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Research Article

Potential production of bioplastics PHAs (polyhydroxyalkanoates) from paper–mill wastewater

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Abstract: The extensive use of petroleum–based synthetic plastics is leading to various environmental problems such as ecological impact, and untreated plastic waste, particularly micro–plastic pollution. Bioplastics are, therefore, considered one of the prospective solutions to replace conventional plastics. PHAs (polyhydroxyalkanoates), one of the widely studied and applied bioplastics, is synthesized by microorganisms when their living conditions are unfavorable. Many species of microorganisms capable of synthesizing PHAs are found in different environments. In the study, we focus on isolating and screening bacteria capable of synthesizing PHAs from activated sludge of paper mill wastewater treatment system. The results have shown two types of bacteria that have the highest synthesizing productivity: Bacillus Megaterium BP5 and Alcaligenes Aquatilis BP6. Their highest yield of PHAs synthesis was reached at the time 48 hours of incubation, which their dry biomass of 41.19% and 49.11%, respectively.

Keywords: PHAs; Polyhydroxyalkanoates; Polyhydroxybutyrate; Wastewater; Bioplastic.

1. Introduction

The widespread application of non–biodegradable petroleum–based synthetic plastics leads to many environmental pollution consequences [1–2] such as plastic pollution in continents and oceans, especially micro–plastic pollution. Due to their biodegradability, bioplastics are acknowledged to have the potential material to replace synthetic plastics, and thus reduce adverse environmental impacts. Bioplastics is considered a potential solution to replace conventional plastics. They are bio–based polymers synthesized from organic sources, with little impact on the environment [3]. One of the bioplastics that has attracted a lot of attention from researchers is Polyhydroxyalkanoates (PHAs). PHAs have similar physical properties to conventional plastics such as heat resistance up to 170^{o}C, high strength, and more importantly almost no toxicity [4]. PHAs are a large family of polymers that are biosynthesized by bacteria when their growing conditions are disadvantaged, such as too high a carbon–to–nitrogen ratio living environment [5–6].

PHAs are classified into two groups based on the length of the polymer units, namely short–chain PHA (poly HASCL) and medium–chain PHA (Poly HAMCL) (Figure 1). In the short–chain group, they are composed of 3 or 5 carbon atoms commonly known as 3HB (hydroxybutirate) and 3HV (hydroxyvalerate). The other group is composed of 6 or more carbon atoms [7]. PHAs has similar physical properties to conventional plastics [8] but is readily biodegradable, so PHAs is considered a potential alternative to synthetic resins [9]. PHAs are applied in many fields such as disposable plastic products, food packaging, agriculture, construction, pharmaceuticals, and medicine [10–11].

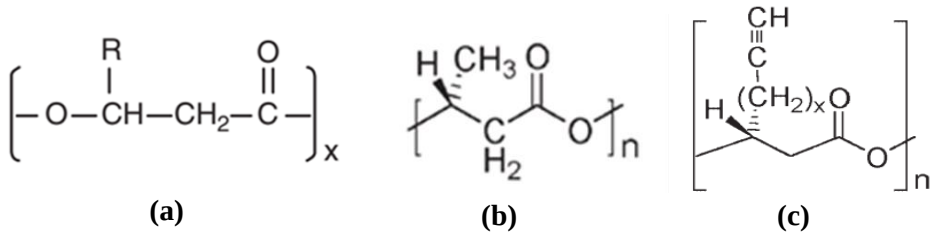

Figure 1. PHAs' chemical formulas: (a) PHAs; (b) poly HASCL (3HB); (c) Poly HAMCL.

Currently, although there have been many studies on the production of PHAs, the application of PHAs in practice is limited due to high manufacturing costs [12]. To reduce production costs, the microbial strains for synthesizing PHAs must meet the following criteria such as fast growth, high biomass of PHAs, and affordable culture materials. Other prerequisites in PHAs production include controlled living conditions, simple culture settings, uncomplicated extraction process, and reasonable cost [13–14]. To date, more than 300 species of microorganisms have been found to be able to synthesize PHAs [3]. Some common types of bacteria such as Pseudomonas, Azotobacter vinelandii Alcaligenes eutrophus, Alcaligenes latus, Bacillus megaterium, etc. are of great importance to many scientists due to their high yield of PHAs biosynthesis [15].

In the study, we isolated and screened bacterial strains capable of biosynthesis of PHAs, from activated sludge of a paper mill wastewater treatment system. The study also evaluated the capability of these bacterial species to PHAs biosynthesize under laboratory conditions. Besides, one of the main differences of this study compared to the previous ones is that we found the optimal culture time for the bacteria to synthesize PHAs.

2. Materials and Methods

2.1. Materials

Isolation materials: Nutrient agar, consist of Peptone 5 g/L; NaCl 5 g/L; Meat glue 1.5 g/L; Mushroom extract 1.5 g/L; Agar 15g/L (Figure 2).

PHAs biosynthesis environment: Improved nutrient broth, including Peptone 2.5 g/L; NaCl 2.5 g/L; Meat glue 0.75 g/L; Mushroom extract 0.75 g/L; Glucose 4 g/L.

Bateria source: the activated sludge sample taken from Minh Hung paper mill (Binh Phuoc province, Vietnam).

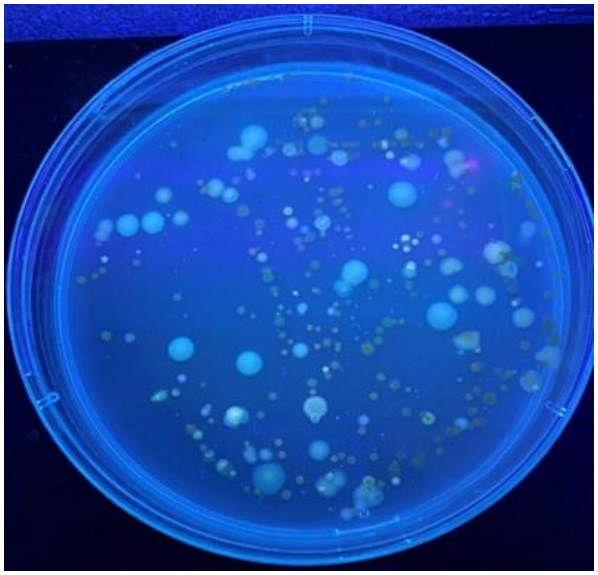

Figure 2. Isolation and screening of PHAs bacteria.

2.2. Methods

2.2.1. Isolation and screening of bacteria capable of biosynthetic PHAs

The activated sludge sample was diluted one million times before spreading on Nutrient Agar (NA) and incubated at 32^{o}C for 72 hours. Colonies of bacteria species capable of synthesizing PHAs were identified by staining with Sudan Black B (black color) [16]; then confirmed by Nile Blue A dye. Under UV light, fluorescence colonies were detected. These colonies were selected to be capable of synthesizing PHAs [17–18].

2.2.2. Evaluation of the PHAs synthesizing capability of the isolated bacterial strains

The screened bacteria were further cultured in the Nutrient broth to motivate PHAs synthesis. The culture process was carried out at 32^{o}C, pH = 7.0, shaking at 150 rpm for 72 hours. Biomass at 24, 48, and 72 hours was collected and measured to determine the highest PHAs yield duration.

The extraction method of Gulab Singh et al was applied to evaluate the PHAs' content in the biomass [19]. The process includes taking 50 ml of culture solution, centrifuging at 7800 rpm for 15 minutes, then removing the solution to collect biomass. The obtained biomass was then incubated with 10 ml of NaOCl solution (2%) at 50^{o}C for one hour. The solution was then centrifuged at 7800 rpm for 20 min. The supernatants were then removed, and washed with distilled water, alcohol, and acetone. The filtered solid substance (obtained polymer) was dissolved in hot chloroform and screened through Whatman No.1 filter paper, then dried at room temperature. Dissolve the obtained PHAs in 10 ml of concentrated H_2SO_4 and incubate at 100^{o}C to convert all the PHAs to crotonic acid. The compound was measured the optical density (OD) at 235 nm with PHAs control (sigma) to determine accumulated PHAs content in bacterial cells. The accumulation of PHAs in bacterial cells was calculated according to the following formula:

$$\text{PHAs} = \text{PHAs content/dry biomass content} \quad (1)$$

2.2.3. Bacteria identification by 16s–rRNA sequence analysis

After checking the capability of PHAs productivity, selected bacterial strains were identified by 16S–rRNA fragment analysis with primers 16sF 5'– AGA GTT TGA TCC TGG CTC AG –3' and 16sR 5'– ACG GCT ACC TTG TTA CGA CTT – 3'.

The experiment was conducted at the laboratory of the Center for Biotechnology, Ho Chi Minh City. The 16S rRNA gene sequences were compared (blast) with the US database, Genebank (NCBI) for identification.

3. Results and discussion

3.1. Isolation and screening of bacteria capable of PHAs biosynthesis

From the activated sludge sample, we isolated 32 strains of bacteria with symbols BP1 to BP32. Screening by Sudan Black B and Nile Blue A, there were two strains fluorescented under 365nm UV lamp, labeled BP5, BP6. Both of them were lighted orange on the second day of incubation. They are considered the potential bacteria of PHAs biosynthesis (Figure 3 to Figure 5).

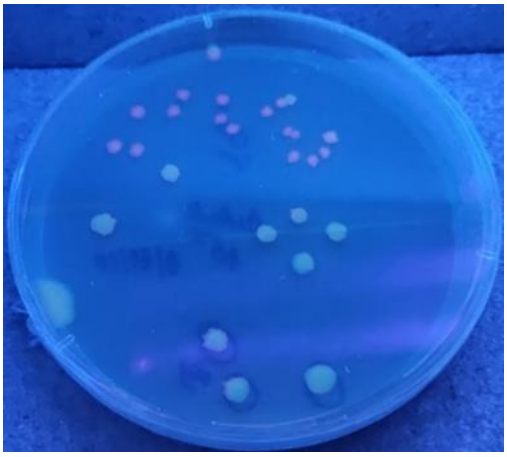

Figure 3. Fluorescent bacteria on NA medium supplemented with Nile blue A dye under 365nm UV lamp.

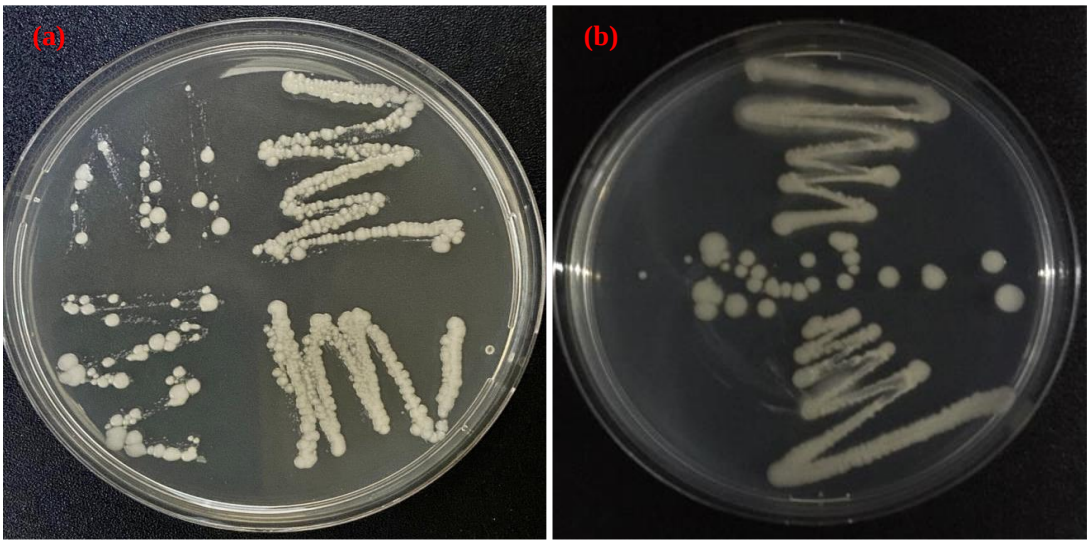

Figure 4. (a) Colony of BP5 bateria; (b) Colony of BP6 bateria.

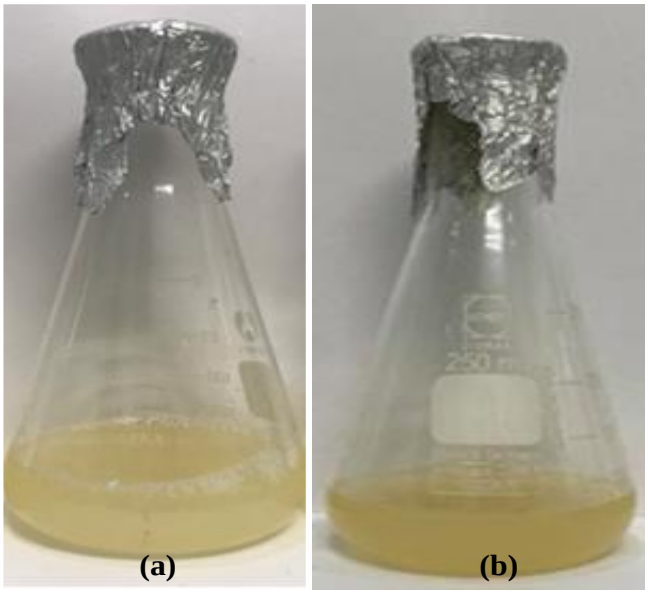

Figure 5. Nutrient broth of bacterial strain BP5 (panel a) and BP6 (panel b).

3.2. Evaluation of the PHAs synthesizing capability

The results of measuring the optical density (OD) of the 2 selected samples after culture and treatment are shown in panel (a) of Figure 6. This result shows that both selected strains have the highest PHAs synthesis yield at 48h of culture.

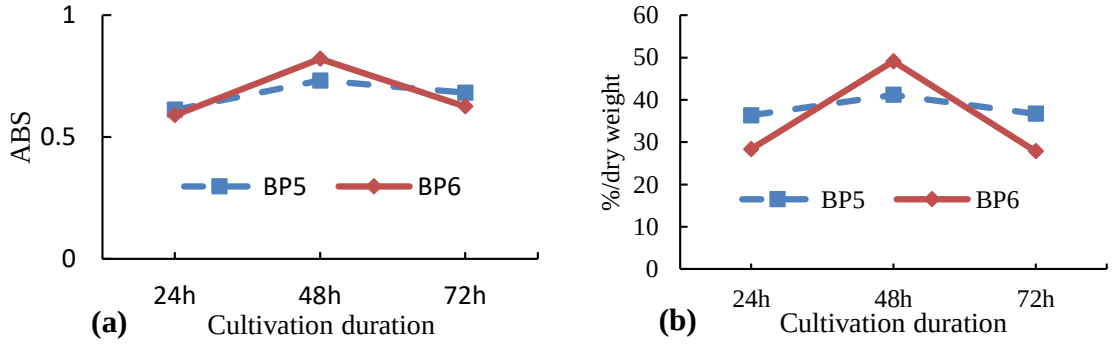

Figure 6. PHB cultivation results: (a) Optical density measurement results at the time of culture; (b) Accumulated PHAs content in bacterial cells.

Biomass from the culture solutions of the two strains at 24h, 48h, and 72h was dried and extracted PHAs according to Gulab Singh process [19]. The accumulated PHAs content on the dry biomass of bacteria is shown in Figure 5.

The assessment results on the synthesizing PHB capability of the two strains BP5 and BP6 showed that the ability to accumulate PHB of these two strains was quite high, 41.19% and 49.11% respectively, which was higher than the average level when compared with other strains (Table 1).

Table 1. PHAs biosynthesis yield in improved nutrient broth environment for 48h.

No.	Strains of bacteria	Dry biomass (g/L)	PHAs contents (g/L)	PHAs productivity (%/dry biomass)
1	BP5	0.378	0.1557	41.19%
2	BP6	0.79	0.388	49.11%

Similar ratios have been published by some other authors. One of the studies on optimizing conditions for culturing some strains of *Bacillus sp*. resulted in PHAs accumulation from 20.3% to a maximum of 70.04% of the dry biomass [6]. Rituparna Das et al (2022) studied that the *bacillus pumilus* AHSD 04, isolated from parts of an oilseed plant *(Oleaginous Plant Arachis)* can produce from 26.2% to 76.5% of the PHB dry biomass [20].

The study also showed that the optimal culture time for the two bacterial strains on PHAs biosynthesis was 48 hours (Figure 7). This result also showed that when the culture time is longer, the bacteria use PHAs as a substrate source for growth. This research results show a similarity in optimal PHAs time of synthesizing on strain *Rhizobium gallicum* R602 [23].

Figure 7. PHA extracted from BP5.

Besides, the yield of PHAs synthesis of BP6 was higher than that of BP5. From these results, it can be assessed that the potential of the two bacterial strains obtained in the study is very promising. Their capability to synthesize PHAs is quite high in sub–optimal conditions, together with their ability to adapt well to living in a wastewater environment.

3.3. Bacterial identification by 16s–rRNA sequence analysis

These bacterial strains (BP5 and BP6) were inoculated for purification on a petri–dish containing Nutrient agar. After culturing at 32^{o}C for 48 hours, the bacterial colonies were sent to the Center for Biotechnology, Ho Chi Minh City for DNA extraction and PCR amplification of the 16S rRNA fragment. Sequences were searched on BLAST NCBI (http://blast.ncbi.nlm.nih.gov/).

The results showed the match of the BP5 gene fragment with the 16S rRNA fragment of *Bacillus megaterium* mj1212 with accession number KJ451626.1. Similarly, the gene sequence of strain BP6 matches the gene fragment of strain *Alcaligenes aquatilis* RC43 with accession number MT572474.1 (Figure 8).

Figure 8. Phylogenetic tree based on NCBI data.

This result shows that the isolated bacterial strains are also the species that have been evaluated to have good PHAs synthesis ability in previously published studies.

4. Conclusion

From the activated sludge sample from the wastewater treatment system of Minh Hung paper factory (Binh Phuoc province, Vietnam), we isolated two strains of bacteria with potential for PHAs biosynthesis, particularly PHB. They are *Bacillus Megaterium* BP5 and *Alcaligenes Aquatilis* BP6. The biosynthesis research results show that the optimal time of culture for the biosynthesis of PHAs of these two strains in the modified Nutrient broth is 48h. This is one of the main findings contributing to the knowledge of PHAs biosynthesis of bacteria.

Since the research was carried out in the laboratory, further studies on the evaluation of the yield of PHAs production in practice, such as the paper mill wastewater environment, should be researched, particularly the optimal incubation time. Besides, as in this study improved nutrient broth was applied to stimulate the bacteria to synthesize PHAs, more studies on affecting living conditions of the bacteria in practice need to be implemented to improve the effectiveness. Consequently, the study's results can apply to the pilot scale.

Author contribution statement: Conceived and designed the experiments; Analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; manuscript editing: M.K.Q.H.; Performed the experiments; contributed reagents, materials, analyzed and interpreted the data, wrote the draft manuscript: T.D.N., T.T.M.N.

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Competing interest statement: The authors declare no conflict of interest.

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