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Empirical analysis of amylolytic and proteolytic activities of microbial isolates recovered from deteriorating painted wall surfaces in Lagos Nigeria

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Abstract

The biodeterioration of painted walls have been associated with several biological mechanisms such as organic acid production and enzymatic activity of microorganisms amongst other factors. Therefore, this study aims to reveal the involvement of amylases and proteases from indigenous microbes on biodeteriorating painted walls. Microbial strains isolated from biodeteriorating painted walls of selected buildings in Lagos, Nigeria and previously characterized as belonging to the genera *Pseudomonas, Candida, Fusarium, Aspergillus, Cerrena and Meyerozyma* were used in this study. Amylolytic and proteolytic activities at varying conditions of temperature, pH, incubation time and substrate concentrations were tested. To bridge the knowledge gaps regarding statistical quantification of enzymatic mechanisms in biodeterioration, the Wilcoxon signed rank sum test was used to test the hypothesis that amylolytic/proteolytic activities are equal at all conditions tested. The conditions for optimal activity were observed to be 24h, 37°C, pH 2 and 0.01% substrate concentration and 48h, 25°C, pH 2, and 1% substrate concentration for amylase and protease respectively. Wilcoxon signed rank test revealed that amylolytic and proteolytic activities do not impact aesthetics on painted walls equally at all environmental conditions considered.

INTRODUCTION

Biodegradation of surface coatings and painted walls is occasioned most importantly because microorganisms utilize any class of material as substrate and have very simple requirements for proliferation (Allsopp *et al.*, 2003). Some microorganisms are also particularly very amenable to horizontal gene transfer which ehances their adaptation to different environments. This results in production of a wide variety of enzymes, such as amylases, and proteases that facilitates their gross biodeterioration activity (Rojas *et al.*, 2008). Microorganisms derive carbon sources as nutrients from the different organic constituents of paints which help to stimulate their growth in liquid and applied paints. The resulting effect is a loss of aesthetics of the paint surface facilitated by discoloration, reduced adhesion and detachment of the paint layer (Da Silva, 2003). Microbial enzymes therefore, remain an important cause of discoloration (Gaylarde and Morton, 2001). Amylases are an important group of enzymes involved in the hydrolysis of polysaccharides such as starch into simple sugar components (Mitchell and Lonsane, 1990; Akpan *et al.*, 1990a; Wahlstron and Suurnakki, 2015). Their hydrolytic properties lead to breakdown of glycosidic linkages of paint starch components into products such as maltose, glucose and maltotriose units which are of low molecular weights (Raini *et al.*, 2016). Fungi belonging to the genus *Aspergillus* have been most associated with the production of α-amylase (Shivaramakrishnan *et al.*, 2006). Microbial proteases are also of great commercial importance (Gupta *et al.*, 2002; 2005). For centuries, proteinaceous substances such as glues including albumin, casein and animal gelatins have been used in the paint industry as binding media in paintings to bind pigments and to protect the outer layer of finished paintings (Peris-Vicente *et al.,* 2005). Rojas *et al.,* 2012. reported the ability of *Aspergillus* strains, *Penicillium chrysogenum, P. glabrum*, and *Cladosporium oxysporum* to degrade such protein-based components in paints.

In particular, proteases of *Aspergillus* species have been extensively studied due to the capacity of *Aspergillus* species to secrete high levels of these enzymes (Biesebeke *et al.*, 2006). Peptidases are involved in breaking either specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein, or the breaking down of a complete peptide to amino acids (unlimited proteolysis) (Hooper, 2002). Peptidase activity may be an activation of a function, a signal in a signaling pathway or a destructive change abolishing a protein's function or digesting it to its principal components (Hooper, 2002). This study aims at elucidating the involvement and impact of proteases and amylases on aesthetics of painted walls from a non-parametric point of view.

MATERIALS AND METHODS

Microbial isolates and culture conditions

In this study, microbial strains isolated from biodeteriorating painted walls of selected buildings in Lagos, Nigeria previously characterized as belonging to the genera *Pseudomonas, Candida, Fusarium, Aspergillus, Cerrena and Meyerozyma* were used. Amylolytic and proteolytic activities at varying conditions of temperature, pH, incubation time and substrate concentrations were tested simultaneously. To bridge the knowledge gaps regarding statistical quantification of enzymatic mechanisms in biodeterioration, the Wilcoxon Signed Rank Sum test was used to test the hypothesis that amylolytic/proteolytic activities are equal at all conditions tested.

The following microorganisms which include: *Pseudomonas aeruginosa* CH01 (KY511067.1), *Meyerozyma caribbica* CBS:5674 (KY104219.1), *Meyerozyma guillermondii* MB14B1 (LT615287.1), *Cerrena* sp. N10CC2a (FJ010208.1), *Candida tropicalis* UZ31_13 (KM361510.1), *Aspergillus* sp. SL2 (KC178662.1), *Aspergillus aculeatus* A1.9 (EU833205.1) and *Fusarium proliferatum* 2705 (EU272509.1) isolated from discolored painted buildings and submitted to GenBank in an earlier study (Obidi and Okekunjo, 2017) were used. The bacterial isolates were cultured in nutrient broth (Sigma-Aldrich) containing yeast extract, 3 g/litre; glucose, 1g/litre; peptone, 15g/litre; and NaCl, 6g/litre at 37°C while the fungal isolates were suspended in Sabouraud Dextrose broth (Sigma-Aldrich) containing peptone 10g/litre and dextrose 20g/litre. Inoculum was standardized with 0.5 McFarland standard (1.5 x 10^8 CFU/mL) and microbial growth monitored at 600 nm

(OD$_{600}$) with UV Spectrophotometer (Microfield SM23A, Microfield Instrument England).

Enzyme production

Nutrient broth and Potato dextrose broth (300ml each) were sterilized and dispensed into 500ml conical flasks together with 5g of starch (for amylase) and 5g of casein (for protease). The nutrient broth was used for *Pseudomonas aeruginosa* CH01 (KY511067.1) while the potato dextrose broth was used for *Meyerozyma caribbica* CBS:5674 (KY104219.1), *Meyerozyma guillermondii* MB14B1 (LT615287.1), *Cerrena* sp. N10CC2a (FJ010208.1), *Candida tropicalis* UZ31_13 (KM361510.1), *Aspergillus* sp. SL2 (KC178662.1), *Aspergillus aculeatus* A1.9 (EU833205.1) and *Fusarium proliferatum* 2705 (EU272509.1) respectively. Standardized suspension (1.5x10^8 CFU/mL) of the culture organisms were then inoculated into each of the flasks, plugged with non-adsorbent cotton wool and incubated at 37°C and 25°C for 24h and 5d respectively in an incubator shaker at 150rpm. At the end of the fermentation period, the culture medium was centrifuged at 1500 rpm for 20min to obtain the crude extract.

Enzyme activity

Amylase activity was assayed following the method of Bernfeld (1995) using 3, 5-dinitrosalicylic acid. The activity was assayed by measuring the reducing sugar formed by the enzymatic hydrolysis of soluble starch. The reaction mixture containing 1 ml of 1% (w/v) soluble starch in citrate phosphate buffer (pH 6.5) and 1 ml cell-free enzyme extract was incubated at 40^{0}C for 30 minutes. The reaction was terminated by addition of 2 ml of dinitrosalicylic acid (DNS) reagents. The reaction mixture was then heated for 5 min in boiling water bath and absorbance was read at 540 nm to estimate reducing sugars released. Subsequently, the solution's optical densities were read against the specific sample blank (glucose for amylase and tyrosine for protease assay) using UV Spectrophotometer (Microfield SM23A, Microfield Instrument England).

Protease activity was determined as described by Chopra and Mathur (1985). The reaction mixture which contained 5 ml of casein in 50 mM of Tris buffer (pH 8.0) and an aliquot of 1.0 ml of the enzyme solution was incubated for 30 min. The reaction was stopped by adding 5 ml of trichloroacetic acid solution (TCA) (0.11M). After 30 minutes, the mixture was filtered, 2ml of the filtrate was then added to 5.0 ml of 0.5 M sodium carbonate and 1.0 ml of Folin-Ciocalteu's phenol reagent and kept for 30 min at a temperature of 37°C. Absorbance was read at 660nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per ml per min under assay conditions.

Optimization for enzyme production

Incubation time: The experiment was carried out for each organism after leaving the culture extract at various incubation periods; 24, 48 and 96h. The pH was left constant at 7, temperature at 25°C and substrate concentration at 1%. The enzyme assay was done after 24, 48 and 96h of incubation.

Temperature: The effect of temperature on enzyme production was studied after incubation of the culture extract at various temperatures of 15, 25 and 37°C. The pH was made constant at 7, incubation time was 24h and substrate concentration was 1%. The enzyme assay was carried out after 24h of incubation.

pH: The effect of pH on amylase and protease production was determined by subjecting the culture extract to different pH levels. The experiment was carried out for each organism at pH 2, 6, and 9 while incubation time was held constant at 24h, temperature at 25°C and substrate concentration at 1%. The enzyme assay was carried out after 24h of incubation.

Substrate concentration: The optimal production of amylase and protease by the selected experimental microorganisms was determined by the addition of starch and casein to the culture extract at different substrate concentrations which are 0.01, 0.1 and 1%. The pH was held constant at 7, temperature at 25°C and incubation time was 24h. The enzyme activity was measured after 24h of incubation. Subsequently, glucose and tyrosine standard curves (Figures not shown) were plotted to calculate the reduced sugar and tyrosine concentrations for amylase and protease respectively.

Data management and statistical analysis

Data management and statistical data analysis for this study was carried out with the use of R software (R Core Team, Version 4.1.1). Descriptive statistics such as boxplots and line charts were used to summarize the relative levels of amylase and protease production with respect to various conditions. Wilcoxon Signed Rank Test was used to investigate whether amylolytic and proteolytic activities impact aesthetics on painted walls equally. All tests were carried out at 5% level of significance.

RESULTS

The eight isolates used in this study which were represented as A, B, C, D, E, F, G, H in the graphs include *M. guillermondii* MB14B1 (LT615287.1), *C. tropicalis* UZ31_13 (KM361510.1), *P.aeruginosa* CH01 (KY511067.1), *Cerrena* sp. N10CC2a (FJ010208.1), *F. proliferatum* 2705 (EU272509.1), *A. aculeatus* A1.9 (EU833205.1), *Aspergillus* sp. SL2 (KC178662.1) and *M. caribbica* CBS:5674 (KY104219.1) respectively. They produced amylase and protease at varying conditions. The results showed that of the microorganisms (*M. guilliermondii, F. proliferatum, Aspergillus sp, Cerrena sp* and *M. caribbica*) had optimum production of amylase at 24h while *C. tropicalis, A. aculeatus* and *P. aeruginosa*) had optimum production at 48h (Fig 1A). *M. guillermondii, M. caribbica, F. proliferatum, A. aculeatus, Aspergillus sp, Cerrena sp* and *C. tropicalis* had optimum production of amylase at pH 2 while *P. aeruginosa* had the optimum production of amylase at pH 6 (Fig 1B). *P. aeruginosa, Cerrena sp., C. tropicalis* and *F. proliferatum* had the optimum production of amylase at 37^0C, *M. caribbica and A. aculeatus* had optimum amylase production at 25^0C while *M. guillermondii* and *Aspergillus* sp had optimum amylase production at 15^0C respectively (Fig1 C). *M. guillermondii, M. caribbica and Cerrena sp.* had optimum amylase production at 0.1% substrate concentration while *P. aeruginosa, C. tropicalis* and *Aspergillus* sp had the optimum production of amylase at 0.01% substrate concentration. *A. aculeatus and F. proliferatum* however, had optimum amylase at

1% substrate concentration (Fig 1D). For protease production, *M. guillermondii, F. proliferatum, P. aeruginosa, Aspergillus sp.* and *A. aculeatus* had the optimum production of protease in 48hours while *C. tropicalis, M. caribbica* and *Cerrena sp* had the optimum production of protease at 96 hours respectively (Fig 2A). *M. guillermondii, C. tropicalis, P. aeruginosa, Cerrena sp, F. proliferatum and M. caribbica* had the optimum production of protease at pH 2 respectively. *A. aculeatus* and *Aspergillus sp* had the optimum production of protease at pH 6 (Fig 2B). Also, *C. tropicalis and Cerrena sp.* had the optimum production of protease in 15^0C while *M. guillermondii, P. aeruginosa, F. proliferatum, A. aculeatus, Aspergillus sp* and *M. caribbica* had optimum production of protease in 25^0C (Fig. 2C).

P. aeruginosa and F. proliferatum had the optimum production of protease at 0.01% substrate concentration while *M. guillermondii, C. tropicalis, A. aculeatus, Aspergillus sp, Cerrena sp, and M. caribbica* had optimum protease production at 1% substrate concentration (Fig. 2D).

Optimization for enzyme production

Incubation time: Result of the effect of incubation time revealed that optimum amylase production was obtained at 24h for 62.5% (5) of the microorganisms while 37.5% (3) showed optimum production at 48 hours. Proteolytic activity was observed to be optimum at 48h for 62.5% (5) of the microorganisms while others (37.5%) (3) showed optimum production at 96 h (Fig. 1A and 2A).

pH: Amylolytic activity was optimum at pH 2 (87.5%) and 6 (12.5%) for all the microorganisms. The effect of pH showed optimum protease activity at 2 for 6 of the microorganisms (75%) and two of the organisms (25%) had optimum pH of 6 (Fig. 1B and 2B).

Temperature: 37.5% of the microorganisms showed optimum amylase activity at 37^oC. Also, 37.5% (3) showed optimum amylase activity at 15^oC while 25% (2) of the microorganisms had optimum amylase activity at 25^0C. Protease activity was optimal at 25^oC for 75% (6) of isolates while others (25%) showed optimum protease activity at 15^0C (Fig. 1C and 2C).

Substrate concentration: The quantity of substrate plays a great role in enzyme fermentation. All the microorganisms had different optimum amylase activity which are 0.01% (37.5%), 0.1% (37.5%) and 1% (25%). For protease, 75% (6) had 1% substrate concentration as optimum while 25% (2) of the isolates had 0.01% optimum substrate concentration (Figs. 1D and 2D).

Results of Wilcoxon rank sum test

In this section, we present the results obtained from the various tests of hypotheses to test whether enzyme activities are equal at the various environmental conditions considered at 5% significant levels.

Hypothesis one.

H_0: enzyme (amylase and protease) activities are equal at the time levels (24h, 48h and 96h).

H_1: enzyme (amylase and protease) activities are not equal at the time levels (24h, 48h and 96h).

Figure 3A shows the boxplot of the distribution of amylase and protease at the various times. It reveals that the location parameter (median) of the two enzymes is not the same.

To verify this difference, Wilcoxon signed rank test result (W = 559, p-value = 2.434e-08) showed that amylase and protease activities are not equal at the time levels (24hours, 48hours and 96 hours).

Hypothesis two.

H_0: enzyme (amylase and protease) activities are equal at the temperature levels (15°C, 25°C and 37°C).

H_1: enzyme (amylase and protease) activities are not equal at the temperature levels (15°C, 25°C and 37°C).

To verify this variation statistically, Wilcoxon Signed Rank Test result (W =576, p-value = 2.891e-09) reveals that enzyme (amylase and protease) activities are not equal at the temperature levels (15°C, 25°C and 37°C) since the p-value is greater than 0.05.

Hypothesis three.

H_0: enzyme (amylase and protease) activities are equal at the substrate levels (0.01%, 0.1% and 1%).

H_1: enzyme (amylase and protease) activities are not equal at the substrate levels (0.01%, 0.1% and 1%).

To verify this hypothesis, Wilcoxon Signed Rank Test result (W = 569.5, p-value = 6.797e-09) showed that enzyme (amylase and protease) activities are not equal at the substrate levels (0.01%, 0.1% and 1%).

Hypothesis four

H_0: enzyme (amylase and protease) activities are equal at different pH levels (2, 6 and 9).

H_1: enzyme (amylase and protease) activities are not equal at pH levels (2, 6 and 9).

To verify this difference statistically, Wilcoxon Signed Rank Test result (W = 576, p-value = 3.04e-09) showed that the test is significant. We therefore reject H_0 and conclude that enzyme (amylase and protease) activities are not equal at the pH levels (2, 6 and 9) since the p-value is greater than .05. The above tests show the comparisons between the effects of amylase and protease activities on the measured environmental parameters. The results from the box plots showed amylase activity to be more prominent for all the environmental conditions considered, suggesting clearly that amylolytic and proteolytic activities do not impact aesthetics on painted walls equally. All the tests were significant.
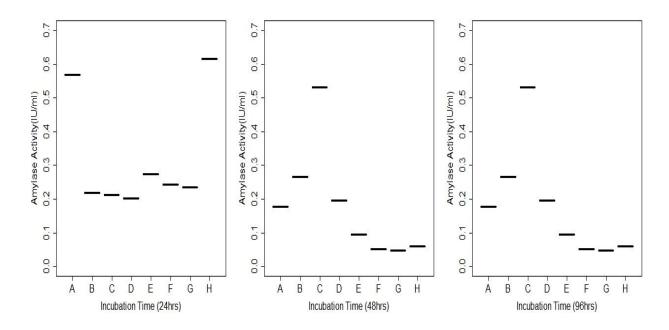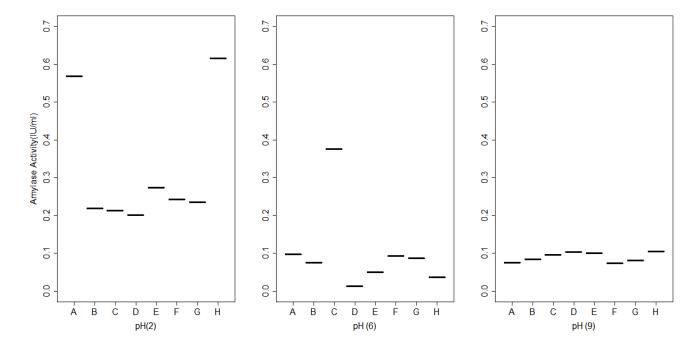
Fig. 1A. Effect of incubation time on amylase activity
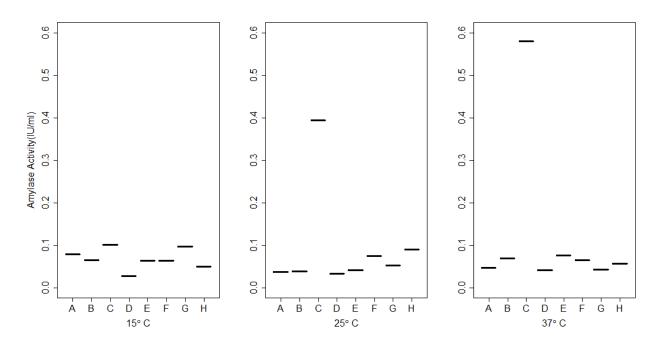

Fig. 1B. Effect of pH on amylase activity
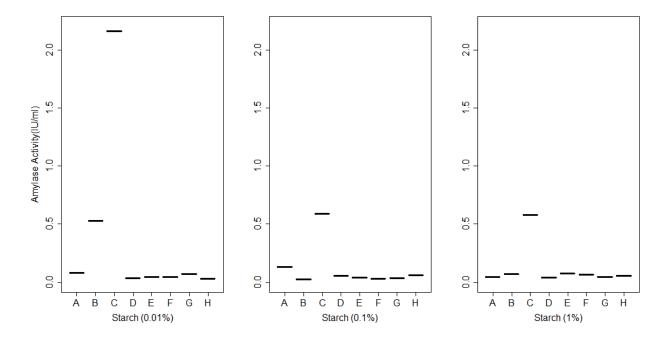
Fig. 1C. Effect of temperature on amylase activity

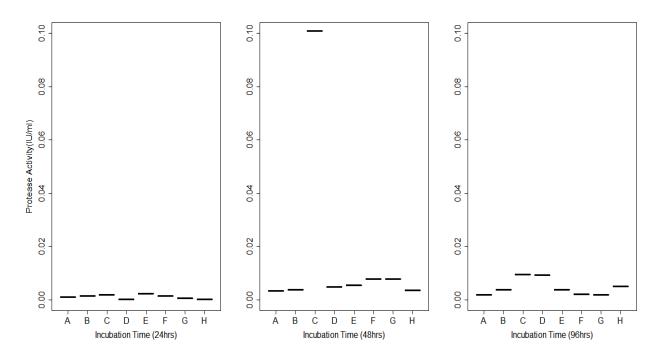**Fig. 1D. Effect of substrate concentration on amylase activity**

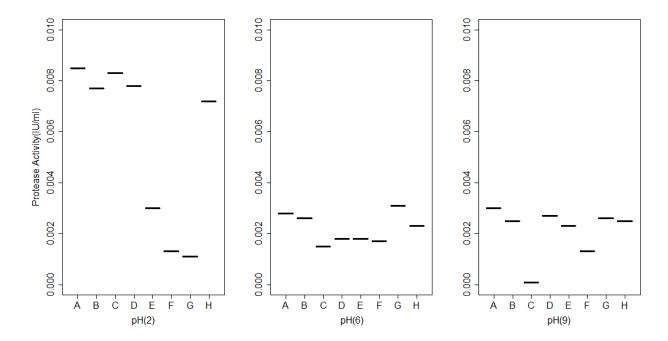Fig. 2A. Effect of incubation time on protease activity

Fig. 2B. Effect of pH on protease activity

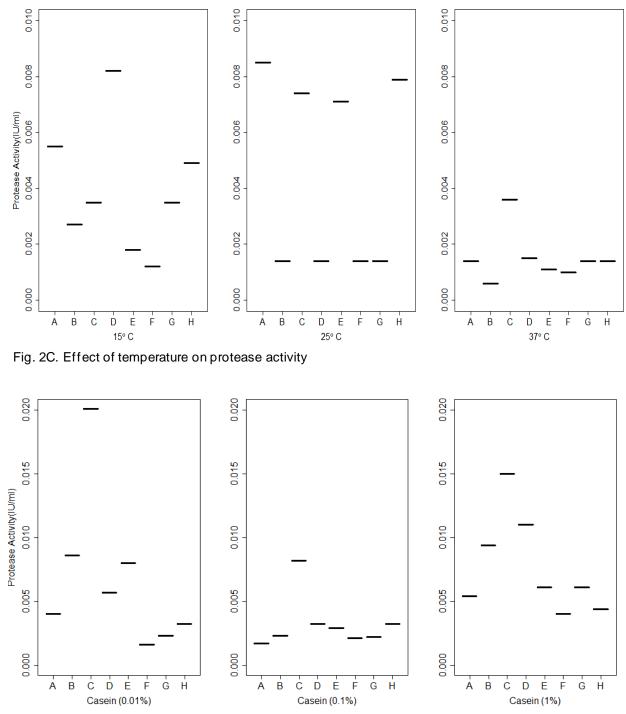Fig. 2C. Effect of temperature on protease activity

Fig. 2D. Effect of substrate concentration on protease activity

A, *M. guillermondii* MB14B1 (LT615287.1); B, *C. tropicalis* UZ31_13 (KM361510.1); C, *P. aeruginosa* CH01 (KY511067.1); D, *Cerrena* sp. N10CC2a (FJ010208.1); E, *F. proliferatum* 2705 (EU272509.1); F, *A. aculeatus* A1.9 (EU833205.1); G, *Aspergillus* sp. SL2 (KC178662.1) and H, *M. caribbica* CBS:5674 (KY104219.1)
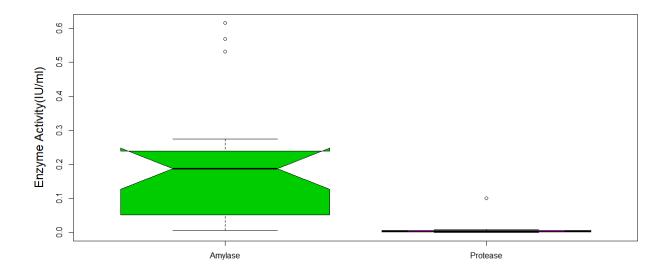
Fig. 3A. Boxplot of Enzyme Activity: Amylase vs Protease

DISCUSSION

Profound enzymatic activities have been linked to biodeterioration and biodegradation (Teyjgeler, 2001; Cappitelli and Sorlini, 2005; Rojas et al., 2008). This paper describes a novel research into the authentication of the deterioration mechanism as well as quantifying its contribution to obvious discolorations and stains using a non-parametric approach. Such studies have not been previously reported in Nigeria, and this represents the first study that reports such investigations. Three of the microorganisms (37.5%) used in this study produced amylase optimally at 37^{0}C. Amylase activity has been reported to be optimum at 35°C (Singh and Kumari, 2016) in an assay involving temperature ranges of 35°C, 40°C and 45°Crespectively. Similarly, protease was produced optimally by 75% (6) of the organisms at 25^{0}C. This suggests the contributions of these enzymes to the heavy discoloration on the painted walls where they were isolated. At the isolation sites, the prevailing temperature ranged from 25-30^{0}Cwhich supports active metabolism and optimum enzymatic production by the producing organisms leading to obvious discolorations (Obidi and Okekunjo, 2017). The fact that amylases are produced optimally at such temperature range shows that amylase activity would decrease as the temperature increases

above 37°C. In this study, majority of the microorganisms (62.5%) (5) screened had their optimum incubation time to be 24 hours for amylase production. This is in line with the study of Koel et al. (2014) who reported an optimum amylase production at 24 h for bacteria. We observed an optimum pH of 6 for *P. aeruginosa.* Similarly, Raju and Divakar (2003) in a previous study, observed a maximum amylase production at pH of 7.0 for *Pseudomonas aeruginosa*. The substrate concentration for optimum amylase production, however, varies with 37.5% (3) having 0.01; 37.5% (3) having 0.1 and 25% (2) having 1% respectively. Previous reports on effects of other physico-chemical parameters such as substrate concentration on amylase production varied when compared with our study. This observation agrees with an earlier report that optimum amylase production differ in other physicochemical properties depending on their origin (Arti et al., 2010.).

Pseudomonas aeruginosa CH01 (KY511067.1), the only bacterium tested in this study produced protease optimally at 25°C while the fungi had temperatures ranging from 15-25^{0}C. This agrees with the study of Sharma et al. (2017) who reported an optimum temperature range of 28°C-30°C for fungi and lower temperature of 25°C for some bacteria during protease production. Also,

our study showed an optimum protease production at incubation time of 48 hours for *Aspergillus sp.* while no microorganism showed optimum protease production at an incubation time lesser than 48 hours. This agrees with previous studies that reported that the time generally, for optimum protease production by bacteria or fungus was 48 h to 9 days (Sharma *et al.*, 2017). The study reported optimum incubation time of 48 hours and 72 h for *Aspergillus oryzae* and *Aspergillus niger* respectively. Furthermore, the study reported an optimum pH range between 5.0 and 10 as optimum for protease production. In the present study, all the microorganisms except *A. aculaetus* and *Aspergillus sp.* had an optimum pH of 2 for protease production. Most studies did not report lower (acidic) pH probably because the pH range was not extended to a lower value during laboratory experiments as it was done in the present study. Protease activity increased as substrate concentration increased for *Cerrena sp.* N10CC2a (FJ010208.1), *M. carribica and Candida tropicalis*. This corroborates the report by Priyanka *et al.* (2015). This study also revealed that the microorganisms utilized minimal substrate concentrations for their amylolytic and proteolytic activities and produced optimally within the range of 0.01% to 1% substrate concentration respectively. This is particularly important since wall paints contain several organic and inorganic compounds which are utilizable as substrates [Rojas et al., 2008; Obidi et al., 2009a] by the isolated microorganisms. Previous workers have shown that microorganisms on painted surfaces utilize nutrients, substrates and carbon sources which enhances their proliferation and enzymatic activities resulting in aesthetic discoloration on painted walls (Ravikumar et al., 2012).

M. guilliermondii had the highest amylolytic and proteolytic activity at 15°C and 25°C respectively. *C. tropicalis* had the highest proteolytic activity at 15°C. This corroborates their greater abundance on deteriorated wall paints as reported by Obidi and Okekunjo (2017). The study detected them (*M. guilliermondii* and *C. tropicalis*) at 0.4-0.6 x 10^2CFU/g compared to others which had their population densities ranging from 0.02- 0.2 x 10^2CFU/g. This agrees with the fact that prevailing environmental conditions in such tropical environments (temperature of ~ 26°C, average rainfall of ~186.8mm and humidity of ~

86% (Obidi and Okekunjo, 2017) will favour the growth of *M. guilliermondii* and *C. tropicalis*. The present study shows that amylolytic and proteolytic activities were achieved within an optimum period of 24-48h suggesting that most painted surfaces in tropical countries with such environmental conditions are at risk of microbial colonization as well as susceptible to serious discoloration over time**.**

Lagos is a typical tropical city which experiences a tropical savanna climate according to the Köppen climate classification because there are four months under 60 millimetres or 2.4 inches of rain (Awe and Gil-Alana, 2021). Environmental conditions such as 37°C, pH 2-6 and substrate concentration of 0.01%during a period of 24 hour provides optimal amylase production. Similarly, a temperature range of 15-25°C, pH 2 and substrate concentration of 1% during a 48-hour period provides optimal protease production in organisms isolated from discolored painted buildings with visible stains. Wilcoxon Signed Rank test however, revealed that amylolytic and proteolytic activities in these organisms do not impact aesthetics on painted walls equally at all environmental conditions considered.

CONCLUSION

The study concludes that variations in environmental conditions affect the amylolytic and proteolytic activities of various microorganisms differently.

Conflicts of interests:

The authors have no conflict of interest to declare

Author Contributions

O.F.O conceived, designed the work and wrote the manuscript. F.O.O carried out the bench work in conjunction with M.N.I and participated in the writing. O.O.A analyzed and interpreted the data. All authors read and approved the final manuscript.

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