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Mesophile PLA-Degrading Bacteria Isolated from Pekan, Pahang, Malaysia Landfill Soil

J.C. Jalani*, Z.I.M Arshad, R.C. Man, S.M. Shaarani, S.K.A. Mudalip and S.Z. Sulaiman

Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang Al-Sultan Abdullah, Pahang, Malaysia

One of the most recent studies on bioplastic focused on bacteria that break down PLA that were isolated from soils. Environmental problems develop when PLA waste fails to decompose completely in Malaysian landfills due to a lack of external heat. This research isolated and screened bacterium that can break down PLA at room temperature. Samples of soil were taken from a nearby landfill in Pekan, Pahang, Malaysia, and isolated in PLA-emulsified agar at 30°C. Two isolates, B8 and A10B, were chosen as possible mesophile PLA-degraders. They were identified as *Brevibacillus parabrevis* and *Renibacterium salmoninarum* using 16S rRNA identification analysis. The result of streaking on skim milk agar showed that both microorganisms were able to produce protease enzyme. The relative halo zones quantified on the skim milk agar by B8 and A10B were 1.33 and 3.86 mm, respectively. Additionally, amylase, lipase, and protease enzyme assays were carried out. The results showed that isolate B8 had 114.49 U/mL of protease, 15.36 U/mL of lipase, and 3.17 U/mL of amylase. Isolate A10B had 120.45 U/mL of protease, 9.23 U/mL of lipase, and 6.56 U/mL of amylase. In addition, B8 and A10B were tested for PLA biodegradation in soil at 30°C for eight weeks, resulting in a weight loss of 37.8% and 32.6% of PLA film, respectively. These results showed that *Brevibacillus parabrevis* and *Renibacterium salmoninarum*, which can degrade PLA at room temperature, can be isolated from Malaysian landfills. We suggest studying these stains to use them to combat bioplastic waste in municipal landfills.

Keywords: Polylactic acid (PLA); Biodegradation; PLA-degrading bacteria; PLA-degrading enzyme

I. INTRODUCTION

Biodegradable plastic has been introduced as an alternative to combat the plastic waste problems generated by conventional plastic.

Bioplastic such as polylactic acid (PLA), has been at the forefront of most research because it can be produced from renewable resources such as corn, starch, and cassava. Several PLA-based technologies have recently emerged, with the goal of achieving chemical, mechanical, and biological qualities that are comparable to or better than traditional polymers (Li *et al*., 2016). PLA has made a breakthrough in the industry as a packaging thermoplastic for general packaging applications due to material qualities that are similar to synthetic thermoplastics. PLA, on the other hand, poses difficulties in the setting of post-disposal deterioration. It means that when PLA-based products are used and dumped in the environment, the time it takes for them to decompose is longer than for other bioplastics like PHA or PEG (Hamad *et al*., 2014). Environmental issues arises when PLA waste fails to decompose fully due to the absence of external heat in Malaysian landfill.

In order to tackle this problem by taking into consideration the Malaysian climate, this research was conducted with the objective to isolate bacteria that can degrade PLA at room temperature, characterise and identify the PLA-degrading bacteria, and test the biodegradability of the bacteria to degrade PLA.

In this research we address the limitation of slow degradation process of polylactides in the landfill with no

*Corresponding author's e-mail: joycecynthia18@gmail.com

additional heat applied. The research will approach local Malaysian landfill, specifically in Pekan, Pahang, to collect soil samples and will attempt to isolate bacteria that can degrade PLA at normal temperature. These bacteria will need to be characterised in for Gram staining, morphological study, catalase test, and protease activity. Bacteria that show the highest protease activity will be further studied on growth curve, and 16S rRNA gene sequencing analysis. After bacteria are identified, this research will continue to evaluate the performance of selected PLA-degrading bacteria of its efficiency to degrade PLA films in soils.

II. MATERIALS AND METHODS

These procedures will be routinely utilised in the Faculty of Chemical and Process Engineering Technology laboratory. Appropriate sterile techniques will be applied to all procedures, and universal precautions and lab safety protocols will be followed.

A. Chemicals and Consumables

All chemicals and reagents were purchased from Gibco, Sigma-Aldrich Chemicals (St. Louis, MO, USA) and Merck Chemicals (Darmstadt, FR, Germany). All chemicals were of analytical grade. The chemicals used are as follow:

¼ Ringer's tablet, Dichloromethane, Tween80, Bacto agar powder, PLA powder, Yeast extract, Malt extract, Glucose, Agarose powder, Skim milk powder, Nutrient agar, Nutrient broth, Gram staining kit (or Crystal violet/ Iodine/ Acetone/ Safranin), Bibulous paper, Bromocresol purple, Disposable Petri dish, Pipette tips (200/1000µL), Disposable dropper, Filter paper, Ethanol, Laboratory gloves, Spatula, Weighing boat, Parafilm tape, 2-mm sieve.

B. Methods

1. Soil samples collection

A landfill located in Pekan, Pahang has been chosen as the soil samples collection site. A total of three replicates were procured from the landfill site and collected samples were stored in refrigerator.

2. Isolation of PLA-degrading bacteria

Soil samples were suspended in ¼ Ringer 's solution and diluted via serial dilution up to dilution factor (DF10) 10^{-10}. Diluted soil samples from DF6 until DF10 were chosen to be spread on PLA-emulsified agar containing 0.1%(w/v) PLA. The Agar plates were incubated for 5 days at 30°C and every bacterial colony was restreaked on to new PLA-emulsified agar to obtain pure colony.

3. Protease presence and relative enzyme activity

Skim milk agar were used to detect the presence of protease enzyme by streaking isolates on agar and observing for a formation of halo zones (Bubpachat *et al.*, 2018). The isolates that were able to form halo zones on skim milk agar were restreaked on new plates to observe the diameter of halo zones formation and calculate the relative enzyme activity using Eq. (1) (Bradner *et al.*, 1999).

$$Relative\ enzyme\ activity = \frac{(X - Y)}{Y} \qquad (1)$$

where,

X = diameter of halo zones

Y = diameter of colony

4. Identification of bacterial isolates via 16S rRNA amplification and sequencing

Pure isolates were sent for bacterial identification by a commercial laboratorial research service, Apical Scientific Sdn. Bhd. Samples were prepared using electrophoresis of 1µL of each sample and PCR were run on 1% TAE agarose gel at 100V for 60 minutes. The test helps identify if the isolate were bacterial or not for further identification process. Negative results were discarded, and only positive bacterial results were taken to be further analysed for DNA sequencing. The sequenced DNA were blasted against NCBI 16S ribosomal RNA sequence (Bacteria and Archaea) Database to further identify the bacteria and its closely related strains.

5. Growth curve of bacterial isolates

Isolates were cultured overnight in Basal Salt Medium (BSM) broth containing 1g of PLA as carbon source. The flasks were incubated in an orbital shaker at 30°C and 180 rpm. Aliquots

of the medium were taken aseptically within a regular interval of 1 hour and was measured using a UV-spectrophotometer at 600nm and using BSM as blank. The optical density (OD) of the samples were recorded for a duration of 4 days of growth. The OD600 values were plotted against time to create a growth curve of bacteria (Hall *et al.*, 2014).

6. *Enzyme assay of bacteria isolates*

Lipase activity assay was conducted based on Abd-Elhakeem's methodology with slight modifications (Abd-Elhakeem *et al.*, 2013). The calibration curve was prepared using phenol standard solutions containing concentration of phenol from 1 to 100 µmole/L and the absorbance was obtained via UV-Vis Spectrophotometer at 765nm. Similarly, the absorbance of the samples was also measured using UV-Vis Spectrophotometer at 765nm against a blank. The concentration of phenol at the respective absorbance value was determined and therefore the activity of lipase was expressed as Units/mL as per shown in Equation (1).

$$Lipase\ activity\ \left(\frac{U}{mL}\right) = \frac{Phenol\ equivalent\ (\mu mole) \times dilution factor}{Volume\ of\ enzyme\ (mL) \times Reaction\ time\ (mins)} \quad (1)$$

Amylase activity assay was conducted by using 3,5-dinitrosalicylic acid (DNSA) method according to Alina's methodology with slight modification (Rasit & Mohammad, 2018) using 1% starch as substrate. The standard solution was prepared using maltose with concentration ranging from 0.1 to 15.0 g/L. The value of absorbance for standard curve and sample was measured using UV-Vis Spectrophotometer at 546nm. The absorbance obtained was used to calculate the amylase activity by using Equation (2) below.

$$Amylase\ activity\ \left(\frac{U}{mL}\right) = \frac{Maltose\ equivalent\ (\mu mole) \times dilution factor}{Volume\ of\ enzyme\ (mL) \times Reaction\ time\ (mins)} \quad (2)$$

The last enzyme test conducted was protease activity assay which in accordance with the Universal Protease Activity Assay, provided by Sigma Aldrich (Cupp-Enyard & Aldrich, 2008), with slight modification. L-tyrosine was used for the standard curve of this assay with concentration ranging from 0.1 – 0.5 µmole. The absorbance for the standard curve and enzyme sample was measured at 660nm using UV-Vis Spectrophotometer. The protease activity was calculated using Equation (3).

$$Protease\ activity\ \left(\frac{U}{mL}\right) = \frac{Tyrosine\ concentration\ (\mu mole) \times olume\ of\ eaction\ solution(mL)}{Volume\ of\ enzyme\ (mL) \times Reaction\ time\ (mins) \times Volume\ of\ sample\ in\ cuvette\ (mL)} \quad (3)$$

7. *Biodegradation assessment of PLA by isolated bacteria*

Soils were prepared by mixing landfill soil and compost at a mixture of 10:1. The soil mixture was air-dried and sieved through a 2mm-seive. PLA sheets were prepared according to Apinya *et al.* (2015) and were cut at a dimension of 20 × 30 × 1mm (W × L × H) and sterilised with 70% ethanol, before the weight of PLA sheets were taken.

The biodegradation assessment of PLA was conducted according to a modified version of the standard test methods of ASTM D5338-11 and ASTM D5988-12 (Ji Yadav *et al.*, 2020). The experiment was carried out in glass beaker under mesophilic condition (30°C ± 2°C) and thermophilic conditions (53°C ± 2°C) and incubated for 8 weeks duration. The degradation test for each temperature were divided into three soil conditions as follow: (i) sterilised soil mixture (no inoculated bacteria); (ii) sterilised soil mixture (inoculated with bacteria); (iii) non-sterilised soil mixture (control). PLA sheets were buried vertically in 200g of soil mixtures. Meanwhile, the moisture content in the soil were maintained at approximately 50% and will be monitored every 5-7 days throughout the experiment. Water loss were recovered by weighing the weight of beaker every week and comparing with the initial weight and adding distilled water to maintain moisture content in soil. The PLA films were weighted once a week to measure the weight loss of the film throughout the experiment.

8. *SEM analysis of PLA film*

After biodegradation test, the PLA films were cleaned and sent to Fluid Centre, Universiti Malaysia Pahang (UMP) to proceed with scanning electron microscopy (SEM) analysis. The analysis was done to observe the changes on the surface

of PLA films after biodegradation has taken place compared with a controlled PLA film.

III. RESULTS AND DISCUSSION

A. Isolation of PLA-degrading Bacteria

Based on the isolation process, 13 isolates were successfully isolated from soil samples onto PLA-emulsified agar. However, not all isolates were further studied due to a few samples remained dilated incubated at 30°C for 72 hours despite a multiple restreaking. This agrees with the statement by (Kedia *et al.*, 2007) where different microorganisms require different incubation period, and an extended time of incubation will dilate the colony, thus allowing the second organisms to thrive.

From the 13 isolates restreaked, only four were able to form halo zones on skim milk agar, which proves the presence of protease enzyme production by the isolates. Protease enzymes was used as an indicator due to protease being one of the main enzymes responsible in degrading PLA (Decorosi *et al.*, 2019). The relative enzyme activity was calculated using Eq. (1) after restreaking bacteria onto a new skim milk agar plate and incubating for 72 hours at 30°C. Isolates B8A and A10B, showed the highest enzyme activity at 1.33 and 3.86 mm, respectively. The results showed that both isolates produced a significant amount of protease enzyme and therefore were chosen further to be characterised.

B. Identification of PLA-degrading Bacteria

Isolate B8A and A10B were identified *via* 16S rRNA identification analysis as *Brevibacillus parabrevis* and *Renibacterium salmoninarum,* respectively. The analysis showed that isolate B8A has 99.93% similarities with *Brevibacillus parabrevis* consisting of 1368 bases sequence. While the result for isolate A10B showed 1479 bases sequences with 97.59% similarities with *Renibacterium salmoninarum*. The results are shown in Figure 1, where the phylogenetic tree for B8A and A10B which consist of the closely related strains.

C. Characteristics Of Pla-Degrading Bacteria

1. Growth curve

The growth curve for strain B8A is shown in Figure 1. The graph showed that the lag phase was at hour 0 until hour 9. The gradual increment of reading began at hour 10 until hour 56, which reached a stationary phase at hour 56 and hour 57. Continually, the death phase began after hour 57 and continued to decline until hour 82. In the same figure, strain A10B showed the beginning of lag phase from hour 0 to hour 7. The graph began showing an exponential increase starting from hour 7 up to hour 58. The highest peak reading was obtained at hour 58 which stayed consistent until hour 59, before it started to decline until it reaches death phase at hour 82.

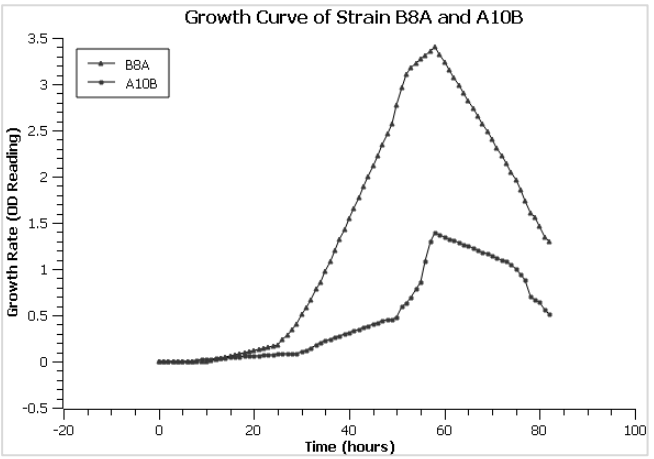
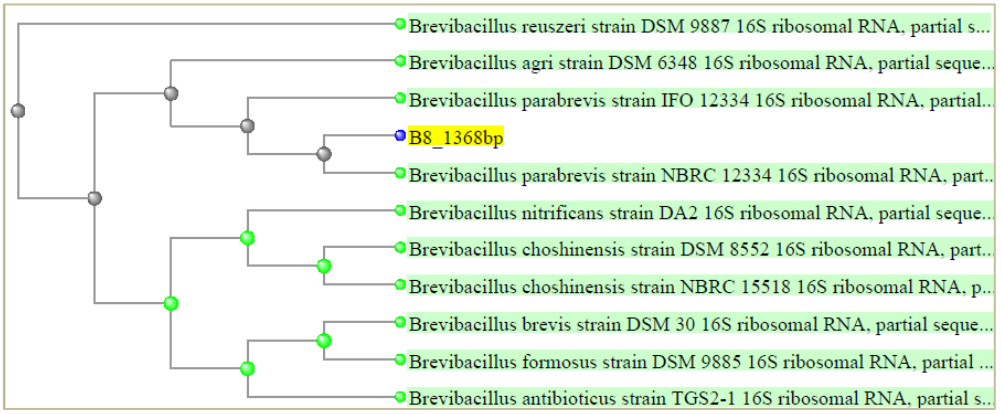
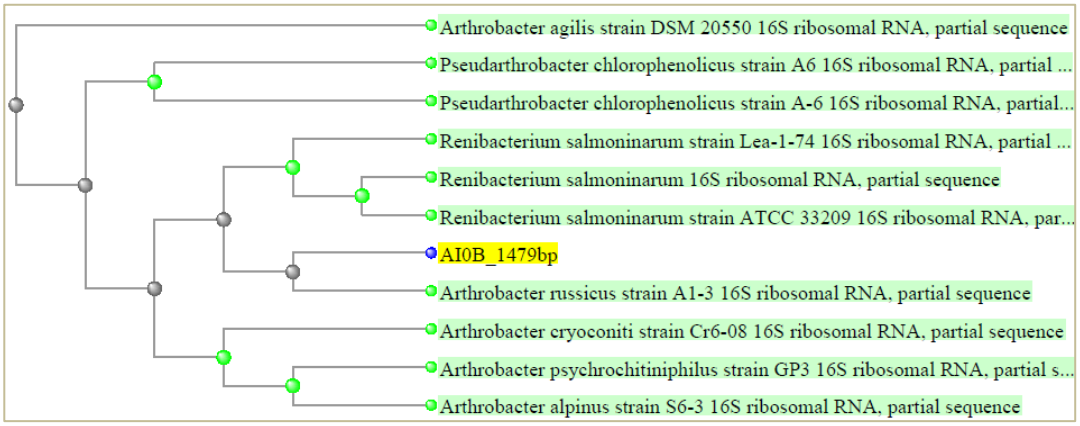


Figure 1. Growth curve of strain A10B and B8A.

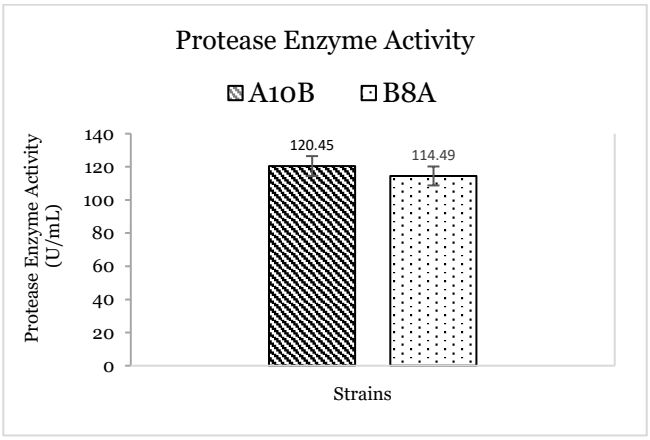


(a)

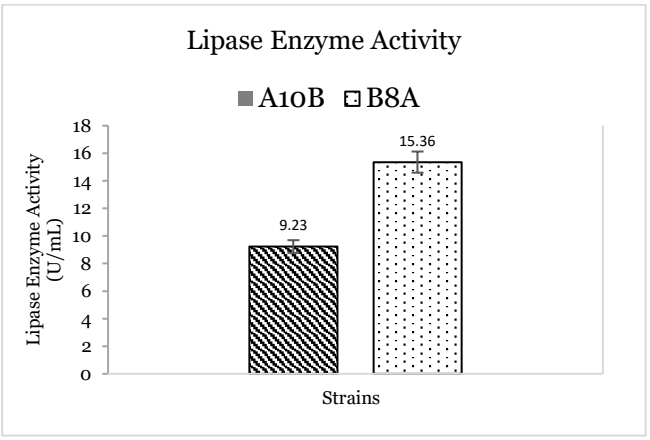


(b)

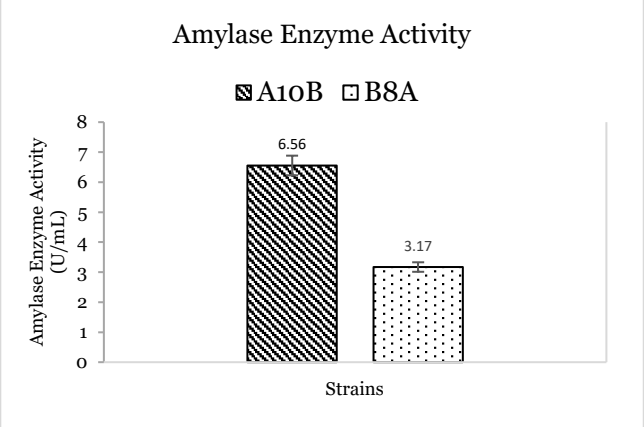
Figure 2. The phylogenetic tree of isolated bacteria and their closely related strains for isolate (a) B8A and (b) A10B.



(a)



(b)



(c)

Figure 3. Enzyme activity for strain A10B and B8A for (a) protease enzyme; (b) lipase enzyme; and (c) amylase enzyme.

2. *Enzyme assay*

The enzyme assay for protease, lipase, and amylase enzymes of strain A10B and B8 are shown in Figure 3. For protease enzyme in Figure 3 (a), it showed that strain A10B had a slightly higher protease enzyme activity which was at 120.45 U/mL, compare to strain B8A that has an enzyme activity of 114.49 U/mL. The high level of protease enzyme activities for both strains supported the claims of several studies (Decorosi *et al.*, 2019; Bubpachat *et al.*, 2018; Hegyesi *et al.*, 2019) which signify the importance of protease as the main enzyme during PLA degradation process.

For lipase enzyme, Figure 3 (b) shows the lipase activities were recorded at 15.36 U/mL and 9.23 U/mL for B8A and A10B, respectively. The number of studies related to this enzyme in PLA degradation is slightly lower and are mostly related to degradation of PLA-copolymers and low molecular PLA (Akutsu-Shigeno *et al.*, 2006; Masaki *et al.*, 2005; Sakai *et al.,* 2001)).

Meanwhile, according to Figure 3 (c), amylase has recorded the lowest enzymatic activity compared to protease and lipase which was recorded at 6.56 U/mL for A10B, and 3.17 U/mL for B8A. This enzyme was mentioned in the degradation of PLA composite such as PLA/RS (Yew *et al.,* 2005). And PLA/PBSA blend (Malwela & Ray, 2015).

D. Biodegradation Test of PLA-degrading Bacteria

After 8 weeks, at mesophilic temperature, the PLA film buried in sterilised soil mixture with inoculated bacteria was shows a 37.8% weight reduction for isolate B8 and 32.6% for isolate A10B. The non-sterilised soil control, the average weight loss is at 27.7%. In addition, for the PLA films buried at thermophilic condition, the highest weight loss by isolate A10B and B8A are 30.5% and 27%, respectively. For sterilised mixed soils without bacteria, the average weight loss of PLA is at 3%. And the control for thermophilic temperature has a weight loss of 12.5%. A sample of control PLA film from non-sterilised soil and a sample of PLA film after 8 weeks in inoculated bacteria soil were sent for SEM analysis. Table 2 shows the difference between the initial PLA film in non-sterilised soils and the at the end of the experiment showing the difference of degradation activity happening on the bioplastics.

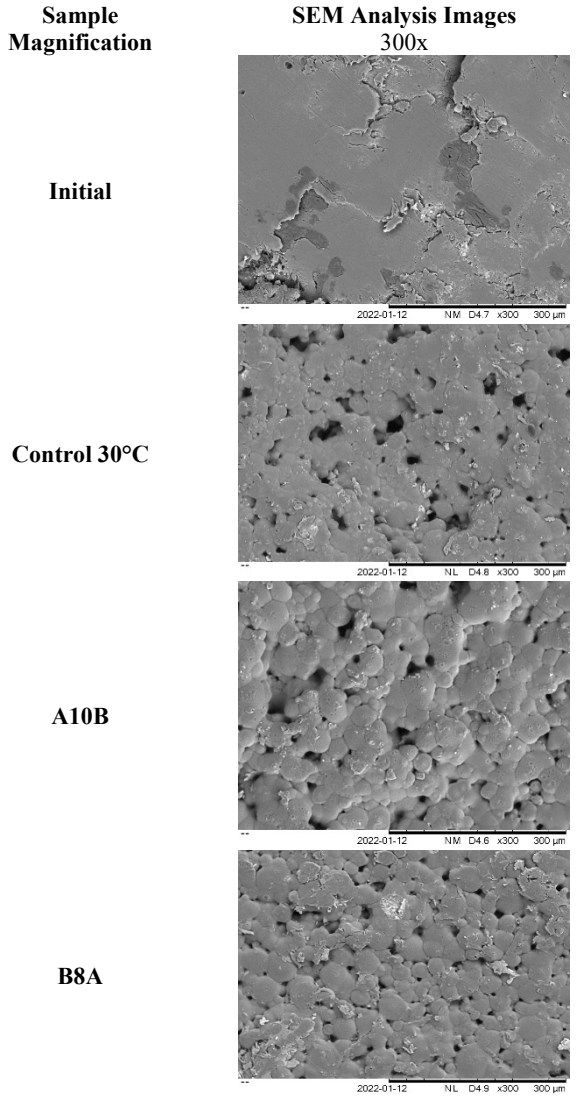


Figure 4. SEM Analysis of surface of PLA films under magnification times 300 for sample A10B, B8, control film and initial film.

IV. CONCLUSION

The identification and characterisation of PLA-degrading bacteria at mesophilic temperature from landfill soil was conducted successfully. Isolate A10B and B8A show highest protease enzyme relative activity thus were further identified. Identification is done via 16S rRNA on the isolates and was identified as bacteria *Renibacterium salmoninarum* and *Brevibacillus parabrevis*. Furthermore, it shows different enzymatic activity the highest being protease enzyme (120.44 U/mL) followed by lipase (9.23U/mL) and lastly amylase (6.66U/mL). Further experimentation shows that, at a span of 8 weeks, the bacteria was able to degrade PLA films with weight reduction of 32.6% and 37.8 for A10B and B8A, respectively from initial weight compared to the control which is at 27.7% weight reduction. This bacteria shows potential to pave further development of biodegradation facilities for bioplastics which will be cost efficient due to the optimal degradation in mesophilic temperature.

V. ACKNOWLEDGEMENT

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VI. REFERENCES

Abd-Elhakeem, MA, Elsayed, AM & Alkhulaqi, TA 2013, 'New Colorimetric Method for Lipases Activity Assay in Microbial Media', *American Journal of Analytical Chemistry*, vol. 2013, no. 09, pp. 442–444.

Akutsu-Shigeno, Y, Adachi, Y, Yamada, C, Toyoshima, K, Nomura, N, Uchiyama, H & Nakajima-Kambe, T 2006, 'Isolation of a bacterium that degrades urethane compounds and characterization of its urethane hydrolase', *Applied Microbiology and Biotechnology*, vol. 70, no. 4, pp. 422–429.

Apinya, T, Sombatsompop, N & Prapagdee, B 2015, 'Selection of a *Pseudonocardia sp*. RM423 that accelerates the biodegradation of poly(lactic) acid in submerged cultures and in soil microcosms', *International Biodeterioration and Biodegradation*, vol. 99, pp. 23–30.

Bradner, JR, Gillings, M & Nevalainen, KM 1999, 'Qualitative assessment of hydrolytic activities in antarctic microfungi grown at different temperatures on solid media', *World Journal of Microbiology and Biotechnology*, vol. 15, pp. 131–132.

Bubpachat, T, Sombatsompop, N & Prapagdee, B 2018, 'Isolation and role of polylactic acid-degrading bacteria on degrading enzymes productions and PLA biodegradability at mesophilic conditions', *Polymer Degradation and Stability*, vol. 152, pp. 75–85.

Cupp-Enyard, C & Aldrich, S 2008, 'Sigma's Non-specific Protease Activity Assay - Casein as a Substrate', *JoVE (Journal of Visualized Experiments)*, vol. 19, pp. e899.

Decorosi, F, Exana, ML, Pini, F, Adessi, A, Messini, A, Giovannetti, L & Viti, C 2019, 'The degradative capabilities of new Amycolatopsis isolates on polylactic acid', *Microorganisms*, vol. 7, no. 12, pp. 1–17.

Hall, BG, Acar, H, Nandipati, A & Barlow, M 2014, 'Growth rates made easy', *Molecular Biology and Evolution*.

Hamad, K, Kaseem, M, Ko, YG & Deri, F 2014, 'Biodegradable polymer blends and composites: An overview', *Polymer Science - Series A*, vol. 56, no. 6, pp. 812–829.

Hegyesi, N, Zhang, Y, Kohári, A, Polyák, P, Sui, X & Pukánszky, B 2019, 'Enzymatic degradation of PLA/cellulose nanocrystal composites', *Industrial Crops and Products*, vol. 141, pp. 111799.

Ji Yadav, SK, Vedrtnam, A & Gunwant, D 2020, 'Experimental and numerical study on mechanical behavior and resistance to natural weathering of sugarcane leave reinforced polymer composite', *Construction and Building Materials*, vol. 262, pp. 120785.

Kedia, G, Wang, R, Patel, H & Pandiella, SS 2007, 'Use of mixed cultures for the fermentation of cereal-based substrates with potential probiotic properties', *Process Biochemistry*, vol. 42, no. 1, pp. 65–70.

Li, F, Tan, L, Zhang, S & Zhu, B 2016, 'Compatibility, steady and dynamic rheological behaviors of polylactide/poly (ethylene glycol) blends', vol. 42919, pp. 1–10.

Malwela, T & Ray, SS 2015, 'Enzymatic degradation behavior of nanoclay reinforced biodegradable PLA/PBSA blend composites', *International Journal of Biological Macromolecules*, vol. 77, pp. 131–142.

Masaki, K, Kamini, NR, Ikeda, H & Iefuji, H 2005, 'Cutinase-

like enzyme from the yeast *Cryptococcus sp*. strain S-2 hydrolyzes polylactic acid and other biodegradable plastics', *Applied and Environmental Microbiology*, vol. 71, no. 11, pp. 7548–7550.

Rasit, N & Mohammad, FS 2018, 'Production and characterization of bio catalytic enzyme produced from fermentation of fruit and vegetable wastes and its influence on aquaculture sludge', *International Journal of Scientific and Technology Research*, vol. 4, pp. 12–26.

Sakai, K, Kawano, H, Iwami, A, Nakamura, M & Moriguchi, M 2001, 'Isolation of a thermophilic poly-L-lactide degrading bacterium from compost and its enzymatic characterization', *Journal of Bioscience and Bioengineering*, vol. 92, no. 3, pp. 298–300.

Yew, GH, Mohd Yusof, AM, Mohd Ishak, ZA & Ishiaku, US 2005, 'Water absorption and enzymatic degradation of poly(lactic acid)/rice starch composites', *Polymer Degradation and Stability*, vol. 90, no. 3, pp. 488–500.