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Isolation of marine bacteria with potential for polyhydroxyalkanoate degradation and optimization for enzyme production

Antika Boondaeng[1], Chanaporn Trakunjae[1], Pilanee Vaithanomsat[1] & Nanthavut Niyomvong[2,3]✉

Plastic materials are widely used because of their strength, light weight, durability, and environmental resistance. However, their decomposition rates are significantly slower than their typical lifespans. The rapid and continuous increase in plastic consumption has caused severe environmental impacts due to the accumulation of plastic waste. We identified potential polyhydroxyalkanoate (PHA)-degrading bacteria from marine environments capable of producing extracellular PHA depolymerases crucial for biodegrading PHAs. Marine debris was collected to screen poly [(R)-3-hydroxybutyric acid] (P(3HB))-degrading bacteria. Six isolates showed the ability to produce clear zones surrounding their colonies by degrading the bioplastic P(3HB). The isolate SS1-2, exhibiting the greatest degradation index of 1.44, was chosen for optimization through the statistical technique. The results indicated that NH_4Cl was the best nitrogen source for enzyme production, and the response surface methodology (RSM) suggested that the greatest P(3HB) depolymerase production could be achieved when the concentrations of substrate loading and NH_4Cl both set at 0.5%. Analysis of the 16S rRNA sequence of isolate SS1-2 revealed similarity to *Pseudooceanicola antarcticus* CGMCC 1.12662 (97.81% similarity). The findings of this study indicate the potential for further exploitation of this depolymerase in enzyme kinetics studies and its application in PHA degradation experiments.

Keywords Bioplastic, Depolymerase, Extracellular enzyme, Surface response methodology

Plastic materials are widely used because they are strong, lightweight, long-lasting, and resistant to various environmental conditions. However, the amount of time required for plastic to break down is much greater than the average lifespan of a plastic object. Almost all these products, including plastic bags, cups, and nearly all forms of liquid packaging, are single-use or meant to be used briefly. The environment has suffered due to the rapid and ongoing increase in plastic use[1], with global plastic waste production reaching 400 million metric tons annually, of which only 9% is recycled and a significant portion accumulates in ecosystems[2]. In addition, the global COVID-19 pandemic significantly increased the demand for single-use plastic packaging and containers, leading to a surge in plastic waste[3,4]. Studies indicate that pandemic-related plastic waste, including medical and food packaging, contributed an estimated 25,000 tons of waste entering the ocean globally[5]. Most plastics used today are derived from petroleum, and their decomposition takes a long time to complete because of their inert nature and inability to biodegrade. As they cannot decompose naturally, they have accumulated over decades, causing various environmental problems[6]. Environmental degradation of plastics through physical, chemical, and biological processes has led to the formation of microplastics and nanoplastics. These particles have been detected in various ecosystems, including marine environments[7–9]. The effects of microplastics on marine pollution have attracted increasing attention in recent years[10] including concerns about toxins from their surfaces[5]. It is also concerning that microplastics that are accumulating these toxins and chemicals may be consumed by fish and plankton, which may then accumulate in mammals as a result of the food chain. Consequently, there has been much interest in the dynamics of microplastic generation, distribution, diffusion, and degradation in marine environments. One approach to address this issue is to use biodegradable plastics that can be decomposed by microorganisms in a short period[11].

[1]Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Bangkok 10900, Thailand. [2]Department of Biology and Biotechnology, Faculty of Science and Technology, Nakhon Sawan Rajabhat University, Nakhon Sawan 60000, Thailand. [3]Science Center, Nakhon Sawan Rajabhat University, Nakhon Sawan 60000, Thailand. ✉email: nanthavut.ni@nsru.ac.th

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by microorganisms as intracellular carbon and energy storage compounds. They are increasingly considered viable alternatives to petroleum-derived plastics due to their complete biodegradability, particularly in marine environments[12]. They are used in industrial applications as alternatives to the synthetic plastics made from petrochemicals. The advantage of PHAs over other bioplastics is their ability to biodegrade fully, particularly in marine environments[13]. Wang et al.[14] reported that the biodegradation rate of PHAs is expected to be 8–20 times faster than abiotic degradation. To date, various bacteria and fungi capable of degrading PHAs have been identified[15]. These PHA- degrading microorganisms have been found in diverse environments, including soil, compost, and marine[16]. Under aerobic conditions, PHA can be degraded into carbon dioxide and water and into carbon dioxide and methane under anaerobic conditions[17]. PHAs can be broken down by various enzymes, depending on the polymer structure and microorganisms involved. Generally, PHA depolymerases are categorized as intracellular or extracellular enzymes, depending on their mode of action on the substrate[18]. Extracellular PHA depolymerase is a keyenzyme in PHA breakdown in nature and is produced by various microorganisms such as thermophiles, archaea, and fungi[19]. Currently, approximately 30 PHA depolymerases with confirmed activity have been identified[19]. The bacteria that have been most extensively studied for their enzyme production belong to the genera *Cupriavidus*[20,21], *Alcaligenes*[22], *Comamonas*[23,24], and *Pseudomonas*[25–28]. These findings suggest that extracellular PHA depolymerases are widely distributed in nature. Most isolated depolymerases specifically target short-chain-length polyhydroxyalkanoates (PHAs), such as 4-hydroxybutyrate (4HB). Noteworthy examples include those derived from *A. faecalis*, *Comamonas* sp., and *P. lemoignei*[29–31]. Bacteria that degrade PHA release extracellular depolymerases, which hydrolyze PHA into water-soluble intermediates that serve as carbon and energy sources for their growth[20,32]. Typically, PHA granules formed after bacterial cell lysis are subject to further degradation; however, some PHA-producing bacteria can also mobilize intracellular PHA in response to stress conditions. The rate of PHA biodegradation is influenced by the characteristics of the polymer, environmental factors (such as temperature and pH), and the dynamics of microbial populations[33]. This study revealed that PHA-degraders produce extracellular PHA depolymerase enzymes in the marine environment. The depolymerase was tested for degradationof poly[(R)-3-hydroxybutyric acid] (P(3HB)) powder. Factors affecting enzyme activity, such as substrate loading and nitrogen source concentration, were evaluated using statistical methods. Partial characterization of the enzyme was also performed. In marine environments, the search for novel PHA-degrading bacteria remains crucial for understanding biodegradation dynamics. *Pseudooceanicola antarcticus*, a marine bacterium originally isolated from Antarctic seawater, has demonstrated significant enzymatic activity in degrading complex polymers[34]. Its ability to survive in cold and saline conditions makes it an ideal candidate for studying PHA degradation in marine ecosystems, where temperature fluctuations and salinity variations influence microbial activity. Moreover, marine-derived bacteria are particularly valuable in bioremediation applications due to their natural adaptation to seawater conditions, enabling efficient breakdown of biodegradable plastics in marine settings[34,35].

This study investigates the extracellular PHA depolymerase activity of *P. antarcticus* in marine environments. The enzyme's efficiency in degrading poly[(R)-3-hydroxybutyric acid] (P(3HB)) powder was assessed, with statistical methods applied to determine key factors affecting its activity, such as substrate loading and nitrogen source concentration. Furthermore, a partial characterization of the enzyme was conducted to better understand its potential for industrial and environmental applications.

Results and discussion

Isolation and screening of P(3HB)-degrading bacteria

Six isolates were obtained by culturing environmental samples in Artificial Sea Water Salts Broth (ASW medium; Himedia India) supplement with 0.1% P(3HB) (ASW-P(3HB)). This Approach was used as an initial screening method for bacteria that can hydrolyze P(3HB) and use it as a carbon source for growth. The isolated strains were extensively tested on ASW-P(3HB) agar to prevent pseudo-positive results caused by the possible existence of additional non-P(3HB)- degrading bacteria that could result in a symbiotic reaction in the presence of positive P(3HB) degraders. The development of clear zones surrounding the bacterial colonies signified a favorable outcome and validated the capacity of the strain to break down P(3HB)[36]. Microorganisms capable of creating clear zones around their colonies or degrading bioplastic PHAs were observed after incubation at 30 C for 1–4 weeks.

Monitoring of PHB-degrading bacteria under solid and liquid conditions

The emergence of clear zones surrounding the colonies demonstrated that the six bacterial isolates isolated from the aforementioned materials could degrade P(3HB). Six bacterial isolates were identified from the samples, all of which were capable of degrading P(3HB), as evidenced by the formation of clear zones around the colonies. When the degradation index of all isolates was tested on ASW-P(3HB) agar using the point inoculation method (Fig. 1), it was discovered that at one week, isolate SS1-2 had the highest degradation index of 1.44, whereas at four weeks, isolate SS2-2 had the highest degradation index of 2.27. The degradation index, represented as a clear zone around the colony, indicated polymer hydrolysis into water-soluble compounds by the depolymerase enzyme [36]. Microorganisms are classified as PHA degraders if their degradation index is more than 1.00[37]. In the present study, all six isolates showed degradation indices greater than 1.00. However, isolate SS1-2 exhibited the highest degradation index after one week; therefore, it was chosen to investigate the ideal conditions for depolymerase production to degrade PHAs.

Identification of isolate SS1-2 using morphological characterization and molecular methods

Isolate SS1-2 was identified based on its morphological characteristics and 16S rRNA gene sequence. Scanning electron microscopy (SEM) analysis revealed that isolate SS1-2 formed rod-like bacteria that were normally

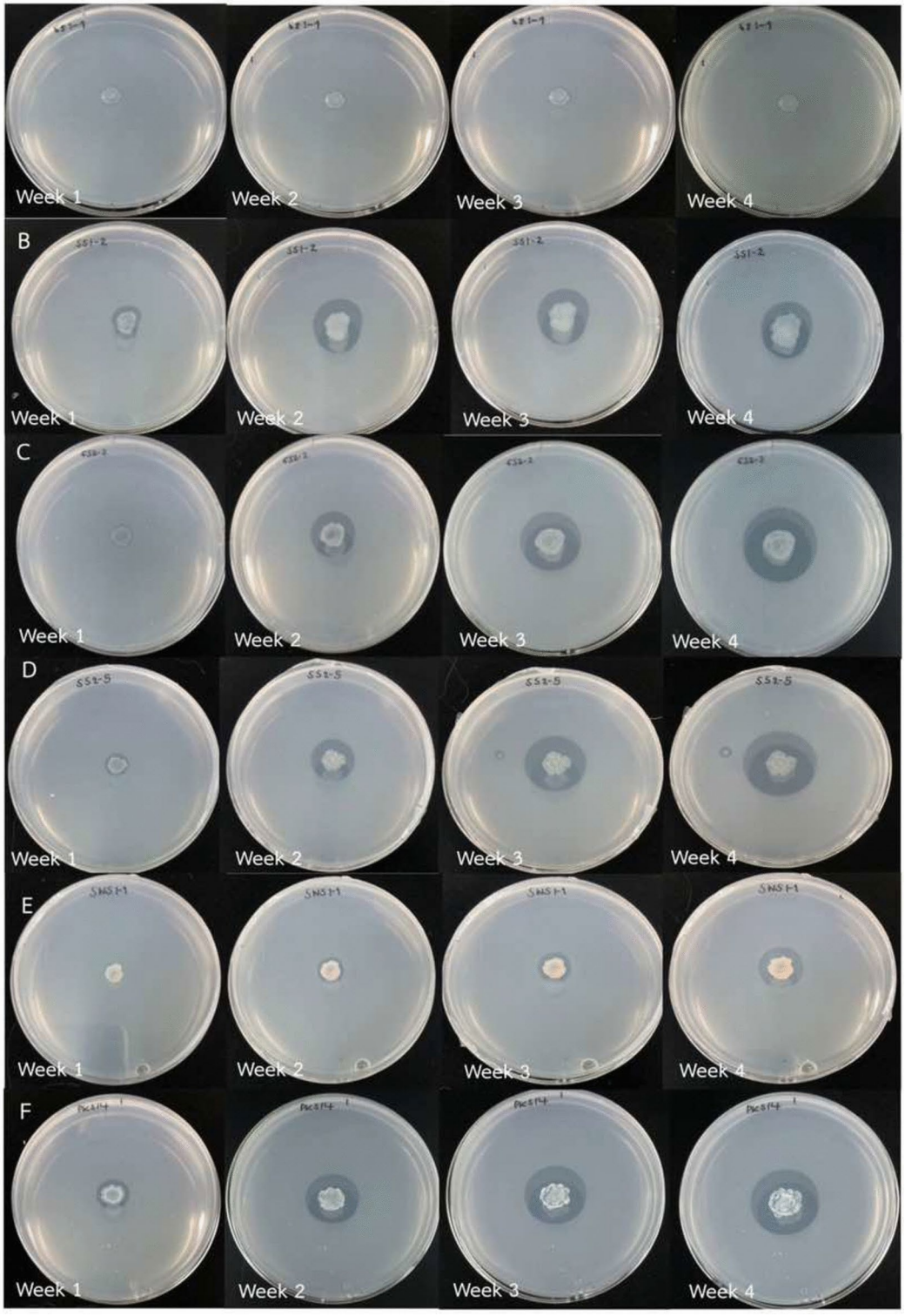

Fig. 1. The change in clear zone diameter produced by isolate SS1-1 (**A**), SS1-2 (**B**), SS2-2 (**C**), SS2-5 (**D**), SWS1-1 (**E**), and PKS14 (**F**) on ASW-P(3HB) plates after 1–4 weeks of incubation.

elongated and cylindrical (Fig. 2). However, these bacteria may form smaller segments or fragments, leading to the loss of rod-like structures. The cells of the isolates appear pink) after Gram staining. To confirm the identity of the isolate, we amplified the 16S rRNA region from its DNA using the universal bacterial primers 20F and 1500R and sequenced the amplicon. The GenBank accession number has been provided as PV162914. BLAST analysis of the 16S rRNA sequence of isolate SS1-2 revealed similarities with *Pseudooceanicola antarcticus* CGMCC 1.12662 (97.81%) , *P. algae* (97.60%) , and *P. aestuarii* (97.60%). Phylogenetic analysis was

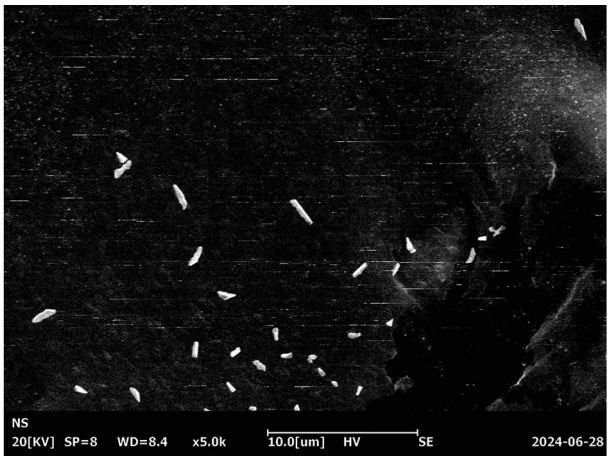

Fig. 2. Morphological features of isolate SS1-2 observed under a scanning electron microscope at 5000× magnification.

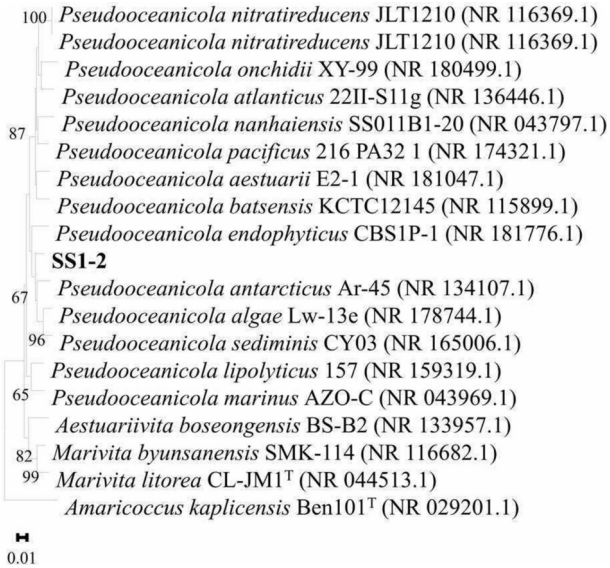

Fig. 3. Neighbor-joining phylogenetic tree generated using maximum likelihood analysis based on 16S rRNA sequences of isolate SS1-2 and related *Pseudooceanicola* species. Bootstrap values ≥ 50% (1000 replicates) are shown at each branch. *Amaricoccus kaplicensis* was used as the outgroup.

performed to determine the taxonomic position of isolate SS1-2 and to compare its 16S rRNA sequence with that of other species of *Pseudooceanicola*. A phylogenetic tree constructed using the NJ approach based on 16S rRNA sequences and comparison with closely related type strains revealed that isolate SS1-2 was related to *P. antarcticus*, *P. algae*, and *P. aestuarii*. However, this was independent of the three species (Fig. 3). These findings suggested that the bacterial sample could be a new species of the genus *Pseudooceanicola*; nevertheless, the genomic DNA sequence should be studied further to compare it to other closely related species.

Optimization of depolymerase production using the one-factor-at-a-time method

The factors in ASW-P(3HB) liquid media were adjusted to increase extracellular depolymerase enzyme production by isolate SS1-2. The effects of various inorganic nitrogen sources on enzyme production were studied (Table 1). These results indicated that NH_4Cl is the best nitrogen source for enzyme production. After 6 days of culture in ASW- P(3HB) liquid medium with 1% NH_4Cl and incubation at 30 °C, enzyme activity was determined to be greatest and statistically significant at 0.027 ± 0.00 mg/mL/min. The next three were $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and the control with enzyme activity levels of 0.023, 0.0094, and 0.003 mg/L, respectively. (Fig. 3). Supplementation with urea did not affect the synthesis of enzymes, but urea was found to be an effective inhibitor of P(3HB) depolymerase[38], as seen by the decline in activity to just 0. 00 U/mL, as opposed to the control (0.0094 U/mL) with P(3HB) as a carbon source. In contrast to our findings, Vigneswari et al.[37] and Bano et al.[38] reported the optimal conditions for enzyme synthesis from *Acidovorax* sp.DP5 and *Paenibacillus alvei* PHB28

Parameters	Depolymerase activity (mg/mL/min)
N-sources	
Urea	$0.00^{e} \pm 0.0$
$(NH_4)_2SO_4$	$0.0225^{b} \pm 0.0001$
NH_4Cl	$0.0267^{a} \pm 0.0001$
$(NH_4)_2HPO_4$	$0.0032^{d} \pm 0.0001$
Control	$0.0094^{c} \pm 0.0001$
Temperature (°C)	
30	$0.0256^{a} \pm 0.0001$
35	$0.0022^{b} \pm 0.0001$
37	$0.0023^{b} \pm 0.0001$
40	$0.0028^{b} \pm 0.0002$
pH	
5	$0.0037^{c} \pm 0.0001$
5.5	$0.0035^{c} \pm 0.0002$
6	$0.0035^{c} \pm 0.0003$
6.5	$0.0034^{c} \pm 0.0002$
6.8	$0.0267^{b} \pm 0.0001$
7.5	$0.0280^{a} \pm 0.0004$

Table 1. Effect of each parameter on P(3HB) depolymerase activity. [a,b,c]Mean values with different letters for each parameter are significantly different at $p \leq 0.05$.

were improved by the addition of urea and $(NH_4)_2SO_4$, respectively. Thus, NH_4Cl is a promising nitrogen source that should be further investigated for the production of depolymerase enzymes in this study.

The ideal temperature range for P(3HB) depolymerase synthesis from isolate SS1-2 was 30–40 °C. At 30 °C, P(3HB) depolymerase was produced at its maximum rate (0.0256 mg/mL/min) (Table 1). Similar findings have been reported by other researchers[37,39], who also discovered that the optimal temperature for the development of the P(3HB) depolymerase enzyme from *B. cepacia* DP1 and *Acidovorax* sp. DP5 is 30 °C.

Table 1 illustrates the effects of temperature on enzyme synthesis. Maximum yields of 0.0280 and 0.0267 mg/mL/min were achieved at an initial pH of 7.5 and 6.8, respectively. The yield was low at initial pH values of 5.0–6.5 (0.0034–0.0037 mg/mL/min). Similarly, changing the initial pH has a noteworthy impact on the production of P(3HB) depolymerase from *Burkholderia cepacian* DP1, according to Azami et al.[39]. They reported that P(3HB) depolymerase synthesis was high at pH 6.8, but low at initial pH values of 5.0 and 7.5. Aly et al.[40] also noted a similar pattern in the synthesis of P(3HB) depolymerase from *Streptomyces lydicus* MM10 with varying initial pH. The synthesis of P(3HB) depolymerase increased as the initial pH was increased to 7.0 but dropped at the initial pH of 10.0. Similarly, Lodhi et al.[41] reported the synthesis of P(3HB) depolymerase by *Aspergillus fumigatus* at various initial pH levels. Based on these results, the synthesis of P(3HB) depolymerase increased when the initial pH increased to pH 7.0; however, it reduced at pH 9.0. The highest amount of PHB depolymerase was produced at pH 8.0, according to Bano et al.[38] however, the enzyme yield dropped at a starting pH of 10–12[38]. Other researchers have reported that the pH range of 7.5 to 10.0 was the greatest for protease synthesis[42–44]. Therefore, the presence of proteases that prevented the formation of enzymes may be the reason for the decrease in P(3 HB) depolymerase production at pH medium, which was higher than pH 7.5[39]. The pH level in a microbial cuture is crucial for depolymerase enzyme synthesis, as each enzyme functions within a defined ideal pH range [45,46]. Variations from this range, whether toward acidic or alkaline environments, may significantly reduce depolymerase production due to disturbances in microbial cell physiology and potential protein denaturation. Each microorganism capable of producing depolymerase possesses a specific pH range that optimizes enzyme synthesis[47]. Extreme pH levels can damage cell membrane integrity, restrict nutrient digestion, and obstruct vital cellular processes necessary for enzyme production[48]. Moreover, deviations beyond the optimal pH range can lead to structural alterations in the depolymerase protein, negatively impacting its activity and stability[48]. Various bacterial species exhibit distinct optimal pH ranges for depolymerase synthesis[45]. Consequently, adjusting the pH of the growth medium is essential for achieving maximal enzyme production and activity[45,49]. As indicated by the results, isolate SS1-2 produced a high concentration of P(3HB) depolymerase enzyme at pH 7.5. Therefore, an initial pH of 7.5 was chosen for further medium improvement tests.

Optimization of depolymerase production using response surface methodology

A two-variable, five-level central composite design (CCD) was used to determine the ideal medium composition for depolymerase production and the interactions between these parameters. Two independent variables, solid loading (X_1) and NH_4Cl concentration (X_2), that affect enzyme production were investigated. Table 2 shows the average of three replicate values for depolymerase production and the predicted response. The results indicated that when the concentrations of substrate loading and ammonium chloride were each set at 0.5% the highest P(3HB) depolymerase production (Run 4) of 0.0350 mg/mL/min was reached. However, when the substrate

Level			Actual level		Enzyme activity (mg/mL/min)	
Run no	X_1	X_2	X_1 (substrate loading, %)	X_2 (NH_4Cl concentration, %)	Observed	Predicted
1	−1	−1	0.10	0.10	0.0137	0.0142
2	1	−1	0.50	0.10	0.018	0.018
3	−1	1	0.10	0.50	0.0199	0.0205
4	1	1	0.50	0.50	0.035	0.0352
5	−1.414	0	0.02	0.30	0.0148	0.0164
6	1.414	0	0.58	0.30	0.0273	0.0296
7	0	−1.414	0.30	0.02	0.0121	0.0126
8	0	1.414	0.30	0.58	0.029	0.0293
9	0	0	0.30	0.30	0.023	0.023
10	0	0	0.30	0.30	0.024	0.023
11	0	0	0.30	0.30	0.022	0.023

Table 2. Experimental design used in response surface methodology of two independent variables: substrate loading (X_1) and NH_4Cl (%) (X_2).

Source	Sum of squares	DF	Mean square	*F*-value	*P*-value
Model	0.000486	5	9.71E−05	125.4888	< 0.0001*
X_1	0.000172	1	0.000172	222.4571	< 0.0001*
X_2	0.000277	1	0.000277	357.8257	< 0.0001*
$X_1 X_2$	2.93E−05	1	2.93E−05	37.9136	0.0016*
X_1^2	3.3E−06	1	3.3E−06	4.265822	0.0938
X_2^2	5.81E−06	1	5.81E−06	7.511533	0.0408*
Residual	3.87E−06	5	7.74E−07		
Lack of fit	1.87E−06	3	6.23E−07	0.62312	0.6642
Total	0.000002	2	0.000001		

Table 3. Analysis of variance (ANOVA) for model regression of P(3HB) depolymerase production. *significance level = 95%; coefficient of determination (R^2) = 0.992; Adjusted-R^2 = 0.9841.

loading and NH_4Cl concentration were 0.30% and 0.02%, respectively, the lowest P(3HB) production (Run 7) was 0.0121 mg/mL/min.

A second-order polynomial model was fitted to the findings of the multiple regression analyses of the CCD experiment. P(3HB) depolymerase production was fitted to the following coded variable model:

$$Y = -0.023 + 0.00464X_1 + 0.00588X_2 + 0.00271X_1X_2 - 0.00102X_2^2 \quad (1)$$

where Y is the P(3HB) depolymerase activity (mg/mL/min), X_1 is the substrate loading (%), and X_2 is NH_4Cl (%).

R^2, a coefficient of determination based on regression, was calculated and used to determine the fit of the model equation to the data. The determination coefficient ($R^2 = 0.992$) accounted for 99.2% of the fit between the created model and the experimental data, with the remaining 0.8% influenced by other variables. However, the adjusted R^2, which accounts for the number of terms and the sample size[50], was 0.9841. The R^2-values ranged from 0 to 1. A model is more powerful and accurate in predicting the response if the R^2 value is high[51]. The importance of each coefficient was evaluated using *p*-values, which aid in deciphering the pattern of mutual interactions between variables[52]. The *P*-value decreased with increasing corresponding coefficient significance[53]. Table 3 displays the estimated F-test and *P*-values that corresponded to it.

The synthesis of P(3 HB) depolymerase was significantly influenced by both substrate loading (X_1) and NH_4Cl concentration(X_2). Table 3 indicates that the quadratic terms of substrate loading (X_1^2) , NH_4Cl concentration (X_2^2) , and the interactions between substrate loading and NH_4Cl concentration (X_1, X_2) were significant. The *F*-value of 0.62 for the lack of fit in Table 3 suggests that the difference between the standard error and the lack of fit is not statistically significant. There was a 66.42 percent probability that noise was the cause of this high lack-of-fit F-value.

The reaction between substrate loading (X_1) and NH_4Cl concentration (X_2) was plotted (Fig. 4) to assess the interaction between various factors and to determine the ideal value of each parameter for maximum P(3HB) depolymerase production. P(3HB) depolymerase activity increased from 0.1 to 0.5% substrate loading, as shown in Fig. 4a and b. P (3HB) depolymerase activity decreased with reduced substrate loading.

The P(3HB) depolymerase activity also increased from 0.1 to 0.5% as the NH_4Cl concentration increased. P(3HB) depolymerase activity drastically decreased with an increase in NH_4Cl concentration (> 0.1%). The P(3HB) depolymerase activity increased as the NH_4Cl concentration rose from 0.1% to 0.5%. However, a further

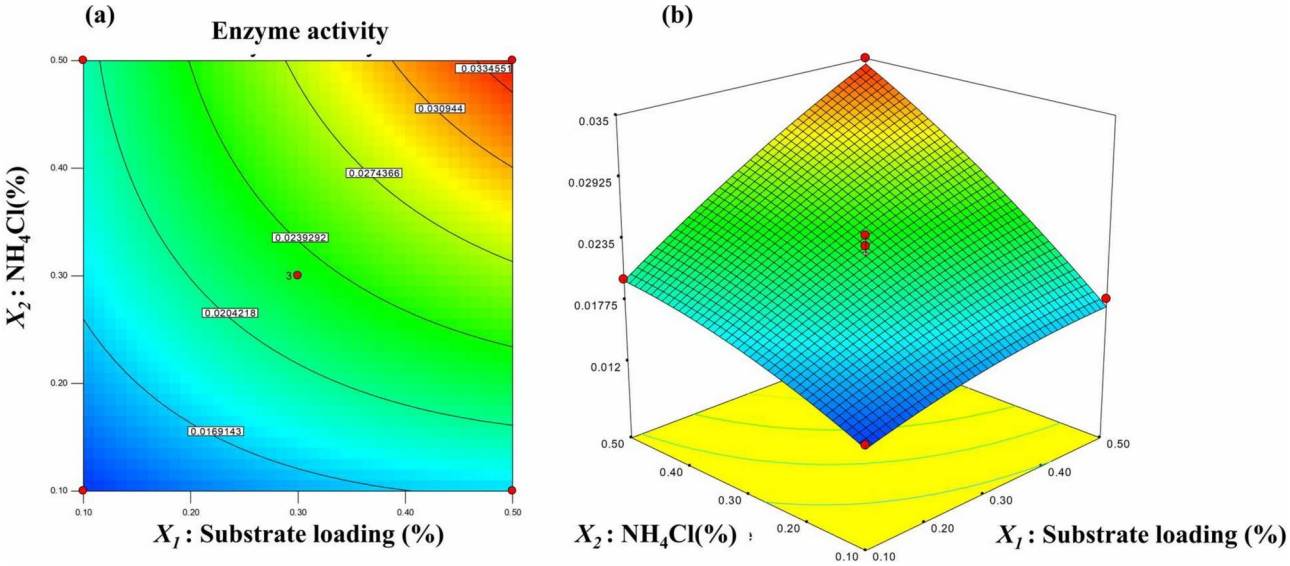

Fig. 4. Response surface (**A**) and contour plots (**B**) of the combined effects between substrate Loading (X_1) and NH_4Cl concentration (X_2) on depolymerase activity.

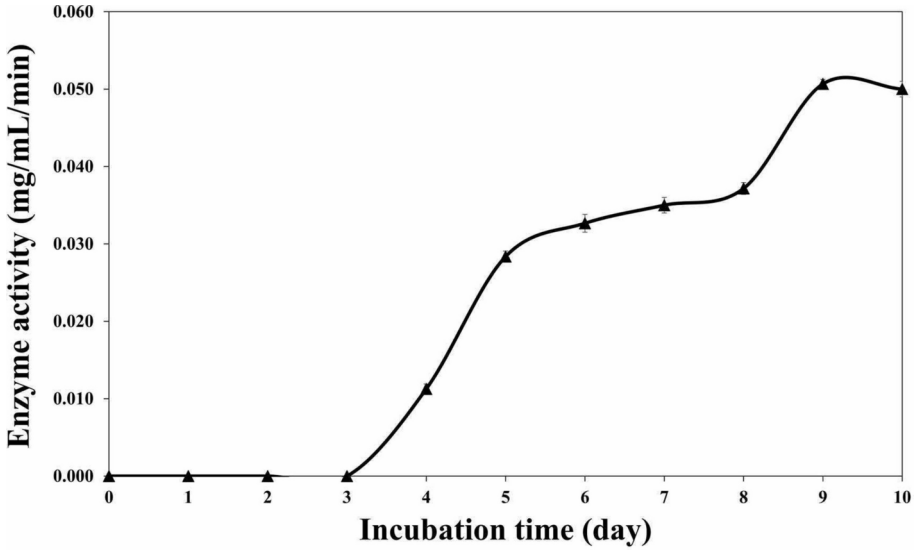

Fig. 5. Time course of depolymerase production.

increase beyond 0.5% led to a significant decline in activity. The 2D contour plots and RSM 3D graphs of substrate loading (X_1) and NH_4Cl concentration (X_2) on P(3HB) depolymerase activity (Fig. 4) revealed that increasing the substrate loading and NH_4Cl concentration from 0.1 to 0.5% significantly improved P(3HB) depolymerase activity. Simultaneously, it decreased when substrate loading and NH_4Cl concentration were below 0.1%.

The model was verified for two independent factors in the design space to validate the optimization predictions. On a 250-flask scale, the RSM-optimized medium composition was tested in triplicate. The findings showed that a maximum P(3HB) depolymerase activity of 0.0329 mg/mL/min was achieved under the following conditions: substrate loading, 0.5%, and NH_4Cl, 0. 5%. These values were close to the predicted P(3HB) depolymerase activity (0.0 34 mg/mL). The residuals were computed after comparing the experimental and anticipated values. A discrepancy of 3.3% was observed between the actual and predicted P(3HB) depolymerase activity. As a result, RSM analysis is a suitable method for forecasting and enhancing fermentation media, and the observed models are quite accurate. The time profile of depolymerase production is shown in Fig. 5. Maximum depolymerase activity of 0.05 mg/mL/min was observed after 9 days of incubation.

The optimal conditions for depolymerase production by isolate SS1-2 were a substrate loading of 0.5% and an NH_4Cl concentration of 0.5%, yielding an enzyme activity of 0.05 mg/mL/min, which was 5.32-fold higher than that of the original medium (0.0094 mg/mL) using a substrate concentration of 0.1%. The findings of

this study are congruent with those of Vigneswari et al.[37], who recovered PHA-degrading bacteria from water and soil samples collected from various habitats in Malaysia. Only 8 of the 18 isolates exhibited the potential to degrade PHA. Isolate DP5, also known as *the Acidovorax* sp. DP5 had the highest degradation index (6.0 after 4–5 days). After 48 h of incubation, depolymerase enzyme activity was highest at 0.035 mg/mL. Using urea at a concentration of 1 g/L as the nitrogen source and P(3HB) at concentrations of 0.1% and 0.25% as the carbon source led to maximum enzyme activities of 0.056 and 0.066 mg/mL/min, respectively, after 72 h of incubation, according to research on ideal conditions by varying the nitrogen sources. While research on the impact of ammonium chloride on depolymerase production is few, ammonium chloride is acknowledged as a significant nitrogen source that affects microbial proliferation and enzyme production. The effect of NH_4Cl is contingent upon the specific microorganism and enzyme type involved. Yu et al.[54] indicated that NH_4^+ supplementation not only augmented cell proliferation but also enhanced the expression of foreign proteins. At a high NH_4^+ concentration of 500 mmol/L, enzyme expression was significantly suppressed. Yang[55] noted that cell density rose with increasing NH_4^+ levels, while growth inhibition was found when concentrations surpassed 600 mmol/L. NH_4Cl serves a dual function in enzyme production, operating as both a stimulant and an inhibitor, contingent upon concentration and environmental conditions. At suitable concentrations, NH_4Cl acts as an available nitrogen supply, increasing microbial growth and enzyme production. Excessive ammonium chloride concentrations can induce catabolite suppression, a regulatory mechanism whereby nitrogen surplus inhibits the expression of genes responsible for enzyme production[56]. In the synthesis of PHA depolymerase, an enzyme that facilitates biopolymer breakdown, elevated nitrogen levels have been observed to redirect microbial metabolism towards growth instead of enzyme secretion, hence diminishing enzyme production[57]. The optimal concentration of NH_4Cl for enzyme synthesis typically varies from 0.1 to 1.0 g/L, however the precise range is contingent upon the specific enzyme and microbial strain employed[56].

NH_4Cl is a critical nitrogen source for enzyme synthesis, necessitating the maintenance of an appropriate concentration. Moderate concentrations promote enzyme synthesis, but high NH_4Cl may inhibit production by prioritizing cellular development over enzyme expression. This suggested that nitrogen is essential for bacterial growth and that the type of nitrogen source influences the extracellular release of enzymes. Vigneswari et al.[37] investigated several nitrogen sources, including NH_4Cl, NH_4SO_4, NH_4NO_3, $(NH_4)_2HPO_4$, and $(NH_2)_2CO$. Urea resulted in the highest depolymerase activity. Similarly, Jendrossek and Handrick [58] reported that microorganisms that degrade PHAs secrete Extracellular enzymes to hydrolyze them into water-soluble intermediates, which are then used as energy sources for microbial development. Bacteria and fungi can break down PHA depending on the secretion of PHA depolymerase. The above information shows that enzyme production depends on various factors such as the type of microorganism, incubation period, substrate concentration, nitrogen source, temperature, and pH[33,59]. Relevant research on PHB degradation includes the study by Amir et al., which reported that potent PHB degraders are screened based on the zone of hydrolysis, with degradation commonly assessed using the soil burial method under conditions like 37 ± 2 °C, pH 7.0, 60% moisture, and 1% microbial inoculum. Their findings demonstrated that *Aeromonas caviae* Kuk1-(34) (MN414252) could effectively degrade PHB even without optimized conditions[60].

Conclusion

The nonbiodegradable nature of petroleum-based plastics and rising consumer demand have brought attention to the environmental impact of these materials. It is essential to properly dispose off discarded plastics to protect the ecology for future generations. The ability of microbes to break down bioplastics has attracted increasing attention. In this study, using statistical methods, we discovered a novel bacterium, isolate SS1-2, that degrades PHB under optimal conditions. Isolate SS1-2, isolated from Tan competently hydrolyzing P(3HB). The depolymerase enzyme produced by this strain showed a high percentage of degradation with P(3HB) film in an alkaline condition (pH 7.5) and at a temperature of 30 °C. The investigation of microorganisms that produce PHB depolymerase holds considerable significance for marine bioremediation. It is essential for decreasing plastic waste in the ocean, improving biodegradation efficacy, and promoting the sustainable application of microorganisms in marine ecosystem rehabilitation. Furthermore, progress in comprehending this enzyme can enable the development of more effective versions or modifications to the genome of bacteria to improve plastic breakdown in marine ecosystems. PHB depolymerase possesses significant potential for utilization in several sectors, notably in environmental management, packaging, medicine, and biotechnology. Enhancing the enzyme's stability and efficiency may augment its commercial viability and promote a sustainable circular economy, facilitating creative approaches to waste management and environmental preservation.

The potential of *Pseudooceanicola antarcticus* in the biodegradation of polyhydroxyalkanoates (PHAs), presenting promising applications in sustainable waste management and the recycling of biopolymers at an industrial level. However, several challenges need to be addressed before its widespread industrial use. Enzyme activity may require further optimization to improve performance under fluctuating industrial conditions. Additionally, scalability remains a concern and requires further research into large-scale operations.

Material and methods

Isolation and screening of poly (R)-3-hydroxybutyric acid (P-3HB)-degrading bacteria

Marine debris and seawater samples were collected from Tan Khu Bay in Trat Province, Thailand. The isolation was performed following the method of Vigneswari et al.[37,61] with modifications. PHB-degrading bacteria in the samples were enriched in Artificial Sea Water Salts Broth (ASW medium; Himedia, India) supplemented with 0.1% P(3HB) ((ASW-3(PHB)) with shaking at 200 rpm and a temperature of 30 °C for 24–48 h. Afterward, the serial dilution technique was conducted on ASW -P(3HB) agar and incubated at 30 °C for 1–4 weeks. In order to generate the 3(PHB) suspension, 3(PHB) powder was sonicated in the ASW medium for 10 min using an

oscillator (Sonics, USA) set to 20 Hz and 750W. The agar plate was created by incorporating 2.0% agar into the PHB mixture. A clear zone surrounding the colonies was observed in the potential bacterial isolates. The pure colony was cultured on a fresh ASW-P(3HB) and kept at 4 °C for further experiment.

Monitoring of PHB-degrading bacteria under solid and liquid conditions

Each isolate was placed on the middle of the plate containing ASW -P(3HB) using point inoculation technique to analyze the characteristics, and the plates were incubated at 30 °C for 1 month, after which the diameter of the clear zones was measured at 1-week intervals[61]. The distance of the clear zone diameter was measured through the center of the colony, and the experiments were performed in duplicate. The degradation index of each isolate was calculated using Eq. (2)[37]

$$Degradation\ index = \frac{Clear\ zone\ diameter\ (cm)}{Colony\ diameter\ (cm)} \tag{2}$$

Each isolate was cultured in ASW-P(3HB) under shaking conditions at 200 rpm at 30 °C for the degradation assay with liquid media. Samples were collected at 24 h intervals over 10 days to analyze enzyme activity. Bacteria that exhibited the highest depolymerase enzyme activity were selected for further experiments.

Morphological and molecular identification

The isolate with the highest depolymerase activity was identified based on the colony morphology on Youschimiau-Kimura (Y-K) medium[62] and microscopic features. The morphological characteristics were examined using a scanning electron microscope (JSM 5600 LV; JEOL, Tokyo, Japan). The bacterial isolate with the highest depolymerase activity was selected for 16S rRNA sequencing. Genomic DNA was extracted from the selected isolate using a Genomic DNA Mini Kit (Blood/Culture Cell) (Geneaid Biotech Ltd., Taiwan). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed with the universal bacterial primers 20F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1500R (5′-GTT ACC TTG TTA CGA CTT-3′)[63]. The PCR was performed on DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories), according to the following conditions: initial denaturation at 94 °C for 3 min followed by 25 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min; these cycles were followed by a final extension at 72 °C for 3 min. The PCR products were purified and sequenced using universal primers. The resulting 16S rRNA gene sequences were matched against the matching sequences of the type strains of bacterial species obtained from the EMBL/GenBank database using BioEdit software version 7.0. Neighbor-joining (NJ) methods were used to generate unrooted phylogenetic trees. Tree topology was assessed using bootstrap studies of NJ data with 1000 resamplings. All analyses were conducted using MEGA 11 software.

Optimization of depolymerase production using the one-factor-at-a-time method

Media and culture conditions, including nitrogen source, temperature, and pH, were optimized for maximum depolymerase production using a one-factor-at-a-time method at three levels[64]. Various nitrogen sources, such as ammonium chloride (NH_4Cl), ammonium sulfate [$(NH_4)_2SO_4$], di-ammonium hydrogen phosphate [$(NH_4)_2HPO_4$], and urea [$(NH_2)_2CO$], were examined for their effects on depolymerase production. In all experiments, each nitrogen source was added at 1% in ASW- P(3HB) under shaking conditions at 200 rpm at 30 °C for 6 days. The control set used the ASW-P(3HB) liquid medium without any nitrogen source. To identify the optimum temperature, the ASW-P(3HB) containing the optimum nitrogen source was incubated at 30 °C, 35 °C, 37 °C, and 40 °C. To study the effect of pH on depolymerase activity, the medium containing the optimum nitrogen source was adjusted to pH 5.0, 5.5, 6.0, 6.8, and 7.5 and incubated at the optimal temperature. Samples were collected at 1-day intervals for 10 days and enzyme activity was measured.

Optimization of depolymerase production

Factors affecting depolymerase production were investigated using surface response methodology (RSM). A CCD was conducted with two factors: solid loading (X_1) ranging from 0.02 to 0.58% and nitrogen concentration (X_2) ranging from 0.02 to 0.58%. Suitable nitrogen concentrations were determined in preliminary experiments. The factors were divided into 5 levels ($-\alpha, -1, 0, +1, +\alpha$) based on the factorial design at two levels. The CCD comprised 2^2 factorial points with four-star points ($\alpha = \pm 1.41$). Three replicates were created at the center point. Eleven trials were conducted to optimize the settings. Equation (3) was used to assess the influence of each independent variable on response.

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_{11} + a_{22}X_{22} \tag{3}$$

The response surface for each variable was evaluated utilizing Design Expert software (Version 11.0; Stat Ease; Minneapolis, MN, USA). The predicted values derived from the software analysis were subsequently validated. A significance level of 95% ($p < 0.05$) was established for the selection of relevant experimental elements.

Enzyme assay

P(3HB) depolymerase activity was analyzed as described by Vigneswari et al.[37]. The assay was performed in 0.1 M phosphate buffer at pH 6.0, with 0.16% P(3HB) granules. The P(3HB) suspension was sonicated for 10 min using a Sonics Vibra Cell (High-Intensity Ultrasonic Liquid Processors, VCX500) (60 kHz, 250 W) for 10 min. The reaction started with a mixture of 0.1 mL of enzyme solution and 0.9 mL of 3(PHB) suspension, which was incubated at 37 °C for 30 min and subsequently measured at 650 nm. One unit of depolymerase activity (mg/mL/min) was defined as the amount of P(3HB) degraded per mL of enzyme per min.

Data availability

Sequence data that support the findings of this study have been deposited in NCBI. The GenBank accession number has been provided as follows: SUB15104994 SS1-2, PV162914. The files can be accessed at https://submit.ncbi.nlm.nih.gov/subs/?search = SUB15104994.

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Author contributions

Author contributions Antika Boondaeng: Conceptualization, Investigation, Writing – original draft, Funding acquisition. Chanaporn Trakunjaea: Methodology, Investigation. Pilanee Vaithanomsata: Formal analysis. Nanthavut Niyomvong: Conceptualization, Writing – review & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to N.N.