Production of phosphatase by microorganisms isolated from discolored painted walls in a typical tropical environment: a Non-Parametric analysis

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1. Introduction

There are varieties of mechanisms involved in the biodegradation of painted materials such as pigmentation, degradation of compounds and hyphal penetration into the matrix. The successive microbial colonization and proliferation on paintings, the excretion of aggressive inorganic or organic metabolic products and the production of extracellular enzymes all contribute to the physical disruption of paint layers and underlying mortars (Rosado et al., 2012). Different enzymes hydrolyse and catalyse specific reactions involved in the biogeochemical transformations of carbon and phosphorus (Rosado et al., 2013) and regenerate inorganic nutrients from organic materials. Since substrates with nutritive support are excellent for most microorganisms, the various components that make up the chemicals used in paintings have also been reported as loci for microbial contamination. Some of the chemolithotrophic microbes that feed on such substrates as inorganic chemical constituents produce extracellular enzymes such as phosphatase. Furthermore, the heterotrophs can also colonize organic materials in the paint matrix releasing enzymes as products of metabolism (Pepe et al., 2010). Phosphatase produced by microorganisms has been reported to be able to mineralize organic phosphates into inorganic phosphates (Stege, Messina, Bianchi, Olsina, & Raba, 2009; Taylor, Wilson, Mills, & Burns, 2002).

Despite the fact that many organisms produce phosphatases and have elaborate mechanisms for regulating their synthesis and activities, it is interesting to find that phosphatase solubilizing bacteria are not in high numbers in the biosphere; hence, the intense competition for phosphate among microbes (Parhamfar, Badoei-Dalfard, Parhamfar, & Rad, 2016). Many extracellular phosphatases are monomers but a few, such as the enzyme from *Thermus aquaticus* 143-kD is a trimer. Phosphatases vary widely in their substrate specificities, phosphatase production are major indices of discoloration on biodeteriorating painted walls in a tropical environment like Nigeria. Kruskal-Wallis non-parametric test revealed that there was no statistically significant difference in phosphatase production at the different environmental conditions examined except at different time intervals.
pH ranges, and metal ion requirements. Some, such as the one from *Escherichia coli*, are periplasmic, some are membrane-associated and some are extracellular (Goldman, Hecht, Eisenberg, & Mvarech, 1990). Phosphatase-producing microorganisms have mostly been isolated from soils (Betty, Dedeh, Tualar, Dw, & Benny, 2011) and marine environments (Yu Plisova et al., 2005) which have been described to be rich in organic phosphates.

The present study aims at establishing the optimal environmental conditions for phosphatase production by certain novel isolates from discoloured painted buildings as well as validating its discoloration role on painted walls. The Kruskal-Wallis k-sample test which is a logical extension of the Wilcoxon-Mann-Whitney test for k = 2 was used in this study.

2. Materials and methods

2.1. Culture preparation and maintenance

The isolates [Meyerozyma guillermontii MB1481 (LT615287.1), Meyerozyma caribbica CBS:5674 (KY104219.1), Candida tropicalis UZ31_13 (KM361510.1), Cerrena sp. N10CC2a (FJ010208.1), Fusarium proliferatum 2705 (EU272509.1), Aspergillus sp. SL2 (KC178662.1), Aspergillus aculeatus A1.9 (EU833205.1) and Pseudomonas aeruginosa CH01 (KYS11067.1)] which were obtained from a previous study (Obidi and Okeunjo, 2017) were suspended in Sigma-Aldrich nutrient broth containing glucose, 1g/l; peptone, 15g/l; yeast extract, 3 g/l; and NaCl, 6g/l at 37 °C for the bacterium and Sigma-Aldrich Sabouraud Dextrose broth containing peptone 10g/l and dextrose 20g/l for fungi. Cell growth was determined by measuring optical density of the culture at 600 nm (OD600) with a UV Spectrophotometer was determined by measuring optical density of the culture at 600 nm (OD600) with a UV Spectrophotometer.

2.2. Identification methods

2.2.1. Conventional characterization

Purified isolates were examined microscopically and morphologically as described by Harrigan and Mc Cance (1976). The isolates were initially identified using conventional tests including Gram stain, growth and morphometric characteristics on selective agar, catalase, oxidase, motility, indole production, MRVP, oxidative fermentative carbohydrate utilization and urease activity. Additional tests included nitrate reduction, citrate utilization, H2S production, hydrolysis of starch, and spore detection test (Cheesbrough, 2008).

2.2.2. 16S rDNA gene sequence and phylogenetic analysis

The 16S rDNA gene sequence determination was carried out for representative pure cultures of the eight isolates according to previously described methods (White, Bruns, Lee, & Taylor, 1990; Blaiotta et al., 2002; Ettenauer, Sterflinger, & Piñar, 2010). Phylogenetic trees were constructed using the MEGA software, version 7.0 (Tamura, Nei, & Kumar, 2004), two tree construction algorithms and the neighbour-joining techniques (Kimura, 1980; Saitou & Nei, 1987). The BLAST tool of the GenBank and EMBL database (http://www.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997) was used to compare the 16S rDNA gene sequences of the test strains.

2.3. Screening for phosphatase producing microorganisms

P-nitrophenylphosphate (0.1 g) together with nutrient broth and potato dextrose broth (300 ml each) were sterilized and dispensed into 500 ml conical flasks. Standardized suspension (1.5 x 10^6 CFU/ml) of the culture suspension were then inoculated into each of the flasks, plugged with non-adsorbent cotton wool and incubated at 37 °C and 25 °C, respectively, for 5 d in an incubator shaker at 150 rpm. After fermentation, the culture medium was centrifuged at 1500 rpm for 20 min to obtain the crude extract.

2.4. Enzyme activity

The phosphatase activity was determined according to the method of Tabatabai and Bremner (1969). The cell-free supernatant (0.1 mL) samples were incubated at 37 °C for 1 h in modified universal buffer (MUB) (12.1 g of Tris-hydrochloric aminomethane, 11.6 g of malic acid, 14. 0 g of citric acid, 6.3 g of boric acid in water and 488 ml of 0.1M NaOH) (pH 5.0) and 0.2 bml of 115 mM p-nitrophenylphosphate (p-PNP). The reaction was stopped by the addition of 0.1 mL of 0.5 M NaOH, and immediate centrifuging for 15 min at 10,000 rpm. The amount of p-nitrophenol released from PNP was measured in the supernatant at 405 nm using p-nitrophenol as standard. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 mM of p-nitrophenol per ml per min under assay conditions.

2.5. Optimization of culture conditions

Incubation time: The culture extract was adjusted to pH 7 with 1% pNPP and left at 25 °C on a rotary shaker. Enzyme assays were carried out after 24h, 48h and 96h.

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

**pH:** The effect of pH on phosphatase production was carried out individually at pH 2, 6, and 9 with 24 h incubation time, temperature set at 25°C and pNPP concentration of 1%. The enzyme assay was carried out after 24 h of incubation.

**Substrate Concentration:** pNPP was added to the culture extract at different concentrations which included 0.01, 0.1 and 1%. The pH was held constant at 7, temperature at 25°C and incubation time at 24 h. The enzyme activity was measured after 24 h of incubation. p-nitrophenol standard curve was used to calculate the p-nitrophenol concentrations for phosphatase assay.

3. Results

3.1. Identification of isolates

The microorganisms monitored for phosphatase production were identified using conventional characterization and the 16S rDNA genetic sequencing method. The microorganisms were *Meyerozyma guilliermondii* MB14B1 (LT615287.1), *Meyerozyma caribbica* CBS:5674 (KY104219.1), *Candida tropicalis* UZ31_13 (KM361510.1), *Cerrena* sp. N10CC2a (FJ010208.1), *Fusarium proliferatum* 2705 (EU272509.1), *Aspergillus aculeatus* A1.9 (EU833205.1) and *Pseudomonas aeruginosa* CH01 (KY511067.1). The analysis of the 16S rDNA sequences confirmed that *P. aeruginosa* CH01 (KY511067.1) was most similar to *P. putida* ATCC 12633 (AF094736.1) (Figure 1) while *M. guilliermondii* MB14B1 (LT615287.1) was most similar to *M. caribbica* CBS:5674 (KY104219.1) and shares closest homology with *C. tropicalis* UZ31_13 (KM361510.1) (Figure 2). The phylogenetic tree also showed that *Meyerozyma*, *Aspergillus*, and *Fusarium* have similar intra-species genetic distances of 0.02 between individual isolates as *Pseudomonas* species (Figures 1 and 2) indicating similar rate of evolution and genetic variation between the isolates.

3.1. Enzyme assay

Table 1 shows that all the tested microorganisms showed an optimum incubation time of 96 h (Figure 3). *M. guilliermondii* MB14B1 (LT615287.1) and *A. aculeatus* A1.9 (EU833205.1) showed the same optimal conditions of pH at 2. However, *M. guilliermondii* MB14B1 (LT615287.1) had 15°C incubation temperature and 1% substrate concentration while *A. aculeatus* A1.9 (EU833205.1) had 37°C optimum temperature and 0.01% substrate concentration for phosphatase production (Figures 4-6). *P. aeruginosa* CH01 (KY511067.1) showed optimum production of phosphatase at 25°C, pH 6, 0.01% substrate concentration (Figures 4-6) while *F. proliferatum* 2705 (EU272509.1) showed optimum conditions pH 9, 37°C and 0.1% substrate concentration (Figures 4 and 6). *P. aeruginosa* CH01 (KY511067.1) was the only organism that had optimum pH at 6. *M. caribbica* CBS:5674 (KY104219.1) and *C. tropicalis* UZ31_13...
showed optimum production at 15°C, 25°C, and pH 2 and 9, respectively (Figures 4 and 5). However, their substrate concentrations vary for optimal phosphatase production. The substrate concentration for optimal phosphatase production was 0.01% and 1% for *M. caribbica* CBS:5674 (KY104219.1) and *C. tropicalis* UZ31_13 (KM361510.1), respectively (Figure 6). *Aspergillus* sp. also had similar optimum temperature for phosphatase production at room temperature (25°C) as *P. aeruginosa*.
However, its optimum pH and substrate concentrations were 9 and 1%, respectively (Figures 4 and 6). *Cerrena* sp. N10CC2a (FJ010208.1) which showed the highest phosphatase activity produced optimally at 15°C, pH 9 and 1% substrate concentration (Figures 4-6).

### 3. Effect of varying conditions on enzyme production

#### 3.2. Effect of incubation time

All the microorganisms showed an optimum incubation time of 96 h (Figure 3).

![Figure 3. Effect of incubation time on phosphatase production.](image1)

![Figure 4. Effect of pH on phosphatase production.](image2)
3.2.2. Effect of pH

Among the various pH ranges, 50% of the microorganisms showed an optimum phosphatase activity at pH of 2, 12.5% at pH 6 and 50% at pH 9 (Figure 4).

3.2.3. Effect of temperature

37.5% of the microorganisms showed an optimum phosphatase activity at 15 °C, 25% at 37 °C and 37.5% at 25 °C (Figure 5).
3.2.4. Effect of substrate concentration

37.5% of the microorganisms showed optimum phosphatase activity at 0.01%, 12.5% at 0.1% and 50% at 1% substrate concentration (Figure 6).

3.3.1. Kruskal-Wallis k-sample test

To verify these results statistically, we employed the Kruskal-Wallis method to test the null hypothesis that phosphatase production is equal at different culture conditions. It is the non-parametric analogue to the F-test used in analysis of variance. While analysis of variance tests depend on the assumption that all populations under comparison are normally distributed, the Kruskal-Wallis test employed in this study places no such restriction, so we assume non-normality and that the observations are independent within each sample.

According to Kruskal and Wallis (1952), the Kruskal-Wallis test statistic for \( k \) samples, each of size \( n_i \), is given as

\[
H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N + 1)
\]

The Kruskal-Wallis test rejects the null hypothesis \( (H_0) \) when \( H \) is large or when \( p \)-value is less than the 0.05 level of significance where \( N \) is the total number (all \( n_i \)) and \( R_i \) is the sum of the ranks (from all samples pooled) for the \( i \)th sample. The null hypothesis of the test is that all \( k \) distribution samples are equal. The alternative hypothesis is that at least two of the populations tend to yield equal values. Whenever the test is significant or the null hypothesis is rejected, we make multiple comparisons between the samples. In all our tests, Bartlett’s Test of Homogeneity of Variance was first conducted to check the assumption that the samples have equal variances.

3.3.2. Results of Kruskal-Wallis test at different environmental conditions

**Hypothesis One**

\( H_0: \) Phosphatase production is equal at different time levels.

\( H_1: \) Phosphatase production is not equal in at least two different time levels.

Kruskal-Wallis H test showed that there was a statistically significant difference in phosphatase production at the different time levels, \( \chi^2(2, N = 24) = 12.255, p = 0.002182 \) (Table 2), hence the null hypothesis was rejected. Bartlett’s Test for equality of variance did not reject the null hypothesis of equal variance. Since the null hypothesis was rejected, we then conducted a pairwise Wilcoxon post-hoc test in order to investigate further using Holm’s correction to adjust for the \( p \)-values. The result is shown in Table 3.

3.3.3. Results of pairwise-Wilcoxon posthoc test at different time levels.

From Table 3, pairwise-Wilcoxon post-hoc test reveals that phosphatase production is different at different time levels. The null hypothesis of equality was rejected since \( p \)-value is less than the 0.05 significant level in all cases.

**Hypothesis Two**

\( H_0: \) Phosphatase production is equal at different temperature levels.

\( H_1: \) Phosphatase production is not equal in at least two different temperature levels.

![Figure 7. Boxplot of phosphatase production at different time levels.](image)

Table 2. Results of statistical tests on effect of phosphatase production at different time levels.

| Test                                | Statistic | Degree of freedom | P-Value       | Decision                |
|-------------------------------------|-----------|-------------------|---------------|-------------------------|
| Bartlett’s Test for Equality of Variance | 0.10894   | 2                 | 0.947         | Do Not Reject \( H_0 \) |
| Kruskal-Wallis H Test               | 12.255    | 2                 | 0.002182      | Reject \( H_0 \) and conduct Post-Hoc Test |

Table 3. P-value results of pairwise-Wilcoxon posthoc test at different time levels.

| Time Level | Statistic | P-Value | Decision |
|------------|-----------|---------|----------|
| 24 hours   | 0.021     |         |          |
| 48 hours   | 0.021     | 0.021   |          |
| 96 hours   | 0.021     | 0.021   |          |
Kruskal-Wallis H test showed that there was no statistically significant difference in phosphatase production at the different temperature levels ($\chi^2(2, N = 24) = 0.3758, p = 0.8286$) (Table 4) hence the null hypothesis was not rejected. Figure 8 shows that the medians of the three temperature levels are virtually equal. Bartlett’s Test for equality of variance did not reject the null hypothesis of equal variance.

**Hypothesis Three**

$H_0$: Phosphatase production is equal at different pH levels.

$H_1$: Phosphatase production is not equal in at least two different pH levels.

Kruskal-Wallis H test showed that there was no statistically significant difference in phosphatase production at the different pH levels ($\chi^2(2, N = 24) = 1.2355, p = 0.5391$) (Table 5), hence the null hypothesis was not rejected. Bartlett’s Test for equality of variance did not reject the null hypothesis of equal variance.

**Hypothesis Four**

$H_0$: Phosphatase production is equal at different substrate levels.

$H_1$: Phosphatase production is not equal in at least two different substrate levels.

Kruskal-Wallis H test showed that there was no statistically significant difference in phosphatase production at the different substrate levels ($\chi^2(2, N = 24) = 0.9625, p = 0.6180$) (Table 6), hence the null hypothesis was not rejected. Bartlett’s Test for equality of variance did not reject the null hypothesis of equal variance. The boxplots of phosphatase production at different time levels, temperature levels, pH levels and substrate levels are shown in Figures 7–10 respectively.

### Table 4. Results of statistical tests on effect of phosphatase production at different temperature levels.

| Test                                | Statistic | Degree of freedom | P-Value | Decision     |
|-------------------------------------|-----------|-------------------|---------|--------------|
| Bartlett’s Test for Equality of Variance | 4.3186    | 2                 | 0.1154  | Do Not Reject $H_0$ |
| Kruskal-Wallis H Test               | 0.37598   | 2                 | 0.8286  | Do Not Reject $H_0$ |

### Table 5. Results of Statistical Tests on Effect of Phosphatase Production at Different pH Levels.

| Test                                | Statistic | Degree of freedom | P-Value | Decision     |
|-------------------------------------|-----------|-------------------|---------|--------------|
| Bartlett’s Test for Equality of Variance | 2.9994    | 2                 | 0.2232  | Do Not Reject $H_0$ |
| Kruskal-Wallis H Test               | 1.2355    | 2                 | 0.5391  | Do Not Reject $H_0$ |

### Table 6. Results of statistical tests on effect of phosphatase production at different substrate levels.

| Test                                | Statistic | Degree of freedom | P-Value | Decision     |
|-------------------------------------|-----------|-------------------|---------|--------------|
| Bartlett’s Test for Equality of Variance | 0.0029    | 2                 | 0.9985  | Do Not Reject $H_0$ |
| Kruskal-Wallis H Test               | 0.9625    | 2                 | 0.6180  | Do Not Reject $H_0$ |

4. Discussion

A major objective of this study is to evaluate phosphatase production from isolates of biodegraded painted walls. Alkaline phosphatase has been traced to phosphatase-producing organisms (Betty et al., 2011). The majority of the microorganisms (50%) produced phosphatase optimally (0.0016–0.0161 IU/ml) between 25 and 37°C. This is in line with previous reports that optimum phosphatase activity (0.029 IU/ml) was at 35–40°C (Prada, Jennifer, & Jean, 1996). Furthermore, our result showed that phosphatase activity was low (0.0014–0.0028 IU/ml) at temperatures below 20°C and also decreased as temperature increases to 37°C. Similar results were obtained by Mahesh, Somashekhar, Preenon, and Puttaiah (2015) who reported that the enzyme activity was very low at 20°C and decreased above 40°C. This suggests and corroborates its contribution to the heavy discoloration on the sites of isolation where the prevailing temperature range was 25–30°C (Obidi and Okekunjo, 2017). Sharma, Kumar, Panwar, and Kumar (2017) also noted an optimum temperature range of 28°C–30°C for fungi and temperature of 25°C for some bacteria. Furthermore, Gonzalez, Esther, Arias, and Montoya (1994) observed an optimum phosphatase production at temperature of 37°C in Mycococcus. The fact that phosphatases are produced optimally at such temperature range shows that phosphatase activity would decrease as the temperature increases above 37°C. On the other hand, an earlier study by Chen, Chen, Zhu, Shi, and Van (1996) showed that the optimum temperature for the hydrolysis of pNPP by alkaline phosphatase was 47°C. The pH range for optimal phosphatase production in the present study was between 2 and 9 which, according to Prada et al. (1996), should not be exceeded. Previous studies on phosphatase activity by Mahesh, Guleria, Rajesh, Somashekhar, and Puttaiah (2010) showed that
optimum pH conditions for phosphatase production is 8.8; Chen et al. (1996) reported pH optimum to be 8.2 while Mahesh et al. (2015), reported optimum pH to be 8. Gonzalez et al. (1994) also noted that alkaline phosphatases are stable between pH 6 and 9. Similarly, the study by Prada et al. (1996) showed that increase in pH up to 11 decreased phosphatase activity by 30%. A study by Yuplisova et al. (2005) reported an optimum pH of 10 for highly active alkaline phosphatase production. However, in this study, 62.5% of the microorganisms had a pH ranging from 6 to 9 except M. guilliermondii MB14B1(LT615287.1), Aspergillus aculeatus A1.9 (EU833205.1) and Meyerozyma caribbica CBS:5674 (KY104219.1) which had an optimum pH of 2. A considerable increase in enzyme activity was observed as the pH changed from

Figure 8. Boxplot of phosphatase production at different temperature levels.

Figure 9. Boxplot of phosphatase production at different pH levels.

Figure 10. Boxplot of phosphatase production at different substrate levels.
6 to 9. Most reports did not report low pH, probably because the pH range was not extended to a lower pH value during laboratory analysis. In addition, we found phosphatase activity to increase as substrate concentration increased. Ali-ul-Qader et al. (2009) observed an increase in phosphatase concentration at high pNPP concentrations up to 20 mM. This is in agreement with Behera et al. (2017) who reported that different pNPP concentrations (0.5–2.5 mg/ml) resulted in an increase in alkaline phosphatase activity with an increase in substrate concentration up to 2 mg/ml. In the present study, we observed an optimum phosphatase production at incubation time of 96 h for all the microorganisms. According to Behera et al. (2017), the time generally for optimum phosphatase production by bacteria or fungi was after 48 h. Mahesh et al. (2015) also reported the optimum phosphatase activity to be 0.688 U/ml after 24 h and noted that phosphatase yield increased with time. These studies are in agreement with our study as all of the tested microorganisms showed increased phosphatase activity with increased incubation time and substrate concentration. Earlier studies have shown that alkaline phosphatase is produced under certain specific conditions. Fraser, Lynch, Entz, & Dunfield (2015) reported a link between alkaline phosphatase activity and gene abundance in the soil. Also, Lahiri et al. (2014) observed the catalysis of the final step in biosynthesis of the anti-stress sugar trehalose in Candida utilis by Trehalose-6-phosphate phosphatase (TPP). Ronald, De Boer, and Maaijen (2016) reported that manganese salts are used in the manufacture of paints as they provide a hard surface to paints particularly glossy paints, suggesting that building paints aid the production of alkaline phosphatase.

5. Conclusion

This study revealed that microorganisms from degraded painted walls exhibit phosphatase activity. These organisms also require minimal (0.1g) of (pNPP) for the least production of phosphatase (0.0168IU) on a painted substrate in a typical tropical environment with an average temperature of 15–37°C within 96 h. Therefore, microorganisms inhabiting degraded painted surfaces are possible considerations for industrially useful phosphatases. Non-parametric analysis showed that there were no statistically significant differences in phosphatase production at the different environmental conditions examined except at different time levels.

Acknowledgements

Anonymous reviewers.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial and not-for-profit sectors.

Disclosure statement

No potential conflict of interest was reported by the authors.

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