The Optimal Condition for Tyrosinase Activity Detecting in *Malassezia* Species

สภาวะที่เหมาะสมสำหรับการตรวจวัดกิจกรรมของเอนไซม์ไทโรซิเนสในเชื้อมาลาสซีเซียสปีชีส์

Apinya Phudornmuang (อภิญญา ภูดรม่วง)* Dr.Kittipan Samerpitak (ดร.กิติพันธุ์ เสมอพิทักษ์)**
Dr.Kunyaluk Chaicumpar (ดร.กัญญลักษณ์ ชัยคำภา)**

ABSTRACT

Malassezia species are microflora and associate with pityriasis versicolor (PV) which is a non-inflammatory disease, characterized by hyper or hypopigmented lesions. At present the mechanisms of PV remain unclear. Tyrosinase is a part of virulence factor of *Malassezia* yeast. This enzyme plays role in melanin synthesis, involving in color change of skin. This study tried to optimize the medium and condition to investigate tyrosinase activity of various *Malassezia*. Test media such as minimal medium (MM), MM with tween 80 (MM+Tween 80) and mDixon, supplemented with L-DOPA were used for optimization. The enzyme activity was detected at incubation times of 5, 7 and 14 days. *Malassezia* isolated from PV patients and reference strains were used in this study. Tyrosinase activity of *M. furfur* in MM was significantly highest on day 14. Thus, tyrosinase activities of various *Malassezia* spp. will be further investigated by using MM on day 14 of incubation.

บทคัดย่อ

มาลาสซีเซีย (*Malassezia*) เป็นเชื้อประจำถิ่นและมีความสัมพันธ์กับการเกิดโรคเกลื้อน ซึ่งเป็นโรคที่ไม่มีการอักเสบ มีลักษณะรอยโรคเป็น รอยโรคที่เป็นวงด่างสีเข้มหรือวงด่างสีจาง ปัจจุบันกลไกการเกิดโรคเกลื้อนยังไม่ชัดเจน เอนไซม์ไทโรซิเนสเป็นปัจจัยหนึ่งในก่อการโรคของยีสต์ เอนไซม์นี้มีบทบาทในการสังเคราะห์เมลานินซึ่งเกี่ยวข้องกับการเปลี่ยนแปลงสีของผิวหนัง การศึกษานี้พยายามหาอาหารเลี้ยงเชื้อและสภาวะที่เหมาะสมสำหรับตรวจวัดกิจกรรมของเอนไซม์ไทโรซิเนสของเชื้อมาลาสซิเซียชนิดต่างๆ อาหารที่ใช้ทดสอบได้แก่ Minimal medium (MM), MM เสริมด้วย tween 80 (MM+Tween 80) และ mDixon ที่เติมด้วย L-DOPA ถูกใช้ในการปรับเลือกอาหารเลี้ยงเชื้อที่เหมาะสมที่สุด กิจกรรมของเอ็นไซม์ถูกตรวจวัดเมื่อบ่มเป็นเวลา 5, 7 และ 14 วัน *Malassezia* ที่แยกมาจากผู้ป่วยโรคเกลื้อนและสายพันธุ์อ้างอิงถูกนำมาใช้ในการศึกษานี้ เอนไซม์ไทโรซิเนสของเชื้อ *M. furfur* ทดสอบในอาหาร MM ให้ค่าสูงสุดอย่างมีนัยสำคัญในวันที่ 14 ดังนั้นในการตรวจวัดกิจกรรมของเอนไซม์ไทโรซิเนสของเชื้อ *Malassezia* ในครั้งต่อไปจะถูกตรวจวัดโดยใช้อาหาร MM เมื่อบ่มเป็นระยะเวลา 14 วัน

Keywords: *Malassezia*, Tyrosinase enzyme, Pityriasis versicolor

คำสำคัญ: มาลาสซีเซีย เอนไซม์ไทโรซิเนส โรคเกลื้อน

* *Student, Master of Science Program in Medical Microbiology, Faculty of Medicine, Khon Kaen University*

** *Assistant Professor, Department of Microbiology, Faculty of Medicine, Khon Kaen University*

Introduction

Malassezia species are lipophilic yeast, except *Malassezia pachydermatis*. They are common found on skin surfaces of human and animals as microflora or pathogenic organisms, and associated the skin diseases such as pityriasis versicolor (PV), atopic dermatitis, *pityrosporum* folliculitis, psoriasis, seborrheic dermatitis and dandruff and even systemic disease in rare cases (Ashbee, 2007; Ashbee, Evans, 2002; Gaitanis et al., 2013; Stephanie, Michael, 2010). *Malassezia* spp. can be found in both yeast and mycelial forms (Ashbee, Evans, 2002). In 1995, genus *Malassezia* has been identified by using morphology, ultrastructure, physiology and molecular biology by Gueho and coworkers (Gueho et al., 1996). At present *Malassezia* includes 14 species; *M. caprae*, *M. cuniculi*, *M. dermatis*, *M. equine*, *M. furfur*, *M. globosa*, *M. japonica*, *M. nana*, *M. obtusa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, *M. sympodialis* and *M. yamatoensis* (Cabanes et al., 2011). Virulence factors of *Malassezia* yeast include metabolites and lipid metabolism resulting in special cell wall features, enzymatic activities, reactive oxygen species and lipoxygenase. Moreover, azelaic acid can inhibit tyrosinase activity; an enzyme involved melanin synthesis, filament production to evade host cell, and pigmentation (Brand, 2012; Hort, Mayser, 2011).

Malassezia causes pityriasis versicolor, a mild and chronic superficial disease, and commonly found on the trunk and upper aspects of the arms (Faergemann, 2000). PV is one of pigmentation disorder, the lesions characterized by hyperpigmentation or hypopigmentation (Harada et al., 2015; Sunenshine et al., 1998). The colors of skin are pink, tan, brown, or black in hyperpigmentation and white or ashy in hypopigmentation (Imwidthaya et al., 1989). However, the mechanisms of different colors remain unclear. Tyrosinase activity is a part of virulence factor of *Malassezia* yeast and the key enzyme involves in melanin synthesis (Chang, 2009; Hort, Mayser, 2011). In 2013, Youngchim and collaborators reported that *Malassezia furfur* is able to produce melanin-like pigment compound with L-3,4-dihydroxyphenylalanine (L-DOPA) substrate (Mayser et al., 1998; Youngchim et al., 2013). However, the culturing of *Malassezia* was difficult since it required lipid for growth (Benham, 1939). Usually, modified Dixon's medium (mDixon) was used to grow *Malassezia* (Guillot et al., 1998). The activity of tyrosinase of *Malassezia* has hardly been investigated for this reason thus different media and incubation times were tested to select optimal condition for further study. Seymour and collaborators studied tyrosinase activity of foreskins from newborn infant and reported that the mean tyrosinase activity for black infants (0.282 ± 0.186) is higher than for white infants (0.126 ± 0.087) ($P << 0.001$) about two and one-fourth times (Pomerantz, Ances, 1975). Activity of this enzyme is directly involved in melanin synthesis, it may involve in different between hyper- and hypopigmented lesions.

Objectives of the study

This study aimed to optimize the condition and select the medium to investigate tyrosinase activity of *Malassezia* species.

Methodology

Fungal strains

Four isolates of *Malassezia furfur* isolated from pityriasis versicolor patients were obtained from M.Sc. student, Miss. Panwad Tongchai, Microbiology Department, Medical Faculty, Khon Kaen University. The reference

strains of *Malassezia*; *M. furfur* CBS 6001, *M. restricta* NBRC 103918, *M. dermatis* CBS 1969, *M. sympodialis* CBS 8740, *M. japonica* CBS 9432, *M. slooffiae* N14, *M. globosa* 10195, *M. furfur* CBS 7019, *M. furfur* CBS 7019 and *M. pachydermatis* CBS 9592 were included in this study.

Tyrosinase activity assay

The tyrosinase activity was performed by following the method of Hideya with modification for *Malassezia* yeast (Hideya, 1995). *Malassezia* yeast were cultured on mDixon agar and incubated at 32° C for 5 days, then yeast cells were washed 2 times and adjusted to an optical density (OD) of 0.1 (2.5 x 10^7 cells/ml) at 540 nm with spectrophotometer. The 2 ml suspension of yeast inoculum was added to 4 ml minimal medium supplemented with L-DOPA as substrate (MM: 1 l of distilled water; 6 g peptone; 10 ml Tween 40 and 1 mM L-DOPA) or minimal medium with Tween 80 supplemented with L-DOPA (MM+Tween 80: 1 l of distilled water; 6 g peptone; 10 ml Tween 40; 10 ml Tween 80 and 1 mM L-DOPA) or modified Dixon medium supplemented with L-DOPA (mDixon+L-DOPA: 1 l of distilled water; 36 g malt extract; 6 g peptone; 20 g ox bile; 10 ml Tween 40; 2 ml glycerol; 2 ml oleic acid; 20 ml olive oil and 1 mM L-DOPA), then incubated in shaking incubator at 32° C, 150 rpm. Tyrosinase activity was measured on day 5, 7 and 14 of incubation with spectrophotometer at 475 nm. To confirm that the activity did not occur from auto-oxidation of L-DOPA, the negative control was performed by incubating media (MM, MM+Tween 80 and mDixon) supplemented with L-DOPA without yeast cell in same conditions of test.

Results

Tyrosinase activity was detected on different conditions. The incubation times and test media were optimized for optimal condition. The enzyme was measured after incubated in different media at 32° C for 5, 7 and 14 days with spectrophotometer at 475 nm. The results showed that tyrosinase activities of *M. furfur* in minimal medium supplemented with L-DOPA substrate at various incubation times, day 5, 7 and 14 were different. The enzyme activity on day 14 was significantly higher than on day 5 and day 7 (Figure 1). In contrast tyrosinase activity of *Malassezia* tested in mDixon supplemented with L-DOPA at different incubation times was not significantly different and showed very low activity (Figure 2). For selecting optimal medium, not only MM and mDixon but also MM supplemented with tween 80 (MM+Tween 80) were used after incubating for 14 days (Figure 3). The result showed that the activities in all media were not significantly different. But when comparing with negative control (media incubated without *M. furfur*), MM medium and MM+Tween 80 showed significantly higher activity than control. However, tyrosinase activity in mDixon between test with and control without *M. furfur,* were not different (Figure 3). Thus, MM was selected as medium for tyrosinase activity testing at day 14 of incubation and tyrosinase activities of various *Malassezia* species including *M. furfur* isolated from PV patients (P53-5, P57-1, P58-1 and P73-1) were preliminarily investigated (Figure 4). The result showed that various species of *Malassezia* had different tyrosinase activities, however of *M. restricta* NBRC 103918 showed significantly highest and tyrosinase activity of *M. pachydermatis* CBS 9592 was higher secondary to *M. restricta* NBRC 103918.

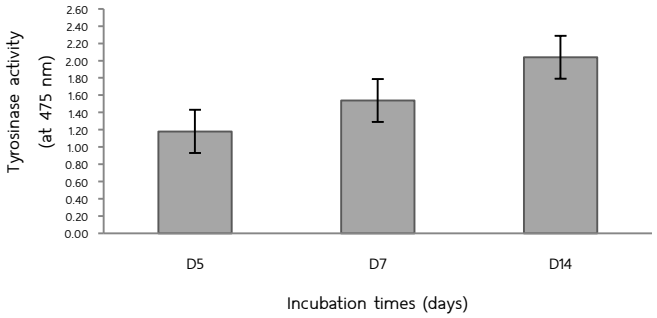

Figure 1 Tyrosinase activity of *M. furfur* isolated from pityriasis versicolor patients in minimal medium (MM) at different incubation times.

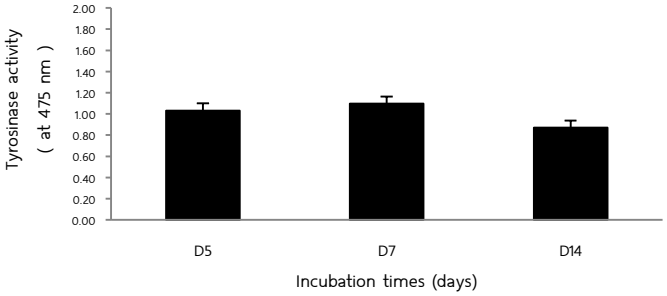

Figure 2 Tyrosinase activity of *M. furfur* isolated from pityriasis versicolor patients in modified Dixon medium (mDixon) at different incubation times.

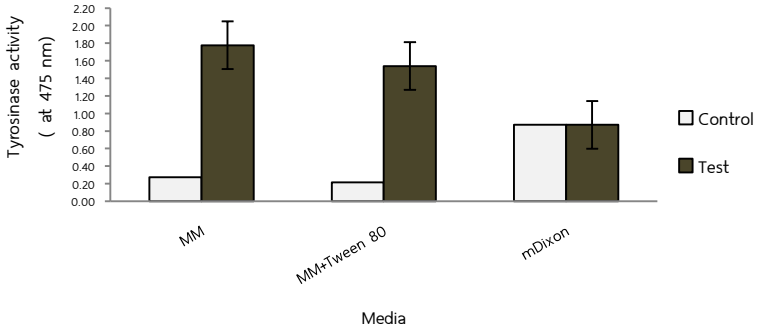

Figure 3 Tyrosinase activity of *M. furfur* isolated from pityriasis versicolor patients in 3 different media on day 14 of incubation. Media without yeast cells were used as negative control. MM; Minimal medium, MM+Tween 80; Minimal medium supplemented with tween 80 and mDixon; modified Dixon medium.

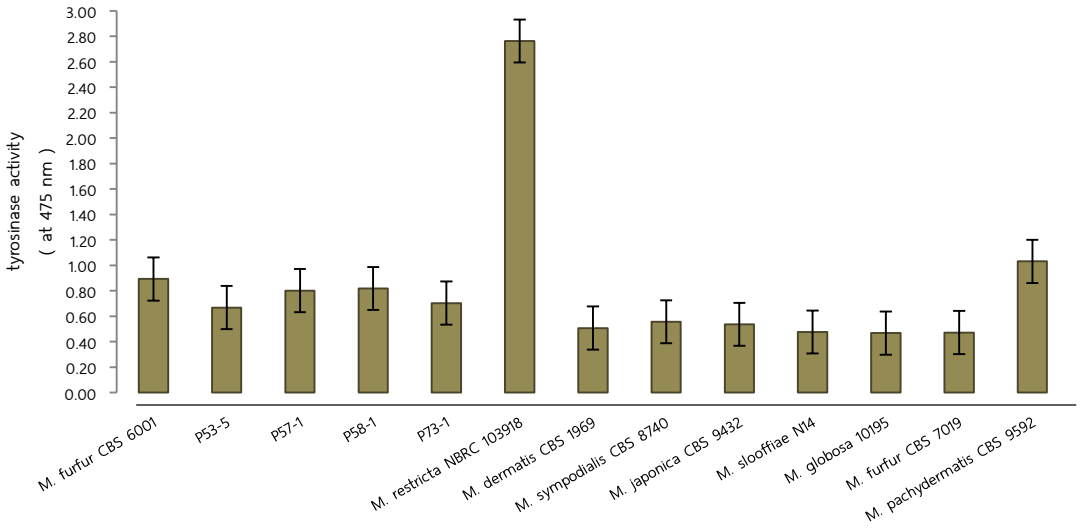

Figure 4 Tyrosinase activity of different *Malassezia* species including *M. furfur* isolated from pityriasis versicolor patients; P53-5, P57-1, P58-1 and P73-1 in minimal medium on day 14 of incubation.

Discussion

Tyrosinase is the enzyme plays role on melanization pathway by converting L-tyrosine or L-DOPA to melanin, this enzyme is present in microorganisms, plants, and animals (Rodriguez-Lopez et al., 1992). *Malassezia furfur* is able to produce melanin or melanin-like compound, which have been reported as a virulence of human fungal pathogen (Hort, Mayser, 2011; Langfelder et al., 2003; Youngchim et al., 2013). This study tried to find out the medium and optimal conditions for tyrosinase activity test of *Malassezia* spp. by using L-DOPA as substrate. We can detect tyrosinase activity of *Malassezia* spp. at all incubation times (5, 7 and 14 days) and in all media, minimal medium (MM), MM supplemented with tween 80 (MM+Tween 80) and mDixon supplemented with L-DOPA by using spectrophotometer at 475 nm. The tyrosinase enzyme of *Malassezia* spp. gradually increasing during a period of incubation times and the activity was highest on day 14. The result correlated with melanization of *M. furfur* yeast cells *in vitro*, colonies of *Malassezia* on medium supplemented with L-DOPA displayed a dark-brown colony and the intensive dark was increased when incubation time was continued (Youngchim, Nosanchuk, Pornsuwan, Kajiwara, & Vanittanakom, 2013). The minimal medium was optimized by adding different lipid substrates, due to *Malassezia* is a lipophilic yeast required lipid for growth (Ashbee, 2007). Tyrosinase activity of *M. furfur* on day 14 in MM was significantly highest. When enzyme activities were compared between test (with *M. furfur*) and negative control (media supplemented with L-DOPA but without *M. furfur*), MM showed distinctly different activities between test and negative control. For tyrosinase test in various species of *Malassezia*, minimal medium supplemented with L-DOPA substrate was used and enzyme activity was detected on day 14 of incubation. Even tyrosinase activity was detected in mDixon medium, but some ingredient in this medium such as olive oil and ox bile may irritate the absorbance. Thus, mDixon was not selected for use in further experiment. For preliminary test, tyrosinase activity of *M. restricta* NBRC 103918 was significantly highest. This result correlated with study of Gaitanis and collaborators that melanin-like pigment in

M. restricta isolated from PV patients with hyperpigmented lesion can be found by Masson-Fontana silver staining but absent in *M. furfur* isolated from PV patients with hypopigmented lesion (Gaitanis et al., 2005).

Conclusions

Tyrosinase activity of *Malassezia furfur* isolated from pityriasis versicolor patients was detected in all incubation times of 5, 7 and 14 days but the activity was highest at day 14. Among minimal medium (MM), MM plus tween 80, and mDixon medium, MM is the best used in tyrosinase activity detection. Thus, MM was selected for preliminary enzyme activity detection in *Malassezia* species after 14 days of incubation and *M. restricta* showed highest tyrosinase activity.

Acknowledgements

The author would like to thank Assoc. Prof. Dr. Sirida Youngchim, Department of Microbiology, Faculty of Medicine, Chiang Mai University who kindly supported of *Malassezia* reference strains, to Miss. Panwad Tongchai for providing *Malassezia* patient isolates, and to Research and Diagnostic Center for Emerging Infectious Diseases (RECIED).

References

Ashbee HR. Update on the genus *Malassezia*. Med Mycol 2007; 45(4): 287-303.

Ashbee HR, Evans EGV. Immunology of Diseases Associated with *Malassezia* Species. Clinical Microbiology Reviews 2002; 15(1): 21-57.

Benham RW. The Cultural Characteristics of *Pityrosporum Ovale*—A Lipophylic Fungus. Journal of Investigative Dermatology 1939; 2(4): 187-203.

Brand A. Hyphal growth in human fungal pathogens and its role in virulence. Int J Microbiol 2012; 2012: 517529.

Cabanes FJ, Vega S, Castella G. *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. Med Mycol 2011; 49(1): 40-48.

Chang TS. An updated review of tyrosinase inhibitors. Int J Mol Sci 2009; 10(6): 2440-2475.

Faergemann J. Management of seborrheic dermatitis and pityriasis versicolor. Am J Clin Dermatol 2000; 1(2): 75-80.

Gaitanis G, Chasapi V, Velegraki A. Novel application of the masson-fontana stain for demonstrating *Malassezia* species melanin-like pigment production in vitro and in clinical specimens. J Clin Microbiol(2005); 43(8): 4147-4151.

Gaitanis G, Velegraki A, Mayser P, Bassukas ID. Skin diseases associated with *Malassezia* yeasts: facts and controversies. Clin Dermatol 2013; 31(4): 455-463.

Gueho E, Midgley G, Guillot J. The genus *Malassezia* with description of four new species. Antonie Van Leeuwenhoek 1996; 69(4): 337-355.

Guillot J, Breugnot C, Barros M, Chermette R. Usefulness of modified Dixon's medium for quantitative culture of *Malassezia* species from canine skin. J Vet Diagn Invest 1998; 10(4): 384-386.

Harada K, Saito M, Sugita T, Tsuboi R. *Malassezia* species and their associated skin diseases. J Dermatol 2015; 42(3): 250-257.

Hideya A. Correlation between the number of melanosome, tyrosinase mRNA levels, and tyrosinase activity in cultured murine melanoma cells in response to various melanogenesis regulatory agents. Koba University Repository: Thesis 1995; 1892: 1-25.

Hort W, Mayser P. *Malassezia* virulence determinants. Curr Opin Infect Dis 2011; 24(2): 100-105.

Imwidthaya P, Thianprasit M, Srimuang S. A study of pityriasis versicolor in Bangkok (Thailand). Mycopathologia 1989; 105(3): 157-161.

Langfelder K, Streibel M, Jahn B, Haase G, Brakhage AA. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol 2003; 38(2): 143-158.

Mayser P, Wille G, Imkampe A, Thoma W, Arnold N, Monsees T. Synthesis of fluorochromes and pigments in *Malassezia furfur* by use of tryptophan as the single nitrogen source. Mycoses 1998; 41(7-8): 265-271.

Pomerantz SH, Ances IG. Tyrosinase activity in human skin. Influence of race and age in newborns. J Clin Invest 1975; 55(5): 1127-1131.

Rodriguez-Lopez JN, Tudela J, Varon R, Garcia-Carmona F, Garcia-Canovas F. Analysis of a kinetic model for melanin biosynthesis pathway. J Biol Chem 1992; 267(6): 3801-3810.

Stephanie HW, Michael B. Pityriasis Versicolor. Arch Dermatol 2010; 146(10): 1132-1140.

Sunenshine PJ, Robert SA, Camila JK. Tinea versicolor. International Journal of Dermatology 1998; 37: 648-655.

Youngchim S, Nosanchuk JD, Pornsuwan S, Kajiwara S, Vanittanakom N. The role of L-DOPA on melanization and mycelial production in *Malassezia furfur*. PLoS One 2013; 8(6): e63764.