Screening of Bacteria Accumulating Polyhydroxyalkanoates from Polluted Water

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Summary

Polyhydroxyalkanoates (PHAs) accumulating bacteria were isolated from polluted water pond. Out of 180 PHA positive isolates, eight highly efficient PHA producing strains were selected with glucose as sole carbon source. PHA granules exhibited a strong orange fluorescence when stained with Nile blue A. Gas chromatography, further confirmed the presence and the concentration of PHA. The optimal growth occurred between 28 and 30°C and at pH 7. The isolates yielded a maximum of 70.74% dry cell weight (DCW) polymer in the medium containing glucose as carbon source, and minimum amount of 47.09% CDW.PHA was analyzed by IR and ^{1}HNMR spectroscopy. The polymers showed the presence of PHA with different monomeric units.

Keywords: polluted water pond; nile blue A; Polyhydroxyalkanoates; copolymer.

Introduction

Polyhydroxyalkanoates (PHAs) are a class of natural polyesters, which can be produced and accumulated by many Gram-positive and Gram-negative bacteria from at least 75 different genera. These polymers are accumulated intracellular under conditions of nutrient stress and act as a carbon and energy reserve [1]. PHA commonly occurs as reserve materials in a wide variety of bacterial species [2, 3, 4]. The chemical composition of PHA depends mainly on the bacterial strain. Hence, there is a need to screen a large number of organisms that accumulate PHA with a combination of monomers, yielding the desirable quality in a sufficiently large amount. Organisms that accumulate PHA have been reported from various environments such as soil [5, 6], sewage sludge, marine

sediments [7], and ponds [8]. Polyhydroxyalkanoates (PHAs) represent a large family of intracellular bacterial storage polyesters with wide range of material properties permitting applications as biodegradable and biocompatible thermoplastics and elastomers [9, 10]. The scope of this work was to search different bacterial isolates capable of producing PHA efficiently from unrelated single carbon source.

Materials and methods

Screening of bacteria from polluted water samples.

Water samples were collected from the polluted water pond, uppal, which is contaminated with domestic wastes and effluents of food related industries. The samples were collected at various sites of polluted pond (Figure1).Samples from four different sites were screened for PHA accumulators. The screening was done regularly on monthly intervals to determine the variation in bacterial flora and the PHA accumulators simultaneously.

Bacterial colony forming units

The screening was done regularly to determine the variation in bacterial flora and the PHA accumulators simultaneously. The water samples 1 ml**,** were measured, and mixed vigorously for 10 min. This was allowed to settle and saline suspension was diluted ten folds before plating. A 0.1 ml sample of each dilution was surface spread on sterile Luria Bertani agar medium. After incubation of 48 h at room temperature, the colony forming units were counted to check the total viable count.

The colonies from the plates were picked at random, purified by surface streaking, and their colony characteristics including the pigmentation were noted. The isolates were replica plated respectively on nutrient agar with 3% (w/v) sodium chloride, tributyrin agar, milk agar and cellulose agar for salt tolerance, lipase, protease and cellulase activities.

Screening of bacteria for PHA accumulation

PHA are polymeric lipids [11] , and hence, the bacterial isolates from water samples were screened microscopically for accumulation of intracellular lipids by staining with Sudan Black B [12]. The lipid positive isolates were then screened for PHA using fluorescence microscopy with the Nile blue A staining method [13]. The bacterial isolates that accumulate PHA from the samples were scored directly using the plate assay. E2 mineral medium agar [14] plates, spot inoculated and incubated for

48 h, were flooded with Nile blue A (1% w/v) in ethanol. On decanting the stain and exposing the plate to ultraviolet light, bright orange colonies were scored as accumulators of PHA.

Cultivation

Each isolate was grown in 250 ml Erlenmeyer flask containing 50 ml E2 mineral medium [14], with 2 % (w/v) glucose as carbon source. The flasks were incubated at 28° C for 48 h on scientific environmental shaker at 150 rpm.

Extraction of PHA

All the initial identified 180 PHA positive isolates screened from various samples, were subjected to extraction of PHA from cells by using the Hypochlorite method [15]. Each bacterial culture was grown in 50 ml E2 mineral medium with 2% (w/v) glucose and 0.04% (w/v) yeast extract for 48 h. The cells were washed once in sterile saline. To the cell pellet, sodium hypochlorite (5 ml, 2% w/v of active chlorine; Qualigens, India) was added and the tubes incubated at 37^0 C for 10 min with constant stirring. The pellet of PHA obtained on centrifugation at 8000 rev/min for 20 min was washed with 10 ml of cold diethyl ether and assayed with concentrated sulphuric acid [16]. PHA were estimated from individual sediment samples collected from the four sites by the method used by [17]. Lyophilized sediment samples were sonicated for 10 min with chloroform and refluxed with 125 ml chloroform for 2 h in a boiling water bath. The chloroform was removed by rotary evaporation. The polymer was redissolved in hot chloroform and filtered through glass wool. The chloroform was dried in a stream of nitrogen. The polymer was washed twice in 2 ml ethanol and diethyl ether and assayed with sulphuric acid.

Characterization of PHA

Extracted PHA samples were also subjected to FTIR. For this, extracted PHA was dissolved in chloroform (AR) and placed on KBR window. After evaporation of the solvent, the film spectrum was taken (GC-FTIR spectrometer; Perkin Elmer, USA) at 400–4000 cm_1. Standard PHA [from Sigma] were used for comparison. Extracted PHA samples were also subjected to gas chromatography (GC) analysis by converting it to methyl esters of monomers [18]. The above-mentioned standards were also used for comparison. Conditions used for GC detection were: Carbowax column PEG M 20 (60–80 mesh, Shimadzu, Japan), injector temperature 230^0C; detector temperature 275^0C, initial column temperature 80^0 C for 4 min followed by temp ramp of 8^0 C per min and 160^0 C per 6 min. The ^{1}H NMR analysis of the polyester samples was carried out on Varian-300 spectrometer (USA). The 300 MHz ^{1}H NMR spectra were recorded at 24°C in CDCl$_3$ solution of polyester (50 mg/ml) with a acquisition time of 2.0480 seconds, sweep width of 4000 Hz. Tetra methyl saline was used as an

internal chemical shift standard. The spectra was recorded for commercial PHA (Sigma-Aldrich, USA) and for the polymer extracted from test strains.

Results and Discussion

General characteristics of the bacterial flora of the polluted water pond

During the screening (figure 1.a)of polluted water samples for PHA accumulating bacterial isolates, the number of bacterial isolates and colony forming units (CFU) were varied from month to month. 450 bacterial isolates were screened for PHA accumulation. 180 isolates were identified as positive isolates for PHA accumulation out of 450 isolates screened. The highest value of 2.38×10^9 (per ml) of Colony Forming Units (CFU) was reported. In and all, mostly Gram positive, as well as Gram negative bacteria to some extent were isolated from the samples. The Gram positive outnumbered the Gram negative. Almost all the isolates were rod shaped either long or short, besides few irregular rods and sometimes rods in chains. The cocci forms of bacteria were also found in the samples, but to a very negligible extent. Amylase, lipase, cellulase and protease activities were observed in the selected isolates. Isolates with multiple enzyme activity, though small in number, are important in such ecosystems due to their involvement in foliage waste matter degradation.

The plate assay method (figure1.b, c) was preferred to screen PHA accumulators, as it is a more rapid technique. The isolates showing the characteristic orange color fluorescence under the UV light of plate assay (Figure 2.3a and 2.3b) were selected for further studies. It was further confirmed by Flourescence microscopy of sample showing strong orange flourescence against dark green back ground (Figure2.3d).

The increased intensity of fluorescent internal PHA granules supports, the high (70.74% CDW) PHA accumulation by the isolates. Other isolates also produced PHA internal granules in a considerable amount. A wide variety of bacteria are known to accumulate PHA [19]. These bacteria have been reported from various environments. However the amounts of PHA extracted from 337 bacterial isolates screened from the tropical marine ecosystem accumulated as 1.73g/l PHA [15].

Quantitative assay of PHA

All of the 180 positive isolates selected from the total 450 isolates from different samples were quantified for the polymer accumulation. The amount of PHA formed from different isolates varied considerably. Some of these isolates accumulated negligible amount of PHA, as low as 0.007 g/l and 0.006 g/l, where as some isolates accumulated high amounts of PHA, ranging from 0.990 g/l to 1.840 g/l (table1). Amongst the over all isolates, 8 isolates producing PHA in the highest range were selected for further studies. The selected isolates includeOU6, OU35, OU40, OU50, OU67, OU73, OUA3 and, OUA7.

PHA accumulation in bacterial cells increases as the incubation period increases, and reaches maximum at late exponential stage of the growth curve and declines on further incubation. Since, all the cultures were quantitated after a certain period of 48h, it is possible that the low yield of PHA obtained for certain cultures is probably due to the time of selection of harvesting the cells, which was either prior to late exponential stage of the growth curve or after onset of PHA hydrolysis. Eight such isolates were obtained which accumulated more than 0.9g/l of PHA. These were selected as the potential PHA accumulators for further study. The yield of PHA accumulated by the eight isolates amounted to 47.09 to 70.74% of their cellular dry weight (table1), signifying the potentials of the PHA accumulating bacteria in this ecosystem.

Another method for the screening of bacteria accumulating PHA, the microscopic method was used to see the intensity of fluorescence exhibited by the selected bacterial isolates with the increase in incubation period (table 2). The intensity of fluorescence was seen to increase with the time, followed by a decline. These selected isolates were grown in E2 broth to estimate the active biomass, dry weight, wet weight, amount of PHA and percentage PHA of cell dry weight. All these results have been tabulated in Table 1.

Analysis of PHA

IR Spectra showed two intense absorption bands at 1,730. and 1,280. Cm⁻ 1, corresponding to $C = O$ and C–O stretching groups, respectively. Other absorption bands at 1,370, 1,450, 2,925 and 3,435 cm⁻¹ corresponding to -CH3, -CH2, -CH and O–H groups are shown in Figure 3.

The ¹H NMR spectrums of polymer is shown in Figure 4. The methyl protons (–CH3) appear to have a double resonance at 1.274 ppm, methylene protons (–CH2) appear to have a multiplet resonance at

2.520 ppm, methine proton (–CH) of bacterial polyhydroxybutyrate also has a multiplet resonance at 5.260 ppm. GC analysis clearly indicated that the polymer extracted was mostly PHB with polyhydroxyvalerate (PHV).From this analysis, it was confirmed that the selected isolates were capable of producing polyhydroxyalkanoates.

The selection of 8 isolates out of 180 isolates was based on the highest amount of PHA produced by these isolates after growth with 2% glucose as sole carbon source. In and all mostly Gram positive, as well as Gram negative bacteria to some extent were isolated from the samples. The Gram positive outnumbered the Gram negative. Almost all the isolates were rod shaped either long or short, besides few irregular rods and sometimes rods in chains. The cocci forms of bacteria were also found in the samples, but to a very negligible extent. The bacterial intracellular lipids were found in 60% of the polluted water samples.The study of cultural characteristics of the isolates revealed the presence of significant number of pigmented organisms. Pigments have a great commercial value and are used immensely as a colorant in numerous industries such as plastics, gums, food, dyes and stains etc [20].

Recently Joshi and Jaysawal, isolated PHA producing bacteria belong to *Staphylococcus, Bacillus, Rhodococcus, Nocardia, Pseudomonas,Escherich*ia and *Klebsiella* genera from industrial and domestic sewage respectively. Glucose and ammonium sulphate were found to be the suitable carbon and nitrogen sources for maximum production of PHA [21]. In the present study, nutrient limitation was shown to enhance the PHA accumulation rate to supply additional energy for the biosynthesis of cell constituents. 70.74 %PHA is the highest report of PHA accumulating bacteria from polluted water when grown on the media supplemented with 2% glucose.

Overall, the Gram positive bacteria tend to dominate the sewage water. Isolates with multiple enzyme activity, though small in number are important in such ecosystems, due to their potentiality in industrial applications [22]. Accumulation of PHA occurs in the presence of excess carbon, which is available for the organisms from the degradation products of diverse nutrients, in the water. the isolates also accumulated a significant amount of cellular carbohydrates when glucose was provided in excess under aerobic conditions.

High concentrations of organic and inorganic nutrients at the polluted pond had a clear effect on the composition and diversity of the microbial community compared to the fresh water pond. Growing concern about environmental pollution has renewed interest in the development of PHA, which are

completely biodegradable by bacteria present in most environments. High concentrations of ammonia and organic matter, including lipids, produce toxic effects on bacteria communities. Synthesis of PHB has been proposed as a detoxifying mechanism of bacteria in water with high concentrations of fatty acids. Because PHA genesis is linked to lipid metabolism, PHA-producers are more competitive in these environments [23]. Thus, PHB-production in the microbial mat probably does not function only as a storage material, but also as a mechanism to cope with stressed and imbalanced nutrient environments, such as the polluted pond.

Conclusions

 Among 180 isolates screened, eight PHA accumulating bacterial strains were selected from polluted water samples. All the selected isolates are exhibiting different enzymatic potentials. Though in general, all isolated strains showed a notable capacity to use glucose to accumulate PHA, the PHA amounts produced significantly varied among the eight strains. In this study, PHA producing bacteria were successfully isolated from polluted water. Accumulation of PHB and its co polymers were identified from various isolates under aerobic conditions. In conclusion, this study contributes to the comprehension of the diversity of PHA producers isolated from polluted pond subject to environmental stress by organic pollution of various industries, which contribute to the imbalance of nutrients.

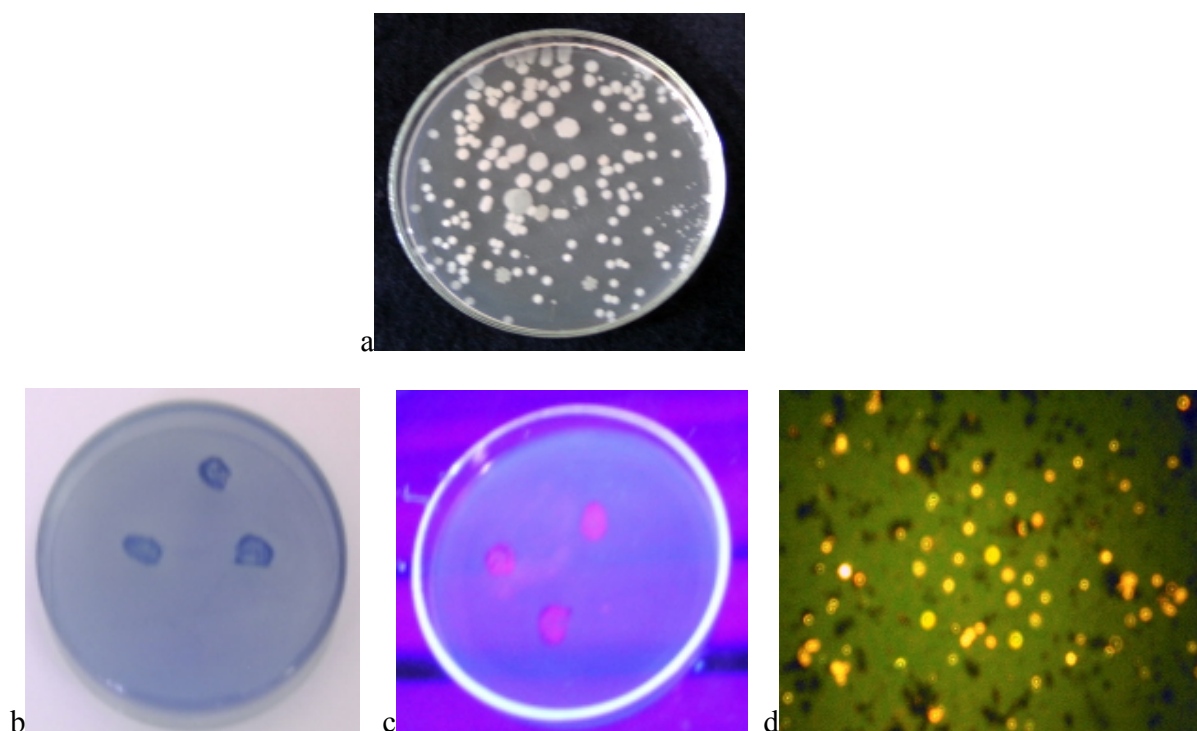**Figure .1 Plan of four sampling sites.1, 2, 3, 4 indicates four different sampling sites of Uppal pond**

Figure 2: a. Screening on LB media   b. Nile blue staining c. E_2 plate with positive PHA producing Colonies d. Florascencse micrograph emitting orange light
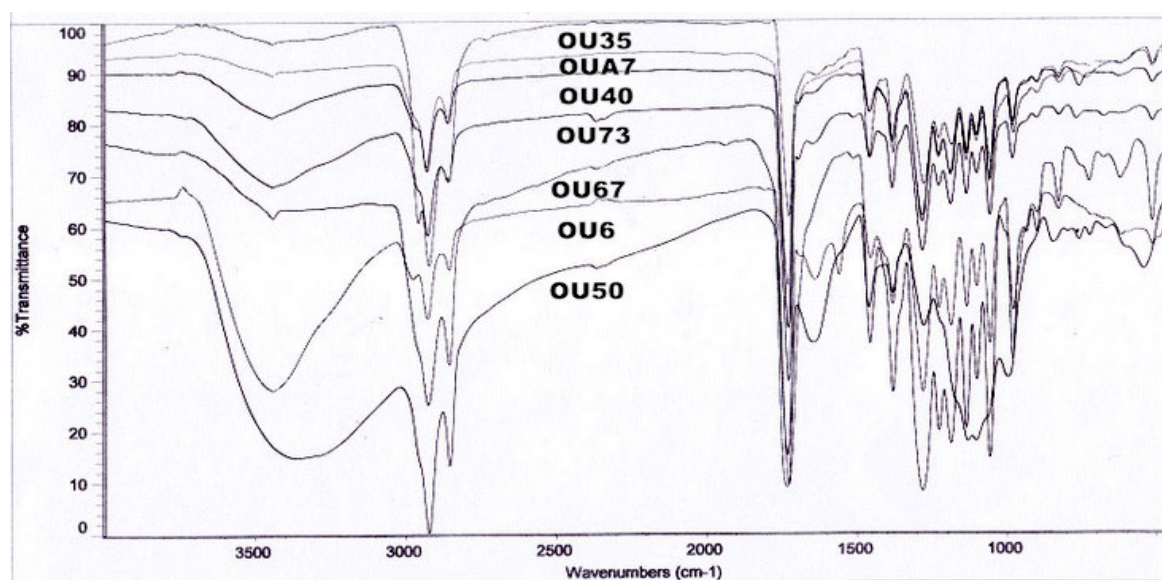

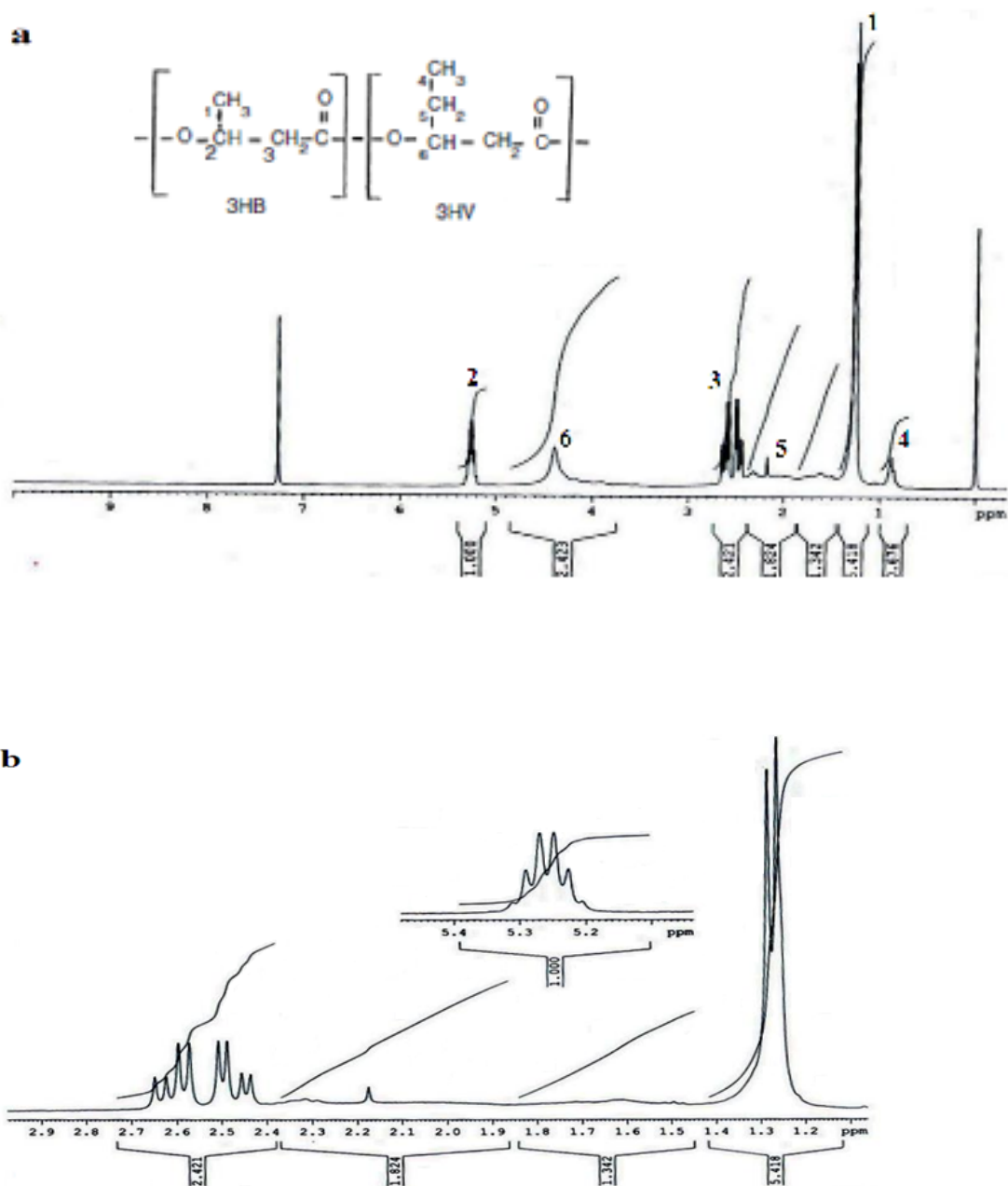**Figure3:** Infrared-spectra of PHA extracted from selected isolates

Figure4: a. ^{1}HNMR spectra of PHA extracted from *Bacillus sp.*OU73;b.Enlarged portion of spectra

Table 1: PHA% accumulated by the selected cultures

Isolates	A_{420}	Dry wt. g/l	Wet wt. g/l	PHA g/l	PHA (% CDW)
OU 35	0.314	2.321	43.50	1.217	52.43
OU 40	0.301	2.754	43.92	1.865	67.87
OU 50	0.290	2.016	25.01	1.096	54.36
OU 06	0.336	2.323	38.90	1.239	55.51
OU 67	0.330	3.015	41.04	1.420	47.09
OU A7	0.311	2.601	41.02	1.840	70.74
OU A3	0.342	2.53 4	44.23	1.321	52.13
OU 73	0.310	2.241	43.62	1.237	55.19

Key: CDW=Cell dry weight;, A_{420} = absorbance at 420 nm,

Table 2: Intensity of fluorescence exhibited by different isolates with increasing incubation period.

Isolates	Day-1	Day-2	Day-3	Day-4	Day-5
OU 35	+	++	++	+	-
OU 40	++	+++	++++	++	+
OU 50	-	+	++	+	-
OU 06	+	+++	+++	+	-
OU 67	+	++	+++	-	-
OU A7	++	++++	+++	++	+
OU A3	+	+++	+++	-	-
OU 73	++	++++	+++	++	-

Key: +→++++ =increasing intensity of fluorescence; - =no fluorescence.

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