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Isolation and production of polyhydroxybutyrate (PHB) from *Bacillus pumilus* NMG5 strain for bioplastic production and treatment of wastewater from paper factories

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Polyhydroxybutyrate (PHB)

Activated sludge

Wastewater

16S rRNA

ABSTRACT

Polyhydroxybutyrate (PHB) has the potential to replace traditional plastics and limit environmental pollution caused by plastic waste. This study combined wastewater treatment with PHB production to reduce costs. Bacteria capable of synthesizing PHB were isolated from paper mill wastewater and identified using Matrix Assisted Laser Desorption/Ionization–Time of Flight (MALDI-TOF) mass spectrometry and 16S rRNA gene analysis. *Bacillus pumilus* NMG5 strain was found to have a good yield in modified Nutrient Broth culture, reaching 42.28% of dry biomass. The PHB product was analyzed using FTIR spectroscopy and ^{1}H NMR spectroscopy. The bacterial strain was also tested for its ability to treat paper mill wastewater, and it showed impressive results in terms of biochemical oxygen demand (COD), total nitrogen, and total phosphorus, with efficiencies of 95.93%, 79.36%, and 83.55%, respectively. The study found that wastewater treatment combined with PHB production was a promising solution to reduce costs and limit environmental pollution. The bacterial strain *B. pumilus* NMG5 had a high yield of PHB, and the PHB product was of high quality, as confirmed by FTIR and ^{1}H NMR spectroscopy. Furthermore, the bacterial strain showed impressive results in treating paper mill wastewater with high COD, total nitrogen, and total phosphorus efficiencies. These results suggest that this harmless bacterium could be used in paper mill wastewater treatment systems to produce PHB, providing a sustainable and environmentally friendly solution.

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1 Introduction

Environmental pollution is a pressing issue, notably pollution caused by industrial wastewater and plastic waste, which directly harms human health and ecosystems and negatively impacts many countries' sustainable development. Industrial wastewater, mainly pulp and paper factory wastewater, contains many highly toxic compounds, with over 250 identified substances, such as dioxins, phenols, and furans (Ali and Sreekrishnan 2001). Therefore, it is crucial and necessary to address thoroughly the problem of water pollution caused by the paper industry's wastewater.

Plastic has become an essential material widely used in daily human life and has been produced on an industrial scale since the 1940s (Ncube et al. 2021). It is a valuable and durable material that is difficult to decompose in the natural environment, and after use, plastic has become one of the significant causes of environmental pollution. Only 9% of the world's nine billion tons of plastic waste is recycled yearly (Obebe and Adamu 2020). In the United States, plastic waste accounts for approximately 12% of total municipal waste, estimated at 30 million tons per year(United State Environmental Protection Agency [EPA], n.d). This figure is predicted to increase as the population and per capita consumption of plastic products rise. In Vietnam, this is equivalent to about 3.1 million tons (The World Bank 2022). These figures show that urgent measures are necessary to manage and solve the problem of plastic waste to minimize adverse environmental impacts. One current solution is to replace traditional plastics that are difficult to decompose with bioplastics that can decompose quickly in a natural environment (Moshood et al. 2022).

Bioplastics are polymers that can decompose into simple molecules such as CO_2, water, CH_4, inorganic compounds, or biomass under the activities of many microorganisms under natural conditions. One bioplastic evaluated with great potential is polyhydroxyalkanoate (PHA), extracted from bacterial cells. PHAs are a group of polymers produced by bacteria that ferment sugars and fats, composed of 10^3 -10^4 monomers, existing as distinct particles within a cell, with sizes ranging from 0.2 to 0.5 µm. After being extracted from the cell, they exhibit properties similar to conventional resins but are biodegradable, insoluble in water, and non-toxic (Bosco and Chiampo 2010). PHA plastic is, therefore, considered suitable for producing disposable items such as food packaging, packaging film, containers, etc.

To simultaneously address the problems related to wastewater from the paper industry and plastic replacement by bioplastic production, this research was carried out to isolate PHAs synthesized bacterial strains from paper factories' wastewater. This is intended to enhance the survivability of isolated bacteria when putting them into practical applications. These isolated bacterial strains were also screened for their ability to treat wastewater, their biosynthesis of PHAs and the characteristics of the obtained PHA products.

2 Material and Methods

2.1 Screening for bacteria isolation

Wastewater samples having a higher concentration of carbohydrates were collected from three paper factories, including Minh Hung (Minh Hung 3 industrial park, Binh Phuoc province, Vietnam), Saigon (Tan Thanh District, Ba Ria Vung Tau Province, Vietnam), and Lee & Man (Chau Thanh District, Hau Giang Province, Vietnam) paper factories. After diluting to the appropriate concentration (10^{-4}), the sample was spread on the solid medium surface to activate synthetic PHAs, which are low in nutrients but high in carbohydrates. In this study, ½ concentration of Nutrient Agar medium was used as per the manufacturer's recommendation (agar 7.5 g/L; meat extract 0.5 g/L; peptone 2.5 g/L; sodium chloride 2.5 g/L; yeast extract 1 g/L) and supplemented with glucose (4g/L) and Nile Blue A dye. Bacterial strains capable of biosynthesized PHAs will fluoresce from pale yellow to orange and pink under UV light (Li et al. 2018; Kung et al. 2007).

2.2 Bacterial identification

All the isolated bacterial strains were quickly identified by MALDI-TOF (Matrix Assisted Laser Desorption/Ionization– Time of Flight) mass spectrometry (Schulthess et al. 2014), using a Bruker Daltonik MALDI Biotyper (Germany) system at the Center of Science and Biotechnology, University of Natural Sciences, Ho Chi Minh City, Vietnam. After identification, bacterial strains causing diseases to humans, animals, plants, etc., will be removed from the collections. The remaining non-pathogenic strains will be further confirmed by analyzing the gene sequence encoding for 16S rRNA with primer pairs 16sF 5′- AGA GTT TGA TCC TGG CTC AG -3′ and 16sR 5′- ACG GCT ACC TTG TTA CGA CTT-3′ (Thirumala et al. 2010). The products obtained after amplification by polymerase chain reaction (PCR) were sequenced at the Biotechnology Center of Ho Chi Minh City, Vietnam and blasted to compare with the US GenBank (NCBI) database for confirmation.

2.3 Enrichment and growth rate determination

Bacterial strains that could biosynthesize PHA were cultured media enriched on 100 mL Luria-Bertani Broth medium (LB) at 37^o C, shaken at 100 rounds per minute (rpm) for 72 hours. The density of cells was checked every 2 hours to determine the growth rate by optical density (OD) measurement at 600 nm. The dried biomass was tested by centrifuging it for 15 minutes at 10,000 rpm and then drying it at 55^{o}C until it reached a constant weight (Hungund et al. 2013). The strains with significant development

Table 1 Synthetic wastewater and minor solution compositions

Synthetic wastewater		Minor solution	
Composition	Content (mg/L)	Composition	Content (mg/L)
Glucose	165	H_3BO_3	50
$NaHCO_3$	270	$ZnCl_2$	50
NH_4Cl	127	$CuCl_2$	30
K_2HPO_4	53	$MnSO_4 \cdot H_2O$	50
$CaCl_2.2H_2O$	30	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50
$MgSO_4.7H_2O$	12	$AlCl_3$	50
$FeCl_3$	3.5	$CoCl_2 \cdot 6H_2O$	50
		$NiCl_2$	50

and high biomass yield would be additionally grown in a modified ½ strength (w/v) Nutrient Broth medium supplemented by 4g/L of glucose to produce PHAs.

2.4 Wastewater treatment capabilities of isolated strains

After the strains were selected and enriched, activated sludge would be produced using these strains. The activated sludge would then be used to evaluate the wastewater treatment ability. In a 12-hour retention time, 30 L of synthetic wastewater was applied to the sequencing batch reactors (SBRs), and the treated wastewater was used as a subtract. The composition of synthetic wastewater was prepared with a C:N ratio of around 20:1 (Johnson et al. 2010), and then 1 mL of minor solution (Wang and Yu 2006) was added, as shown in Table 1. Dissolved oxygen (DO) in the system was maintained at around 4 mg/L by a 58-watt air blower.

Furthermore, the culturing of activated sludge was carried out until the stabilization phase was reached and the mixed liquor suspended solids (MLSS) value was greater than 2.5 g/L. At the end of each of the ten experimental days following the stabilization phase, total COD, nitrogen, and phosphorus parameters were analyzed to determine treatment efficiency. The process of testing and evaluating the treatment efficiency was continued with actual wastewater collected from the Minh Hung paper factory. To prepare the system, we used 2 L of activated sludge for every 20 L of wastewater and allowed it to acclimate for 24 hours. The output wastewater was collected daily and retained for 12 hours before analysis. Simultaneously, activated sludge samples were collected to perform extraction and evaluate the PHAs' synthesizing ability.

2.5 PHA extraction and analysis

To evaluate the synthesis ability as well as product characteristics of the polymerized PHAs product, the selected strain was cultured in 4 g/L Nutrient Broth (Merck 105443) supplemented with glucose (4 g/L). The biomass was collected after 48 hours. After sampling, PHAs were extracted based on the modified method of Singh et al. (2011). The biomass sample was washed and dried using distilled water and then sent to the centrifuge to remove excess water. The 5g of dried biomass was then incubated in 500 mL of NaClO (4,7%) at 85^{o}C for 1 hour. A continuous shaking force was applied throughout the incubation to break down the cells. After 1 hour, the sample was sent to the centrifuge again for 15 minutes at 5,500 rpm to collect pallets. Then, the pallets were washed using distilled water and dried by centrifuge at 5,500 rpm. Afterwards, the pallets were incubated in 0.13 M of ammonium laureate solution for 3 hours at 75 °C (Mannina et al. 2019). Pallets were then collected using the centrifuge for 15 minutes at 5,500 rpm. Finally, the collected pallets were washed by running them through distilled water and ethanol and then centrifuged and dried overnight at 60^o C to collect PHAs.

Continually, the extracted and purified polymer (5 mg) was thoroughly mixed with spectroscopic grade KBr (100 mg) and pelletized. Fourier Transform Infrared (FTIR) spectroscopy analyzed the functional groups of the isolated polymer using a Bruker FTIR spectrophotometer in the range of 4000 to 400 cm^{-1}. Based on proton nuclear magnetic resonance (^{1}H NMR) spectroscopic analysis, the chemical structure of the isolated polymer was interpreted. The polymer samples (5 mg) were dissolved in 0.5 mL of DMSO-d_6 and analyzed with a Bruker ^{1}H NMR (600 MHz) spectrophotometer. The chemical shifts were represented on the δ_H scale [parts per million (ppm)], and TMS (tetramethylsilane) was used as the internal standard.

The PHAs polymer product's thermal stability was tested by the thermogravimetric analyzer (TGA) by Q500, TA Instruments, USA, and the PHAs yield was estimated by conversion to crotonic acid with concentrated H_2SO_4 then measured by absorbance at 235 nm (Law and Slepecky 1961).

Table 2 Identification results by the MALDI-TOF method

No.	Sample name	Species	Pathogen	Source
1	LM2	*Bacillus cereus*	×	Lee & Man Paper Manufacturing LTD.
2	LM5	*Citrobacter braakii*	×	
3	LM3	*Neisseria gonorrhoeae*	×	
4	NMG3	*Bacillus cereus*	×	Saigon Paper Company
5	NMG4	*Bacillus flexus*		
6	NMG5	*Bacillus pumilus*		
7	NMG9	*Bacillus cereus*	×	
8	NMG2-L	*Bacillus cereus*	×	
9	BP1	*Rhixobiumradiobacter*	×	Minh Hung Paper Joint Stock Company
10	BP3	*Bacillus weihenstephanensis*	×	
11	BP5	*Bacillus megaterium*		
12	BP7	*Pseudacidovorax intermedius*		
13	BP8	*Delftiaacidovoran*	×	

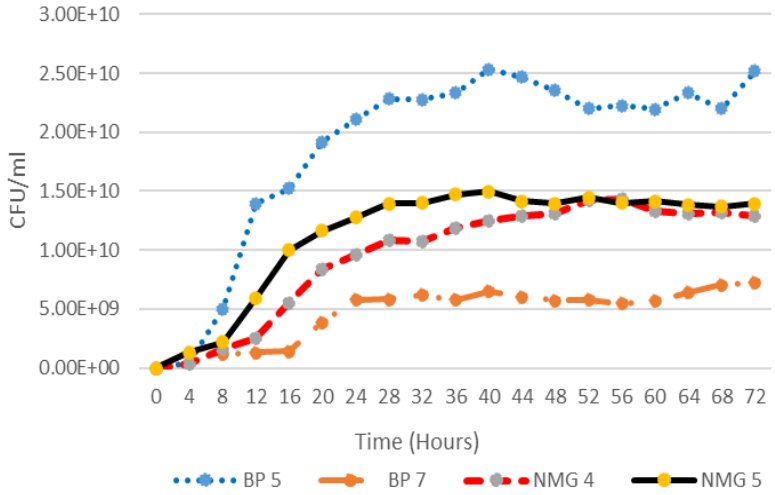

Figure 1 The growth rates of BP5, BP7, NMG4, and NMG5 strains

3 Results and Discussion

3.1 Bacterial Isolation and growth rate

As a result of the screening, 13 bacterial strains capable of synthesizing PHAs were obtained, including three from Lee and Man, four from Saigon, and six from Minh Hung paper factories. From the results shown in Table 2, strains suspected to be pathogenic to humans, livestock, or plants were removed. Growth rate surveys were continued on four remaining strains, including NMG4, NMG5, BP5, and BP7.

The results indicate the presence of beneficial bacteria, particularly those capable of synthesizing polyhydroxyalkanoates (PHAs), even in the toxic conditions found in paper industry wastewater. This finding supports our research team's original aim of coordinating the wastewater treatment and PHA synthesis processes. Furthermore, these bacteria are better suited to adapting and growing in paper mill wastewater than those collected from other sources, making them a promising candidate for PHA synthesis.

The results presented in Figure 1 revealed that all tested strains are approximately at their maximum growth rate after 24 hours. These results are similar to the findings of Seo et al. (2013). Among the four studied strains, the BP5 strain had the highest cell density, reaching 2.5×10^{10} CFU/mL, while the BP7 strain had the lowest cell density at 3×10^{9} CFU/mL. However, the dried biomass result of BP5 was so low (0.38 g/L) compared to NMG5 (2.66 g/L).

These results were not as good as those of a previous study by Getachew and Woldesenbet (2016), which studied optimal conditions for *Bacillus* sp. and obtained biomass ranging from 8.37 to 15.49 g/L. However, because our study focused on using microorganisms in wastewater treatment systems, we could not control certain conditions such as temperature and carbon source, as was done in the study by the authors mentioned above. As a result, we chose to proceed with further trials using strain NMG5.

The sequence of the 16S ribosomal RNA gene segment of strain NMG5 was 1412 nucleotides long and matched 99.72% with the 16S ribosomal RNA fragment of *B. pumilus* DFs 1420 (NCBI Reference Sequence: NR_043242.1). This allows us to confirm that the strain NMG5 has the correct *B. pumilus* identifier. This beneficial aerobic bacterium is being utilized broadly in many industrial and agricultural fields, so it is safe and very suitable to direct PHA production in wastewater treatment systems (Maliehe et al. 2016).

3.2 Wastewater treatment capacities

After culturing in the Nutrient Broth medium, the *B. pumilus* NMG5 strain was cultured with synthetic wastewater, and the activated sludge began to appear on the sixth culture day. By the 22nd culture day, the amount of activated sludge in the system had reached the steady phase, around 2.5 g/L of mixed liquor suspended solids (MLSS). The wastewater was stopped from providing oxygen and left to settle for the next 10 days (from the 23rd to the 32nd day), the sludge was separated, and the effluent water was collected to analyze and evaluate the treatment efficiency. The study results show that COD removal efficiency was over 94.5%, tended to increase slightly, and peaked on the 29th day with a treatment efficiency of 97.71%. Similarly, the nitrogen and phosphorus treatment capacities of *B.pumilus* NMG5 achieved the highest treatment efficiencies of 84.6% and 86.5% on the 29th day, respectively (Table 3).

On the 33rd day, real wastewater collected from the Minh Hung paper factory was used. After one day of acclimatization, the results showed that the treatment efficiency for all three parameters was slightly decreased, at 95.93%, 79.36%, and 83.55% for COD, total nitrogen, and total phosphorus, respectively (Table 4).

This lower treatment efficiency can be explained by some microbial inhibitors in the paper mill industry's wastewater. Even so, with such treatment efficiency as *B.pumilus* NMG5 strain, the

Table 3 Treatment efficiency when using analyzed wastewater

Day	COD			Total nitrogen			Total phosphorus		
	Input (mg/L)	Output (mg/L)	Treatment efficiency (%)	Input (mg/L)	Output (mg/L)	Treatment efficiency (%)	Input (mg/L)	Output (mg/L)	Treatment efficiency (%)
23	1400	77	94.50	17.10	4.72	72.40	16.84	3.65	78.33
24		75	94.64		4.47	73.86		3.47	79.39
25		57	95.93		3.58	79.06		2.77	83.55
26		64	95.43		3.8	77.78		3.02	82.07
27		57	95.93		3.53	79.36		2.9	82.78
28		48	96.57		3.37	80.29		2.52	85.04
29		32	97.71		2.63	84.62		2.27	86.52
30		38	97.29		2.67	84.39		2.32	86.22
31		34	97.57		2.65	84.50		2.28	86.46
32		33	97.64		2.63	84.62		2.28	86.46

Table 4 Treatment efficiency when using real wastewater from Minh Hung paper factory

Day	COD			Total nitrogen			Total phosphorus		
	Input (mg/L)	Output (mg/L)	Treatment efficiency (%)	Input (mg/L)	Output (mg/L)	Treatment efficiency (%)	Input (mg/L)	Output (mg/L)	Treatment efficiency (%)
34	680	77	94.50	12.8	4.72	72.40	1.44	3.65	78.33
35		75	94.64		4.47	73.86		3.47	79.39
36		57	95.93		3.58	79.06		2.77	83.55
37		64	95.43		3.8	77.78		3.02	82.07
38		57	95.93		3.53	79.36		2.9	82.78

output wastewater still meets the A standard, according to Vietnam's regulations on industrial wastewater (National Technical Regulation on Industrial Wastewater QCVN 40:2011/BTNMT).

3.3 Polymer production capacity

The quantitative results of the PHA polymer obtained were 2.1g/5g of dry biomass, equivalent to 42.28%. The FTIR spectra of this PHAs polymer (Figure 2) revealed distinct absorption spectrum for esters: –OH bending at 3436 cm^{-1}, C-H stretching at 2933 cm^{-1}, the absorption band of aliphatic carbonyl C=O at 1723 cm^{-1} and the –CH group of the aliphatic compound at 1228–1381 cm^{-1}. This result revealed that the PHA's polymer chain structure consists of hydroxybutyrate (HB) monomers.

The polyhydroxybutyrate (PHB) structure was confirmed by ^{1}H NMR spectroscopy. ^{1}H NMR spectrum of the polymer (Figure 3) displayed three signals characteristic of PHB, including a doublet at δ_H 1.20 ppm (J = 12.0 Hz), which was attributed to the methyl group (-CH_3), a multiplet at δ_H 2.55 ppm, which was assigned to the methylene group (-CH_2), and a multiplet at δ_H 5.11 ppm, which was characterized as the methine group (-CH). The NMR spectrum obtained followed the data reported in the literature (Shamala et al. 2009; Das et al. 2022), which confirmed that the polymer was made from the *B. pumilus* NMG5 strain PHB.

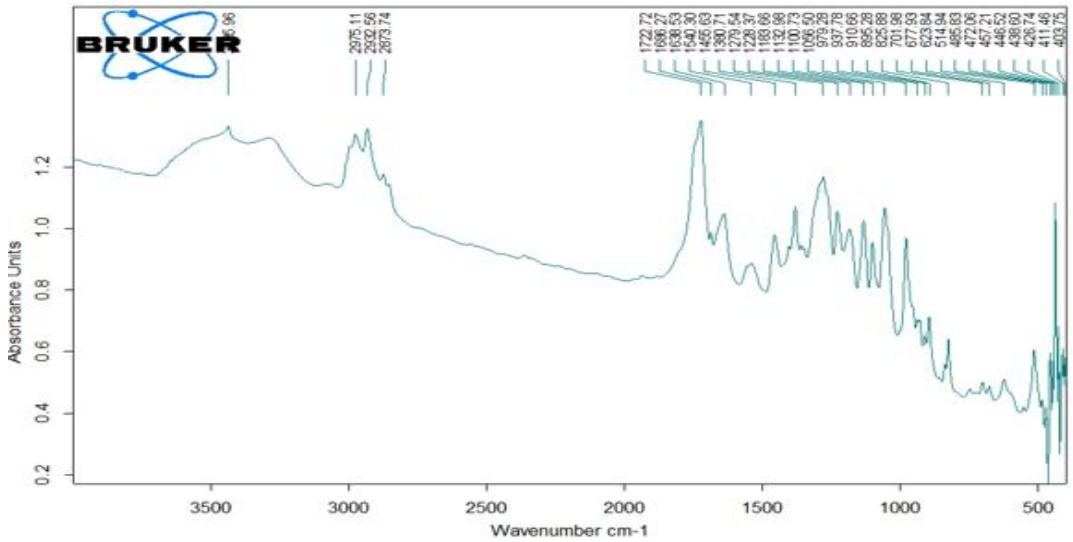

Figure 2 FTIR spectrum of PHA polymer product

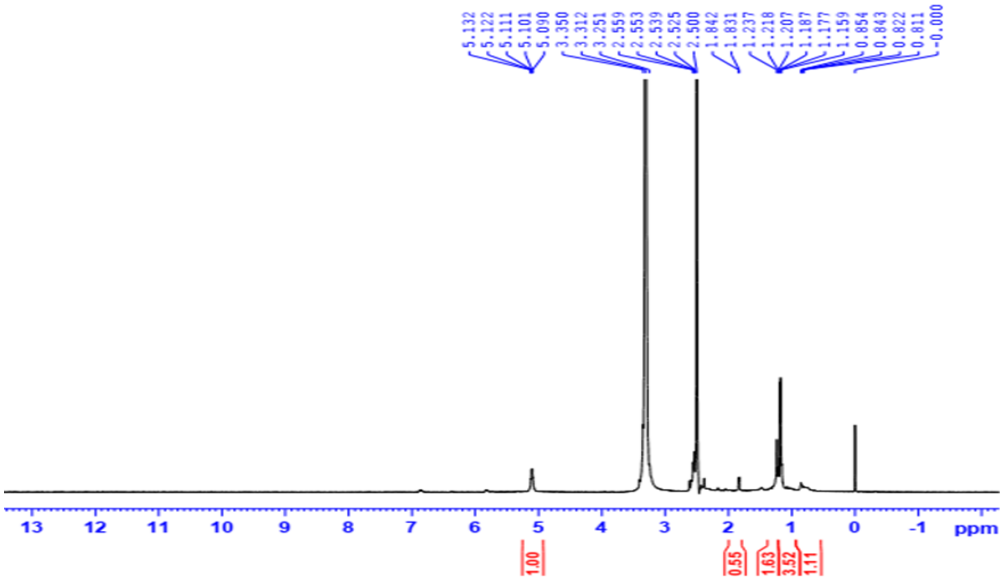

Figure 3 ^{1}H NMR spectral analysis of PHB polymer product in DMSO-d_6

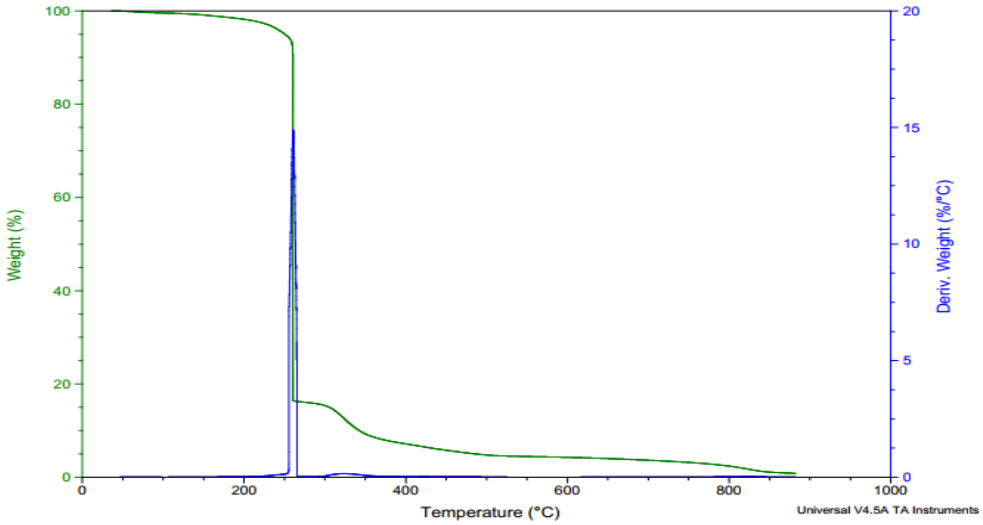

Figure 4 Thermogravimetric (TGA) spectrum of PHB from *B. pumilus* NMG5 strain

Figure 4 depicts PHB TGA test results, demonstrating the material's good stability and the most significant weight loss at 260°C. This outcome is comparable to those reported by Pradhan et al. (2018) and Vahabi et al. (2019) investigations. In which PHB samples that were synthesized from bacteria, as well as standard PHB, were degraded at a temperature between 250°C and 300°C.

Conclusion

The results of this study suggested that the *B. pumilus* NMG5 strain, which was isolated from paper mill wastewater, is safe and capable of synthesizing PHB with a quite good yield of 42.28 %/dry biomass weight. Additionally, this strain has a high ability to adapt and treat paper mill wastewater. However, to integrate PHB production with wastewater treatment in actual practice, a variety set of bacteria should be used to ensure safety and treatment effectiveness. Therefore, we recommend continuing to search, isolate, and evaluate for more bacterial strains capable of biosynthesizing PHB and highly adapted to wastewater environments.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

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