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DIFFERENTIAL ACTIVITY OF PEROXIDASES AND LACCASE OF GUT ISOLATED STRAINS IN THE CHANGING OF TEMPERATURE AND PH

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ABSTRACT

Peroxidases (Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP)) and laccase (Lac) are extracellular enzymes that related to lignin degradation. A number of conditions have been designated to increase or inhibit these enzymes' activities in bacteria. The physical parameters such as pH and temperature are primary importance to enhance the performance of peroxidase and laccase activity. This is due to economy impact and its practicability in the industrial process. The main objective of this research was to identify individual gut bacterial strains; *Klebsiella sp., Serratia sp., Pseudomonas sp.* and *Enterobacter sp.* with maximal peroxidases and laccase enzymes activity manipulative conditions of temperatures and pHs. Enzyme activity assays were conducted for LiP, MnP and Lac using different substrates to specifically measure the activity in all four species. All the recorded data were then analyzed using ANNOVA to demonstrate the significant effect of different temperatures and pHs conditions on enzymes activities in all four species. The results show the optimum temperature for LiP and Lac was 40ºC while for MnP is 30ºC while for pH, LiP and Lac were optimum at pH6 and pH7. Also, among all four species, *Enterobacter sp*. gives the highest performance in both peroxidases and laccase in response to the change of pH and temperature. Then followed by *Pseudomonas sp.* and *Serratia sp.* where the enzymes activity in peroxidases and laccase are above average. *Klebsiella sp.* demonstrates poor performance below average in peroxidases and laccase. In conclusion, individual species has unique response to the changing pH and temperature which provides optimal activity within the gut.

Keywords: wood-feeding insects, lignin, ligninase, production, biodelignification and enzyme activity.

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PERBEZAAN AKTIVITI PEROKSIDA DAN *LACCASE* OLEH BAKTERIA YANG DI ISOLASI DARIPADA USUS DALAM KONTEKS PERUBAHAN SUHU DAN PH

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ABSTRAK

Peroksida (Lignin Peroksida (LiP) dan Mangan Peroksida (MnP)) dan *laccase* (Lac) adalah enzim ekstraselular yang berkaitan dengan degradasi lignin. Beberapa parameter telah ditetapkan untuk meningkatkan aktiviti enzim ini dalam bakteria. Parameter fizikal seperti pH dan suhu adalah penting untuk meningkatkan prestasi aktiviti peroksida dan *laccase*. Ini adalah penting kerana parameter ini boleh memberi impak kepada ekonomi dan kebolehannya dalam proses perindustrian. Objektif utama penyelidikan ini adalah untuk mengenalpasti ketegangan bakteria individu; *Klebsiella sp*., *Serratia sp*., *Pseudomonas sp*. dan *Enterobacter sp*. dengan peroksida maksimum dan enzim *laccase* aktiviti suhu manipulatif suhu dan pHs. Pemeriksaan aktiviti enzim yang telah dijalankan untuk LiP, MnP dan Lac menggunakan substrat yang berbeza untuk secara khusus mengukur aktiviti dalam keempat spesies tersebut. Semua data yang direkodkan kemudiannya dianalisis dengan menggunakan ANNOVA untuk menunjukkan kesan yang signifikan dari suhu dan keadaan pH yang berbeza terhadap aktiviti enzim dalam semua empat spesies. Hasilnya menunjukkan suhu optimum untuk LiP dan Lac adalah 40ºC manakala untuk MnP adalah 30ºC manakala untuk pH, LiP dan Lac adalah optimum pada pH6 dan pH7. Juga, di antara empat spesies, *Enterobacter sp.* memberikan prestasi tertinggi dalam kedua-dua peroksidases dan laccase sebagai tindak balas kepada perubahan pH dan suhu. Kemudian diikuti oleh *Pseudomonas sp*. dan *Serratia sp.* di mana aktiviti enzim dalam peroksidase dan laccase berada di atas purata. *Klebsiella sp*. menunjukkan prestasi buruk di bawah purata dalam peroksidase dan laccase. Kesimpulannya, spesies individu mempunyai tindak balas yang unik terhadap perubahan pH dan suhu yang memberikan aktiviti optimum dalam usus.

***Kata kunci*:** Serangga pemakan kayu,lignin, ligninase, biodelignifikasi dan aktiviti enzim

1.0 INTRODUCTION

Ligninase are classified into two large groups known as peroxidases and laccase. Both peroxidases and laccase are extracellular enzymes that related to lignin degradation [1-2]. Natural lignin degraders such as fungi are well studied for its ability to produce both enzymes [3]. However, fungi are difficult to regulate due to its sensitivity to the shear forces that are presence in the bioreactor and the agitation during the process which inhibit ligninase production and activity [4]. Also, the enzyme activity in fungi is low and requires long reaction time to achieve a maximum enzyme activity [5]. Bacteria from the soil and the wood feeding insects are seen as one of the potentials to identify new novel ligninase enzymes with high enzymatic activity [6]. These organisms act as decomposer in soil [7] and symbiont within the gut of the wood-feeding insects [8]. Lignin-degrading bacteria have the ability to modify and solubilize lignin by expressing different types of lignin-degrading enzymes [9]. And uniquely, red palm weevil (*Rynchophorus ferrugineus*) larva which is known as major pest in many palm species in Malaysia has this ability as they consume massive plant tissues [10-11]. Lignin degradation has allowed the bacteria to access to cellulose and breakdown the cellulose into glucose to be utilized for survival activities [12]. Due to this essential activity, lignin degradation is known as primary metabolic process in bacteria [6] hence, higher activity with better production which is suitable to the industrial activity. A number of conditions have been designated to increase or inhibit the peroxidases and laccase activities in bacteria. This includes the type of strains involved, concentration of carbon and nitrogen source, aeration, agitation speed, pH and temperature of the culture medium. The physical parameters such as pH and temperature are primary importance to enhance the performance of ligninase activity as these two parameters have impact on the economy and practicability in the industrial process. The main objective of this research was to identify individual gut bacterial strains that are having maximal peroxidases and laccase enzymes activity under different conditions of temperatures and pHs.

2.0 METHODOLOGY

2.1 Microorganisms

Four bacterial strains; *Klebsiella sp*., *Serratia sp.*, *Pseudomonas sp*. and *Enterobacter sp*., were previously isolated from The inoculum was prepared by growing the bacterial glycerol stock in LB broth media culture on rotary shaker at 120 rpm at 30 ºC for overnight.

2.2 Submerged fermentation

All four strains were cultured via submerged fermentation with the parameters were set to corresponding temperature, shaking at120 rpm for 7 days in an

incubator shaker (SISTA). All the isolates were freshly inoculated in 100 mL of LB broth with addition of 0.5% lignin in 250 ml Erlenmeyer flask. Initial cell concentration to inoculate is 1x107 and the cells were prepared using MacFallen standard.

2.3 Effect of cultivation pH

The effect of pH on peroxidases and laccases activity was determined by selecting four different range of pH were selected as recommended by Rahman et al. [13]. These include pH 5, pH 6, pH 7 and pH 8. The broth culture was adjusted according to the relevant pH with sodium hydroxide (1M) and hydrochloric acid (1M).

2.4 Effect of cultivation temperature

To determine the effect of temperature on peroxidases and laccases activity, two different range of temperatures were selected as recommended by Rahman et al. [13]. These include 30ºC to 40ºC.

2.5 Crude enzymes preparation

About 6mL of the cultures broth from submerged fermentation of the isolates were collected in 15mL centrifuge tube. The culture broths were centrifuged at 4ºC at 7000 rpm in cooling centrifuge. The supernatant after biomass separation were analyzed for enzyme activity. All the tubes were kept in ice to prevent protein degradation.

2.6 Enzyme activity assays

Lignin peroxidase activity. Reaction mixtures of assay reagent were prepared according to Bholay et.al. [14]. The reagent consist of 1 mL Sodium Potassium Tartarate buffer (50mM, pH 4), 0.1 mL H2O2 (0.1mM), 0.1 mL methylene blue as substrate (32 µM) and 0.5 mL of supernatant (crude enzyme) was added. The solution was incubated for 1 hour at 37ºC and absorbance was measured using UV- Vis (LABOMED) with wavelength of A650. *Manganese peroxidase activity*. Manganese peroxidase activity was assayed using phenol red as substrate as described by Hariharan & Nambisan [15]. The reaction mixture consists of 0.05 mL phenol Red (0.1%), 0.1 mL sodium lactate buffer (250 mM), 0.025 mL manganese sulphate (22mM), 0.1 mL BSA (0.5%), 0.025 ml hydrogen peroxide (0.2 mM) in 0.1 M citrate buffer and 0.5 mL supernatant (crude enzyme). A colour change was measured using UV-Vis (LABOMED) with wavelength at 610 nm.

Laccases activity. Laccase activity was measured using ABTS as substrate as suggested by Rahman et al.[13]. The reaction mixture consist of 0.15 mL ABTS (0.03%), 0.5mL sodium acetate buffer (0.1 M, pH 5.0), 0.5 mL supernatant (crude enzymes). ABTS oxidation was measured by UV-Vis (LABOMED) with an absorbance at 420 nm.

2.7 Statistical Analysis

The data of enzyme activity was subjected to ANNOVA using general linear model. The purpose was to estimate the p-value for significance effect of cultivation temperatures and pHs with the interval confidence 95% and degree of freedom of 1 for temperature and 3 for pH. All experiments were carried out in triplicates and the triplicates data were used in the analysis.

3.0 RESULTS

3.1 Effect of pH and Temperature on lignin peroxidase (LiP) activity

LiP production capacities of different bacterial strains were compared and analyzed using methylene blue for a visual inspection of LiP activity. The results for maximal LiP activity are presented in Table 1. Two important physical parameters (temperatures and pHs) that have influenced on enzymatic activity were tested and the results were presented in Figure 1. As it can be noticed, there are remarkably high correlations between LiP activity with pHs and temperatures. It can be observed that the activities of LiP in all four strains were notably increase at slightly acidic to neutral pH values (pH6 – pH7) especially at high temperatures. *Klebsiella sp*. (11.90 ±0.10) and *Serratia sp.*(14.16 ±0.05) are shown to have maximal activity at neutral condition with an increment of 2-fold compared to pH5 and pH8 and 20 - 30% higher when the temperature reached at 40ºC. Meanwhile, in *Pseudomonas sp.*(15.38 ±0.36) and *Enterobacter sp*. (14.46 ±0.45) the LiP activities reached the highest when the condition at slightly acidic with > 10% differences in pH and >20% in temperature. With the support of statistical analysis, both factors are significantly affected LiP activity in *Klebsiella sp.* (*p-value* < 0.05), *Serratia sp.* (p-value <0.05), *Pseudomonas sp.* (p-value< 0.05) and *Enterobacter sp*. (p-value<0.05).

3.2 Effect of pH and Temperature on Manganese peroxidase (MnP) activity

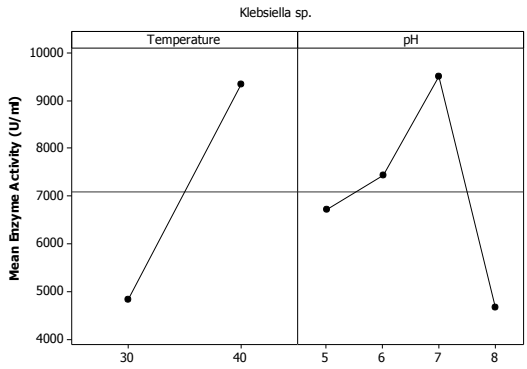
MnP activity was monitored with phenol red which enabled to visually measure using UV-Vis spectophotometer. The enzyme activity were measured and recorded in Table 2 and Figure 2 represents the correlation between MnP with different the pH and temperature. The MnP activities in all four different strains

were not dramatically enhanced by the changing of pH and temperature. In *Klebsiella sp*. and *Enterobacter sp.* the MnP activity was significantly influence by pH (p-value<0.05) as compared to temperature (p-value>0.59, 0.35).

Table 1: Maximal LiP activity measured using methylene blue.

Species		**Maximal Crude Lignin Peroxidase Activity (U/mL)**			
Temp. (°C)	**pH**	***Klebsiella sp.* (x10³)**	***Serratia sp.* (x10³)**	***Pseudomonas sp.*(x10³)**	***Enterobacter sp.*(x10³)**
30	5	3.80 ± 0.09	3.64 ± 0.15	4.20 ± 0.14	7.92 ± 0.16
40		9.67 ± 0.12	7.94 ± 0.06	8.14 ± 0.40	6.00 ± 0.35
30	6	5.08 ± 0.43	8.67 ± 0.10	11.1 ± 0.21	9.90 ± 0.23
40		9.78 ± 0.07	11.31 ± 0.26	15.4 ± 0.36	14.46 ± 0.45
30	7	7.11 ± 0.20	10.19 ± 0.52	9.96 ± 0.40	8.80 ± 0.96
40		11.90 ± 0.10	14.16 ± 0.05	13.44± 0.07	13.74 ± 0.13
30	8	3.40 ± 0.30	4.48 ± 0.18	3.55 ± 0.25	7.97 ± 0.09
40		6.00 ± 0.13	7.46 ± 0.13	8.87 ± 0.65	9.70± 0.19

(a)



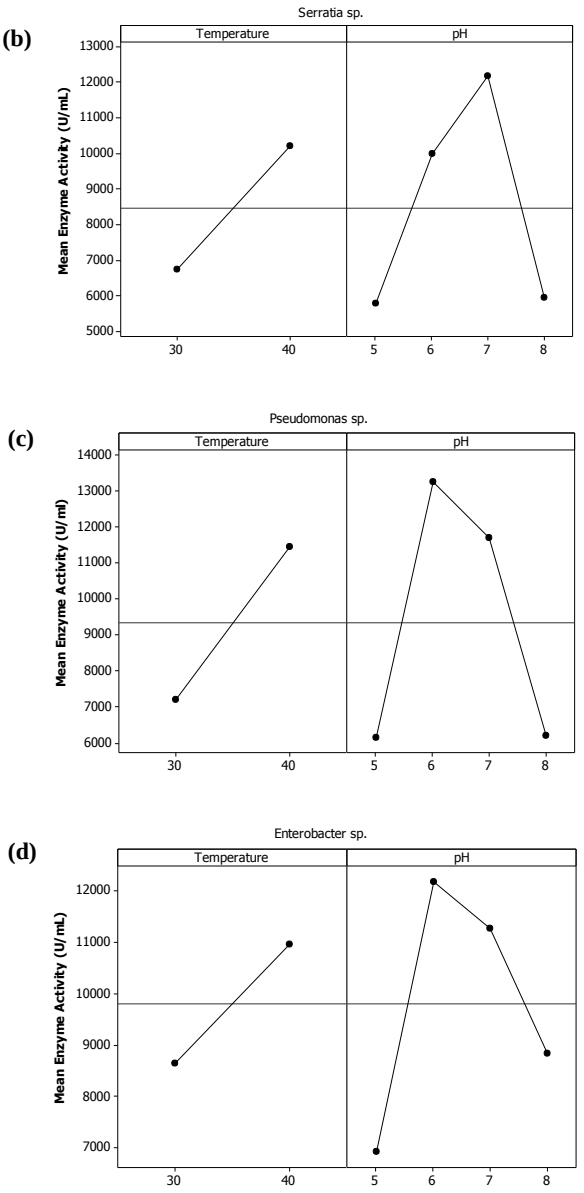
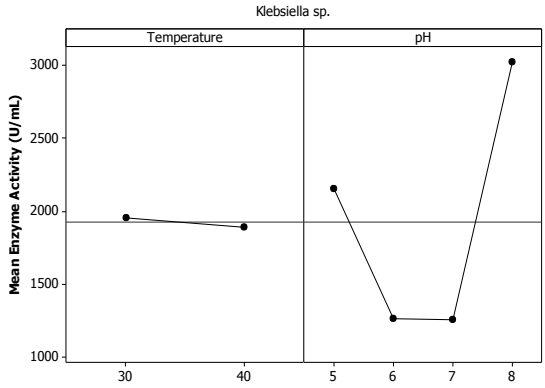


Figure 1**:** Effect of temperatures and pHs on lignin peroxidase for all four different species

Table 2: Maximal MnP activity measured using Mn2+ and phenol red as a substrate

Species		**Maximal Crude Manganese Peroxidase Activity (U/mL)**			
Temp. (°C)	**pH**	***Klebsiella sp.* (x10³)**	***Serratia sp.* (x10³)**	***Pseudomonas sp.*(x10³)**	***Enterobacter sp.*(x10³)**
30	5	2.60 ±0.08	3.90 ± 0.17	2.00± 0.09	5.37 ± 0.03
40		1.71 ± 0.70	2.60 ± 0.02	4.32 ±0.07	7.52 ± 0.05
30	6	1.30 ± 0.03	4.52 ± 0.08	1.71 ± 0.11	2.48 ± 0.03
40		1.21 ± 0.16	2.72 ± 0.02	2.11 ± 0.05	3.11 ± 0.05
30	7	1.11 ± 0.25	3.62 ± 0.03	2.45 ± 0.07	2.96 ± 0.03
40		1.41 ± 0.06	2.93 ± 0.02	2.72 ± 0.03	3.20 ± 0.05
30	8	2.81 ± 0.70	3.80 ± 0.06	3.74 ± 0.04	5.85 ± 0.03
40		3.24 ±0.06	3.62 ± 0.03	1.97 ± 0.05	3.55 ± 0.11

(a)



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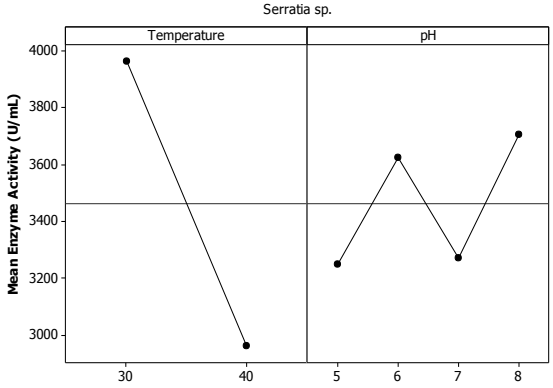


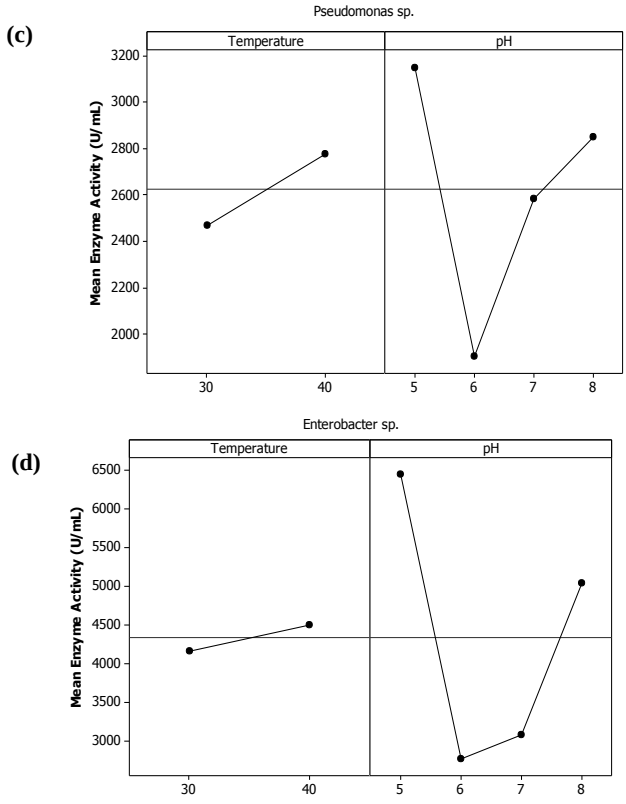
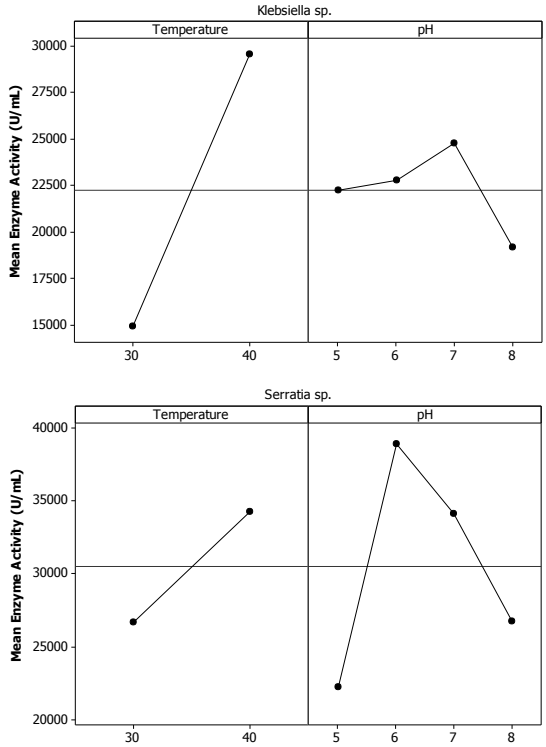


Figure 2: Effect of temperatures and pHs on manganese peroxidase for all four different species.

Table 3: Maximal Lac activity measured using ABTS as a substrate.

Species		**Maximal Crude Laccase Activity (U/mL)**			
Temp. (ºC)	**pH**	***Klebsiellasp.* (x10^3)**	***Serratia sp.* (x10^3)**	***Pseudomonas sp.*(x10^3)**	***Enterobacter sp.*(x10^3)**
30	5	1.74 ± 0.46	16.67 ± 0.11	20.57 ± 0.35	32.57 ± 0.10
40		27.10 ± 0.09	27.8 ± 0.08	31.65 ±0.77	28.57 ± 0.86
30	6	15.60 ± 0.14	37.31 ± 0.03	31.86 ± 0.25	31.30 ± 0.11
40		29.96 ± 0.89	40.40 ± 0.38	43.33 ± 0.43	43.30 ± 0.40
30	7	1.64 ± 0.13	3.19 ± 0.13	32.77 ± 0.38	39.39 ± 0.28
40		33.18 ± 0.23	36.33 ± 0.32	42.07 ± 0.14	44.27 ± 0.45
30	8	10.37 ± 0.07	20.97 ± 0.12	19.22 ± 0.35	27.55 ±0.11
40		28.00 ± 0.02	32.49 ± 0.43	40.03 ± 0.14	43.43 ± 0.97



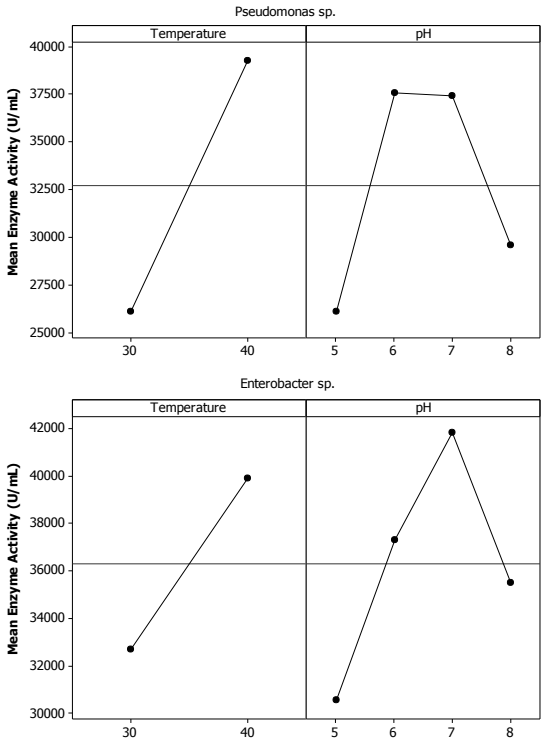


Figure 3: Effect of temperatures and pHs on Lac activity for all four different species

3.3 Effect of pH and Temperature on Laccases (Lac) activity

Influence of temperature and pH on in vitro activity of Lac was carried out using ABTS as a substrate. Data collection was presented in Table 3. The collected data were subjected to statistical analysis to prove the significance effect of these two parameters on Lac activity and the results were illustrated in Figure 3. These results confirmed that Lac activity in all four species is greatly influenced by the pH and the temperature. The Lac activity was enhanced nearly 2-fold with the rising 10ºC in temperatures and this was observed in *Klebsiella sp*. ($p < 0.005$), *Serratia sp*. ($p<0.005$), *Pseudomonas sp*. ($p<0.005$) and *Enterobacter* sp. ($p < 0.005$). Changes in pH significantly affect Lac activity in *Klebsiella sp*. ($p < 0.05$), *Serratia sp.* ($p < 0.05$), *Pseudomonas sp.* ($p < 0.05$) and *Enterobacter sp*. ($p<0.05$). Maximal Lac activity was observed at pH 7 in *Klebsiella sp.*, *Pseudomonas sp.*, and *Enterobacter sp*. at 40ºC. While for *Serratia sp.*, highest Lac activity was observed in pH6 at 40ºC.

4.0 DISCUSSIONS

The activities of peroxidases and laccase enzymes of the gut bacteria in natural environment are primarily controlled by abiotic factors such as pH and temperature (Jiang et al. 2008; Moldes 2006; Rahman et al. 2013; Chai 2008). Changes in abiotic environment of culture conditions affect enzymes activity in complex manner that are difficult to predict (Burns et al. 2013). This is due to complex microbial communities that are exist to support their insect host with crucial carbon source from the ingested materials (König 2006). Observing individual species responding to the changing temperature and pH provide an insight on which strains works best and give the optimal peroxidase and laccase activity.

4.1 Effect of Temperature on LiP, MnP and Laccase activities

Temperature is related to the enzyme thermal stability of the enzymes hence affects the activity greatly. This thermal stability of enzymes is depending on the microbial source. In this experiment, all the four strains were isolated from the gut of red palm weevil. Based on the temperature profile of LiP and Lac activity, the optimal temperature for maximal enzyme activity was 40^0C.This results relevant to the previous findings where microbes that are isolated from the soil (Wang et al. 2010) or gut bacteria (Tartar et al. 2009; Ni & Tokuda 2013) have an optimal temperature of peroxidase and laccase activity at range between 30 – 55ºC. However, these optimal temperatures were considered lower compared to microbes that are isolated from the effluents that are released by industrial activities. For example *Bacillus sp.* VUS which had an optimal temperature for peroxidase activity at 65^0C (Dawkar et al. 2009). Hence, the optimal temperature is similar to the gut which is within 30ºC to 40ºC. In the case of MnP, the enzyme activity is dependent on Mn2+ ion that binds to Mn binding site. The Mn(II) protect manganese peroxidase from thermal denaturation. MnP oxidizes lignin indirectly via oxidation of Mn(II) to Mn(III). The released of Mn(III) act as an oxidizing agent initiate lignin degradation. Therefore Mn ion binding facilitate MnP activity. This explanation fits to the observation whereby higher temperature decrease MnP activity. Limited Mn ion availability might have affected the MnP activity in response to increase temperature.

4.2 Effect of pH on LiP, MnP and Laccase activities

Response of individual species towards changing in pH is possibly related to the localization of individual species within gut compartment. The physiochemical conditions in different gut compartment exhibit variation in pH value. According to Engel and Moran (Engel & Moran 2013), the pH conditions in the

anterior midgut is near slightly alkaline (pH8), whereas in the midgut there is a strong alkaline condition (pH >10) and hindgut is pH7 (Engel & Moran 2013). Interestingly, the majority of microbial resides most at the hindgut where the pH condition is neutral. It is possible that the four species that involved in lignin degradation are localized within the hindgut. Hence, this provides an explanation to the reason for most of ligninase (LiP and Lac) in all four species work best at neutral condition. Optimal pH for laccase are varies depending on the type of substrates used (Margot et al. 2013; Madhavi & Lele 2009). For example, optimal pH for phenolic substrate can be ranged within pH3 to pH7 whereas, for ABTS the optimum pH is more acidic between pH3 and pH5 (Madhavi & Lele 2009). In this study, all the four strains were previously cultured with phenolic substrate (lignocellulosic) but later were tested using ABTS. It is possible the enzymes are adjusting to these different substrates thus given the optimal pH of slightly acidic to neutral. Eventhough the pH preference of MnP is the acidic and alkaline, it is possible that compensation mechanism exist to orchestra the elevation of enzymes within the gut as to ensure optimal nutrient is obtained through lignocellulosic degradation. However, further experiment required to support this theoretical opinion by quantifying the expression of MnP in relative to the expression of LiP and Lac.

4.0 CONCLUSION

In this study, we have discovered that the physical parameters such as pH and temperature are primary importance to enhance the performance of peroxidase and laccase activity. Individual species have responded differently to the changes of temperature and pH in expressing different profile of peroxidases and laccase enzymes activity. Therefore, understanding their optimal temperature and pH is one of the crucial prior knowledge before adding their commercial value in industrial application.

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