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Isolation, Purification and Characterization of Biodegradable Polymer Producing Bacteria *Listeria murrayi*

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Abstract: Using synthetic enrichment medium, fifteen bacterial colonies were isolated, purified, preserved and stained for Poly-β-Hydroxybutyric Acid (PHB) granules with Sudan Black B stain. Among them ten showed PHB positive reaction. An attempt was made for the production, isolation and purification of polymer by the proposed isolate QGR. 7.81% of biodegradable polymer production was recorded by QGR. On the basis of cultural, morphological, physiological and biochemical characteristics the isolate QGR was provisionally identified as *Listeria murrayi*. The chloroform extract of biodegradable polymer of isolate QGR was analyzed by Gas Chromatography Linked to Mass Spectroscopy (GCMS) where it showed eighteen different compounds. Among them, ethyl cyclopropane carboxylate, oleic acid and di-n-octylphthalate are the major compounds.

Key words: Isolation, purification, biodegradable polymer, *Listeria murrayi*

INTRODUCTION

Biodegradable plastic is new and very interesting because of its actual utilization of bacteria to form a biopolymer. Bacterial plastic is usually defined as an exciting new area of research, where naturally synthesized bacterial polymer, such as, the lipid storage material PHB is being used as raw materials for plastic based packaging materials[1]. PHB is a specialized reserve of carbon and energy that accumulates in a variety of microorganisms under appropriate conditions of nutrient limitation[2]. PHB has also been found in numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria[2], gliding bacteria[3], actinomycetes[4], cyanobacteria[5] and many other prokaryotes. This biodegradable polymer can be produced from relatively cheaper substrates, such as, carbon dioxide or ethanol and also by microbes from renewable resources[6]. The main advantage of this type of polymer is that since they are of biological origin, they degrade naturally and completely to CO_2 and H_2O under natural environment by the enzymatic activities of microbes. So, the use of biodegradable polymer as a substitute of synthetic polyester that helps us in improving environmental hazards.

MATERIALS AND METHODS

Isolation, purification and screening of biodegradable polymer producing bacteria: Enrichment media and nutrient agar media were used during isolation. The isolates were purified through repeated plating method in nutrient agar medium. The purified bacterial isolates were then transferred to the nutrient agar slant and preserved as stock culture. The isolates were screened for bacterial polymer by staining with Sudan black B stain (0.3% in 70% alcohol)[7] and observed under microscope (X100x).

Identification of selected isolates: The selected isolates were then identified on the basis of their morphological, cultural, physiological and biochemical characteristics.

Determination of cultural condition for maximum production of bacterial polymer: To observe the effects of culture conditions for maximum bacterial polymer production, cultures were incubated at different incubation period 4, 8, 16, 24, 30, 36, 40 and 48 h at 30°C.

The production of bacterial polymer under different carbon and nitrogen were also studied using liquid synthetic medium, Modified Okamoto Medium (MOM), described by Maeda[8] as basal medium. (Sodium acetate, Sodium malate, Sodium pyruvate, Sodium succinate)/Glucose/Fructose or Methanol were used as carbon source, whereas Ammonium sulfate, Asparagine and Yeast extract were tested for their ability to utilize nitrogen source. Biodegradable polymer production was also carried out in presence or absence of Vitamin solution (Thiamine, Nicotinic acid, p-Aminobenzoic acid, Cyanocobalamine, Vitamin-B_6 and distilled water, using MOM as basal medium).

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Cell cultivation: For large-scale growth, 24 h old culture was prepared in nutrient broth medium at 30°C and transferred to 500 mL of nutrient broth in a wide-necked 1 L culture flask, incubated at 30°C with continuous gentle shaking (20 strokes/min).

Biodegradable polymer accumulation in cells: After 24 h of cultivation period, cells were harvested by centrifugation at 8000 rpm for 12 min, washed aseptically with sterile distilled water and resuspended into 1 L culture bottle containing 500 mL of biodegradable polymer production medium, which consist of 0.25 g of K_2HPO_4, 0.25 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 0.02 g of $CaCl_2.7H_2O$ and 0.5 mL of solution 1(2.0 g of EDTA, 2.0 g of $FeSO_4.7H_2O$, 0.1 g of H_3BO_3, 0.1 g of $CoCl_2.6H_2O$, 0.1 g of $ZnCl_2$, 0.1 g of $MnCl_24H_2O$, 0.020 g of $Na_2MoO_4.2H_2O$, 0.020 g of $NiCl_26H_2O$, 0.01 g of $CuCl_2.2H_2O$, 0.010 g of Na_2SeO_3 and 1000 mL distilled water) and 500 mL of distilled water with the addition of different nitrogen and carbon source at a final concentration of 0.1%. Cells were then incubated with gentle shaking (20 strokes/min) at 30°C for 48 h.

Harvesting of cells: After incubation, cells were harvested by centrifugation at 8000 rpm for 12 min, washed in sterile water and recentrifuged similarly. Pellets were collected aseptically, dried at 60°C until constant weight and weight was measured.

Biodegradable polymer extraction: Allumina was used to crush the cells with mortar and pestle. From the white powdery mass of the crushed cells, the polymer was extracted with chloroform ($CHCl_3$). The chloroform mixtures were vigorously shaken, left for 1 h; the clear solution was carefully decanted into another clean test tube and evaporated. The powdery mass along the test tube wall was collected. Dry weight of the biodegradable polymer and percentage (w/w) of it against cell dry weight was measured.

GCMS analysis: The GCMS analysis was carried out in Bangladesh Council of Science and Industrial Research (BCSIR) Laboratories, Chittagong using Electron Impact Ionization (EI) method on GC-17A gas chromatograph (Shimadzu, Japan) coupled to way GCM-QP 5050A mass spectrometer (Shimadzu); fused silica capillary column, 30 m x 0.25 mm i.d., coated with DB-5, 0.25 μm film thickness; column temperature 100-280°C at the rate of 4°C/min., injection port temperature 250°C, constant pressure of carrier gas (helium) 100 kPa, flow rate (mL/min) 20, acquisition parameters full scan, scan range 60 to 550 amu, searched library NIST 127 and 147, Shimadzu corporation, sample dissolved in chloroform.

RESULTS AND DISCUSSION

Fifteen bacterial isolates were collected from various marine and fresh water sources and then purified, preserved and tested for their biodegradable polymer content by staining with Sudan Black B stain. Among the positive isolates, QGR was grown in liquid culture media for extraction of polymer content.

Identification of the selected isolates: The isolate QGR was characterized on the basis of their morphological, cultural and biochemical properties (Fig. 1a and b). All these properties were then compared with the standard characteristics described in Bergey`s Manual of determinative bacteriology, 8th Edn.[9]. The bacterial isolate QGR was found to belong to the genus *Listeria* and was provisionally identified as *Listeria murrayi*[9].

Effect of vitamin, carbon and nitrogen on biodegradable polymer production: Table 1 shows that maximum

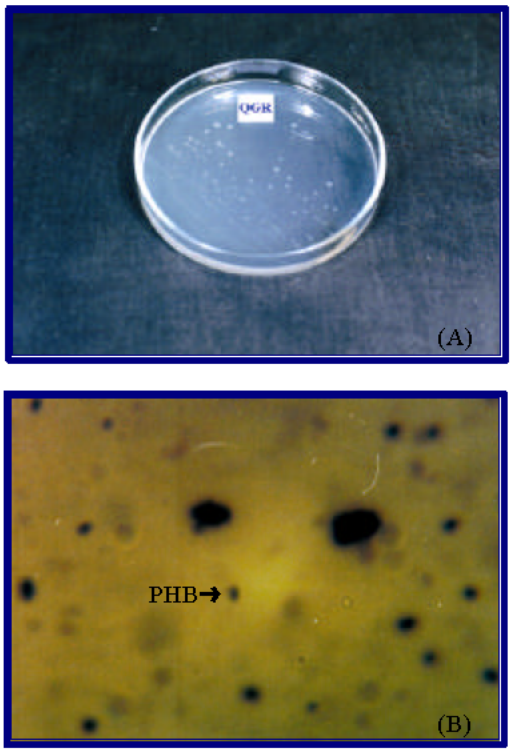

Fig. 1: Photographs showing (A) Colonies on NA plate (B) Sudan black staining of the isolate QGR (*Listeria murrayi*)

Table 1: Effect of carbon, nitrogen and vitamin on the biomass and biodegradable polymer production of isolate QGR (*Listeria murrayi*) .

		Nitrogen sources			
Carbon sources		Ammonium sulfate	Asparagine	Yeast extract	Yeast extract+Ammonium sulfate
Sodium acetate+					
Sodium malate+	Vit +	0.01	0.01	0.04	0.04
Sodium pyruvate+	Vit -	0.02	0.01	0.00	0.08
Sodium succinate					
Glucose	Vit +	0.01	0.19	0.21	0.17
	Vit -	0.04	0.10	0.28	0.23
Fructose	Vit +	0.05	0.09	0.33	0.08
	Vit -	0.04	0.11	0.51	0.07
Methanol	Vit +	0.01	0.08	0.17	0.12
	Vit -	0.07	0.10	0.14	0.13

Table 2: Cell dry weight and biodegradable polymer content (%) of the isolate QGR (*Listeria murrayi*)

Total cell dry weight (g)	Biodegradable polyester weight (g)	Biodegradable polyester content (%)
1.2798	0.1	7.81

Table 3: Percentage of different components with MW and MF present in Chloroform extract of biodegradable polymer of isolate QGR (*Listeria murrayi*)[9]

Peak no.	Retention time	% Total	Name of compound	Molecular formulae	Molecular weight
1	3.853	1.24	2-Octaneal	$C_8H_{14}O$	126
2	5.771	1.12	2-Nonenal	$C_9H_{16}O$	140
3	15.657	12.75	Ethyl cyclopropane carboxylate	$C_6H_{10}O_2$	114
4	17.205	1.10	Cyclohexyl butyl pthalate	$C_{18}H_{24}O_4$	304
5	18.982	2.16	Hexadecanoic acid 2-11	$C_{16}H_{30}O_2$	254
6	19.475	33.70	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
7	20.028	2.94	Ethyl Palmitate	$C_{18}H_{36}O_2$	284
8	20.729	2.34	Oleic acid	$C_{18}H_{34}O_2$	282
9	21.134	0.58	Ethyl-9-hexadecanoate	$C_{18}H_{34}O_2$	282
10	21.708	1.36	Oleic acid	$C_{18}H_{34}O_2$	282
11	21.757	3.83	Oleic acid	$C_{18}H_{34}O_2$	282
12	21.925	0.41	Oleic acid	$C_{18}H_{34}O_2$	282
13	22.058	0.29	Oleic acid	$C_{18}H_{34}O_2$	282
14	22.167	0.90	Oleic acid	$C_{18}H_{34}O_2$	282
15	22.264	12.03	2, 7-Dimethyl, 5-Octenadiol	$C_{10}H_{22}O_2$	174
16	24.617	0.71	Phenol-2, 4-bis (1-methyl-phenylethyl)	$C_{24}H_{26}O$	330
17	24.792	13.54	Di-n-octylphthalate	$C_{24}H_{38}O_4$	390
18	27.591	9.00	Hexadecanoic acid 2-11	$C_{16}H_{30}O_2$	254

biomass and biodegradable polymers were produced by QGR when fructose used as the carbon source and yeast extract used as the nitrogen source, respectively. Anderson and Dawes[10], Braunegg *et al.*[11] showed accumulation of PHB by *Alcaligenes faecalis* using fructose as the carbon source. Use of yeast extract as the nitrogen source has been reported by other scientists[12-14]. No considerable effect of vitamins on biomass and biodegradable polymer production was observed.

Biodegradable polymer production, isolation and purification: An attempt was made for the production, isolation and purification of biodegradable polymer of QGR. Biodegradable polymer was extracted from the crushed cells with chloroform, dried and measured. The ratio of the biodegradable polymer was determined and recorded as 7.81% in case of the isolate QGR (Table 2). Similarly, biodegradable polymer extraction with chloroform from bacterial biomass was also reported by many other researchers[10,11,14-19].

GCMS analysis: The chloroform extract of biodegradable polymer were dried and analyzed by GCMS (Qp5050A). Table 3 shows, the result of GCMS analysis, where

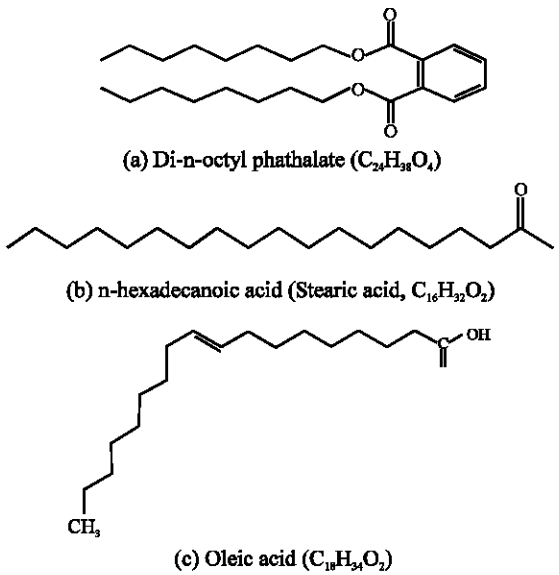

Fig. 2: The structure of (a)-Di-n-octyl Phthalate, (b)-n-hexadecanoic acid (Stearic acid) and (c)-Oleic acid recovered from chloform extraction of biodegradable polymer

eighteen different biodegradable compounds were found from chloroform extract. This method of analysis was also followed by Wallen and Rohwedder[16], Findlay and White[17], Suzuki *et al.*[18]. The major compounds among the analyzed compounds were ethyl cyclopropane carboxylate, oleic acid and di-n-octylphthalate (Fig. 2). Di-n-octylpthalate is an aromatic (pthalic acid) butyl cyclohexyl ester. This biodegradable polyester family was due to hydrolysable ester bonds[20,21].

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