International Journal of Research (IJR)

e-ISSN: 2348-6848
p-ISSN: 2348-795X
Vol. 10 Issue 06
June 2023

Screening and Characterization of Lignolytic Enzymes Produced by *Escherichia coli* Exposed to Polyethylene

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Abstract

Several research studies has opined lignolytic enzymes to perform significant roles in bioremediation of polyethylene. However studies unveiling the physicochemical properties of these pivotal group of enzymes are limited. Hence, this study focused on assaying and characterizing manganese peroxidase and laccase produced by *Escherichia coli* isolated from plastic polluted site under exposure to polyethylene based mineral medium. The result showed that *Escherichia coli* produced optimum manganese peroxidase and laccase activities on the third day. Study of their physicochemical properties revealed that manganese peroxidase activity was optimum and stable at pH 3.0 and pH 5.0 while laccase activity was optimum and stable at pH 9.0 and pH 7.0. Manganese peroxidase displayed optimum temperature and stability at 40°C. While laccase displayed optimum temperature at 60°C and was most stable at 50°C. *Escherichia coli* produced thermostable lignolytic enzymes that were either optimum at acidic or alkaline region making it activity relevant for biodegradation of polyethylene either in acidic or alkaline medium, therefore it can be utilized for polyethylene biodegradation.

Keywords: Manganese peroxidase, Laccase, *Escherichia coli*, Bioremediation, Polyethylene.

1. Introduction

Plastics usage is increasing with an annual production of about 300 million tons. Four percent of oil produced globally is used as raw material and energy for producing different plastic polymer. Almost all aspects of daily life involves the use of different plastics (Zuriash *et al*., 2023). Plastics are used in fabrics, foot wear, food and drinks producing companies, transport and telecommunications sector (Zuriash *et al*., 2023). There are different types of plastic polymers such as polyethylene terephthalate, polyethylene, polypropylene, polystyrene, polyvincyl chloride e. t. c; However, Polyethylene (PE) is the most widely manufactured synthetic plastic polymer globally with about 80 million metric tons produced per annum (Danso *et al*., 2019). This

Received: *04 May 2023*
Revised: *21 May 2023*
Final Accepted: *03 June 2023*

DOI: https://doi.org/10.5281/zenodo.8079547

enormous production of PE has limited adoption of appropriate disposal system (Nanda *et al*., 2010). Several PE are dumped on open air environment while others are either incinerated or burnt (Divyalakshmi, 2016). These methods are not ecologically friendly hence the need for cheap and an environmentally friendly disposal method (Divyalakshmi, 2016). For several decades bioremediation methods have been implemented as an environment friendly strategy for eradicating recalcitrant environmental pollutant such as synthetic plastics (PE) thereby, reducing severe pollution produced from conventional remediation methods (Azeko *et al*., 2015).

Bioremediation of PE involves the use of biological agents such as insect, microorganism and microbial enzymes etc. (Liu Ren *et al*., 2019) to break complex PE polymer into short chain monomers while water and carbon monoxide are released as end product. Microorganism such as fungi and bacteria have been revealed to be involved in biodegradation of both natural and synthetic plastics such as PE (Danso *et al*., 2019). Fungi have been displayed as significant group of organism due to the remarkable function they perform in in vivo degradation of plastic polymer because of their ability to form mycelial network and produce different enzyme activities especially lignolytic enzyme activities (Michael *et al*., 2013).

Lignolytic enzymes are versatile enzymes associated with degradation of complex recalcitrant plastic polymer such as PE. This enzyme activity is linked with three enzymes, such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP). Peroxidases possess heme structure and require H_2O_2 for catalysis, while LiP has the ability to oxidize non-phenolic structures related to lignin. Moreover, MnP facilitates the decomposition by generating phenoxy radicals from phenolic rings (Maurya *et al*., 2020). Laccases are multicopper oxidoreductase glycoproteins with the ability to oxidize phenolics as well as the non-phenolic compounds in presence of the mediators, such as ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6- sulphonic acid). The availability of the bacterium *Escherichia coli* in different environment made it suitable for bioremediation process (Santacoloma *et al*., 2019). The objective of this study was to screen, produce and characterize lignolytic enzymes (manganese peroxidase (MnP) and laccase (Lac)) produced by *Escherichia coli* isolated from PE polluted dung site under polyethylene exposure.

2.0 Material and Methods

2.1 Materials

Media constituents and other chemicals used were of analytical grade and products of Sigma-Aldrich (St. Louis, MO, USA).

2.2 Polyethylene (PE) Preparation

Polyethylene sheets were purchased from a local market were disinfected in sterile distilled water followed by placing in 70% ethanol for 30 min. The sterile polyethylene sheets were cut into small pieces after which it was milled.

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2.3 Screening of Soil for Fungus

Soil sample was collected from local dung site in Akure, Nigeria. About 1 g of soil was aseptically scrapped from the surface of different plastic polymer found at different soil depth (approximately 5-10 cm) and transported to laboratory in sterile Ziploc plastic bag within 24 h. The cell suspension containing washed cells (0.5 mL) was obtained from the soil sample and homogenously spread on sterile mineral salt medium (MSM) agar plates and sterile PE sheet was placed in the center. Inoculated MSM plate without PE film was used as control to check whether the isolates could grow on MSM alone (Yang *et al.*, 2014). Plates were incubated at 37°C. Growth on PE sheets was preliminarily determined by observing the formation of colony and biofilm after 2–7 days.

2.4 Identification of Fungus and Production of Lignolytic Enzymes

The microorganism used for this study was isolated from soil samples collected from a local dung site in Akure, Ondo state, Nigeria. Isolation and identification was carried out at Microbiology Laboratory, Elizade University, Ondo state, Nigeria. The fungus was identified using Gram staining and Vitek 2 Identification System according to manufacturer's instructions (VITEK 2 Compact, Biomerieux, France). Seed inoculum was prepared by growing a loopful of slant culture in sterile nutrient broth containing glucose (0.2g/20ml), NH_4NO_3 (0.04g/20ml), KH_2PO_4 (0.016g/20ml), K_2HPO_4 (0.004g/20ml), $MgSO_4$ (0.01g/20ml) and yeast extract (0.04g/20ml) at pH 6.0 for 24 h at 180 rpm in a shaking incubator (Stuart, UK). 5% v/v inoculum was taken from the seed culture for PE based medium (PBM). The PBM contained 1% PE powder as sole carbon source incubated in a shaking incubator at 180 rpm at 30 °C. PE based medium (PBM) contained of NH_4NO_3 (0.34g/170ml), KH_2PO_4 (0.136g/170ml), K_2HPO_4 (0.034g/170ml), yeast extract (0.34g/170ml), $CuSO_4.5H_2O$ (0.0425g/170ml), $MnSO4.H_2O$ (0.0425g/170ml), $MgSO_4.7H_2O$ (0.085g/170ml) and glycine (0.51g./170ml) at pH 6.0 incubated at 30˚C and 180 rpm for 7 days to obtain optimum days of manganese peroxidase and laccase activities in *Escherichia coli*. Fresh cultures of *Escherichia coli* were prepared for manganese peroxidase and laccase production on PBM incubated at 30˚C and 180 rpm for 4 days. At the end of the cultivation period, the broths were centrifuged at 10,000 rpm for 20 min at 4˚C. Clear supernatants were recovered and assayed for manganese peroxidase and laccase activities.

2.5 Manganese Peroxidase Assay

Manganese peroxidase activity was determined using modified method of Marion *et al*. (1998). The oxidation of 3.6 mM 2,2'-azino-di-[3~ethyl benzothiazoline-6-sulphonic acid] (ABTS) buffered with 0.2M Tris-Hcl buffer (pH 7) in the presence of 5 mM H_2O_2 at 414 nm was monitored for 5 minutes in a visible spectrophotometer. The reaction mixture (3 mL) contained 1 mL ABTS, 1 mL of culture filtrate and 1 mL H_2O_2. One unit (U) of manganese peroxidase activity was defined as the amount of enzyme oxidizing 1 µmol ABTS per minute at pH 7.0 and 30 °C with a molar

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extinction coefficient for the ABTS radical cation (the reaction product) of ε414 nm = 31100 M^{-1} cm^{-1}.

2.6 Laccase Assay

Laccase activity was determined using modified method of Marion *et al.* (1998). The oxidation of 3.6 mM 2,2'-azino-di-[3~ethyl benzothiazoline-6-sulphonic acid] (ABTS) buffered with 0.2 M Sodium acetate buffer (pH 5) at 420 nm was monitored for 5 minutes in a visible spectrophotometer. The reaction mixture (2 mL) contained 1 mL ABTS and 1 mL of culture filtrate. One unit (U) of laccase activity was defined as the amount of enzyme oxidizing 1 µmol ABTS per minute at pH 5.0 and 30 °C with a molar extinction coefficient for the ABTS radical cation (the reaction product) of ε420 nm = 36000 M^{-1} cm^{-1}.

2.7 Protein Content Determination.

Protein concentration was determined by the method of Bradford, 1976 using bovine serum albumin (BSA) as standard. In the assay, 100 of diluted dye reagent was pipetted into 5 uL of sample solution. The mixture was then incubated at room temperature for 15 minutes to allow proper colour development. The absorbance was measured at 595 nm against blank. The specific activities of manganese peroxidase and laccase were expressed as U/mL protein.

2.8 Characterization of Manganese Peroxidase and Laccase from *Escherichia coli*

2.8.1 Effect of pH on Manganese Peroxidase and Laccase Activities and Stabilities

The effect of pH on activities of manganese peroxidase and laccase were determined using various buffers over a pH range of 3.0 – 11.0 (glycine-HCl (pH 3.0), sodium acetate (pH 5.0), Tris-HCl (pH 7.0) and glycine-NaOH (pH 9.0 and 11.0) using the standard activity assay for each enzyme earlier described. pH stability was done by incubating manganese peroxidase and laccase with the relevant buffer solutions for 24 hours and their residual activities were determined according to the standard assay procedure.

2.8.2 Effect of Temperature on Manganese Peroxidase and Laccase Activities and Stabilities

The activities of the manganese peroxidase and laccase at different temperatures were determined by incubating the reaction mixture at temperatures ranging from 30 °C to 80 °C for 30 minutes before determining enzyme activity. To determine their stability at different temperature, the crude enzymes were incubated at temperature ranging from 30 °C to 80 °C for 3 hours, respectively and their residual activities were determined according to the standard assay procedure at 30 minutes interval.

2.9 Statistical Analysis

Results are expressed as mean ± standard deviation. The data were analyzed by one-way analysis of variance. Means values were compared using Duncan test. The SPSS statistical package by IBM version 16 was used.

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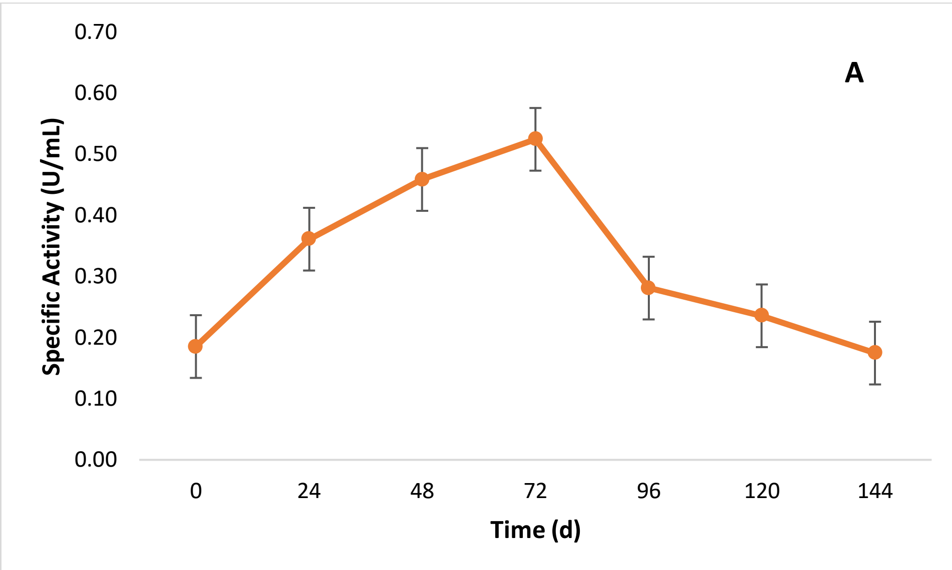
Vol. 10 Issue 06
June 2023

3. Results and Discussion



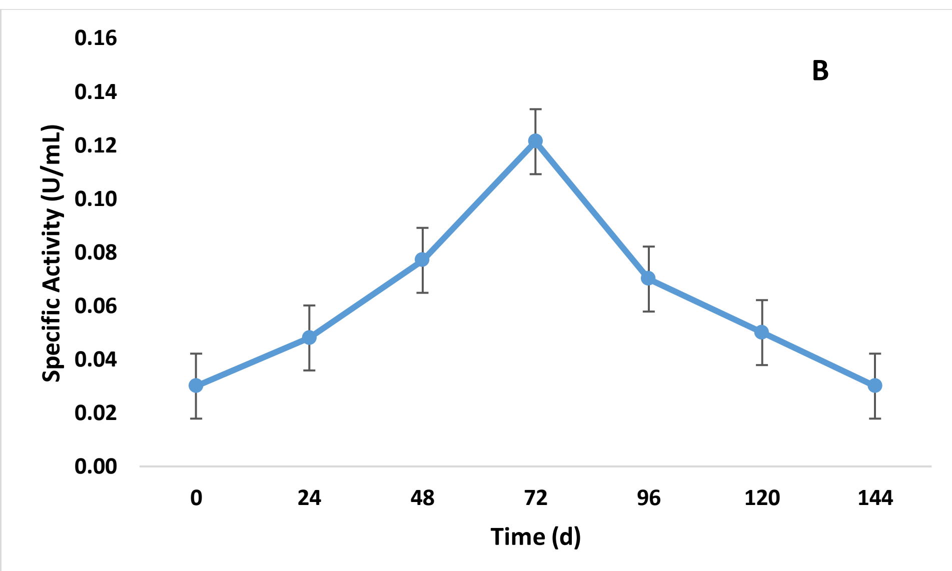


Figure 1: Production of (a) Manganese peroxidase and (b) Laccase from *Escherichia coli* (*Error bars* represent Mean ± standard deviation)

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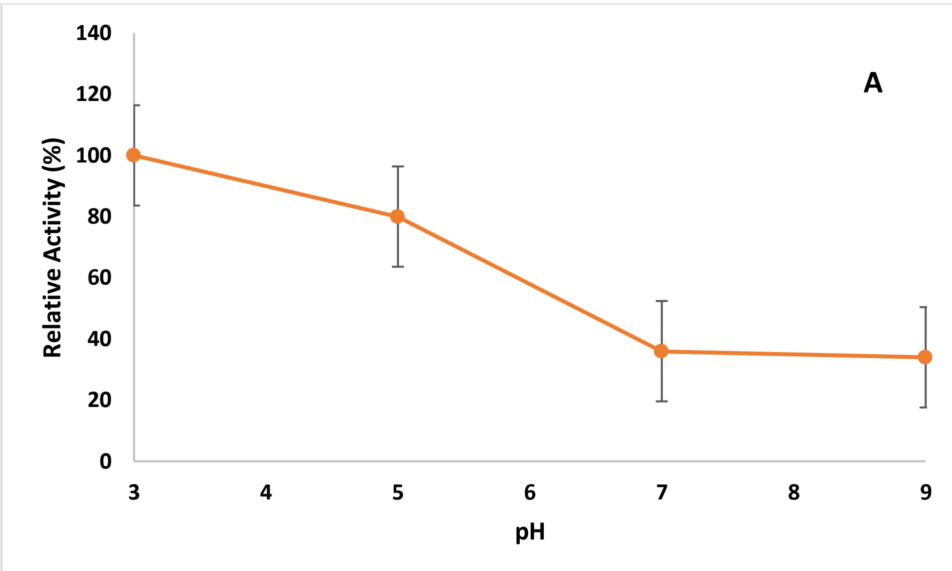

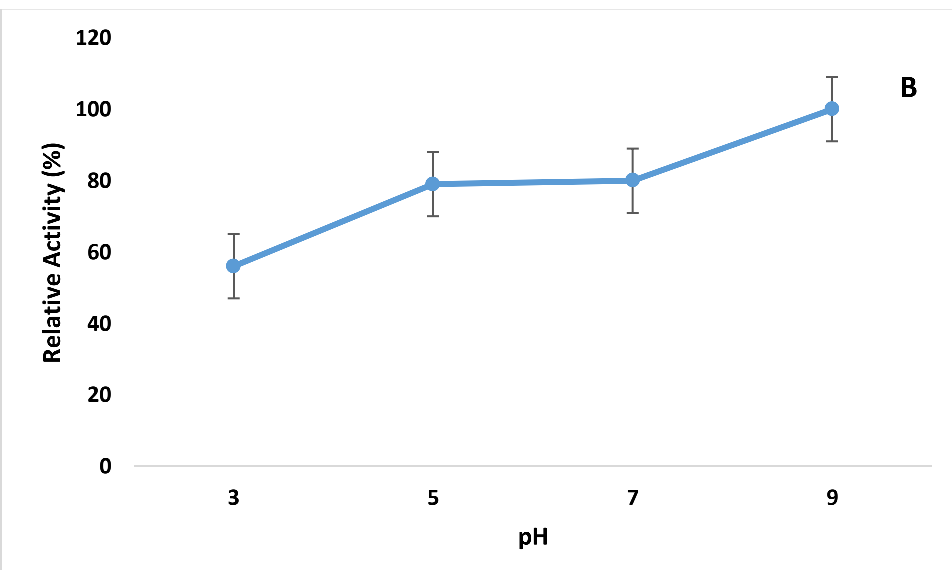

Figure 2: Effect of pH on activity of crude (a) Manganese peroxidase and (b) Laccase from *Escherichia coli* (*Error bars* represent Mean ± standard deviation)

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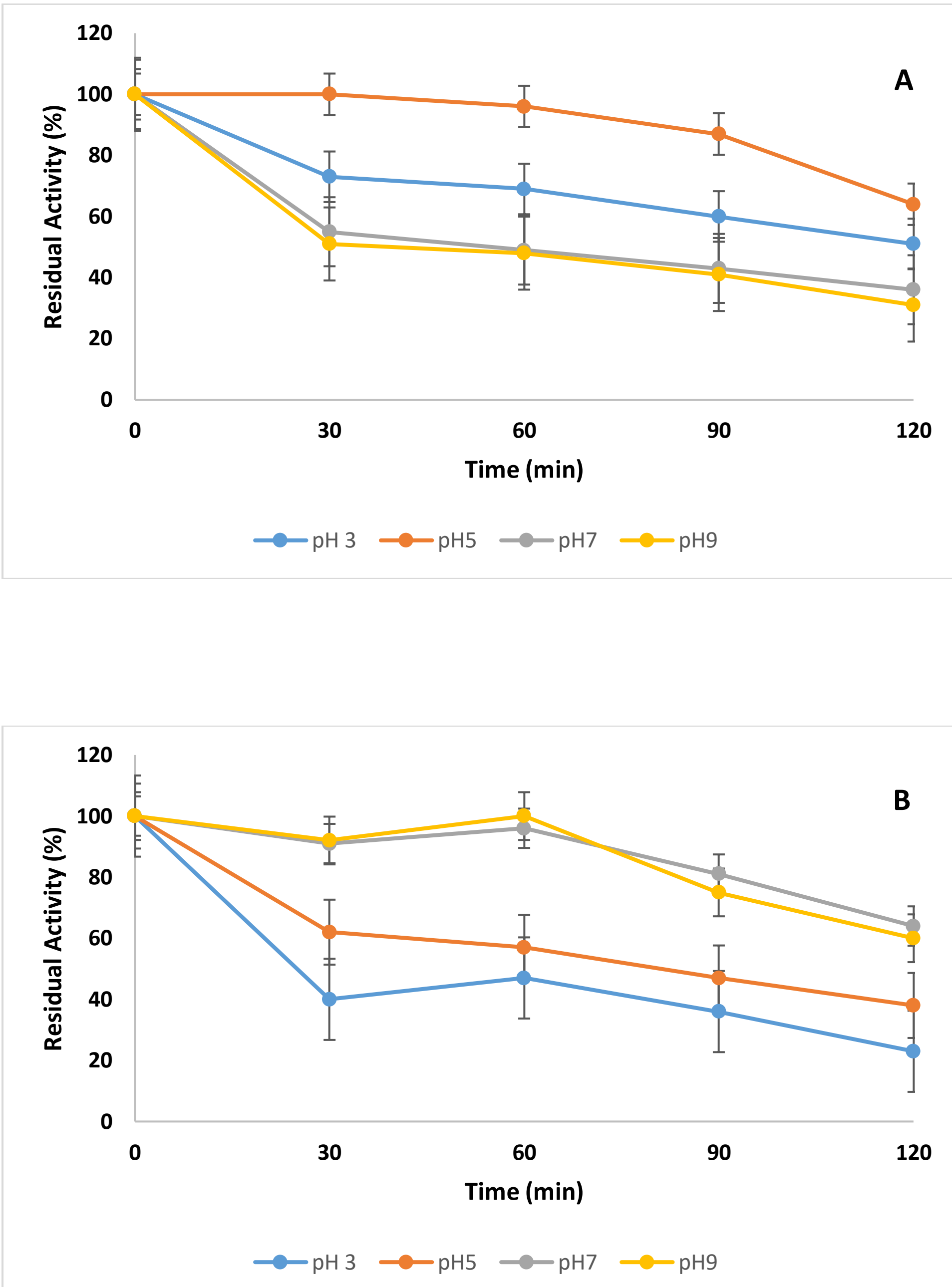

Figure 3: Effect of pH on stability of crude (a) Manganese peroxidase and (b) Laccase from *Escherichia coli* (*Error bars* represent Mean ± standard deviation)

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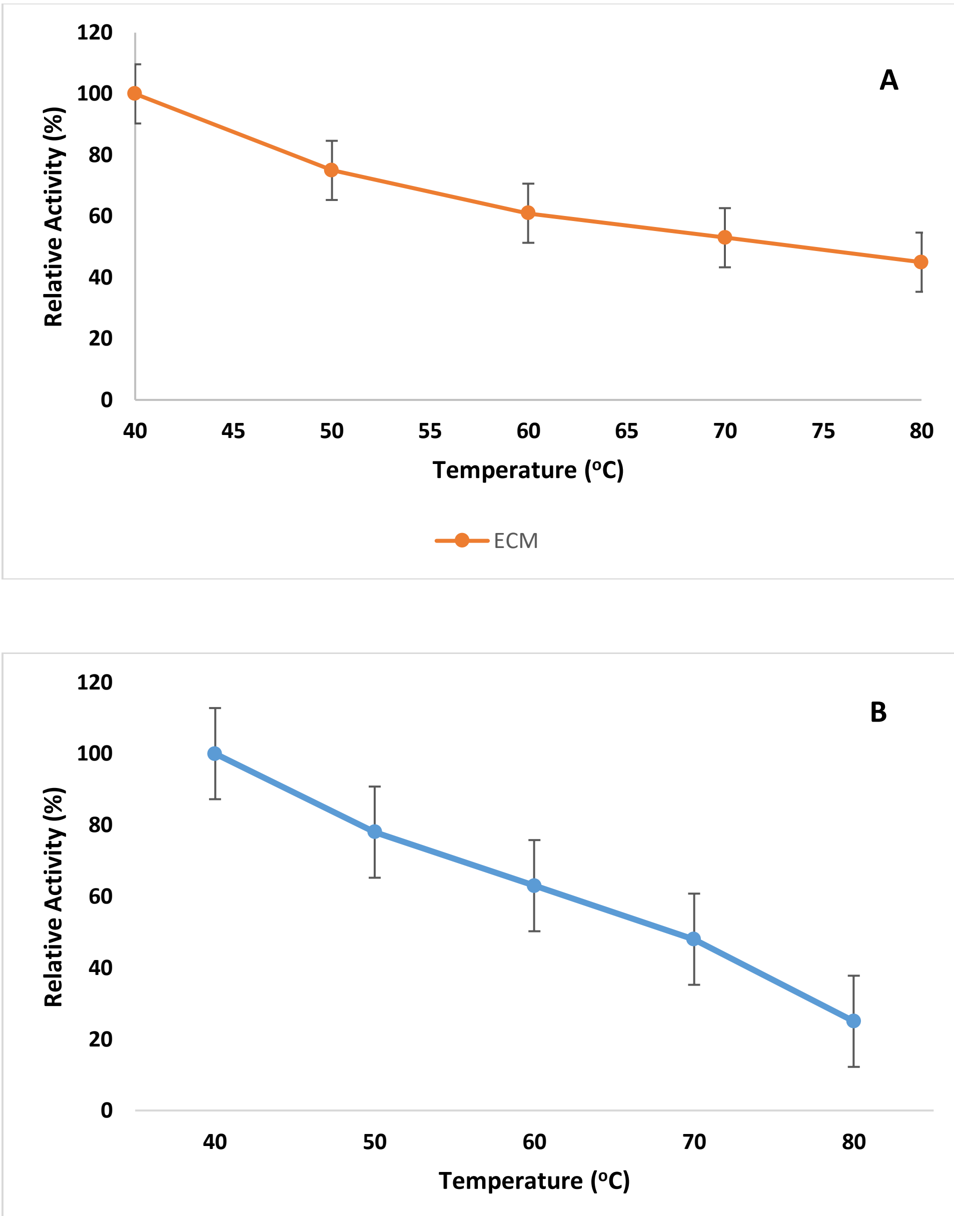

Figure 4: Effect of temperature on activity of crude (a) Manganese peroxidase and (b) Laccase from *Escherichia coli* (*Error bars* represent Mean ± standard deviation)

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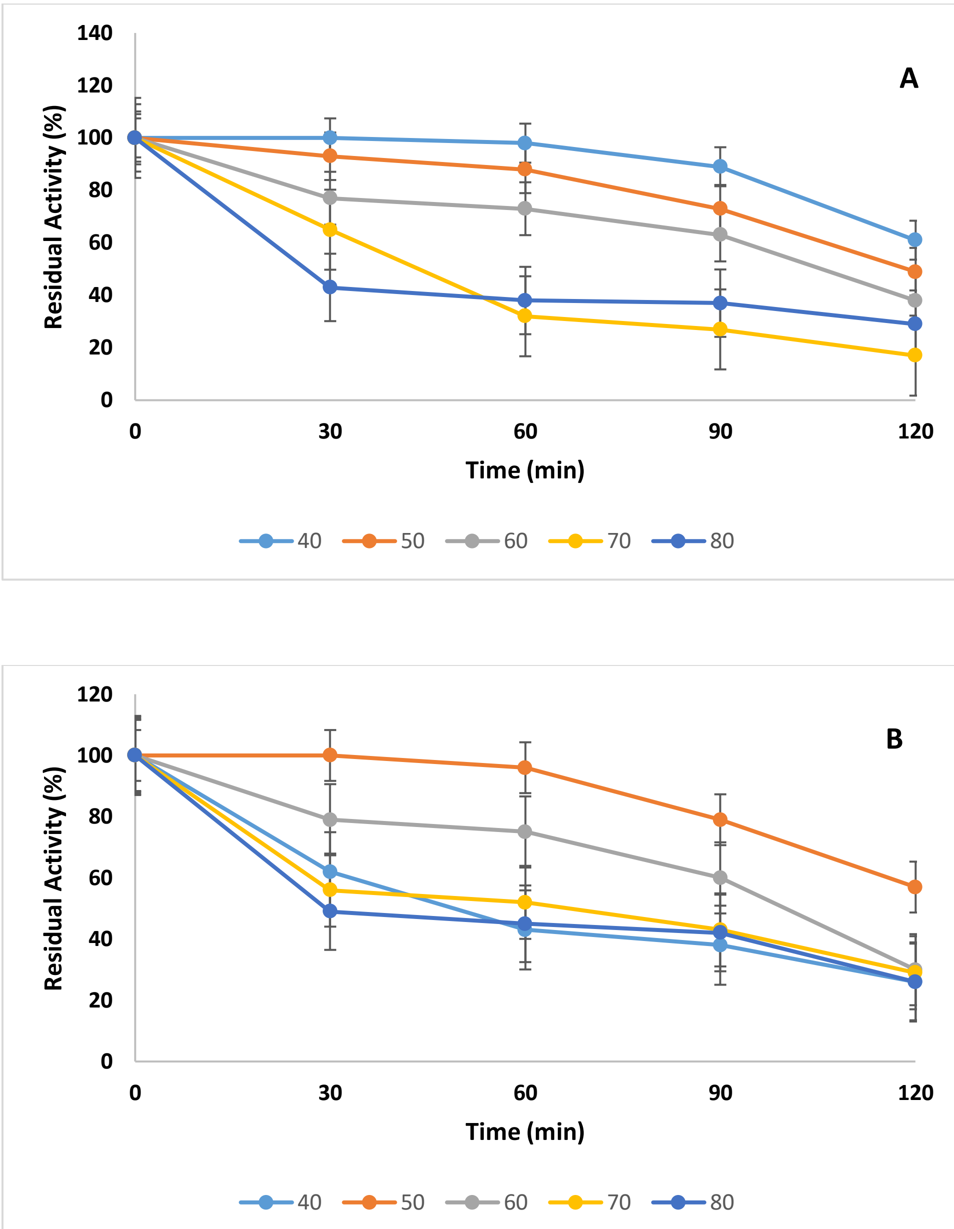

Figure 5: Effect of temperature on stability of crude (a) Manganese peroxidase and (b) Laccase from *Escherichia coli* (*Error bars* represent Mean ± standard deviation)

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3.1 Identification and Characterization of Microorganism

Gram-staining and biochemical characterization was carried out on the isolated bacterium with prominent growth on polyethylene. It was tentatively identified as *Escherichia coli* and chosen for further studies. The result of the characterization test is shown in Table 1.

Table 1: Biochemical characterization of *Escherichia coli*

Gram's reaction	-ve Rods
Pigmentation/color	White
Reactivity	
Catalase	+
Oxidase	-
Indole production	-
Hydrogen sulfide	-
Nitrogen reduction	-
Casein reaction	-
Fermentation test	
Urease	+
Lactose	+
Fructose	+
Maltose	+
Galactose	+
Glucose	+
Arabinose	-
Mannose	+
pH optimum	6.5
Temperature optimum	37^{o}C

3.2 Screening and Enzyme Production by *Escherichia coli*

Production of extracellular enzymes plays an important role in polymer degradation into smaller sub units (Pathak and Navneet, 2017) which can be assimilated into microbial cells (Sahadevan *et al*., 2013) and utilized as carbon sources leading to production of energy, water and carbon dioxide (Hamilton *et al*., 2014). Several studies have shown that lignolytic enzymes are essential for PE degradation hence, in this study manganese peroxidase (MnP) and laccase (Lac) activities were screened in *Escherichia coli* exposed to polyethylene. The strain showed MnP maximum production on the third day with an activity of 0.52 U/mL in the submerged culture fermentation. The results support previous observation of Elisashvilli, 2008 that that opined that MnP an activity is widely spread in different species. In this study, Laccase (Lac) activity was also optimum on the third day with an activity of 0.12 U/mL in the submerged culture fermentation. This support previously observation that Lac is produced under growth limiting conditions especially nitrogen

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Revised: *21 May 2023*
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which has negative impact on the enzyme yields (Valle *et al*., 2014). Results are also in agreement with the study of Christopher *et al.*, 2014 that discovered low laccase activities in most native fungi and Sumandano *et al*., 2015 that showed no laccase activities in 19 fungi strains. Therefore laccase gene cloning and expression is essential for improved activity and productivity. However, manganese peroxidase activity was higher than laccase activity *Escherichia coli.*

3.3 Effect of pH on MnP and Laccase Activities and Stabilities

Enzymes are sensitive to the pH of the culture medium as sudden alterations affects the ionization states enzymes, which in turn affects their activity and substrate selectivity (Devi *et al*., 2014). The result of the effect of pH on MnP activity showed optimum activity at pH 3.0 (Figure 2a). The residual activity was optimum (with 64 %) at pH 5.0 and had 51 % and 31 % residual activities at pH 3.0 and pH 9.0 after 3 hours incubation at room temperature (Figure 3a). This shows that the MnP produced by *Escherichia coli* is acidic. Statistical analysis by one-way ANOVA test for the data on effect of temperature on MnP activity and stability indicated a highly significant variation ($p < 0.05$). This result is contrary to the findings Oliveira *et al.* 2009 but in agreement with Huy *et al.*, 2017 that showed MnP optimum at acidic region. Laccase activity produced by *Escherichia coli* exhibited 100 % activity at pH 9.0 (Figure 2b) and was most stable at pH 7.0 with 64 % residual activity, 23 % and 60 % residual activities were recorded at pH 3.0 and pH 9.0 (Figure 3b) while the residual activity reduced with increase in pH. This is in disagreement with Muthusamy *et al*., 2017 that opined that laccase with optimum activity in acidic pH are efficient in biodegradation of recalcitrant polymers. Asgher *et al*., 2016 also reported that increase in pH above optimum pH causes rapid decrease in activity. However, this agrees with Chun-lei *et al*., 2010 that illustrated laccase activity was optimum at pH 8.0 during plastic polymer degradation. Statistical analysis by one-way ANOVA test for the data on effect of pH on laccase activity and stability indicated a highly significant variation ($p < 0.05$).

3.4 Effect of Temperature on MnP and Laccase Activities and Stabilities

The effect of temperature on crude MnP activity from *Escherichia coli* is illustrated in Figure 4a. The optimum temperature for MnP activity was at 40 °C. After 3 hours of incubation of enzyme solution at different temperature, the enzyme was also most stable at 40 ºC retaining approximately 61 % of its activity while it showed 49 % and 29 % residual activities at 50 °C and 80 °C respectively. (Figure 5a). This is in agreement with Huy *et al*., 2017 that showed MnP isolated from *Trametes spp with* an optimal activity at 60ºC. This is in accordance with Bilal *et al*., 2016 that reported that MnP shows better activity at higher temperatures, which may be due to the strengthening of the protein molecule's structure when it binds to the solid support, reducing it molecular flexibility (Muthusamy *et al.*, 2017). Statistical analysis by one-way ANOVA test for the data on effect of temperature on MnP activity and stability indicated a highly significant variation ($p < 0.05$).

The optimum temperature for laccase activity was at 60 °C. After 3 hours of incubation of enzyme solution at different temperature, the enzyme was most stable at 50 ºC retaining approximately 57 % of its activity while it showed 26 % and 29 % residual activities at 60 °C and 80 °C respectively. (Figure 5a). This is was also observed by Vignesh *et al*., 2016 that observed that the optimum

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e-ISSN: 2348-6848
p-ISSN: 2348-795X
Vol. 10 Issue 06
June 2023

activity of laccase was found at 50 ºC. Also optimum temperature was observed at 60°C by Chun-lei *et al.*, 2010. Statistical analysis by one-way ANOVA test for the data on effect of temperature on laccase activity and stability indicated a highly significant variation ($p < 0.05$).

4. Conclusion

Escherichia coli used in this study produced thermostable lignolytic enzymes and should be explored for plastics degradation especially biodegradation of polyethylene. However, further studies are needed to characterize this strain for maximum growth while genetic cloning and heterologous expression of enzymes are needed for maximum activity and production.

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Received: *04 May 2023*
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Vol. 10 Issue 06
June 2023

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Received: *04 May 2023*
Revised: *21 May 2023*
Final Accepted: *03 June 2023*

DOI: https://doi.org/10.5281/zenodo.8079547