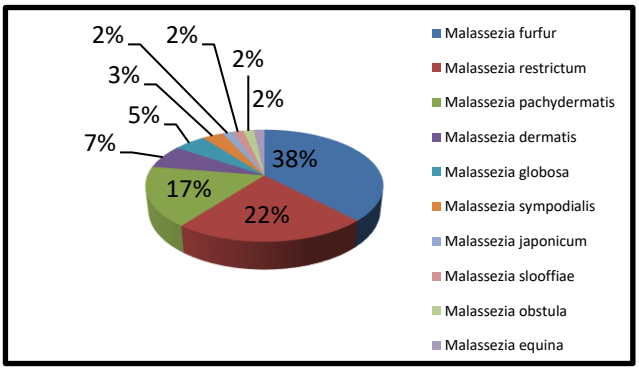

Fig. 6: Percent distribution of mDA and GPA with olive oil isolates of *Malassezia* species

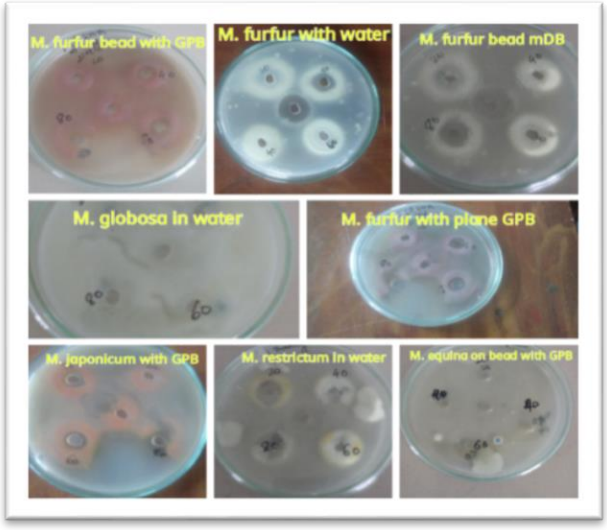

Fig.7: Result confirmation after six months

Fig.8: Sugar fermentation, Tween assimilation and preservation at -20C

One hundred and three individuals were enrolled for scalp content study in this piece of research. Figure 1 shows suspected cases with increasing severity and were classified under rare, mild, moderate and severe categories. Distribution of individuals also done on the basis of age and gender. There was no significant difference as 30 male and 28 females were found to be infected with this opportunistic pathogen. The infection stage was high at the age group 21–23 and 24–26 (Figure 2). Based on the presence of both hyphae and spores as found under direct microscopic examination of scalp scraping with 20 % KOH and Gram staining, *Malassezia* species were isolated in 74.35 % of total cases and 25.64 % was negative for *Malassezia spp.* but positive for other fungal species when cultured on mDixon agar (Table 1 and 2).

The study identified ten different species of *Malassezia* on the basis of morphological characteristics and biochemical tests (Table 3 & 4 and Figure 3 & 4). The size of the colonies was found in the range of 2–5 mm. Most of the colonies were emulsifiable and having monopolar budding and other distinct features of *Malassezia* species as reported by others (Table 4). Sugar fermentation tests for the identified and cultured species of *Malessezia* as per Table 5 showed that *M. furfur* is positive to dextrose, xylose and maltose. *M. restrictum* is positive to fructose, maltose and mannitol and *M. globosa* to dextrose, maltose and mannitol. Rest all species have negative response to fermentation of

sugars. Further biochemical characterization and identification were done by using enzymatic tests, tween assimilation, TE, EL slant, growth patterns at various temperature condition on mDA and GPA (at 32^{0}C, 37^{0}C and 40^{0}C). Only *Malassezia furfur* and *Malassezia pachydermatis* showed their growth at 40^{0}C, while other species did not grow (Table 3). For confirmation of the identification, molecular identification study was done based on ITS region. A decreasing distribution percent of *Malassezia* species was found among 58 isolates viz.*Malassezia furfur* (38 %), *Malassezia restrictum* (22 %), *Malassezia pachydermatis* (17 %), *Malassezia globosa* (5 %), *Malassezia sympodialis* (3 %), *Malassezia japonicum, Malassezia sloffiae, Malassezia obstula and Malassezia equina* (2 % each) from infected scalp (Figure 6). The cultures were confirmed for *Malassezia* yeast fungus from molecular analysis by the research laboratories of Pune too.

Malassezia species shows low viability when preserved in culture for long time storage. In the present attempt, freezing the culture at -20^{0}C was found as a suitable method for the long-term storage of *M. furfur.* Also, all prominent species like *M. restrictum* and *M. pachydermatis* along with *M. dermatis, M. japonicum, M. globosa* and *M. equina* survived when the cultures were stored at -20^{0}C on ceramic beads inoculated with mDixon optimized broth and water. Some changes in characteristic growth were observed when glucose peptone broth (GPB) containing olive oil and plane GPB were used in the medium. Interestingly, only *M. furfur* was preserved in all described preservation medium and other species survived with changing their properties in GPB broth when analysed by using Tween assimilation method (Figure 7). *M. furfur* was stored at -20^{0}C and retained its viability after six months storage on beads coated with mDixon broth. It was also stored two months successfully with GPB containing olive oil. In sterile water, the culture also survived after six months. However, the culture did not revive in GPB media after two months. *Malassezia pachydermatis* remained active in sterile water too. But *M. pachydermatis* as per biochemical studies showed tween assimilation negative around the well containing Tween 20 and 40 before and after two months preservation at -20^{0}C. At Tween 80 and cremophor wells, weak assimilation around the wells observed. This indicates possibility of mutational changes in presence of GPB containing olive oil making survival possible. In case of *M. equina,* -20^{0}C preservation with GPB coated beads and repeated thawing of same vial showed observable changes (Figure 7).

DISCUSSION:

Malassezia yeasts are lipophilic under basidiomycetes fungi and have been known to medical microbiology for its association with human skin and scalp up to 75- 98 % of healthy individuals (16). Recently, different *Malassezia species* have been considered as etiological agents of seborrheic, atomic dermatitis and dandruff. Its distribution and frequency are variable and depend on different climatic, occupational and socio-economic conditions (12). Seborrhoeic dermatitis and dandruff (SD/D) are perhaps the most common diseases associated with *Malassezia* species, with SD occurring in 1–3% and dandruff in greater than 50% of the general population (17,18). *Malassezia* species are identified and categorized as per the morphological, biochemical and molecular characterization. The present study was carried out to identify *Malassezia* species from scalp infected individuals suffering from hair disorders. The infection status was classified under rare to mild, moderate and severe categories. *Malassezia furfur*, *M. sympodialis*, and *M. slooffiae* are physiologically very similar, and uncertainty remained regarding correct identification of these species on the basis of tests for utilization of tween compounds in the simple media (7). Recently, Mayser et al. (6) have reported the use of additional tests, such as cremophor EL assimilation. Characterization of ß-glucosidase activity has also been considered to resolve this uncertainty (19). In our study, out of the 78 scalp infected individuals that were inoculated, 74 % yielded of *Malassezia* in culture. Amongst these, the most prominently isolated species were *Malassezia furfur* (37.93 %), *Malassezia restrictum* (22.41%), *Malassezia pachydermatis* (17.24%), *Malassezia dermatis* (7%), *Malassezia globosa* (5%), *Malassezia sympodialis* (3%), *Malassezia japonicum* (2%), and *M. sloffiae* (2%). Tween assimilation allows differentiation of different *Malassezia* species. Tween assimilation pattern and esculin agar test results show different observations in comparison to earlier works of others (18) but in case of *M.furfur, M. globosa, M. obstula and M. equina* species, our observations with these tests showed only variable percent than earlier reports.

The low viability of *Malassezia* cultures is one of the main difficulties in continuing regular study on this genus. Yeast cultures are best maintained on a medium which contains glucose as the only source of carbon. Glucose in the medium reduces the risk of change in growth and fermentation patterns during storage and is to avoid mutational changes (14, 20). However, it has also been noted that many basidiomycetes yeasts do not survive well during prolonged storage on a glucose-peptone medium, although they grow well on mDixon (21). Four different preservation methods for a few *Malassezia* species with some variable inclusions have been reported earlier; like- freezing at -80^{0}C, freeze drying and storage at room temperature, preservation at -80^{0}C in distilled water and use of different culture media with storing at 4^{0}C (22,23). In present study, storage of the freeze-dried cultures at room temperature was found as an unsuitable method for preservation of *Malassezia* spp. According to present findings, overcoming freezing temperature from -80^{0}C to -20^{0}C by using selective broth coated ceramic beads and alternatively keeping only water as medium to achieve viable storage for longer time duration are significantly important immediately after the first isolation in order to avoid the loss of isolates.

CONCLUSIONS:

The significant new dimensional features observed from the present study, which could be of very useful for future research endeavours and drug management are concluded in the followings:

- *Malassezia* was not found as a constituent of heavily infected dandruff individuals but found even in very less dandruff contents of individuals showing peculiarities of *Malassezia* yeast.
- Most of the *Malassezia* species other than *M. furfur, M. restrictum* and *M. globosa* are negative to various sugar fermentations indicating species diversity in their mode of action.
- Preservation with mDixon coated ceramic beads at -20^{0}C for more than six months showed successful revival of *Malassezia* culture.
- mDixon media coated beads used for the cultures if kept at normal refrigeration, no retrieval of the species is possible.
- Preservation with ceramic beads coated for GPB containing Olive oil and stored at -20^{0}C for two month showed successful retrieval too.
- Plain distilled water preservation of *Malassezia* colonies at -20^{0}C further enhanced the possibilities of its easy retrieval and low-cost preservation.
- However, the above preservation trials and findings were most successful with *Malassezia furfur* species.

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