IJERSD
INTERNATIONAL JOURNAL OF EDUCATION, RESEARCH AND SCIENTIFIC DEVELOPMENT
https://www.ijresd.org Print: 2992-5665 and Online: 2992-5673
Impact Factor: 5.2 Vol 4, Issue 2 December 2 2023

ASSAY OF EXTRACELLULAR ENZYMES OF DERMATOPHYTES AND NON-DERMATOPHYTES ISOLATED FROM RICE FARMERS IN EBONYI STATE, NIGERIA

***Nnagbo, P. A., *Nwachukwu, I. O., Nwachukwu, M. I and Akabogu, O.I**
***Imo State University, Owerri, Imo State, Nigeria**

Abstract

Dermatophytes and non-dermatophytes are the major cause of cutaneous mycoses. The infection is highly contagious and represent significant public health problem in Nigeria and the world at large. These groups of fungi invade and grow in dead keratin. They are capable of producing several enzymes for their survival within a wide range of substrates and causing severe reactions. The ability of dermatophyte and nondermatophyte fungal species isolated from rice farmers in parts of Ebonyi State to secrete extracellular enzymes was screened on solid media. This was determined by their ability to develop clear zone formation. Highest keratinase (23 mm), protease (24 mm), lipase (22 mm) and collagenase (17 mm) activities were observed in *Trichophyton rubrum* while the highest xylanase (17 mm) and cellulase (22 mm) activities were observed in *Trichophyton tonsurans* and *Trichophyton mentagrophytes* respectively. Of the nondermatophytes isolated, highest keratinase (15 mm), protease (16 mm), lipase (18 mm), xylanase (11.5 mm) and cellulase (14 mm) activities were observed in *Aspergillus flavus* while the highest collagenase (10.5 mm) activity was observed in *Aspergillus nomius*. The ability of these fungal organisms to produce these enzymes could explain their ability to degrade the keratinized tissues of the skin, hair and nails, indicating the importance of these enzymes in the pathogenesis of cutaneous mycoses.

Keywords: cutaneous mycoses, contagious, keratin, extracellular enzymes, public health

Introduction

Cutaneous mycosis is one of the most common fungal infections and is caused by filamentous keratinophilic fungi called dermatophytes and some fungi that use keratin as nutrient source during skin, hair and nail infection. Keratin is a fibrous protein molecule of high molecular weight, rich in cysteine, whose disulfide bridges and acetamide bonds guarantee its stability. This protein is produced by humans and other animals and is the main component of skin,

nails and shells, having the function to protect and cover (Fraser and Parry, 2005).

Dermatophytic and nondermatophytic fungi secrete varieties of enzymes that have different substrate specificities such as keratinases, protease, lipase and cellulase. These fungal pathogens secrete these enzymes to obtain the nutrients to develop and survive. The macromolecules that are present in the host tissue are used as a source of carbon, nitrogen, phosphorus and sulfur for dermatophytes and associated fungi (Peres *et al*., 2010). The keratinases secreted by these fungal pathogens catalyse the degration of keratin present in the host tissue into oligopeptides or amino acids that may be then assimilated by the fungi. Moreover, it had been suggested that released enzymes from these fungal pathogens also act as antigens and induce various degrees of inflammation (Jensen *et al*., 2007).

Among the wide variety of enzymes secreted by dermatophytes and nondermatophytes, protease enzymes are the most studied and are the major enzymes from dermatophytes and associated fungi involved in invasion and utilization of the stratum corneum of the host (Liu *et al*., 2014). It has been suggested that these pathogens secrete proteases in response to the presence of the components of the skin during tissue invasion. Some authors suggest that these pathogens secrete proteases to facilitate and are even necessary for an efficient adhesion of these pathogens to the host tissue. Furthermore, secreted proteases from these pathogens also trigger immune response (Peres *et al*., 2010). Although protease enzymes from these pathogens were initially studied, other enzymes apart from proteases also needed to be studied. The aim of this study was to screen fungal pathogens associated with cutaneous mycoses among rice farmers in parts of Ebonyi State for the production of extracellular enzymes.

Materials and Methods

Collection and Processing of test fungi

The test fungal organisms were isolated from rice farmers examined for cutaneous mycoses in Ebonyi State. The test fungi were maintained on potato dextrose agar (PDA) slants at 25^{o}C. The spore suspensions were prepared by adding 10ml of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop. The dermatophyte species included *Trichophyton tonsurans*, *T. mentagrophytes, T. rubrum, T. soudanense, Microsporum gypseum* and *M. canis* while non-dermatophyte species included *Aspergillus flavus*, *A. tamarii* , *A. nomius* and *Candida albicans.*

Screening of Extracellular Enzymes

1. Keratinase Production

The modified method of Wawrzkiewicz *et al*. (1991) was used to screen the test fungi for keratinase production on mineral salt media. Keratin azure (Sigma, USA) was used in place of chicken feathers as a source of keratin. Four (4) mg finely chopped keratin azure was suspended in 1 ml dimethylsulphoxide (DMSO) and the resulting suspension was added to 200 ml mineral salt medium. 15ml of this medium was dispensed aseptically into Petri dishes. The plates were inoculated with spore suspension of the test fungi and incubated at 25ºC for 7 days. All test fungi were assayed in duplicate. Keratinase activities of the test fungi were detected as clear zones around the colonies. The diameter of clear zone was measured to quantify activity.

2. Protease Production

The test was performed using the technique described by Vijayaraghavan and Vincent (2013). A 200 ml PDA incorporated with 10 g casein (Sigma, USA) as protein substrate was sterilized and dispensed into sterile Petri dishes. The plates were inoculated with spore suspension of the test fungi and incubated at 25ºC for 7 days. Colonies which showed clear zones around them indicated protease production. All test fungi were assayed in duplicate.

3. Lipase Production

Screening for lipase production was carried out using the gel diffusion assay described by Rua *et al.* (1993). Ten (10) µl of spore suspension of the test fungi was inoculated at the center of a 20ml PDA media incorporated with 4ml of olive oil emulsion as lipid substrate. The olive oil emulsion consisted of 25% of olive oil and 75% of a 10% (w/v) gum arabic (Plus Chem., India) solution in water, mixed in a homogeneizer on an ice-bath for 15min. The plates were incubated at 25°C for 7 days. A clear zone of hydrolysis beneath and around the colony indicated a positive lipase activity.

4. Xylanase Production

The screening of xylanase enzyme was carried out as described by Dhulappa and Lingappa (2013). Ten plates containing PDA were added oat meal xylan 1% (v/v) as carbon source and inoculated with mycelia and spores of the test fungi. Crude xylan was recovered from breakfast oat meal purchased from a grocery store. The plates were incubated at 25ºC for 7 days. Colonies which showed clear zones around them were considered positive, regarding to xylanase enzyme production.

5. Cellulase Production

The test fungi were subjected to screening for their cellulolytic properties using the

plate assay method of Whitaker *et al.* (2002). Ten (10) µl of spore suspension of each isolate was inoculated onto the center of PDA incorporated with 1% carboxylmethyl cellulose (CMC; Sigma) as carbon source. The plates were incubated at 25°C for 7 days before flooding with 0.4% Congo red and observed for lysis. The hydrolysis zones on the plate media were measured and recorded.

6. Collagenase Production

The ability of the test fungi to synthesize collagenase was screened on agar plates containing 20 g/L of peptone agar and 10 g/L of chicken sternal cartilage collagen as substrate. This method is a modification of the method described by Cooper and Davidson (1965) and it differs in the use of chicken sternal cartilage collagen as substrate instead of calf skin collagen. Chicken sternal cartilage collagen was prepared and kept in 0.47 M acetic acid at 4°C. Prior to use, the collagen was clarified by centrifugation and dialysed against 0.1 M sodium phosphate buffer, pH 7.6. The collagen was poured over the surface of peptone agar plates and allowed to gel. The plates were inoculated with the fungal strain and incubated at 25°C for 7 days. Collagenase-positive colonies were identified by a zone of hydrolysis around the colonies.

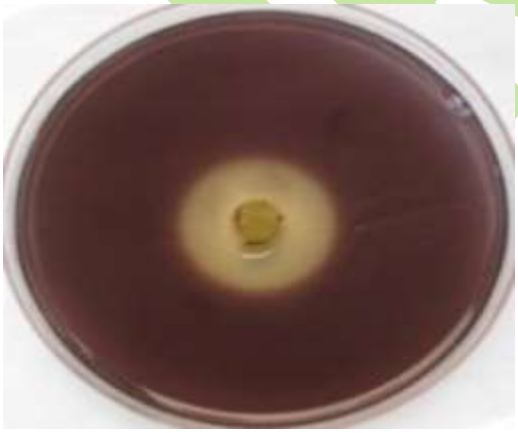
Statistical Analysis

Statistical computation was done using Statistical Package for Social Science (SPSS) version 22 for windows.

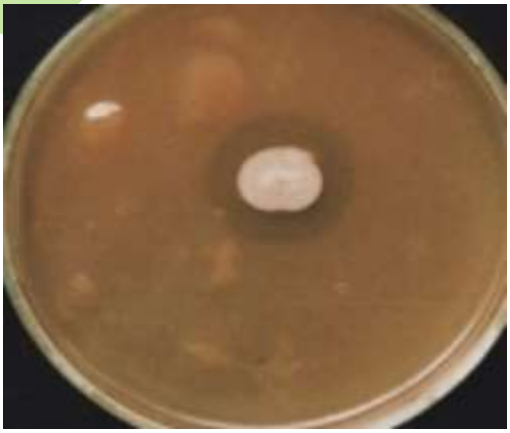
Results

The ability of the test fungi to secrete extracellular enzymes was screened on solid media (Figure 1). Highest keratinase activity (23 mm) was observed in *T. rubrum,* followed by *T. tonsurans, T. mentagrophytes* and *M. gypeum* (22 mm). *T. soudanense* recorded the least keratinase activity (16 mm) as enumerated in Figure 2. Highest protease activity (24 mm) was observed in *T. rubrum,* followed by *M. canis* (23 mm). Protease activity recorded for *T. mentagrophytes* was the least (16 mm). The highest lipase activity of 22 mm was recorded for *T. rubrum,* followed by *T. tonsurans* (21 mm). The least activity was observed in *M. canis* (15 mm). In screening dermatophyte species for their xylanase activity, the figure shows that *T. tonsurans* had the highest xylanase activity of 20 mm, followed by *T. soudanense* (18 mm). *T. mentagrophytes* and *T. rubrum* had the least xylanase activity (16 mm). *T. mentagrophytes* and *T. rubrum* had the highest (22 mm) and least (10 mm) cellulase activity respectively. The collagenase

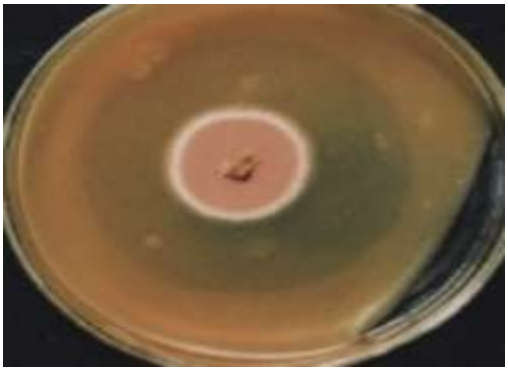
activity of 17 mm recorded for *T. rubrum* was the highest and the least was 14 mm for *M. canis*. Highest keratinase, protease, lipase and collagenase activities were observed in *T. rubrum* while the highest xylanase and cellulase activities were observed in *T. tonsurans* and *T. mentagrophytes* respectively. In screening non-dermatophyte species for their ability to secrete extracellular enzymes, Figure 3 shows that *A. flavus* produced the highest keratinase enzyme (15 mm) followed by *A. nomius* (10 mm). *C. albicans* produced the least keratinase (5 mm). *A. flavus* and *A. nomius* had the highest (16 mm) and least (10 mm) protease activity respectively. The lipase activity of 18 mm recorded for *A. flavus* was the highest, followed by 10 mm for *A. tamarii* and the least was 5.5 mm for *A. nomius*. Highest xylanase activity (11.5 mm) was observed in *A. flavus*, followed by *A. tamarii* and *C. albicans* (10 mm). Xylanase activity recorded for *A. nomius* was the least (8.5 mm). The highest cellulase activity of 14 mm was obtained for *A. flavus*, followed by *C. albicans* (10 mm). The least activity was observed in *A. nomius* (5 mm). *A. nomius* produced the highest collagenase (10.5 mm) while *A. tamarii* produced the least collagenase (6 mm). Highest keratinase, protease, lipase, xylanase and cellulase activities were observed in *A. flavus* while the highest collagenase activity was observed in *A. nomius*. The results of this study showed that the isolated cutaneous mycotic fungi produced keratinase, protease, lipase, xylanase, cellulase and collagenase virulence enzymes.



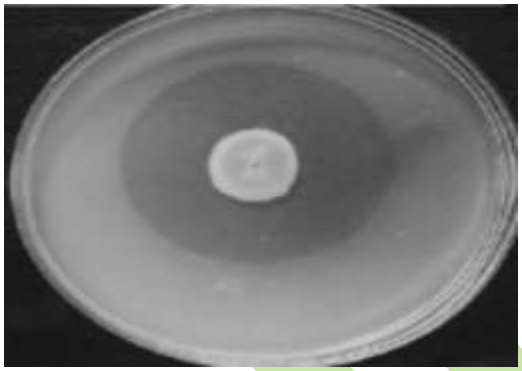
Keratinase secretion by *A. flavus*



Protease secretion by *T. mentagrophytes*

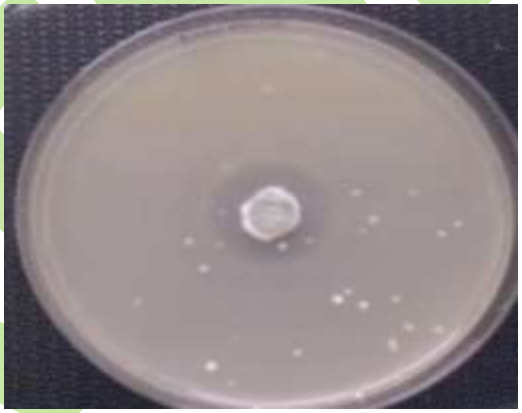


Lipase secretion by *T. rubrum*



Xylanase secretion by *T. tonsurans*

Cellulase secretion by *Candida albicans*



Collagenase secretion by *M. gypseum*

Figure 1: Extracellular enzyme activities of test fungi on solid media showing zones of clearance

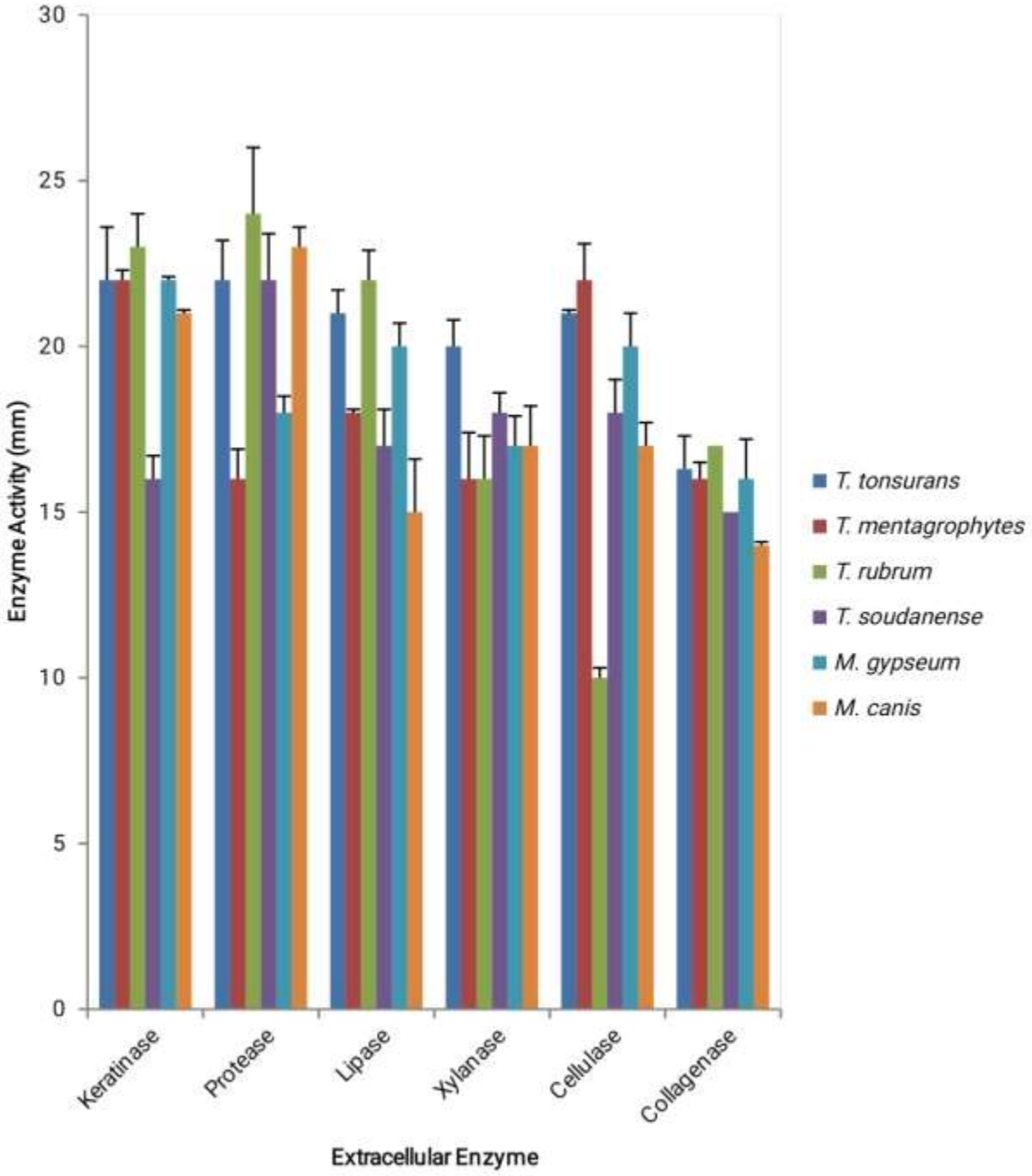


Figure 2: Relative enzyme activities of dermatophyte species on solid media

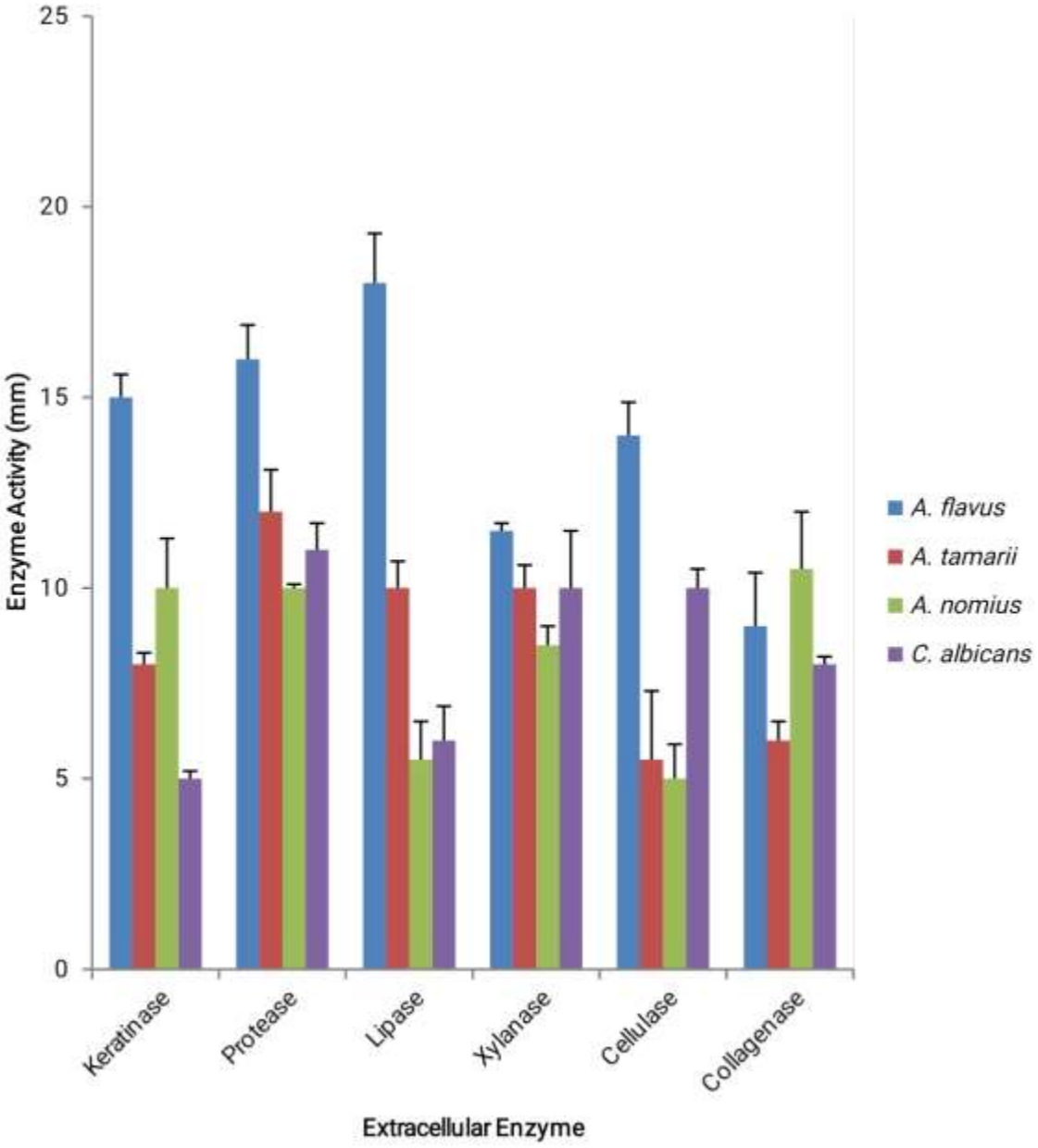


Figure 3: Relative enzyme activities of non-dermatophyte species on solid media

DISCUSSION

Dermatophytes and other fungi are the major cause of cutaneous mycoses (Weitzman and Summerbell, 1995). These fungi have the capacity to invade keratinized tissue of humans or animals to produce infections that are generally restricted to the corneocytes of the skin, hair and nails. The keratinolytic activity of keratinophilic fungi is important for ecology and has attracted many researchers' attention around the world. Keratinophilic fungi play an important role in the natural degradation of keratinized residues in the soil (Fillipello, 2000). Some of these fungi live in soil and could be transmitted to humans as well as animals and cause cutaneous mycotic infection (Spiewak and Szostak, 2000). The results of this study showed that the isolated dermatophytes and associated fungi produced keratinase, protease, lipase, xylanase, cellulase, and collagenase enzymes. Highest keratinase activity was observed in *T. rubrum* and *A. flavus*. In a similar study by Sharma *et al*. (2012), *T. rubrum* produced the highest activity of keratinase that concurs with our findings. Contrarily, in another study by Muhsin and Salih (2001), high keratinase activity was expressed by *T. mentagrophytes* and *M. gypseum*. They also showed high protease activity that was contrary to our result. In overall, the difference in extracellular enzyme activities in the different fungal species was shown to be statistically significant. The ability of these organisms to produce the above mentioned enzymes could explain their ability to degrade the keratinized areas of the skin, indicating the importance of these enzymes in the pathogenesis of cutaneous mycoses.

Conclusion and Recommendations

Our results indicated the importance of keratinase, protease, lipase, xylanase, cellulase, and collagenase enzymes in the pathogenesis of cutaneous mycoses. An in vivo study of adherence and invasion of cutaneous mycotic fungi to the stratum corneum would be further studied. The study will contribute to a better understanding of the nature of the interaction between dermatophytes and associated fungi and skin cells in cutaneous mycoses process. In addition, future studies to characterize these enzymes and the sequences of their DNA where they are produced is necessary for the proper understanding of how these fungi behave during infection of their hosts as well as its function as saprophytes in the natural environment.

References

Cooper, D. R. and Davidson, R. J. (1965). The effect of ultraviolet irradiation on soluble collagen. *Biochemical Journal* 97: 139-147.

Dhulappa, A. and Lingappa, K. (2013). Xylanase screening and biosynthesis from *Aspergillus tamarii*. *International Journal of Current Microbiology and Applied Science* 2(7): 79-83.

Fillipello, M. V. (2000). "Keratinophilic fungi: their role in nature and degradation of keratinic substrates," In: *Biology of Dermatophytes and Other Fungi*, Kushawaha, R. K. S. and Guarri, J. (Eds.), Revista Iberoamericana de Micologia, Bilbao, Spain. p. 77–85.

Fraser, R. D. and Parry, D. A. (2005). The three-dimensional structure of trichocyte (hard alpha) keratin intermediate filaments: features of the molecular packing deduced from the sites of induced crosslinks. *Journal of Structural Biology* 151: 171-81.

Jensen, J. M., Pfeiffer, S., Akaki, T., Schröder, J. M., Kleine, M., Neumann, C., Proksch, E. and Brasch, J. (2007). Barrier function, epidermal differentiation and human β-defensin 2 expression in Tinea corporis. *Journal of Investigative Dermatology* 127: 1720-7.

Muhsin, T. M. and Salih, T. H. (2001). Exocellular enzyme activity of dermatophytes and other fungi isolated from ruminants in Southern Iraq. *Mycopathologia* 150: 49-52.

Peres, N. T. A., Maranhão, F. C. A., Rossi, A. and Martinez-Rossi, N. M. (2010). Dermatophytes: Host-pathogen interaction and antifungal resistance. *Annals of Bras Dermatology* 85: 657-67.

Rua, M. L., Diaz-Maurino, T., Fernandez, V. M., Otero, C. and Ballesteros, A. (1993). Purification and characterization of two distinct lipases from Candida cylindracea. *Biochimica et Biophysica Acta, 1156: 181-189.*

Sharma, A., Chandra, S. and Sharma, M. (2012). Difference in keratinase activity of dermatophytes at different environmental conditions is an attribute of adaptation to parasitism. *Mycoses* 55: 410-415.

Spiewak, R. and Szostak, W. (2000). Zoophilic and geophilic dermatophytoses among farmers and non-farmers in eastern Poland. *Annals of Agricultural Environmental Medical* 7(2): 125-129.

Vijayaraghavan, P. and Vincent, S. G. P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. *Journal of Biochemical Techniques* 4(3): 628-630.

Wawrzkiewicz, K., Wolski, T. and Lobarzewski, J. (1991). Screening the keratinolytic activity of dermatophytes in vitro. *Mycopathologia* 114:1-8.

Weitzman, I. and Summerbell, R. C. (1995). The dermatophytes. *Clinical Microbiology Review* 8: 240-59.

Whitaker, J. R., Voragen, A. G. J. and Wong, D. W. S. (2002). *Handbook of Food Enzymology*, Routledge, USA, p. 879-886.