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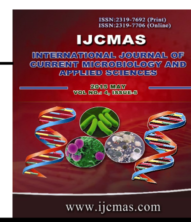

Original Research Article

A study of growth and physiological characteristics of *Malassezia furfur* on indigenously developed Coconut milk agar medium

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ABSTRACT

The limitations of both oil-overlay and lipid-incorporated media available for culture of *Malassezia furfur* call for introduction of a better substitute. As coconut milk contains most of the ingredients required for growth of lipophilic fungus, an in-house developed medium coconut milk agar was introduced and compared to modified Dixon's agar in terms of colony formation and suitability to assess growth and different physiological properties of *M. furfur*. By the study of oxygen requirement and tolerance to incremental temperature, pH, salt, urea and UV light, the growth conditions were optimized. Some biochemical tests like hemolysis, production of β-glucosidase, catalase, urease and utilization of Tweens were also performed on the proposed medium. Agglomeration of the yeasts around the incorporated skin scales into the medium and observation of their in-vivo like morphological changes to short hyphal forms on modified slide culture was correlated with the pathogenic potentiality of the strain.

Introduction

Malassezia furfur, the important causative agent of pityriasis versicolor, seborrhoeic dermatitis, dandruff and other diseases[1-5] essentially requires fatty acids for growth. Previously Sabouraud's glucose agar medium overlaid with olive oil (SGA-O) was used for its cultivation, but with drawbacks like oil leaking, smudged (uncountable) colonies and altered cellular morphology[6]. Newer media e.g. lipid-incorporated modified Dixon's agar (mDA) were efficient yet multifarious (with limited availability of ingredients), expensive and deeply tinted making use of indicators

implausible[7]. 'IMU-Mf', a proposed medium[6] almost free of unwanted hue was also very complex for regular use.

The in-house manufactured coconut milk agar (CMA) medium[8] was devoid of most of these obstacles and here, growth patterns along with demonstration of physiological properties of *M. furfur* on CMA were evaluated against mDA.

Materials and Methods

The coconut milk used here was from a commercial source (Hommade® coconut milk, Dabur India Ltd, 8/3 Asaf Ali Road, New Delhi, India, 110002) towards ensuring the uniformity of study purpose. It was composed of coconut cream 41.3% and water along with emulsifying and stabilizing agents. Per 100 ml, it contained protein 0.8 g, carbohydrates 1 g (in the form of natural sugars), fat 11.5 g and calculated to give energy of 112 kCal. Mild lot variation noted was minimal and within acceptable limit. The packets were preserved as per manufacturer's instructions.

Four separate clinical isolates of *M. furfur* were confirmed by methods described by Guillot*et al*[9] and were grown on plates containing mDA and CMA. The inocula used here were0.5 ml of a cell suspension containing 1.0 X 10^3 cells ml^{-1} (counted in a NeuberHemocytometer) which were poured on the surface of the medium and spread over the entire surface. The plates were incubated in a humidified incubator (BOD type) at 32^0C. They were examined daily for evidence of growth along with size of the colonies up to 7 days. The assessment was recorded by two different observers independently and the average scores were utilized. Additional to actual counts, colony production on media was evaluated on a five-point scale (0=nil, 1=haze, 2=few, 3=good, 4=confluent). Means of the three observations with SD were recorded. Size of the colony was measured in millimeters and in addition, interpreted using Dot-Font size (Times New Roman) Template (technique proposed by co-author Maiti PK). Ten isolated colonies were calculated accordingly and the mean was documented.

Oxygen requirement was studied by incubating inoculated plates of both media aerobically, in a candle jar (with low oxygen and high carbon dioxide content) and in anaerobic jar (with Gaspac®, HiMedia Laboratories Pvt. Ltd., 23, Vadhani Industrial Estate, LBS Marg, Mumbai-400086, India). The plates were observed after 7 days for presence of growth. However, the plates from candle jar and anaerobic jar were further incubated aerobically for 48 h to inspect whether the inhibition of growth was absolute or partial.

Temperature tolerance studies were performed at 18 ^{0}C, 22 ^{0}C, 25 ^{0}C, 32 ^{0}C, 37 ^{0}C, 40 ^{0}C and 44 ^{0}C. Absence of growth even after 48 h was considered as inhibitory.

Effect of pH of the medium was assessed on plates with pH 4.0 to 9.0 at the intervals of 0.5 which were prepared by adding 0.1N HCl or 0.1N NaOH as required during preparation. Absence of growth at 48 h was inferred to be inhibitory.

The media under study with different concentrations of NaCl, from 0.0% to 5.0% at intervals of 0.5% were tested for salt tolerance. Absence of growth at 48 h was recorded as inhibitory.

Urea (available as 40% solution in filter sterilized vials) was added to sterilized media at 50 ^{0}C (still liquid) at a final concentration of 0.0% to 5.0% at intervals of 0.5%. The mixtures were immediately

distributed in plates and used for inoculation. Absence of growth after 48 h was considered as inhibitory.

Effect of ultra violet (UV) light (with power of 40 µwattssq. cm^{-1} at 254 nm) was studied by exposing plates immediately after inoculation in the cabinet for 0, 5, 10, 15, 20, 25 and 30 min at a fixed distance. After the required UV exposure, the plates were incubated and studied for growth after 48 h.

Now, attempts were made to demonstrate some biochemical properties of *M. furfur* on CMA (with addition as appropriate). First of all, hemolytic activity was shown after adding 5% sheep blood aseptically to molten CMA held at 50 ^{0}C. The plates were inoculated and observed for hemolytic zone around colonies after 48 h.

For detection of β-glucosidase activity of *M. furfur*, 5% coconut milk was added to aesculin agar. The plates were heavily streaked and examined daily for 5 days for darkening of the medium[10]. An inoculated plate of Tween 60–aesculin agar medium served as positive control.

Ability to utilize Tweens 20, 40, 60 and 80 were studied following the method described by Guillot *et al*[9]. Eighteen ml of molten CMA held at 50 ^{0}C was seeded with 2 ml of yeast cell suspension (1.0 X 10^4 cells ml^{-1}) and solidified on plates as in 'pour-plate method'.

With the help of a sterile 2 mm diameter punch, four wells were dug in each quadrant of the plate and were filled with 5 µl of Tweens each. The plates were observed daily for 7 days for growth and / or precipitate formation around the wells[10].

Production of the enzyme catalase was assessed by adding a portion of the colony (taken on an edge of a cover slip) cultivated on CMA to a drop of 3% H_2O_2 solution on a slide and noting prompt effervescence.

Urease test was done by supplementing Christensen's urea agar base with 5% coconut milk and adding readymade, sterile 40% urea solution after autoclaving. *Candida albicans*(MTCC 227) served as negative control. The result was seen daily for 48 h[11].

Effects of human stratum corneum cells on growing *M. furfur* were assessed by utilizing a modification of Dalmau culture method. Cast off skin scales from human volunteers were washed, dried and added at 1% concentration to the media (mDA and CMA) before autoclaving.

The media, when in a liquid state, were distributed on the surface of sterile glass slides as a thin film and solidified. Linear streaks of pure cultures of *M. furfur* were made on the surface of those slides, covered with cover slips and incubated at 37 ^{0}C in sterile Petri dishes with bent glass rod platform on a moist filter paper[12].

The slides were examined daily under microscope for formation and nature of growth with particular attention to any attachment of the yeast cells to human epithelial cells.

All the tests were done on three sets of experiments to ensure comparability and reproducibility. Unless mentioned otherwise, the source of all media and reagents used in the study were from HiMedia Laboratories Pvt. Ltd., 23, Vadhani Industrial Estate, LBS Marg, Mumbai-400086, India.

Statistical methods used: Percentage, Student's *t* test.

Results and Discussion

Appearance of colonies was evident from second day onwards on both mDA and CMA (1=haze). However, at the end of 7 days, mDA showed larger colonies (2-3 mm diameter, average Dot-Font size 60) in comparison to CMA. The colonies on CMA (on days 5-7) comprised of white to cream colored, 1-2 mm diameter (average Dot-Font size 40), circular with slightly irregular margins, smooth, soft, friable (like refrigerated butter) and no specific smell. Lactophenol cotton blue mount from growth on both of the media demonstrated oval or spherical yeast cells of ~5-6 μ diameter with budding on a broad base. On similar cultural environments (in optimal range), there was no significant difference of colony counts on the two media when counted on days 2 or 7 ($P > 0.05$); but significant increase in counts were observed on day 7 than that on day 2 ($P < 0.05$). Colony morphology was identical on both the media tested.

Requirement of oxygen was a must for multiplication of the fungus, though scanty growth (scale=1-2) was seen on candle jar where the environment was conducive for organisms capable of growing in low oxygen concentration only. On aerobic re-incubation of the plates, the ones from the candle jar showed light growths (scale=2) supporting the inference. No growth was seen on plates incubated anaerobically – in anaerobic jar with Gaspac. A novel method of combination of rapid and slow combustion of oxygen described by Maiti*et al*[13] was also tried and the findings matched with those of anaerobic jar itself.

The fungus had grown optimally through a range of temperatures of 32 – 37 ^{0}C and pH of 5.5 – 7.5. Sparse growth (scale=1) were seen in plates kept at 22 ^{0}C & 40 ^{0}C and those having pH of 4.5 & 8.0. Outside the abovementioned range, no growth was detected. Salt (NaCl) and urea concentrations of 5% and 3.5%, respectively, had shown to inhibit growth of *M. furfur*. In our experimental setup, these fungi could tolerate up to 20 min of UV light exposure as thin layers of growth were observed on plates exposed for 20 min, but no growth on plates kept for 25 min. The statistical significances of comparisons between media were $P > 0.05$ vis-à-vis colony counts on day 2 and 7 were $P < 0.05$ in all the above mentioned tolerance studies.

Good growth (scale=4) of *M. furfur* was obtained on 5% blood-coconut milk agar plates, but no hemolysis was observed. Aesculin agar mixed with coconut milk showed weak development of brown color. Tween 60- aesculin agar plates showed similar results. The four strains of *M. furfur* used in the experiment utilized all Tweens 20, 40, 60 & 80 well and produced the enzymes catalase and urease on CMA.

In modified slide culture technique, enhanced growth of the fungi around the added skin scales on both mDA and CMA were observed. Some of them showed a tendency to form elongated, pseudomycelium like structures resembling hyphal conversion.

Malassezia furfur, the most commonly known member of the genus *Malassezia*, is considered as a part of commensal flora of human integument[1]. It is also related to / causes pityriasisversicolor, seborrhoeic dermatitis, dandruff, onychomycosis, Malassezia folliculitis, atopic dermatitis, psoriasis etc[2, 3]. Upcoming of reports of systemic infections in immune-compromised patients, especially neonates on total parenteral nutrition (TPN) renewed its importance[4, 5].

As this fungus is lipophilic, traditionally Sabouraud's glucose agar medium with olive oil overlay (SGA-O) was used for cultivation of *Malassezia* species – both for primary isolation as well as for further processing and preservation. But this medium gets easily contaminated and overgrown by saprobic fungi and bacteria. The colonies grown are smudged and not quantifiable. The medium undergoes 'syneresis' – the oil is separated from the medium and seeps out of the plates[6] in addition to being volatile and inflammable. Use of overlaid oil, which is almost always more than just a thin film and remains as several pools formed over the surface of the medium, results in incorporation of more lipids in the yeast cell bodies resulting in morphological changes[6] and making identification difficult.

Towards overcoming these difficulties, many media has been formulated. Of them, the most commonly used is modified Dixon's agar with added chloramphenicol and cycloheximide (mDA). This is a complex, lipid-incorporated medium giving an early visible (within ~24 h) growth, individual colony formation (in ~4 days) and seems capable of sustaining growth for a short span of time. But it suffers from the deficiencies of being costly, has many ingredients some of which are heat labile (hence not autoclavable), of deeply bile coloured (greenish-brown) – hence not allowing addition of indicators to the medium (thereby encumbers some biochemical tests). Antifungal susceptibility testing, which is the call of the day in the background of progressively shifting patterns, is also very difficult to interpret on this medium.

Further propositions, made by several researchers in this field suggested substitution of bile salts in mDA by taurocholic acid, reducing the malt agar content etc[7]. One medium developed at International Medical University, Kuala Lumpur, Malaysia and described as IMU-Mf medium[6] required 10 ingredients of which L-asparagine is also heat labile.

It is therefore necessary to carry on the efforts towards compounding a low cost, easily available, simply formulated, lipid incorporated, colourless / white, effective medium with heat stable components which are less or non-reacting with reagents needed for physiological studies as well as antifungal susceptibility tests.

Coconut milk, the white colored juice available from the copra of mature fruits of coconut (*Cocosnucifera),* contains sugars like glucose and fructose, proteins (chiefly the essential amino acids) as well as high oil content (unsaturated fatty acids including oleic acid). These promote growth of lipophilic fungi like *Malassezia.* The ability of medium containing coconut water or coconut milk (5%) with agar powder (2%) to obtain and sustain good growth of common pathogenic yeasts has already been demonstrated and documented[8]. Under these circumstances, we considered coconut milk agar (CMA), as an alternative to other medium for culture of *M. furfur* and systemically compared the growth patterns on CMA vis-à-vis mDA.

The interpretation of the study that both mDA and CMA were efficient to grow *M. furfur* both for primary isolation as well as for subculture correlated well with that of Maiti*et al*[8]. The larger colony size at seventh day on mDA might represent a relative deficiency of one or some specific nutritive material in CMA (as mDA was a complex composition of various specific synthetic nutrients in contrast to CMA which depended on natural resources only).

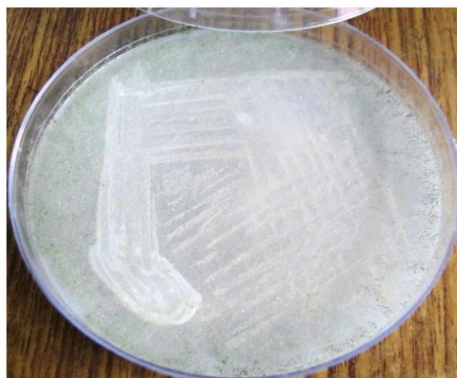
Table.1 Influence of different physical conditions on growth of Malasseziafurfur on modified Dixon's agar (mDA) and coconut milk agar (CMA) on second and seventh day

Culture Environment			Visible colony count											
			2nd Day						7th Day					
			m	D	A	C	M	A	m	D	A	C	M	A
Temperature (^{0}C)														
1		8		2			1			3			3	
2		2	1	4	9	1	1	4	2	1	1	1	6	9
2		5	2	7	6	2	5	9	3	1	4	3	0	1
3		2	2	8	5	2	6	7	3	2	1	3	1	9
3		7	2	8	1	2	7	0	3	3	3	3	2	3
4		0	2	5	1	2	4	4	2	7	7	2	6	5
4		4		6			3			7			5	
p		**H**												
	4		1		1		5		1		5	1		2
4	.	5	1	8	4	1	1	9	2	1	3	1	9	2
	5		2	1	7	1	9	8	2	3	0	2	1	3
5	.	5	2	2	2	2	0	3	2	5	6	2	4	5
	6		2	4	7	2	3	5	2	6	0	2	5	8
6	.	5	2	5	1	2	1	4	2	7	2	2	2	5
	7		2	5	4	2	4	6	2	8	8	2	5	7
7	.	5	2	5	9	2	4	8	3	1	3	2	8	9
	8		2	2	1	2	0	6	2	5	7	2	4	1
8	.	5	1		7	1		2	2		6	1		9
	9			3			0			4			3	
UV light (min)														
	0		3	6	7	3	1	4	3	8	6	3	5	1
	5		3	6	5	3	1	9	3	8	2	3	4	0
1		0	3	1	9	2	8	1	3	7	5	3	2	6
1		5	2	5	1	2	4	4	2	7	6	2	6	1
2		0	1	7	4	1	1	9	2	0	2	1	7	7
2		5	2		0	1		1	2		6	1		3
3		0		2			0			2			1	

Table.2 Salt (NaCl) and urea tolerance of Malasseziafurfur on modified Dixon's agar (mDA) and coconut milk agar (CMA) on second and seventh day

Concentration of chemical used (w/v)	Visible colony count			
	2nd Day		7th Day	
	mDA	CMA	mDA	CMA
NaCl (%)				
0	299	268	317	297
0.5	303	275	320	281
1	306	298	319	301
1.5	296	281	322	307
2	316	288	319	305
2.5	318	301	328	309
3	296	284	306	287
3.5	313	295	347	308
4	317	302	337	311
4.5	254	216	275	235
5	18	20	26	26
Urea (%)				
0	314	304	357	324
0.5	320	302	368	321
1	304	301	352	316
1.5	310	268	325	296
2	296	267	327	311
2.5	306	298	334	308
3	252	221	274	261
3.5	22	13	26	18
4	23	10	25	12
4.5	19	11	21	11
5	3	0	4	0

Figure.1 Comparative growths on Coconut milk agar (A) & modified Dixon's agar (B) plates on day seven



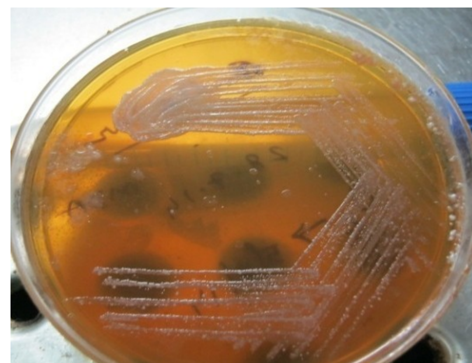


Figure.2 Modified Candle jar with Double combustion technique (A) & Appearance of Coconut milk agar plate after incubation for 48 h in it (B)

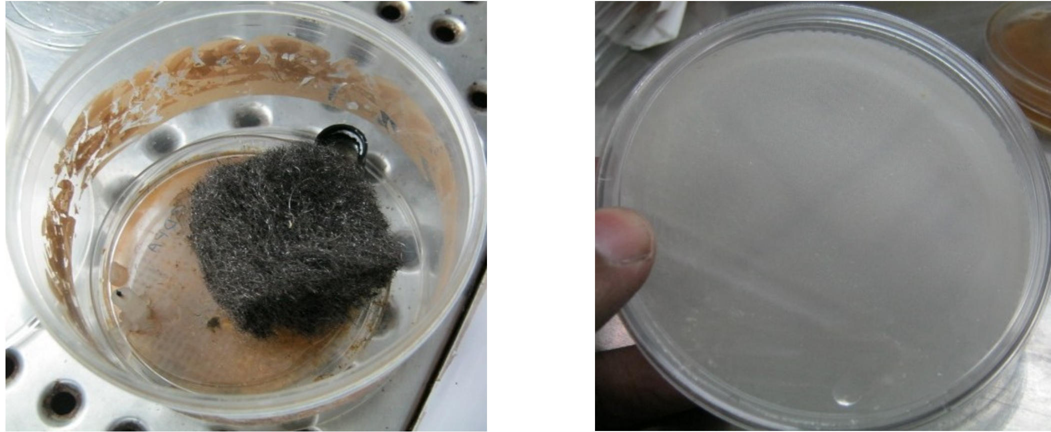
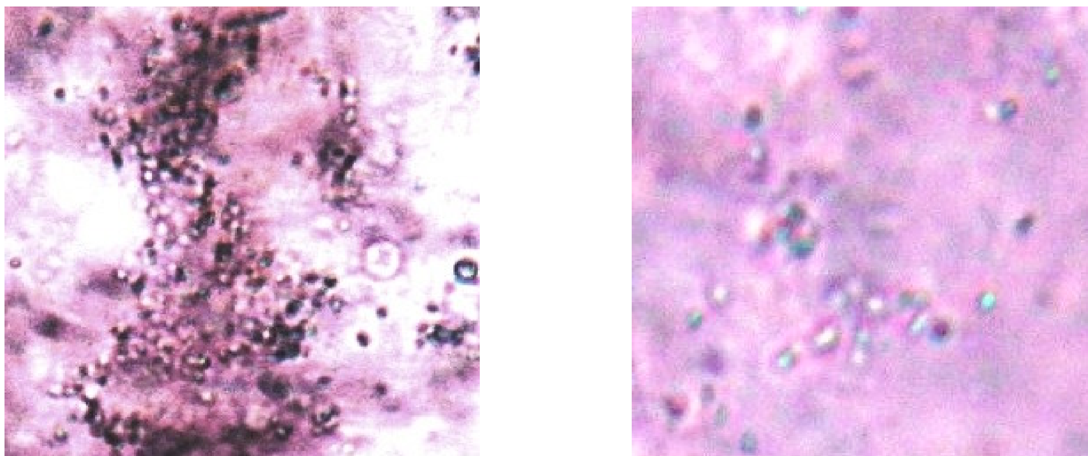


Figure.3 Non-hemolytic colonies of Malasseziafurfur on Blood-Coconut milk agar medium

Figure.4 Budding yeast cells in Modified Slide Culture: A – 400X (Agglomeration near skin scale more pronounced), B – 1000X (Some yeasts show precurrent budding)



The maintenance of colony profiles specific to the medium on which the yeasts from different sources were cultured negated any potential strain variation.

The findings of study of oxygen requirement considered together might suggest the mechanism why *Malassezia* species remain alive inside a bottle of fatty acid suspension (used for intravenous infusion[5]) having a very small amount of air inside. Complete elimination of oxygen from the container by replacement with nitrogen or some other inert gas (as some free space inside the bottle is a necessity to withstand expansion or contraction with variations in temperature during storage or transport), thereby maintaining an absolutely anaerobic environment may go a long way towards prevention of fungaemia in recipients of total parenteral alimentation. Regarding tolerance of temperature, pH, salt, urea and UV light the comparability as per available literature was varied. Leeming*et al*[14] found that at 34 ^{0}C, *M. furfur* yielded high growth although they noted reports of optimal development of colonies at 35 – 37 ^{0}C. Vijayakumar *et al*[15] reported optimum temperature of 30$\pm$ 2 ^{0}C, pH of 7 – 9 and salinity of 40 ppt, but their study design was based on so widely distributed parameters (like temperature of 10, 20, 30 & 40^{0}C and salinity of 20, 40, 60, 80 & 100 ppt) that no comparison could be made. So far as our knowledge goes, no publication reviewing the effect of urea and UV light on *M. furfur* was available in the literature for comparison.

Utilization of Tween compounds as well as production of the enzymes catalase and urease on CMA supported the description of Khosravi*et al*[10] and Guillot*et al*[9] respectively. This pronouncement of growth and morphological alterations of those yeasts around skin scales corroborated with the observations of Manna *et al*[12]. Such morphological feature produced by pathogenic strains may be considered as the hallmark of microscopical diagnosis of active infection, if compared with strains isolated from normal skin. This intensified colonization of *Malassezia* in the vicinity of lifeless crusting of the integument might be an effect of some biofilm-like colonies by these fungi and needs proper evaluation in the future. The property of adherence and incidences of clinical recurrences on the same site as a result of persisters might be better explained in the light of biofilm.

This study reiterated the need to develop an efficient, economic, easily availed, minimally complex media for cultivation of *M. furfur* and observed the comparability of mDA and CMA towards the same. Though the sizes of the colonies were smaller, CMA proved to be a good substitute to mDA. Further research with other *Malassezia* species are required to substantiate acceptability of this media in routine use for culture of these lipophilic fungi.

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References

1. Gao Z, Perez-Perez GI, Chen Y, Blaser MJ. Quantitation of major human cutaneous bacterial and fungal populations.*J ClinMicrobiol* 2010; 48: 3575-3581.

2. Gupta AK, Batra R, BluhmR, Boekhout T, Dawson TL, Jr. Skin diseases associated with Malassezia species. *J Am AcadDermatol* 2004; 51(5): 785-798.
3. Baroni A, Paoletti I, Ruocco E, Agozzino M, Tufano MA, Donnarumma G. Possible role of *Malasseziafurfur* in Psoriasis: Modulation of TGF-beta1, integrin, and HSP70 expression in human keratinocytes and in the skin of psoriasis-affected patients. *J Cut Pathol* 2004; 31: 35-42.
4. Barber GR, Brown AE, Kiehn TE, Edwards FF, Armstrong D. Catheter related *Malasseziafurfur*fungemia in immunocompromised patients. *Am J Med* 1993; 95: 365-370.
5. Dankner WM, Spector SA, Fierer J, Davis CE. Malasseziafungemia in neonates and adults: complication of hyperalimentation. *Rev Infect Dis* J 1998; 7(6): 398-401.
6. Chua KB, Chua I, Chong KH, Chua KH. A modified mycological medium for isolation and culture of *Malasseziafurfur.Malaysian J Pathol* 2005; 27(2): 99-105.
7. Buick SF. Development of an *in vitro* diagnostic technique for *Malasseziafurfur*. Thesis for MS (Microbiology), University of Canterbury.Accessed on 26.1.2013.
8. Maiti PK, Bose M, Ganguly S, Ghosh S, Manna A. Coconut based in-house developed medium for fungal growth. *J Mycopathol Res* 2008; 46(2): 275-277.
9. Guillot J, Gueho E, Lesourd M, Midgley G, Chevrier G, Dupont B. Identification of Malassezia species: a practical approach. *J Mycol Med* 1996; 6: 103-110.
10. Khosravi AR, Eidi S, Katiraee F, Ziglari T, Bayat M, Nissiani M. Identification of different *Malassezia* species isolated from patients with *Malassezia* infections. *World J Zool* 2009; 4(2): 85-89.
11. Chander J. *Textbook of Medical Mycology*, Third Edition, New Delhi, India: Mehta Publishers, 2013:92-105, 512.
12. Manna A, Manna J, Ganguly S, Maiti PK. Form conversion of *Malasseziafurfur* and its pathogenic significance. *J Mycopathol Res* 2011; 49(1): 123-126.
13. Maiti PK, Haldar J, Mukherjee P, Dey R. Anaerobic culture on growth efficient bi-layered culture plate in a modified candle jar using a rapid and slow combustion system. *Indian J Med Microbiol* 2013; 31: 173-176.
14. Leeming JP, Notman FH. Improved methods for isolation and enumeration of *Malasseziafurfur* from human skin.*J ClinMicrobiol* 1987; 25(10): 2017-2019.
15. Vijayakumar R, Muthukumar C, Kumar T, Saravanamuthu R. Characterization of *Malasseziafurfur*and its control by using plant extracts. *Indian J Dermatol* 2006; 51(2): 145-148.