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# Studies on Biodegradation of Medium Chain Length PHAs by Soil Bacterial Isolates

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**Abstract:** Growing environmental awareness has imposed a paradigm shift from biostable materials to biodegradable ones that are compatible with the environment to ensure an eco-friendly atmosphere. On account of that, in the present study the biodegradation of Polyhydroxyalkanoates was monitored by open window composting method. Under the prevailing condition (pH 6.8-7.2 and temperature 34-37$^{o}$C) during open window composting it was observed that, 96 % of PHAs disc degradation occurred within 20 days. In toto fifteen aerobic, bacteria were isolated from the soil and surface of PHA discs, of which three observed as depolymerase producers. These depolymerase producing bacterial isolates were grown abundantly by utilizing PHAs as sole carbon source in Minimal salt medium for four days at pH 7 and temperature 37$^{o}$C. The *in-silico* analysis revealed, *Alcaligenes* sp. B1 shown a strong evolutionary closeness with *Alcaligenes faecalis* which is supported by 99 % bootstrap value during phylogenetic tree construction. The identified bacterium is *Alcaligenes* sp. B1 (KT784806). Thus, further study is also essential for exploitation of the potent bacterial isolate for biodegradation of biopolymer or bio-based polymer.

**Key words:** Composting, Polyhydroxyalkanoates, Depolymerase, Evolutionary, Bio-based.

## Introduction

Synthetic plastics have become an imperative part in our day to day lives. However, these plastics are non-biodegradable and accumulate at the rate of 25 million tons per year [1]. The radical increasing in accumulation of synthetic plastics has become one of the foremost environmental hazards. This problem is compounded with the fact that the resources for crude oil is also decreasing. Moreover, in the modern scenario when global warming, climate change and dwindling fossil carbon resources have taken a centre stage; researchers are looking for eco-friendly alternatives to petrochemical based plastics. This alternative has come in the form of biodegradable plastics such as polyhydroxyalkanoates (PHAs) [1, 2]. Nutrient limitations play a pivotal role in PHAs production by many bacterial species, which is further assimilated by bacteria under carbon starvation condition. When carbon and energy are required, PHAs is normally depolymerized to D-hydroxybutyric acid and then metabolized to acetoacetate and acetoacetyl-CoA[3, 4, 5]. The unique properties of PHAs are their bio-

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degradability in natural environments. PHAs are high molecular weight biopolymer that cannot be transported through the cell wall, thus microbes such as bacteria and fungi excrete extracellular PHAs depolymerases that hydrolyze PHAs into water soluble monomer and oligomers as a result, the low molecular weight products are transported into the cell and subsequently metabolized as carbon and energy sources [6]. Several PHAs depolymerases have been purified from various microbes such as *Ralstonia pickettii* T1 [7], *P. stutzeri* [8], *C. acidovorans* [9], *Streptomyces* sp.[10], *Rhodococcus* species [11].

Extracellular PHAs depolymerases fall in two categories as, short chain length PHAs (scl-PHAs) depolymerases and medium chain length (mcl-PHAs) depolymerases, which differ with respect to substrate specificity for scl-PHAs or mcl-PHAs. Although a few bacteria have been reported to degrade both scl-PHAs and mcl-PHAs by producing two types of depolymerases [10], majority of PHAs degrading bacteria produce only one type of PHAs depolymerase. PHAs depolymerase producing bacteria of different taxa are widely distributed in various ecosystems such as soil, sewage sludge, compost and marine water [12, 13]. The scl-PHAs degrading bacteria in soil have been estimated to be 2 to 18 % [14]. However, few reports concerning the abundance and diversity of mcl-PHAs degrading bacteria in natural environments have been documented [15]. Keeping the above facts in view, in the present investigation the authors have made an attempt to study the biodegradation mcl-PHAs using bacteria isolated from soil and surface of biopolymer disc during open window composting in order to exploit their biotechnological potential.

## Materials and methods

### *Media and chemicals*

Chemical and culture media used in this investigation analysis were procured from Sigma-Aldrich and Hi-media laboratories Pvt. Ltd., respectively.

### Study sites and collection of sample

In our previous work the PHAs was extracted from *Bacillus* sp. S1 (2013b) and it was molded to prepare biopolymer disc [16]. The PHAs disc was subjected to biodegradation in the OUAT agriculture field (*Latitude*: 20. 264354N, *Longitude*: 85.798695E) by open window composting method. Briefly, 0.772 gm of disc was composted 10 cm below the surface of soil. Then, disc was visually inspected for changes in their morphology and weight loss at different time interval of 10 and 20 days.

### Bacteriological analysis

Aerobic, heterotrophic and mesophilic bacteria were isolated and enumerated using standard microbiological method on Nutrient Agar (NA) and incubated at 37°C for 24 hours. All the experiments were carried out in triplicates. Pure cultures of the isolates were maintained at 4°C on nutrient agar slants in our laboratory. Similarly, bacteria attached with the surface of biopolymer discs were isolated by swab culture method on NA medium. Identification of the isolates were carried out based on their colony characteristics, Gram's reaction, through an array of biochemical tests, enzymatic activities and antibiotic sensitivity test as per Bergy's manual of determinative bacteriology [17] and PIBWin software [18].

### Screening of the isolates for depolymerase activity

Depolymerase activity of the isolates was screened following the standard method described elsewhere [19]. Briefly, PHB-NA (most common Homopolymer of PHAs) plates were prepared by supplementing 1 % [W/V] PHB powder to NA medium. All the isolates were spot inoculated on the plates and incubated at 37°C for 24 hours. Growth of bacteria accompanied by the formation of clear zones around colonies indicated depolymerase producer.

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### Effect of pH and temperature on growth of bacterial isolates

To find out the optimum pH and temperature for growth, depolymerase producing bacterial isolates were revived in Minimum Salt Medium (MSM). Ten ml aliquots of MSM was taken in different tubes and the pH was adjusted from 5-9 separately. 100 µl of the overnight culture was dispensed into the test tubes and incubated at 37°C for 24 hours. Similarly, 10 ml of MSM (pH 7) was

taken in different tubes to which 100 µl of the overnight culture was inoculated into the test tubes and incubated at different temperature (23°C, 30°C, 37°C, 44°C, 51°C) for 24 hours. Then the optimum growth of the isolates as a specific pH and temperature was determined by taking O.D at 600 nm.

**Biodegradation of PHAs by the isolates**

*In-vitro* PHAs degradation was studied using selected depolymerase producing bacterial isolates for four days. Briefly, 100 ml of MSM was taken in a conical flask and 1 % extracted PHAs powder was added to it as sole source of carbon. Then, pH of the culture medium was adjusted to 7.0 and 10 ml/L of inoculum was added to the culture medium and incubated at 37°C in shaker incubator. The, PHAs degradation was observed in terms of bacterial growth at 24 hours interval by taking OD using U.V visible spectrophotometer at 600 nm. Control was maintained in the same manner in MSM without PHAs.

**Molecular identification of potent PHAs degrading isolate**

Molecular identification of the potent PHAs degrading bacterial isolate was carried out by sequencing of 16S rRNA gene (carried out at Xcelris Genomics, India) followed by submission of sequence to NCBI GenBank. The phylogenetic tree was constructed using Mega 6 and resultant tree topologies were evaluated by bootstrap analysis of UPGMA data sets based on 2000 resamplings. The final sequence was submitted to NCBI GenBank.

**Result and discussion**

In the present study the biodegradation PHAs was monitored using bacteria isolated from soil and surface of biopolymer disc during open window composting method.

**Physico-chemical and bacteriological analysis of soil**

Environmental parameters and soil properties are the major factors that influenced abundance of soil microbial communities as well as biopolymer degradation in the study site. The pH of soil samples was 6.8-7.2 and environmental temperature was 34-37°C during open window composting. Under this prevailing condition it was observed that, PHAs disc was degraded to 96 % (0.782-0.031gm) within 20 days. Notably, bacterial load of soil sample was increased from $9.8x10^5$ to $1.03x10^6$ and then reduced to $9.7x10^5$ CFU/gm during composting. Altogether fifteen aerobic, bacteria (B1-B15) were isolated from the soil and surface of PHAs disc and were selected for further study. In accordance to our findings studies have shown that 85 % of PHAs were degraded in seven weeks and some of the PHAs have been reported to degrade in aquatic environments within 254 days [20, 21, 22]. Moreover, a degradation rate of PHB was higher than other monomer, which is due to variation in surface structure and properties of different monomer of PHAs [23]. In our study, the prevailing environmental condition such as pH and temperature of the microhabitat favors growth of numerous bacteria, which is due to presence of abundant carbon source in form of PHAs [24].

**Screening of depolymerase activity of the producing isolates**

The selected bacterial isolates were then screened for the presence of depolymerase enzyme activity by following the conventional clear zone technique. Amongst all, three isolates (B1, B2 & B15) were found to have depoly-merase enzyme activity that constitutes about 20 % of the bacteria isolated. In the similar way 7.5-10 % of bacterial isolates found to be depolymerase positive from the total soil bacteria isolated during degradation of PHAs [25]. Moreover, in our study presence of higher magnitude of depolymerase producing bacterial isolates in the soil or study site was increased the rate of degradation.

**Biochemical characterization of the selected bacterial isolates**

Depolymerase producing bacterial isolates were subjected to biochemical characterization. These bacterial isolates were found to be Gram negative (B1 & B15) & Gram positive (B2) and the corresponding biochemical tests results (Table 1) were analyzed by PIBwin software & Bergey's manual of determinative bacteriology that confirmed up to genus level. On the basis of morpho-physiological and biochemical characteri-

**Table 1. Biochemical characterization of the isolates**

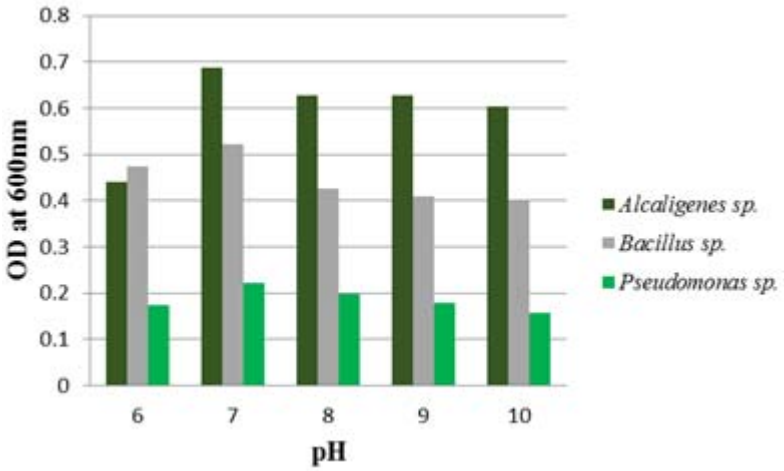
| Biochemical test | $B_1$ | B2 | B15 |
|---|---|---|---|
| Gram staining | - | + | - |
| Indole | - | - | + |
| Methyl Red | - | + | + |
| Voges Proskar | - | - | + |
| Citrate Utilization | + | - | - |
| Nitrate Reduction | - | - | + |
| Urease Activity | + | - | + |
| Catalase Activity | + | + | + |
| Oxidase | + | + | + |
| Manitol | - | + | - |
| Growth at RT | + | + | + |
| Growth in Mac-Conkey | + | - | + |
| Gelatin Liquefaction | + | - | + |
| Arginine Hydrolysis | + | + | + |
| 10 % Lactose | + | + | + |
| Esculin test | + | + | - |
| Assigned to genus | *Alcaligenes* sp. | *Bacillus* sp. | *Pseudomonas* sp. |

NB: (+) Positive and (-) Negative for the particular test

zations these bacteria B1, B15 and B2 were ascertained to be member of the genus *Alcaligenes*, *Pseudomonas* and *Bacillus* respectively.

**Effect of pH and temperature on growth of the isolates**

Microbial growth rate and activity of enzymes are significantly affected by pH and temperature. The variation in pH and temperature of the culture medium causes changes in the ionic form of the active site of enzyme and affects its activity. In the present investigation, bacterial growth was observed in the pH range 6-10, however, optimal growth was observed at pH 7 further increase of the pH of the medium a decline in growth rate was recorded (Graph 1). Similarly, increased growth rate of the isolates was observed when incubated at 23 to 37°C, and a decreased growth rate was reported beyond 44°C. Moreover, the optimum growth of these isolates was observed

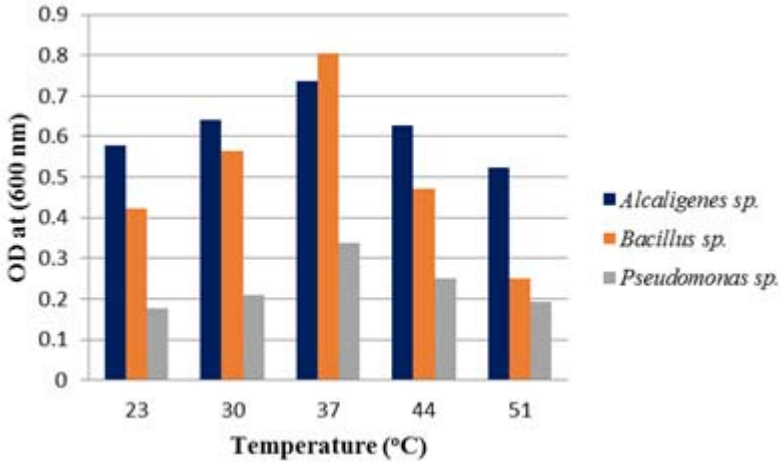



**Graph 1.** Effect of pH on growth of bacterial isolates

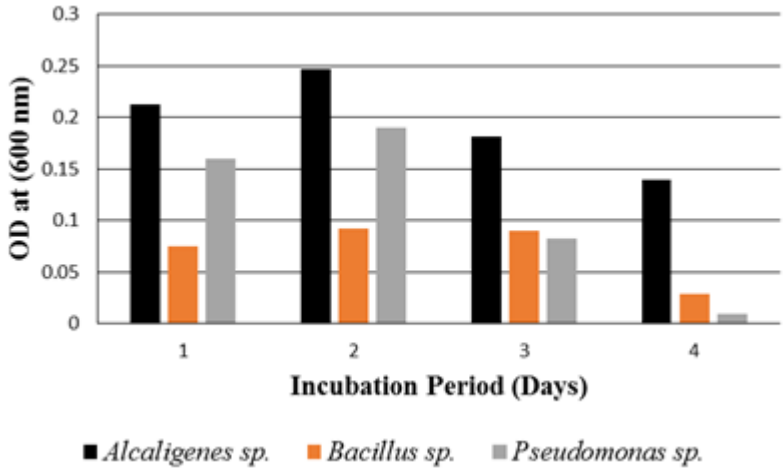
at 37°C indicating their mesophilic nature (Graph 2). In corroboration to our results researchers [26,27] also observed the optimal growth rate of depolymerase producing bacteria such as *Variovorax, Spenotrophomons, Acinetobacter, Pseudomonas, Bacillus, Xanthomonas, Alcaligenes* at pH 7.2-8 and a temperature range of 30-37°C. This could be attributable to the soil pH and temperature of the study site that varied from 6.8-7.2 and 34-37°C respectively during open window composting.

### *In-vitro* degradation of PHAs by depolymerase producing bacterial isolates

*In vitro* degradation studies revealed that the biopolymer PHAs undergo hydrolytic cleavage which started at the surface and that aged with time. The biodegradation of PHAs was observed in terms of bacterial growth obtained in the MSM for four days. These depolymerase producing bacterial isolates grew abundantly by utilizing PHAs as sole carbon source. Amongst, three bacterial isolates *Alcali-genes* species grew faster and utilized maximum amount of PHAs than species of *Pseudomonas* and *Bacillus* (Graph 3). Previous findings also conveyed utilization of PHAs through synthesis of depolymerase by different genera such as *Alcaligenes, Pseudomonas* and *Bacillus* [28, 27]. PHAs degrading bacteria that have been described in the literature belong to various genera such as *Bacillus, Pseudomonas, Alcaligenes, Syntro-phomonas, Brevibacillus, Agrobacte-rium, Ralstonia, Rhodoferax, Acinetobacter, Azospi-rillum, Streptomyces* [29,30,31,32] in support to our findings as observed in this investigation. Most soil PHAs




**Graph 2.** Effect of temperature on growth of bacterial isolates




**Graph 3.** Effect of carbon source (PHAs) on growth of bacterial isolates

degraders are believed to be capable of degrading scl-PHAs and few of them can degrade mcl-PHAs that could be attributable to the substrate specificity of extracellular PHAs depolymerase [13, 15].

**Molecular identification of potent depolymerase producing bacterial isolate**

Out of the three isolates studied, the isolate B1 was observed to be the most potent depolymerase producer and was identified and assigned to the genus *Alcaligens* through conventional microbial identification technique in our laboratory. Further identification of the isolate was done by16S rDNA sequencing followed by BLAST search analysis. The *in-silico* analysis revealed, that *Alcaligenes* sp. B1 99 % similar with *Alcaligenes faecalis* (Fig. 1), which indicate strong evolutionary closeness between *Alcaligenes* sp. B1 and *Alcaligenes faecalis*, where *Bacillus subtilis BCRC* which was taken as an out group. The identified bacterium is *Alcaligenes* sp. B1 (KT784806). Furthermore, rates of degradation of PHB by *Alcali-genes faecalis* were higher than PHV [26, 25].

**Conclusion**

During our studies we observed that *Alcaligenes* sp. B1 to be the most potent PHAs degrader in comparison to the other two isolates *Pseudomonas* and *Bacillus* spp. We further, place in record that the biodegradation of PHAs in soil is influenced by various biotic and abiotic factors as observed in our study. This particular soil environment facilitated bio-degradation of biopolymer PHAs up to 97 % within 20 days by open window composting method. However, further studies are essential for exploiting the biotechnological potential of these isolates for biodegradation of biopolymer or bio-based polymer.

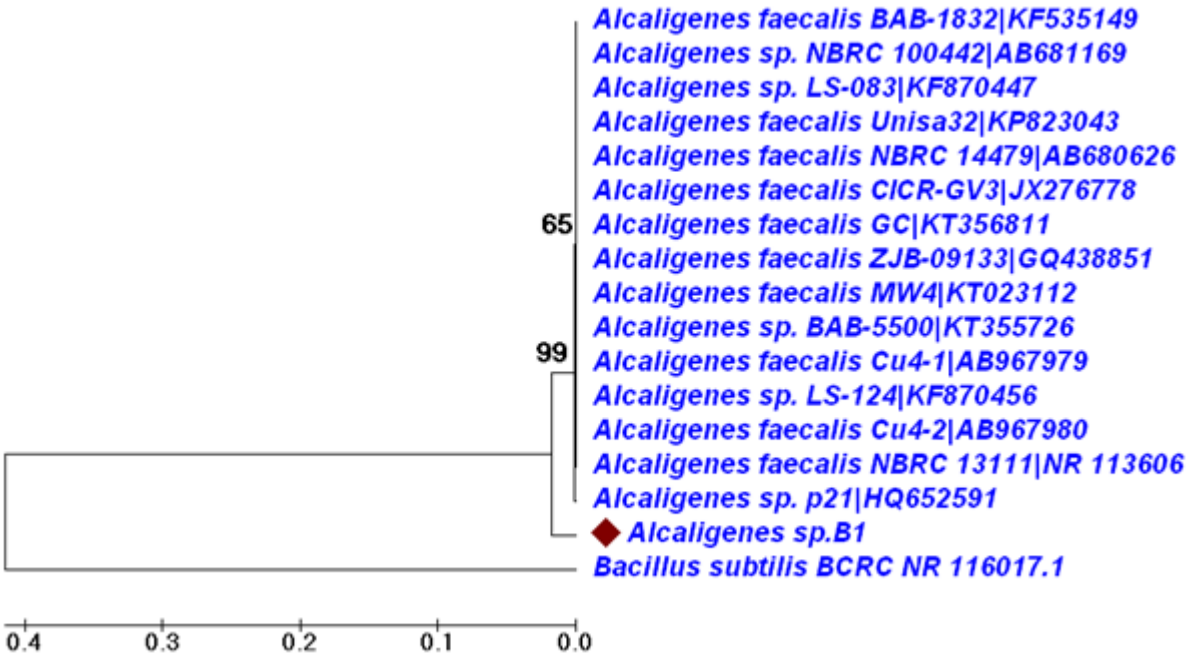



**Fig. 1.** Phylogenetic relationship of *Alcaligenes* sp. B1 with other bacterial isolates

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