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Degradation of Poly(Butylene Succinate) and Poly(Butylene Succinate)/Poly(Lactide) Blends using Serine Protease Produced from *Laceyella sacchari* LP175

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Abstract

The thermophilic filamentous bacterium *Laceyella sacchari* LP175 was cultivated in a 10.0 L airlift fermenter to produce serine protease at 50 °C. Maximal serine protease activity at 1,123.32 ± 15.8 U/mL was obtained for cultivation at 0.6 vvm aeration rate for 36 h. The crude enzyme was applied for degradation of poly (butylene succinate) (PBS), and poly (butylene succinate)/poly(lactide) blend (PBS/PLA) powders at 50 °C for 48 h with different substrates and enzyme concentrations. Results showed that serine protease produced from *L. sacchari* LP175 degraded PBS and PBS/PLA at 46.5 ± 2.05 and 49.8 ± 1.45 %, respectively, at an initial substrate concentration of 100 g/L with 1,200 U/mL of serine protease activity. Percentage degradation of PBS and PBS/PLA was improved to 51.4 ± 1.06 and 56.9 ± 1.42 %, respectively, when upscaled in a 2.0 L stirrer fermenter with 200 rpm agitation rate. Degradation products evaluated by a scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR) confirmed that serine protease produced from *L. sacchari* LP175 degraded both PBS and PBS/PLA polymers. Results showed that microbial enzyme technology could be used to degrade PBS and PBS/PLA blend polymers and reduce the accumulation of waste.

Keywords: Poly(butylene succinate) (PBS), Polybutylene succinate/poly(lactide) (PBS/PLA) blend, *Laceyella sacchari* LP175, Serine protease production, Biodegradation

Introduction

Nowadays, biodegradable aliphatic polyesters have replaced conventional non-degradable materials that caused environmental problems from the limitations of landfill capacity [1]. Poly(butylene succinate) (PBS) and poly(butylene succinate)/poly(lactide) blend polymers (PBS/PLA) with low production costs are used as substitutes for routine plastics. Compared to PLA material, they have reduced brittleness and improved toughness and degradation ability [2-4]. PBS is an aliphatic polyester polymer, produced from the condensation of succinic acid (SA) and 1,4-butanediol (BD) [5,6]. At the same time, PLA is a polymer of lactic acid obtained from the fermentation of various renewable materials, and produced by the condensation polymerization process [7]. PBS and PLA take a long time to completely degrade in natural environments. Shaiju *et al.* [8] reported that PBS degraded by less than 5.0 % after 10 weeks of seawater immersion. Other processes are required to accelerate the degradation of these polymers to reduce the volume of waste generated from consumer utilization. Recent reports on PBS and PBS/PLA degradation by microbial enzymes such as lipase from *Cryptococcus* sp. MTCC 5455 [1], cutinase from *Fusarium* sp. [9] and protease from *Amycolatopsis* sp. strain SCM_MK2-4 [10] show an increase in the degradation rate of polyester biodegradable plastics.

Laceyella sacchari LP175 showed PLA-degrading enzyme production characterized as serine protease activity [11,12]. This utilized low-cost substrates for growth and enzyme production in a 3.0 L airlift fermenter, yielding 94.4 U/mL [13]. Crude enzyme obtained from *L. sacchari* LP175 degraded PLA polymer and poly(lactide)/thermoplastic starch blend film at 50 °C. Optimal conditions for

degradation of PLA polymer at 100 g/L by crude enzyme produced from *L. sacchari* LP175 were reported at pH 9.0, with 0.2 M of buffer concentration yielding 68 % degradation [14]. However, upscale for enzyme production in a 10.0 airlift fermenter by the LP175 strain and application for PBS and PBS/PLA degradation has not been reported.

Each microorganism has optimal growth conditions. Aeration rate is a highly important factor in an airlift fermenter that does not contain an agitation system compared with a stirrer fermenter [12,13]. Thus, the effect of aeration rate on serine protease production by *L. sacchari* LP175 in a 10.0 L airlift fermenter was investigated. Optimal conditions for degradation of PBS and PBS/PLA blend powders by crude enzyme were also assessed.

Materials and methods

Microorganism

The bacterial strain, *L. sacchari* LP175 was grown in nutrient broth (NB) at 50 °C for 24 h as described by Lomthong *et al.* [15]. A cell suspension of LP175 strain (1×10^7 CFU/mL) after the centrifugation at 10,000 rpm, at 4 °C was suspended in sterile 0.85 % NaCl solution and used as the inoculum.

Enzyme production in a 10.0 L airlift fermenter

Serine protease production was carried out in a 10.0 L airlift fermenter using 7.0 L working volume of the optimized medium; 4.64 g/L cassava chips, 1.53 g/L soybean meal and 0.31 g/L PLA powder, pH 7.0 [13]. The fermentation was conducted at 50 °C with different aeration rates of 0.2 - 1.0 vvm for 36 h. Protease activity was determined using 0.6 % (w/v) casein in 0.1 M Tris-HCl, pH 9.0 as substrate, modified from the method of Jarerat *et al.* [16]. The reaction of 0.5 mL of enzyme sample and 2.5 mL of substrate was incubated at 50 °C for 10 min and terminated by adding 5 mL of 0.3 M trichloroacetic acid solution. An aliquot of 2.0 mL of clear solution after 20 min termination was reacted with 5 mL of 6 % (w/v) Na_2CO_3 and 1.0 mL of 1.0 M Folin-Ciocalteu phenol reagent (Sigma). The reaction was incubated at 30 °C for 30 min and liberated L-tyrosine was determined at 660 nm using L-tyrosine as standard. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of L-tyrosine per min.

The time course of enzyme production and growth of *L. sacchari* LP175 in a 10.0 L airlift fermenter using the optimal aeration rate was investigated. Samples were determined every 12 for 36 h. The obtained enzyme was concentrated using a freeze dryer (Oeron FDU-8624, Korea) and dissolved in a 0.2 M Tris-HCl buffer, pH 9.0 as an enzyme for degradation of PBS and PBS/PLA in further experiments [14].

Enzymatic degradation of PBS and PBS/PLA blends

Powder preparation of PBS and PBS/PLA blends

PBS powder was prepared by dissolving 2.0 g of PBS pellets (FZ91PD, Mitsubishi Chemical Corporation, Japan) in 100 mL of chloroform (Merck, Germany). The dissolved solution was poured into a stainless steel tray which contained aluminum foil covered on the surface. The reaction was dried overnight at room temperature. The obtained dried powder was used to investigate degradation ability by the serine protease produced from *L. sacchari* LP175.

PBS/PLA blends were prepared by dissolving 1.0 g of PBS and 1.0 g of PLA pellets (Terramac TP-4000, Unitika. Co., Ltd., Japan) in 100 mL of chloroform (Merck, Germany), following the modified method of Hu *et al.* [2]. The dissolved solution was poured into a stainless steel tray and the obtained powder was also used for testing degradation by serine protease produced from *L. sacchari* LP175.

Effect of substrate concentrations on degradation of PBS and PBS/PLA blends

The hydrolysis of PBS and PBS/PLA was conducted in 250 mL Erlenmeyer flasks containing 50 mL of crude enzyme serine protease produced from *L. sacchari* LP175 at 400 U/mL in 0.2 M Tris-HCl buffer, pH 9.0 with PBS and PBS/PLA at 10 - 100 g/L. The reaction mixtures were incubated in a shaking incubator at 150 rpm and 50 °C for 48 h. Dry weight of the obtained powder after filtration through Whatman® No. 1 filter paper and drying at 50 °C for 12 h was used to calculate the percentage degradation according to the equation below, following Lomthong *et al.* [17].

$$\text{Percentage Degradation} = \frac{(\text{Initial film weight - Retained film weight}) \times 100}{\text{Initial film weight}}$$

Effect of enzyme concentrations on degradation of PBS and PBS/PLA blends

The effect of enzyme concentrations on the degradation of PBS and PBS/PLA blends was investigated at 400-1600 U/mL of serine protease activity in 0.2 M Tris-HCl buffer, pH 9.0 with 100 g/L of PBS and PBS/PLA powders. Reaction mixtures were incubated at 150 rpm and 50 °C for 48 h, and then the dry weight of the retained powder was used to calculate percentage degradation as described previously.

Biodegradation of PBS and PBS/PLA in a 2.0 L stirrer fermenter

To upscale the degradation of PBS and PBS/PLA blend powders by crude serine protease produced from *L. sacchari* LP175, the degradation of each bioplastic was conducted in a 2.0 L stirrer fermenter with 1.0 L working reaction volume. Enzyme solution in 0.2 M Tris-HCl buffer pH 9.0 was added to the fermenter with 100 g/L of each PBS and PBS/PLA blend powders. The reaction was operated at 50°C for 48 h with 200 rpm agitation rate, as reported by Lomthong *et al.* [14]. Clear supernatant, after filtration through Whatman® No. 1 filter paper, was used to determine the pH of the reaction. Both native and digested PBS and PBS/PLA blend powder morphologies were qualitatively assessed by scanning electron microscopy, SEM (model SU8020; Hitachi, Tokyo, Japan). At the end of the reaction, the degraded bioplastic was washed with distilled water, dried at 50 °C for 24 h, and examined under SEM at 10.0 kV. Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet is5, USA) was used to determine the degraded product spectra of PBS and PBS/PLA blends using a scanning range of 4000 to 500 cm^{-1} [1].

Statistical analysis

Results were reported as means of 3 determinations (n = 3), and the significance of the data was analyzed by one-way analysis of variance (ANOVA) (SPSS 21.0, USA). Values were considered significant at $p < 0.05$ using Duncan's multiple range tests.

Results and discussion

Serine protease production by *L. sacchari* LP175 in a 10.0 L airlift fermenter

Results of serine protease production by *L. sacchari* LP175 at different aeration rates are shown in **Figure 1**. Maximal enzyme production at 1,123.32 ± 15.8 U/mL with productivity of 31.2 U/mL/h was found at aeration rate of 0.6 vvm. Aeration rate was an important factor for cultivation of *L. sacchari* LP175 in the airlift fermenter as it improved the heat and mass transfer in the reactor. An airlift fermenter does not contain an agitator compared to a stirrer fermenter [13]. Nadeem *et al.* [18] reported that the increased aeration rate improved oxygen transfer and nutrient mixing in the fermentation system. *L. sacchari* LP175 is a filamentous bacterium that cannot grow under the shear stress of agitation in a stirrer fermenter. This explains why an airlift fermenter was appropriate for cultivation of *L. sacchari* LP175 [11]. Low aeration rate of 0.1 - 0.4 vvm gave insufficient mixing of the reaction and adversely impacted heat and mass transfer. Likewise, high aeration rate of 0.8 - 1.0 vvm stimulated growth and lysis as characteristic of this bacterial species [11]. Nadeem *et al.* [18] reported that higher aeration rates than the optimal flow decreased the growth of bacterial cells and alkaline protease production as a result of shear stress and heterogeneous mixing effects.

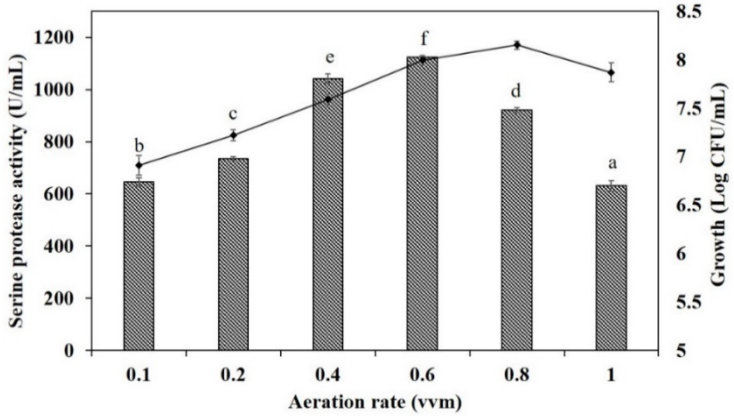

Figure 1 Effects of aeration rate on serine protease production (bar graph) by *Laceyella sacchari* LP175 in 10.0 L airlift fermenter at 50 °C for 36 h. ◆: Growth of *Laceyella sacchari* LP175. Error bars = ± SD, and different lowercase letters above columns indicate significant ($p < 0.05$) difference among means.

Time courses of batch fermentations for serine protease production by *L. sacchari* LP175 are shown in **Figure 2**. The serine protease production was started in the early log phase, increasing exponentially during the first 24 h of fermentation and giving a steady production. This is a progression for serine protease production in a 10.0 L airlift fermenter using low-cost agricultural products as substrate. Compared with previous reports, enzyme production was obtained at 1330.2 U/mL with productivity of 27.7 U/mL/h by *L. sacchari* YNDH using yeast extract as the main substrate, after the incubation at 45 °C for 48 h [19]. Results of this study indicated the feasibility of upscaling future serine protease production by *L. sacchari* LP175 at the industrial level using low-cost agricultural products.

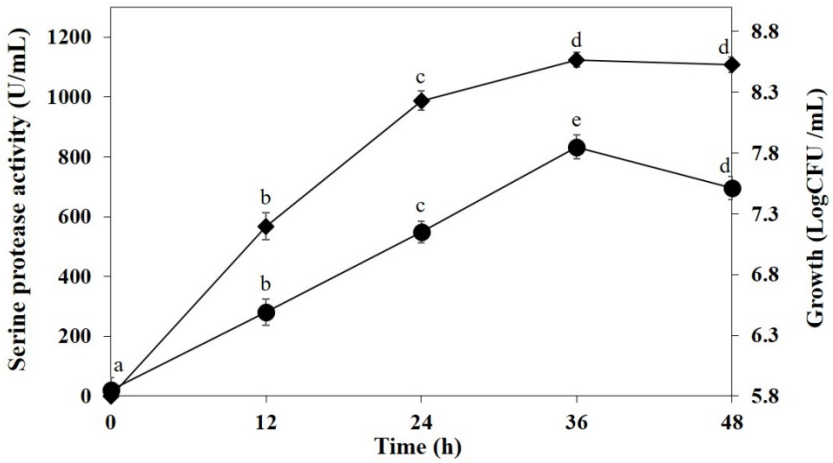

Figure 2 Time courses of batch fermentations for serine protease production by *Laceyella sacchari* LP175 in a 10.0 L airlift fermenter. ◆: Serine protease activity, ●: Growth of *Laceyella sacchari* LP175. Error bars = ± SD. Different lowercase letters indicate significant ($p < 0.05$) differences among means.

Enzymatic degradation of PBS and PBS/PLA blends

Effect of substrate concentrations on degradation of PBS and PBS/PLA blends

Bioplastics can be degraded by various microbial enzymes such as esterases, lipases, cutinases and proteases [10,20]. Serine protease was reported to degrade polyester polymers such as PBS and PLA by doing the hydrolysis of the ester bonds by Lim *et al.* [21]. In this study, serine protease produced by *L. sacchari* LP175 was used to investigate the feasibility for the degradation of PBS and PBS/PLA blend powders as described above. Serine protease produced from *L. sacchari* LP175 degraded both PBS and PBS/PLA blend powders as shown in **Table 1**. Highest percentage degradation was found using 10 g/L of

PBS and PBS/PLA blend powders at 77.00 ± 1.41 and 84.00 ± 2.83 %, respectively. Highest weight loss was found using PBS and PBS/PLA blend powders at 100 g/L, with reduction of 29.15 ± 0.21 and 32.35 ± 0.49 g, respectively. Lower percentage degradation at a higher substrate concentration was caused by reduced enzyme activity in the reaction. Further investigations are required to determine the effect of enzyme concentration on percentage degradation of PBS and PBS/PLA at higher substrate concentrations.

Table 1 Effects of substrate concentration on degradation of PBS and PBS/PLA blends by serine protease produced from *Laceyella sacchari* LP175.

Substrate concentration (g/L)	Weight loss (g)		Percentage degradation	
	PBS	PBS/PLA	PBS	PBS/PLA
10	7.70 ± 0.14[a]	8.40 ± 0.28[a]	77.00 ± 1.41[d]	84.00 ± 2.83[d]
20	13.50 ± 0.42[b]	15.15 ± 0.21[b]	67.50 ± 2.10[c]	75.75 ± 1.06[c]
50	21.60 ± 0.71[c]	26.10 ± 0.42[c]	43.20 ± 1.41[b]	52.20 ± 0.85[b]
100	29.15 ± 0.21[d]	32.35 ± 0.49[d]	29.15 ± 0.21[a]	32.35 ± 0.49[a]

Different letters within the same column indicate statistical difference at $p < 0.05$.

Effect of enzyme concentrations on PBS and PBS/PLA degradation

Maximal degradation of PBS and PBS/PLA blends was found at enzyme concentration of 1,200 U/mL, yielding 46.5 ± 2.05 and 49.8 ± 1.45 %, respectively when incubated at 50 °C for 48 h, as shown in **Table 2**. Lower enzyme concentration gave a lower degradation due to enzyme hydrolysis limitation of the substrate. In a previous study, Lomthong *et al.* [13] reported that enzyme concentration affected hydrolysis efficiency of PLA polymer. Optimal enzyme concentration improved hydrolysis efficiency compared to lower and higher enzyme concentrations [13]. To correlate with other studies, 2.5 g/L of PBS was degraded by lipase produced from *Cryptococcu*s sp. After the incubation at 30 °C for 96 h with shaking at 120 rpm [1]. Hu *et al.* [2] reported that PBS and PBS/PLA blends were degraded by the enzymatic hydrolysis process, with most PLA components degraded by protease enzyme, while the PLA component in the PBS/PLA blends was not degraded completely. This was explained by the different chemical structure and specificity of the enzyme. PBS/PLA is easier to degrade by serine protease compared to PBS polymer.

Table 2 Effects of enzyme concentration on percentage degradation of PBS and PBS/PLA blends by serine protease produced from *Laceyella sacchari* LP175.

Enzyme concentration (U/mL)	Percentage degradation	
	PBS	PBS/PLA
400	29.80 ± 1.46[a]	32.60 ± 1.64[a]
800	42.10 ± 1.17[b]	46.40 ± 0.94[b]
1200	46.50 ± 2.05[c]	49.80 ± 1.45[c]
1600	46.30 ± 1.89[c]	49.80 ± 2.36[c]

Different letters within the same column indicate statistical difference at $p < 0.05$.

Biodegradation of PBS and PBS/PLA blends in a 2.0 L stirrer fermenter

The degradation of PBS and PBS/PLA blend powder was performed in the 2.0 stirrer fermenter with 1.0 L working volume of the total suspension as described above. Optimal pH and temperature were 9.0 and 50 °C, respectively as reported by Lomthong *et al.* [13]. Maximal degradations of PBS and PBS/PLA blend powders in a 2.0 stirrer were 51.4 ± 1.06 and 56.9 ± 1.42 %, respectively (**Figure 3**). The degradation in 2.0 stirrer fermenter increased the hydrolysis efficiency of the reaction due to agitation from the impeller as the main component of the stirrer fermenter that improved bioplastic and enzyme mixing in the reaction. Moreover, the shear force of the impeller stimulated the breakdown of bioplastic structures. Panyachanakul *et al.* [22] also found that agitation in the stirrer fermenter positively impacted the degradation of bioplastics. Lomthong *et al.* [14] reported that increasing the agitation rate in the stirrer reactor to 200 rpm accelerated the degradation of PLA polymer (100 g/L) by up to 68 %.

The scanning electron micrographs showed that residues of PBS and PBS/PLA samples had reduced rigidity, with the appearance of fractures on the surface structure compared to native samples (**Figure 4**). Serine protease produced from *L. sacchari* LP175 degraded PLA polymers by hydrolysis, causing loss of rigidity and appearance of fractures [14,23]. Proteolytic enzymes hydrolyzed ester bonds of the bioplastic polyesters due to the similarity in chemical structure between the l-lactic acid unit in PLA and the l-alanine unit in protein [24].

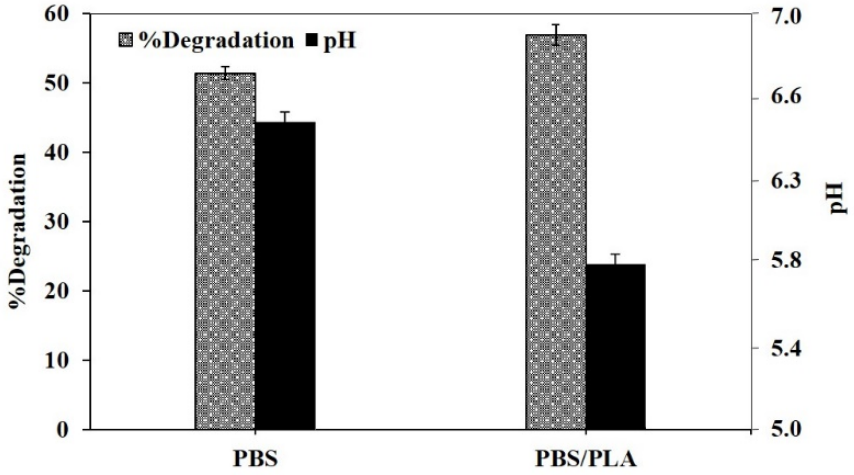

Figure 3 Degradation of poly(butylene succinate) (PBS) and poly(butylene succinate)/poly(lactide) blend polymers (PBS/PLA) by serine protease produced by *Laceyella sacchari* LP175 in a 2.0 L stirrer fermenter at 50 °C for 48 h.

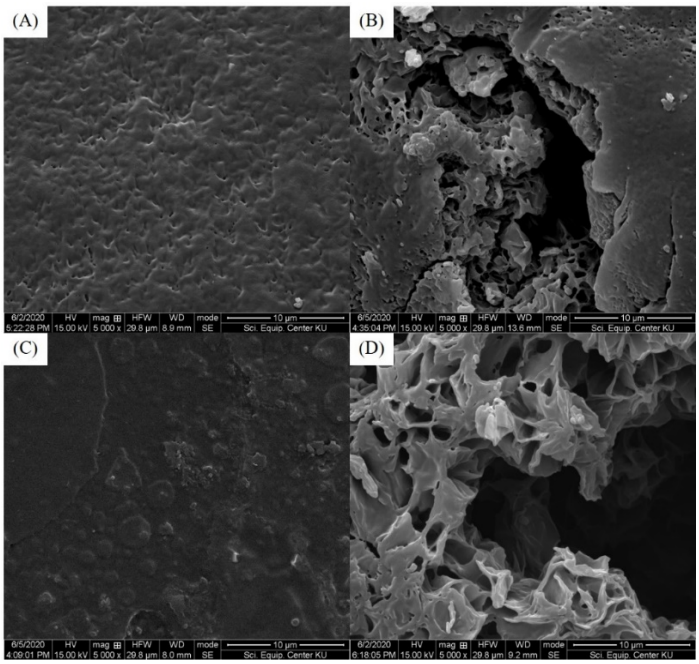

Figure 4 Scanning electron micrographs of native and digested PBS (A, B) and PBS/PLA (C, D) after degradation by serine protease produced from *Laceyella sacchari* LP175 at 50 °C for 48 h.

FTIR spectra of the native and degraded products of PBS and PBS/PLA samples are shown in **Figure 5**. Both native samples of PBS and PBS/PLA blend powders showed similar patterns. The peak at wavenumbers 1708 - 1714 cm^{-1} was attributed to the presence of C=O, while the peak at wavenumbers

1150 - 1164 cm^{-1} was assigned to the –C–O–C– bond by Thirunavukarasu *et al.* [1]. The C–H bond was found at wavenumber 1327 - 1335 cm^{-1}. Digested PBS and PBS/PLA samples showed significant changes in transmittance intensity at wavenumbers 1708 - 1714 and 1150 - 1164 cm^{-1} compared to the native products without enzymatic hydrolysis. Results revealed changes in the chemical structure of PBS and PBS/PLA due to degradation of the C=O and –C–O–C– bonds, concurring with Umare *et al.* [25] and Thirunavukarasu *et al.* [1].

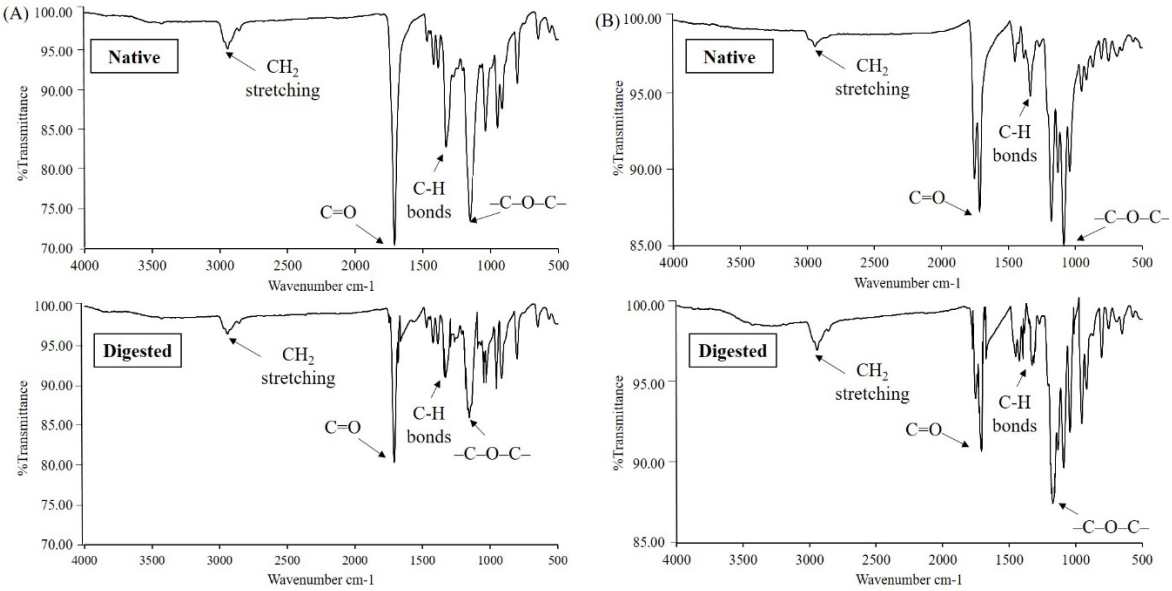

Figure 5 FTIR spectra of native and digested PBS (A), PBS/PLA (B) at wavenumbers 4000 to 500 cm^{-1}.

Conclusions

The serine protease production was upscaled in a 10.0 L airlift fermenter using 7.0 L of working volume. Maximal value was achieved at 0.6 vvm, yielding 1,123.32 ± 15.8 U/mL using a low-cost agricultural products of cassava chips and soybean meal as substrates. The crude enzyme degraded both bioplastics PBS and PBS/PLA blend powders under a shaking flask and a 2.0 L stirrer fermenter. Enzyme concentration at 1200 U/mL accelerated the degradation of PBS and PBS/PLA blends by up to 51.4 ± 1.06 and 56.9 ± 1.42 %, respectively. Results showed a potential application of enzymatic hydrolysis under submerged condition as an alternative method to reduce future accumulation of PBS and PBS/PLA bioplastic wastes.

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