Production of Ligninolytic Enzymes from Thermophilic Bacterial Strains Isolated from Palm Oil Wastes

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Abstract. The conversion of lignocellulosic biomass into bioethanol or biochemical products requires a crucial pre-treatment process to break down the recalcitrant lignin structure. Biological depolymerization of lignin using microbial enzyme appeared to be a promising pre-treatment alternative as it offers environmentally friendly treatment with lower energy requirements. In this study, three (3) thermophilic bacterial strains (S2, S11Y, S23) with lignin-degrading potential were previously isolated from palm oil wastes and identified as *Stenotrophomonas* sp., *Bacillus subtilis*, and *Aeribacillus* sp., respectively. These isolates demonstrated the capability to grow in a medium containing AL as the sole carbon source. Most isolates also demonstrated enzymatic activities toward lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) enzymes within a short period of 72 h. The highest LiP was secreted by strain S2 (70.97 ± 0.059 U/L), followed by S11Y (63.570 ± 0.021 U/L) and S23 (60.880 ± 0.042 U/L). MnP was generated with low activities of approximately 1.313 ± 0.037 U/L by S11Y, 0.364 ± 0.004 U/L by S2, and 0.215 ± 0.021 U/L by S23. For the Lac enzyme, low activities were detected only in S2 and S11Y, with 2.075 ± 0.612 U/L and 1.463 ± 0.116 U/L, respectively. This preliminary study appeared to show that the isolated thermophilic bacteria have the potential to be applied for lignin degradation application.

1. Introduction

Malaysia's palm oil sector has contributed to the most significant biomass generation in the country, with nearly 143 million tons annual production and predicted to increase further with the expansion of palm oil plantations [1]. Utilizing the palm oil biomass, which currently has little use in the industries, for higher value-added downstream applications, such as bioethanol and bio-based chemicals, can significantly contribute to gross national income. However, the initial pre-treatment step of breaking down the lignin component of the biomass structures before transforming it into industrial products is challenging. The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. [2]. Among them, lignin has the most complex structure, which causes it to be challenging to degrade.

Microbial enzymes to degrade lignin appear to be a potential pre-treatment strategy since it provides an environmentally friendly treatment with lesser energy requirement. Some microorganisms are predicted to have the ability to develop a complex system of oxidative enzymes that could subsequently degrade the lignin structures [3]. The enzymatic decomposition of lignin is primarily mediated by two types of ligninolytic enzymes: phenol oxidase (laccase, Lac) and peroxidases such as lignin peroxidase,

LiP, and manganese peroxidase, MnP [4]. Some white-rot and brown-rot fungi have been proven to decompose lignin, and various researches on the synthesis of fungal lignin-degrading enzymes have been reported [5,6]. However, fungi have more complex genetic and protein expression characteristics [7] than bacteria. Bacteria are well-known for their rapid growth, metabolic flexibility, and their lignin metabolism can be investigated further. However, most enzymatic lignin degradation from bacteria, especially thermophilic bacteria, is underexplored. Thermophilic bacteria are expected to secrete enzymes with better stability and resistivity to denaturation than the mesophiles [8], which require further investigation. This study evaluates three isolated thermophilic bacterial strains (S2, S11Y, S23), previously identified as *Stenotrophomonas* sp., *Bacillus subtilis,* and *Aeribacillus* sp., respectively, for their lignin-degrading potential. The strains were further examined to evaluate their ligninolytic enzyme activities.

2. Materials and Methods

2.1. Cultivation of bacteria strains and Determination of cell growth

In this study, three thermophilic bacteria strains (S2, S11Y, S23) were previously isolated from Palm Oil Empty Fruit Bunch and identified as *Stenotrophomonas* sp. strain S2, *Bacillus subtilis* strain S11Y, and *Aeribacillus* sp. strain S23, respectively. These bacterial strains were utilized to evaluate their lignin-degrading potentials. Bacterial isolates were grown on agar plates comprising of 2.5 g/L of Alkali lignin (AL) in minimal salt medium (AL-MSM) for bacterial growth [9], where AL was used as the only carbon source. Further screening was performed with Azure-B dye [6] by supplementing the agar plate with 0.01 %w/v Azure B. The appearance of a clear zone around the colony was used to indicate peroxidase enzyme activity [10].

2.2. Inoculum Preparation

A single colony of all bacteria strains was initially grown on 20 mL Luria-Bertani (LB) broth at 50 ^{o}C, shaking at 160 rpm for 24 hours to obtain a final OD600 of 1.0 [9]. The cells were harvested by sequentially centrifuged at 8,000 ×g for 10 mins. The cell pellets were washed twice with a 10 mL MSM. The bacteria cells were then resuspended in 5 mL containing the same washing media. 0.25 mL of the bacteria cells were collected and added to 10 mL of lignin fermentation medium containing AL-MSM medium to set the initial OD_{600} ~ 0.1. The reaction was performed by incubating at 160 rpm and 50 ^{o}C for 7 days. Sample (1 mL) was taken daily to evaluate the cell growth by evaluating the OD_{600} of the degraded samples. Samples collected were centrifuged to separate the supernatants from the pellets at 8,000 ×g for 5 mins. The supernatants were collected and used as crude enzymes to measure the ligninolytic enzyme activity.

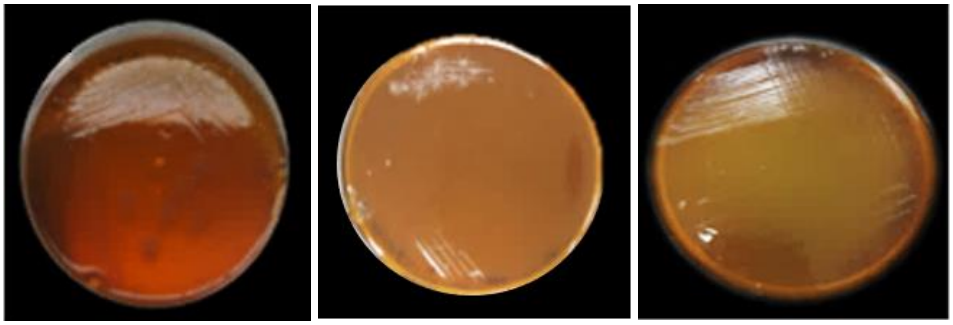
2.3. Enzymatic Assays for Ligninolytic Enzyme Production

Lignin peroxidase (LiP) enzyme was measured based on the oxidation of dye Azure B [11] at 651 nm ($\epsilon = 48800\ M^{-1}cm^{-1}$). The reaction mixture (total volume, 1.0 mL) contained 200 µl of culture filtrate in 125 mM Na-tartrate buffer (pH 3.0) and 0.160 mM azure B. The addition of 2 mM H_2O_2 initiated the reaction. One unit of enzyme activity is expressed as a decrease of 0.1 units per minute of the culture filtrate. Manganese peroxidase (MnP) activity was determined based on Phenol red assay [10]. A total reaction volume of 1.0 mL contained 50 mM Na-tartrate buffer (pH 4.5), 0.2 mM $MnSO_4$, 0.1 mM H_20_2, 0.0025% phenol red and culture filtrate. The reaction was monitored at 431 nm ($\epsilon = 22000\ M^{-1}cm^{-1}$). Laccase (Lac) activity was assayed through the oxidation of 2,2'-azinobis-(3-ethylbenzethiazoline-6-sulphonate) (ABTS) [12]. The enzymatic mixture consisted of 0.03% ABTS, 0.1 M Na-acetate buffer (pH 5.0), and culture filtrate, making a total volume of 1 mL. Oxidation of ABTS was measured by an increase in absorbance at 420 nm ($\epsilon = 36000\ M^{-1}cm^{-1}$).

3. Results and Discussion

3.1. Bacterial growth and screening for ligninolytic activity

In this study, three thermophilic bacterial strains (S11Y, S23, and S2) were evaluated for their ability to degrade lignin structure. Ligninolytic bacteria were reported as the bacteria that can utilize lignin as their primary carbon source and do not require the addition of other carbon sources as co-substrates. [13]. In this study, all bacterial strains belonging to different genera (*Stenotrophomonas* sp., *Bacillus subtilis*, and *Aeribacillus* sp.) were grown on solid and liquid medium containing MSM with AL as the sole carbon source. AL is a semi-degraded (average M_w ~ 10,000) chemically modified derivative of high-molecular-mass lignin, used as a lignin model [14]. Based on Figure 1, all bacterial strains demonstrated growth on AL-MSM agar plate medium when grown for 7 days, indicating their capability in utilizing AL for growth. Strain S11Y demonstrated prominent growth, followed by strain S2 and S23.



(a) (b) (c)

Figure 1. Growth of the isolated bacterial strains in solid agar plate for (a) *Stenotrophomonas sp.* strain S2, (b) *Bacillus subtilis* strain S11Y, and (c) *Aeribacillus sp.* strain S23, using AL as sole carbon source.

Growth on the liquid culture in OD_{600} was also conducted to confirm the bacterial capability in utilizing lignin. Figure 2 demonstrates the growth patterns for all bacterial strains in the AL-MSM. The rapid growth of S11Y and S23 was observed in the first 72 hours. Maximum growth was seen after 144 and 168 hours for both strains, respectively (Figure 2). On the contrary, strain S2 showed slower growth, but maximum growth was seen at 120 hours incubation period. The results indicated that the bacteria metabolized AL during the exponential growth phase to provide carbon and energy sources. However, in the previous reports, most bacteria strains, such as *Nonomuraea gerenzanensis* [14] and *Bacillus ligniniphilus* L1[15], and *Bacillus* sp. [16] utilize glucose or mannose as carbon sources at the initial growth stage and subsequently use lignin as co-substrate. Besides, compared to the alkaliphilic bacterium *Bacillus ligniniphilus* L1 [15], *Bacillus subtilis* strain S11Y in this study showed better growth when AL was used as the sole carbon source. Thus, in contrast to the previous reports, AL can be used as the sole carbon source by all strains in this study. Besides, since these bacterial strains can metabolize AL throughout the entire life cycle for their growth, better efficiency and lignin degradation rate might be attained than the bacteria strains that use lignin as co-substrate.

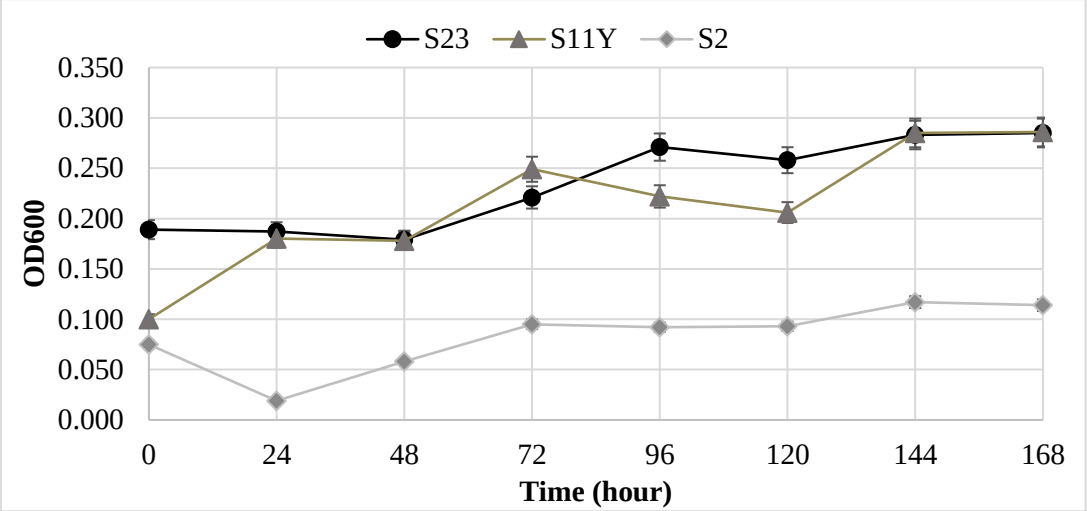


Figure 2. Growth of the isolated bacterial strains (*Stenotrophomonas* sp. strain S2, *(2.) Bacillus subtilis* strain S11Y and *(3.) Aeribacillus* sp. strain S23) using AL as sole carbon media.

The Azure B plate decolorization is commonly used as a qualitative method to screen for bacterial strains with the lignin-degrading ability [17]. Thus, this analysis was performed on the isolated bacterial strains, as presented in Figure 3.

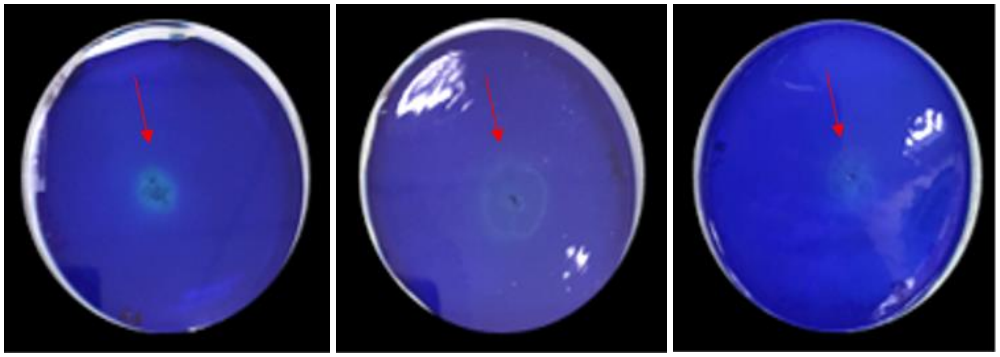


Figure 3. Azure B agar plate decolorization of (a) *Stenotrophomonas* sp. strain S2; (b) *Bacillus subtilis* strain S11Y; (c) and *Aeribacillus* sp. strain S23.

In this study, all bacterial strains showed decolorization on agar plates containing MSM incorporated with Azure B as the lignin polymeric dye (Figure 3). Based on the results, strain S2 and S11Y demonstrate a clear zone of decolorization. A similar observation was also reported for *Enterobacter hormaechei* and *Bacillus licheniformis* [18] and *Nonomuraea gerenzanensis* [14], demonstrating the presence of ligninolytic enzymes in each isolate.

3.2. Production of ligninolytic enzymes by bacterial strains

Several ligninolytic enzyme assays were performed for each isolate to ensure the lignin-degrading ability of each strain. The studied enzymes include LiP, MnP, and Lac, which were previously reported as ligninolytic enzymes. Among all the enzyme assays performed, LiP and MnP were secreted by all bacterial strains (Table 1), with LiP being the predominant enzyme being detected. Highest LiP were secreted by strain S2 (70.968 ± 0.175 U/L), followed by S11Y (63.570 ± 0.021 U/L), and S23 (60.880 ± 0.042 U/L). Based on the observation, strain S23 showed an absence of Lac activity and generated

low enzyme activities compared to the rest of the strains. In most bacterial strains, maximum enzyme production was generated at 72 hours incubation period, except for the production of MnP by S11Y, where the highest enzyme production was secreted at 168 hours of incubation.

Table 1. Highest LiP, MnP, and Lac enzyme activities produced by bacterial strains studied.

Isolates	LiP Enzyme Activity (U/L)	Period (h)	Lac Enzyme Activity (U/L)	Period (h)	MnP Enzyme Activity (U/L)	Period (h)
S23	60.880 ± 0.042	72	ND*	-	0.215 ± 0.021	72
S11Y	63.570 ± 0.021	72	1.463 ± 0.116	72	1.313 ± 0.037	168
S2	70.968 ± 0.059	72	2.075 ± 0.612	72	0.364 ± 0.004	72

*ND: Not detected

Table 2. Comparison of bacterial LiP, MnP, and Lac enzyme activities with previous studies.

Isolates	Enzyme activities (U/L)			References
	LiP	MnP	Lac	
Bacillus subtilis WPI	-	-	2.28	[19]
Bacillus sp. strain CS-1	-	-	0.60	[20]
Brevibacillus agri AN-3	3.10	-	1.90	[21]
Stenotrophomonas maltophilia	9.22	7.84	3.77	[22]
S23 (*Aeribacillus* sp.)	60.88	0.22	-	This study
S11Y (*Bacillus subtilis*.)	73.57	1.31	1.46	
S2 (*Stenotrophomonas* sp.)	70.97	0.36	2.08	

Table 2. shows the comparison of the studied bacterial LiP, MnP, and Lac enzyme activities with the previous reports on ligninolytic enzyme productions by bacteria of similar species with this study. Most of these strains only secreted Lac, and no apparent LiP activity was observed when strains were grown on lignin substrate [19] [20]. In contrast, our results demonstrated the highest enzyme activity for LiP by each strain when grown in AL, and only low Lac activities were observed in S11Y and S2. Besides, most of the previous related studies for *Bacillus* sp. did not detect the presence of MnP activities [19] [20] [21], but it was detected in our study. Thus, it can be seen that the enzyme activities studied are comparable with the previous studies, indicating that the studied bacterial strains present promising results towards the production of lignin-degrading enzymes. Besides, limited reports on the ligninolytic enzymes and their function in lignin degradation by bacteria of genus *Stenotrophomonas* sp. are available [22]. No previous study on lignin degradation by *Aeribacillus* sp. was also reported. Therefore, there is a new potential for future research on attaining novel sources of lignin-degrading enzymes.

4. Conclusion

In conclusion, the thermophilic bacteria reported in this study demonstrated potential for lignin degradation since they can generate the main ligninolytic enzymes (LiP, MnP, Lac). Future work on analyzing the draft genome sequencing of the isolated bacteria could be done to validate the presence of the genes corresponding to ligninolytic enzymes and study the actual mechanism of lignin degradation by bacteria. Exploring bacterial enzymes for lignin degradation will yield fundamental insights into necessary enzymes and could be applied by industrial enzymes for the lignin degradation process.

Acknowledgment
The authors gratefully thank Dr. Hirofumi Hara (University of Tokyo) and Dr. Nor'azizi bin Othman (Universiti Teknologi Malaysia) for their excellent technical assistance. The authors would also like to express appreciation for the support of the sponsors Vot No.: R.K130000.7343.4B400.

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