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Research Article

Extracellular Enzymatic Activity of *Malassezia* spp. Isolated from Pityriasis Versicolor Patients and Healthy Individuals

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I N F O

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A B S T R A C T

Introduction: Malassezia spp. is incapable of synthesising fatty acids. It takes external lipids as a nutritional source for survival by secreting various lipase enzymes, which degrade sebum to produce and uptake fatty acid. During this process, it produces certain extracellular enzymes; lipase, phospholipase, and protease which may act as virulence factors. We intend to determine the extracellular enzymatic action of *Malassezia* spp. isolated from individuals with pityriasis versicolor and healthy individuals.

Methodology: One hundred strains were obtained from healthy individuals and pityriasis versicolor (PV) patients each. The enzymatic activity was determined by phenotypic methods.

Results: Phospholipase production of *Malassezia* spp. was found to be high in PV patients (n = 85) as compared to healthy individuals (n = 38). Among these isolated, *M. globosa* showed the maximum production of phospholipase in both PV and healthy individuals (n = 49 and n = 12).

Conclusion: The extracellular enzymes produced by *Malassezia* spp. (lipase, protease, phospholipase, haemolysis) exhibit virulence factors which are involved in the pathogenicity of disease caused by *Malassezia,* but our findings showed no significant difference in isolates from healthy individuals and PV patients.

Keywords: Extracellular Enzymes, Phospholipase, Protease, Malassezia, Pityriasis Versicolor

Introduction

Malassezia is a lipophilic yeast which resides as commensals in healthy skin. There are 18 species of *Malassezia* which have been identified, among which *Malassezia* pachydermatis is lipid-independent.[1] *Malassezia* may cause superficial infections such as atopic dermatitis, pityriasis versicolor, seborrheic dermatitis, folliculitis and cradle cap. It may cause systemic infection in immunocompromised individuals and in patients on total parenteral nutrition. The pathogenesis of *Malassezia* yeast-associated infection

is unclear except for pityriasis versicolor.[2] There are four key factors responsible for the skin disease caused by *Malassezia*; i) the activation of the immune system, ii) the production of irritant fatty acids by a fungal lipase, iii) irritant lipid metabolite by lipid peroxidase, and iv) a toxin.[3]

Myristic acid, which is required for *Malassezia* to flourish, cannot be produced by the organism. It generates a variety of lipolytic enzymes, including lysophospholipase, lipase, esterase, and phospholipase,[3] which help in the utilisation of essential fatty acids from exogenous lipid sources. Extracellular enzymes serve as potent virulence factors.

Lipases catalyse the hydrolysis of ester bonds of triacylglycerol. Phospholipase hydrolyses the ester linkage in glycerol-phospholipids of host cell membranes as it triggers the haemolytic action. Yeasts produce phospholipase and zymogen. This causes *Malassezia* to transition from a saprophytic stage to a pathogenic state. To achieve this, the host complement system is activated. Pathogenic fungi can create phospholipase, which can harm cell membranes and trigger immune reactions in the host.[4]

Bacteria like *Listeria* and *Staphylococcus* produce haemolysin which has a cytotoxic effect on the membrane of erythrocytes and phagocytes, and pore-forming and lytic effect on eukaryotic cell and cellular structures like bacteria. *Malassezia* also causes haemolysis which is attributed as one of the virulence factors.[5] *Malassezia* has a tendency to form biofilm which is a serious health threat and leads to sources of persistent infections.[6] Biofilm production by *Malassezia* spp. is responsible for the emergence of antifungal resistance.

Hence, we intended to study the virulence factors of *Malassezia* spp. isolated from pityriasis versicolor patients and healthy individuals.

Methodology

After receiving approval from the institutional ethics committee (IEC No: AV/IEC/2020/118) and obtaining informed consent from the participants, this prospective study was conducted between January 2021 and January 2023 in the Microbiology Department of the Aarupadai Veedu Medical College and Hospital, Puducherry.

Sample Collection

A total of 100 strains from healthy individuals and 100 strains from pityriasis versicolor patients collected in a previous study were used in the present study.[7]

Virulence Factors

A. Phospholipase Production[8]

On egg yolk agar medium, a 10 µl broth suspension of the *Malassezia* isolate was deposited, and it was incubated at 32 °C for five days. The zone of precipitation was observed. Production of phospholipase (Pz value) was calculated by the following formula:

$$\text{Pz value} = \frac{\text{Colony diameter}}{\text{Colony diameter + Zone of precipitation}}$$

The five types of Pz values for phospholipase activity are listed ahead. Phospholipase activity is negatively correlated with a Pz value of 1, weakly correlated with a Pz value of 0.90 to 0.99, poorly correlated with a Pz value of 0.80 to 0.89, moderately correlated with a Pz value of 0.70 to 0.79, and intensely correlated with a Pz value of 0.70.

B. Lipase[9]

Malassezia was inoculated onto sorbitan monolaurate agar and incubated at 32 °C for 5 to 10 days. As the *Malassezia* is a slow grower, the incubation period may vary. The formation of an opaque precipitation zone is a sign that lipase enzyme is being produced.

C. Protease[9]

Malassezia isolate was inoculated on the selective medium for protease production. The media contained beef extract (3 g), peptone (5 g), gelatin (8%), agar (15 g) and distilled water (1000 mL). The isolates were incubated at 32 °C for seven days. The halo precipitation zone around the colonies indicated a positive reaction.

D. Blood Agar Test[10]

Tryptone soya agar with 5% human blood was used to determine the haemolytic activity of *Malassezia*. Isolate was inoculated on the media. Olive oil was applied to the blood agar surface, and after 7 days of incubation at 30 °C, a haemolysis zone was visible. To detect the haemolytic activity (halo formation) clearly, grown yeast cells were removed from the plates before observation.

E. Biofilm Production[11]

Malasesszia species' biofilm development was carried out utilising the crystal violet staining (CVS) method. A *Malassezia* isolate was added to the peptone extract yeast broth and incubated for three days at 32 °C. 150 µl of suspension was taken from the broth and placed in each well of a 96-well microtiter plate. They were then left to incubate for 24 hours at 32 °C. The suspension was aspirated, and each well was washed twice with 150 µl of phosphate buffered saline (PBS, pH 7.2), before being thoroughly dried by air drying for 45 minutes. Wells that had been cleaned were stained for 45 minutes with 150 µl of 0.5% aqueous crystal violet solution, washed twice with 200 µl of sterile distilled water, then destained for 45 minutes with 200 µl of 95% ethanol, and measured at 595 nm. The strains were examined twice, and optical density was used to calculate the average absorbance value. The optical density of the culture increased due to the accumulation of cells and the extracellular matrix.

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Results

A total of 200 *Malassezia* isolates were used in this study, of which 100 were obtained from healthy individuals and 100 from pityriasis versicolor patients. The species distribution of *Malassezia* species is shown in Table 1. In healthy individuals, most isolates were of *M. furfur* (n = 30), followed by *M. globosa* (n = 27) and *M. pachydermatis* (n = 20). In pityriasis versicolor patients, *M. globosa* (n = 52) was the maximum, followed by *M. furfur* (n = 32).

Phospholipase production of *Malassezia* spp. was high in PV patients (n = 85) as compared to healthy individuals (n = 38). Among the isolates, *M. globosa* showed the maximum production of phospholipase in both PV and healthy individuals (n = 49 and n = 12, respectively) as shown in Figure 1.

On overall enzymatic action, it was seen that the maximum number of *Malassezia* spp. from PV patients produced the extracellular enzymes (Table 2).

Table 1.Distribution of *Malassezia* spp. Among Healthy Individuals and Individuals with Pityriasis Versicolor

Species	Lipase		Protease		Haemolysis		Biofilm	
	Healthy	PV	Healthy	PV	Healthy	PV	Healthy	PV
M. furfur	5	13	15	32	0	0	28	30
M. globosa	10	23	10	52	22	29	25	53
M. pachydermatis	7	5	12	5	0	0	20	5
M. sympodialis	3	3	12	0	0	0	13	3
M. slooffiae	0	2	0	0	0	0	0	2
M. restricta	0	2	0	0	0	0	0	2
M. obtusa	0	1	0	0	0	0	0	0

Table 2.Enzymatic Activity of *Malassezia* spp. Isolated from Healthy Individuals and Individuals with Pityriasis Versicolor

Species	No. of Isolates from Healthy Individuals	No. of Isolates from Individuals with Pityriasis Versicolor	Total	Percentage
M. furfur	30	32	62	31.0
M. globosa	27	56	83	41.5
M. pachydermatis	20	5	25	10.0
M. sympodialis	16	3	19	9.5
M. slooffiae	4	2	6	3.0
M. restricta	2	2	4	2.0
M. obtusa	1	0	1	0.5
Total	100	100	200	100.0

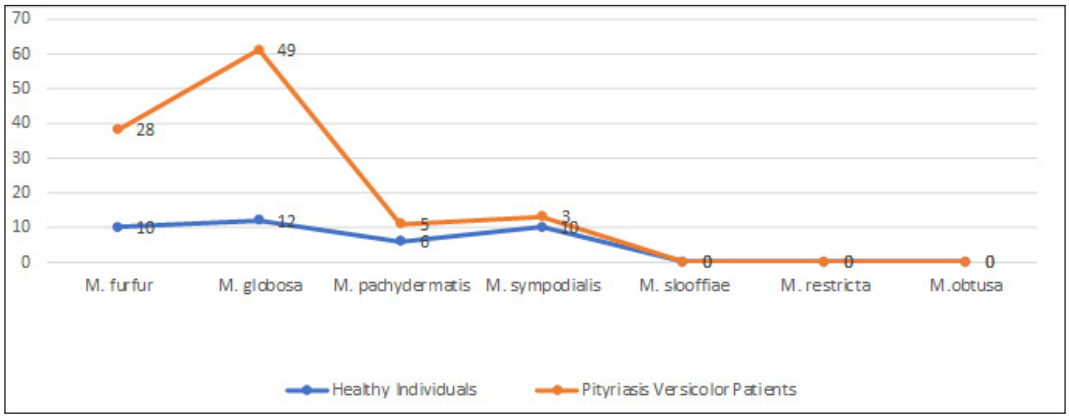

Figure 1.Phospholipase Production of *Malassezia* spp. Isolated from Healthy Individuals and Pityriasis Versicolor Patients

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Discussion

Malassezia yeast, known to cause dandruff in individuals of Indian origin, lacks extensive epidemiological data. In a study conducted in southern India, *M. sympodialis* was reported to be the predominant species.[12] Another study, which examined the geographical and seasonal influences on dandruff sufferers, found that *Malassezia globosa* and *Malassezia restricta* were the most frequently isolated species.[13] In a study of 100 pityriasis versicolor patients in central India, the most common isolates were M. globosa (57.5%), *M. sympodialis* (17.2%), and *M. furfur* (6.7%).[14]

Our own study yielded slightly different results, indicating that the top three species were *M. furfur* (31%), *M. globosa* (41.5%), and *M. pachydermatis* (10%), with the remaining species being *M. sympodialis, M. slooffiae, M. restricta,* and *M. obtusa* (9.5%, 3%, 2%, and 1%, respectively). In another study, *M. furfur* accounted for 64.7% of the isolates, followed by *M. restricta* and *M. slooffiae* (14% each), and *M. sympodialis* and M. globosa (4% each).[12] Our findings align with those from Iran, where seborrheic dermatitis patients receiving therapy were most likely to have *M. furfur*.[14] It is evident from global epidemiological data that the three most common species, *M. globosa, M. furfur,* and *M. sympodialis*, are responsible for pityriasis versicolor across different nations. Variations in the distribution of *Malassezia* species are influenced by sebum lipid secretion, which impacts the growth of lipophilic organisms, and individual susceptibility to different species.[15]

However, it's worth noting that epidemiological research concerning *Malassezia* can be challenging due to inconsistent isolation rates on laboratory media, partly because of longer incubation durations and viability loss. While conventional plating, biochemical, and morphological testing can identify *Malassezia* isolates with some precision, molecular confirmation procedures like PCR-RFLP are more reliable and accurate.[14,16]

Malassezia species are unable to synthesise fatty acids and rely on external lipids as nutritional sources for their survival. They achieve this by secreting various lipase enzymes that degrade sebum, producing and taking up fatty acids. These lipase enzymes, which hydrolyse triglycerides and other lipid molecules found on the skin's surface, are significant virulence factors.[17,18] By breaking down host skin lipids and gaining access to fatty acids, *Malassezia* promotes its growth and survival. The overproduction of lipases by *Malassezia* can disrupt the skin's lipid barrier and lead to skin conditions such as seborrheic dermatitis and pityriasis versicolor.

In addition to lipase enzymes, *Malassezia* species also produce phospholipase enzymes, which are involved in the hydrolysis of phospholipids – major components of cell membranes. These enzymes enable *Malassezia* to alter host cell membranes, facilitating colonisation and invasion. The disruption of the skin's natural barrier by phospholipase activity can trigger host immune responses, contributing to skin inflammation and irritation.[19]

The pathogenic role of *Malassezia* is associated with alterations in the physical, chemical, or immunological mechanisms of the skin, which may either enhance or downregulate the yeast's virulence factors.[20,21] Microbial attachment to the host is crucial for colonisation and infection. Biofilm production by *Malassezia*, which is observed in most species in our study, plays a key role in drug resistance and increased virulence. Biofilms consist of surface-associated microbial cells enclosed in an extracellular polymeric substance matrix.[22–24] The production of biofilms by *Malassezia* is believed to contribute to the emergence of antifungal resistance. Understanding antifungal drug resistance may lead to new treatments for biofilm-based diseases. Biofilm resistance has multiple mechanisms.[19,25] Unlike some biofilm bacteria and fungi that resist antimicrobials through metabolic quiescence, our data showed that *Malassezia* biofilm cells are metabolically active, similar to *Candida albicans* and *C. parapsilosis.*[21] This suggests that biofilm production by *Malassezia* is unlikely to promote antifungal resistance.[25]

In a study on the identification and pathogenicity of *Malassezia* spp. obtained from healthy individuals and those with macules, it was found that 38 *Malassezia* spp. isolates from both groups produced lipase, phospholipase, and protease.[26] Similarly, our research showed that isolates from individuals with pityriasis versicolor and those with healthy skin both produced lipase, phospholipase, and protease. This suggests that the pathogenic properties of *Malassezia* species isolated from healthy and affected skin are similar.

Most notably, *M. globosa* isolates from healthy individuals and pityriasis versicolor patients in our study demonstrated lipase enzyme production. These lipases are crucial for breaking down monoglycerides, diglycerides, and triglycerides found in human skin sebum into saturated and unsaturated fatty acids. *Malassezia* utilises saturated fatty acids for growth, while unsaturated fatty acids accumulate on the stratum corneum. This buildup can affect the skin barrier's permeability, potentially leading to various skin disorders.[7] Furthermore, after digesting the saturated fatty acids in sebum, the remaining oleic acid and unsaturated fatty acids may contribute to dandruff development, particularly in individuals prone to this condition, highlighting the role of lipases in *Malassezia*'s pathogenesis.[23]

In addition to lipases, *Malassezia* spp. produce lipolytic enzyme phospholipase, which facilitates the utilisation

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of essential fatty acids from an exogenous lipid source.[26] The release of beta-endorphin, a protein, encourages the production of phospholipase by *Malassezia* species. Various factors, including changes in the skin's microenvironment (e.g., pH, stress, bacterial microbiota, salts, melanocytes, sebocytes, and keratinocytes), can trigger the release of endorphin in susceptible individuals. The activation of the -opiate receptor found on the cell wall of *Malassezia* species results in the overproduction of phospholipase. This enzyme degrades the intercellular lipid of the stratum corneum and enters host cells, potentially causing dysfunction in the stratum corneum and contributing to skin disorders.[7,24]

Conclusion

The extracellular enzymes produced by *Malassezia* spp. (lipase, protease, phospholipase, and haemolysis) exhibit the virulence factors of this organism, which are involved in the pathogenicity of disease caused by *Malassezia*, but our findings showed no significant difference in isolates obtained from healthy and pityriasis versicolor patients.

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Conflict of Interest: None

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