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Isolation and Screening of PHA (Polyhydroxyalkanoates) Producing Soil Bacteria: A Comparative Study

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Abstract: **Use of plastic has increased significantly over other type of materials because of their chemically inertness, lightweight, durability, comfort ability, versatility of shape and size. But due to their non biodegradability since last decades there has been a growing demand of environment friendly products. Government, now promoting the development of biodegradable materials based on biopolymers as lipids, polysaccharides and proteins, which have been studied being renewable raw materials and inexpensive. Biopolymers such as PHA (Polyhydroxyalkanoate) are organic polymers that are produced by different biological organisms. Screening of PHA producing bacteria was carried out by two sequential steps, qualitative followed by quantitative. Qualitative screening was conducted by Nile red dye. The quantitative screening was conducted by measuring the intracellular material of bacterial cell by gravimetric method.**

Keywords: Polyhydroxyalkanoates, Microrganisms, Staining

I INTRODUCTION

The use of plastic is increasing in every sphere of human life. The increased use of plastic was driven due to its lightweight, flexible, sustainable, strong, stable, transparent, not easily broken and resistant to chemical materials[24]. Plastics in the market are petroleum-based plastics so they are not easily degraded biologically by microbes [25]. As a result plastic waste will accumulate and create environment problem. Best way to avoid this situation is to substitute these with environmentally friendly plastics that can be degraded by microbes, known as biodegradable plastic or bioplastic[29]. PHA (Polyhdroxyalkanoates) is one of biodegradable plastics that have great potential and is now being developed to replace the widely used hydrocarbon plastics. This biodegradable plastic will decompose completely into carbon dioxide and water after burial for several months in the soil[4].

II. POLYHYDROXYALKANOATES (PHA)

Polyhydroxyalkanoates (PHA) are microbial produced polyester synthesized by various types of bacteria and accumulated in cytoplasm as energy and carbon reserves in the form of granules when the condition of excess carbon with nutrients (phosphorus, nitrogen, sulphur , oxygen) are limited [(26,28,30)]. There are various microbes from negative and positive bacteria that can accumulate PHA as an energy reserve material including Alcaligenes, Nocardia, Bacillus, Pseudomonas, Azotobacter, Rhizobium, Cupriavidusnecator, Ralstoniaeutropha, Corneybacterium B.sirculans, B.thuriengiensis and Micrococcus sp [(9-13)] . Different microorganism will produce a different composition of PHA[(26,27)] and the carbon source consumed by each microorganism also determines the type of PHA produced [(5)]. The microorganism can be obtained from landfill, palm oil mill effluents and others [(2,29)] .

Different carbon sources were used for the production of PHA. Ralstonia picketti produce PHA in a medium containing fructose as a carbon source [(4)]. Bacillus subtilis and E.coli were grown on cane molasses to produce PHA, while Ralstonia eutropa was grown on hydrolyzed sago starch medium as carbon source [(25)]. Various other carbon source such as molasses, sewage waste, wheat flour, corn flour, rice bran were also used for production of PHA. The cost of production is based on the source of carbon used. The main aim of this paper is to obtain potential bacterial strains for PHA production.

III. MATERIALS AND METHODS

A. Sampling and isolation of bacteria

Isolation of bacterial stains was carried out by using serial dilution plating method. Minimal media used for PHA production was carbon enriched nutrient agar media having glucose 10g, $(NH_4)_2SO_4$ 1g, $Na_2HPO_4.7H_2O$ 6.7g, KH_2PO_4 0.1g, $MgSO_4.7H_2O$ 0.2g, ferrous ammonium citrate 60mg, $CaCl_2.7H_2O$ 10mg and trace elements 1mL. Composition of trace element solution per liter: $MnCl_2.4H_2O$ 1.98g, $CoSO_4.7H_2O$ 2.81g, $CuCl_2.2H_2O$ 0.17g, $ZnSO_4.7H_2O$ 0.29g and $FeSO_4.7H_2O$ 2.78g.

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Maintenance of bacterial cultures: Bacterial cultures grown on nutrient agar, incubated at 30^0 C for 24 hours and stored at 4^0C for further use.

B. Qualitative Screening of PHA producing bacteria

After incubation for 24 hours, Bacterial colonies were screened by pouring 0.02% alcoholic solution of Sudan black B and kept undisturbed for 20min. The excess dye was decanted and the plates were rinsed by adding absolute alcohol. The colonies that had ability to produce PHAs incorporated Sudan black B and appeared bluish black. Colonies which are not PHA producers appeared white [32]. After the primary screening, Sudan black B-positive isolates were further confirmed by viable colony method using Nile blue given by Bhuwal et al [7]. The Sudan black B –positive bacterial isolates were streaked in different petri plates containing carbon rich (1% glucose) nutrient agar along with Nile blue A stain at concentrations of 0.5µg/ml. Nile blue A stain ensures a strong discrimination between PHA positive and PHA negative strains. The PHAs accumulating colonies fluorescence bright orange color after Nile blue A staining on irradiation with UV light and their fluorescence intensity increased with increase in PHA contents of bacterial cells. The isolates which showed bright orange fluorescence on irradiation with UV light after Nile blue A staining shown in fig 1 were elected as PHA accumulator [32]. After that, PHAs accumulating bacteria were streaked to obtain pure culture.

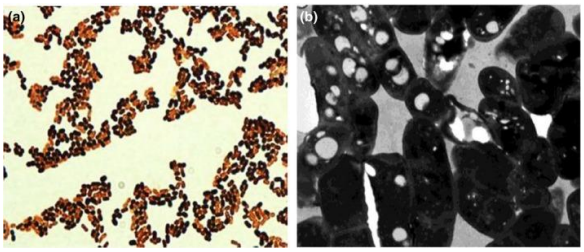

Fig. 1.(a) Fluorescence microscopic view exhibiting bright yellowish-orange color and **(b) electron microscopic (EM) view showing polyhydroxyalkanoate granules inside the cells**

C. *Culture inoculation and bacterial growth*

The PHA positive colonies grown in 100ml sterile nutrient broth containing 1% glucose was used. The composition of nutrient broth was as (g/l): peptone 5.0, sodium chloride 5.0, beef extract 1.5 and yeast extract 1.5. To prepare 100ml of nutrient broth, 1.3g of nutrient broth powder was dissolved in 100ml distilled water. 1% glucose powder was also added to it. pH was 7.0±0.2 and autoclaved. The autoclaved froth was incubate using a loop full of pure culture and allowed to incubate overnight at 37° C with shaking at 120rpm in an incubator shaker. The liquid culture of the bacteria was used as a seed culture for further experiments.

The growth pattern of isolates was observed in mineral salt medium (MSM) containing 1% glucose at an initial pH of 7.0 at 37° C with agitation speed of 120rpm. The composition of MSM was as follow (g/l): urea (1.0), yeast extract (0.16), KH_2PO_4 (1.52), Na_2HPO_4 (4.0), $MgSO_4.7H_2O$ (0.52), $CaCl_2$ (0.02), glucose (40) and trace element solution 0.1ml. The trace element solution contained (g/l): $ZnSO_4.7H_2O$ (0.13), $FeSO_4.7H_2O$ (0.02), $(NH_4)6MO_7O_2.4H_2O$ (0.06) and H_3BO_3 (0.06). Both glucose and trace element solutions were autoclaved separately. Inoculum size of 1% was used to study the growth pattern. Absorbance was observed at regular intervals. Cell growth was monitored over time at regular intervals by measuring absorbance at 600nm. All experiments were performed in triplicates.

10g of carbon substrate per liter was added in it and pH was adjusted to 7.0. The medium was sterilized at 15lb pressure for 20min in an autoclave. After that, fermentation medium was inoculated under sterile conditions using a laminar bench. The inoculated fermentation flasks were kept on a rotatory shaker 96h at 37° C with shaking at 120 rpm [24] .

D. Extraction and quantitative screening of PHA producing bacteria

For PHA extraction, the dried cell biomass was treated with sodium hypochlorite (NaClO) and chloroform ($CHCl_3$) at a ratio of 1:100 (w/v). The mixture was incubated at 60°C for 2 hours under gentle agitation at 100 rpm. Following incubation, the upper aqueous layer containing residual biomass and NaClO was carefully removed, while the lower chloroform phase containing the solubilized PHA was collected and transferred into a glass Petri dish. To remove cellular lipids and impurities, methanol and water were added to the extract in a 7:3 (v/v) ratio, and the mixture was centrifuged at 8500 × g for 15 minutes. The supernatant was discarded, and the resulting pellet was washed twice with 95% (v/v) ethanol. The purified PHA was then air-dried overnight at room temperature and weighed. The residual biomass was determined by subtracting the weight of the purified PHA from the initial dry cell weight. The PHA accumulation percentage was calculated as the ratio of PHA content to total DCW. The experiments were performed in triplicate.

Residual biomass (g/l) = Dry cell weight (g/l) – Dry weight of extracted PHAs (g/l)

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PHA accumulation (%) = Dry weight of extracted PHAs (g/l) x 100% / dry cell weight (g/l)

IV. RESULT AND DISCUSSION

In this research paper we studied a number of bacterial strains with different genera and species of Bacillus, Pseudomonas, Staphylococcus from the genera previously reported. The strains were isolated from various land sources. Out of various bacteria screened with Nile red staining, there were following isolates shown in table 1 having the ability to accumulate PHA. Fluorescence microscope `used to see the shape of PHA granules.

Table 1: Result of screening of PHA- producing soil bacteria strains based on qualitative analysis.

S.No	Genus	Species Example	Gram Strain Nature
1.	Bacillus	*B.megaterium, B.subtilis, B.circulans, B.amyloliquefaciens*	Gram – positive
2.	Pseudomonas	*P. oleovorans, P.spp.*	Gram – negative
3.	Ralstonia	*R.eutropha*	Gram –negative
4.	Azotobacter	*A.beijerinckii*	Gram –negative
5.	Acinetobacter	*A.spp.*	Gram- negative
6.	Rhodococcus	*R.spp.*	Gram-positive
7.	Streptomyces	*S.spp.*	Gram-positive(Actinomycete)
8.	Klebsiella	*K.spp.*	Gram - negative
9.	Staphylococcus	*S.spp.*	Gram -positive

A. Qualitative screening for PHA producing bacteria

Several researches have been done to find the highest PHA producing strain. Table 2 represents the different PHA producing bacteria with varying range of PHA production.

Table2: PHA producing bacterial strain

S.No.	Isolates	Cell dry weight (g/L)	PHA amount	PHA content (%)	PHA type
1.	*Bacillus thuriengenesis*	1.1662	0.3547	~30	P (3HB)
2.	*Bacillus subtilis*	1.2012	0.3128	26	P (3HB)
3.	*Sthapylococcus cohni*	0.8189	0.4261	52	Scl –P(3HB)
4.	*Bacillus cerus*	1.6520	0.3018	~18	P (3HB)
5.	*Staphylococcus caprae*	1.4080	0.3104	22	Scl –P(3HB)
6.	*Cupriavidus basiliensis*	0.6405	0.3617	~56	P (3HB)
7.	*Burkholderia sp B73*	0.7325	0.3080	42	P (3HB)
8.	*Bacillus toyonensis*	1.0316	0.3591	36	P(3HB—co-3HV)
9.	*Aeronomas hydrophylia*	1.1583	0.2385	20	PHBHHx
10.	*Bacillus sp B51*	1.0015	0.3561	~35	P (3HB)
11.	*Brevibacterium sp B45*	1.4561	0.3653	25	P(3HB)
12.	*Bacillus sp B 60*	0.7568	0.2544	~34	P(3HB)
1.	*Brevibacterium sp B46*	1.2576	0.2371	~19	Mcl - PHA
14.	*Pseudomonas aeruginosa*	1.8231	0.2914	16	Mcl -PHA
15.	*Cupriavidus sp B71*	0.9790	0.4613	47	P (3HB)
16.	*Burkholderia sp B64*	1.3831	0.3398	~25	P (3HB)
17.	*Rhodococcus equity*	1.4567	0.3691	25	Scl- PHA
18.	*Enterobacter sp B48*	1.0873	0.3624	33	P(3HB), P(3HB-co-3HV)
19.	*Pseudomonas sp B68*	1.130	0.1621	14	Mcl -PHA

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V. CONCLUSION

Out of the various bacterial strains tested, given strains were able to accumulate PHA by giving a positive reaction to Nile red staining. Quantitative measurement of PHA from these strains presented four potential strains for PHA accumulation ranged from 14-56% of cell dry weight.

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