INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2005/07–1–114–117
http://www.ijab.org

# Isolation, Purification and Characterization of Biodegradable Polymer Producing Bacteria *Pseudomonas pseudomallei*

Q. DAS, J.U. CHOWDHURY† AND M.N. ANWAR[1]
*Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh*
*†BCSIR Laboratories, Chittagong, Bangladesh*
[1]Corresponding author's e-mail: mnanwar54@yahoo.com

## ABSTRACT

Fifteen bacterial colonies were isolated, purified and preserved using synthetic enrichment medium. The isolates were then stained for poly-β-hydroxybutyric acid (PHB) with Sudan Black B stain. With the positive isolates, an attempt was made for the production, isolation and purification of biodegradable polymer, where 1.91% PHB related compounds were recorded from the proposed isolate $QY_1$. On the basis of morphological, cultural, physiological and biochemical characteristics, the isolate $QY_1$ was identified as *Pseudomonas pseudomallei.* Finally, the *chloroform* extracts of biodegradable polymer was analysed by GCMS (Gas chromatography linked to Mass spectroscopy), where 28 different compounds were recorded. Among them, n-Hexadecanoic acid (stearic acid), oleic acid and phenyl isobutyrate are the major compounds.

**Key Words:** Isolation; Purification; Bacterial polymer; *Pseudomonas pseudomallei*

## INTRODUCTION

Biodegradable polymer plays a predominant role in the functioning of as biodegradable plastic due to their potentially hydrolysable ester bonds. This polymer family is made of two major groups- aliphatic and aromatic. Polyhydroxyalkanoate (PHAs) are alilphatic polmer naturally produced via a microbial process on sugar-based medium, where they act as carbon and energy storage material in bacteria. They were first biodegradable polymers to be utilized in plastics. The two main members of the PHA family are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). PHB is accumulated inside a variety of microorganisms under appropriate conditions such as limitation of nitrogen, calcium, magnesium, iron or essential vitamins. PHB has also been found in numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria (Dawes & Senior, 1973), gliding bacteria (Prinsheim & Wiessner, 1963), actinomycetes (Kannan & Rehacek, 1970), cyanobacteria (Carr, 1966) and many other prokaryotes. 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. Keeping all the points in mind, present study has been taken to isolate, purify, characterize and analyze the PHB producing microbes.

## MATERIALS AND METHODS

**Screening of PHB producing bacteria.** Fifteen bacterial colonies were isolated, purified and preserved using enrichment and nutrient agar medium. The isolates were screened for PHB by staining with Sudan black B stain (0.3 in 70% alcohol) (Smibert & Krieg, 1981) and observed under microscope (X100x). The selected isolates were then identified on the basis of their morphological, cultural, physiological and biochemical characteristics.

**Optimization of culture medium and conditions.** 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, 2000] 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, Vitamine-$B_6$ and distilled water, using MOM as basal medium).

**Cell cultivation.** For large-scale growth, 24 h old culture was prepared in nutrient broth medium at $30^0$C and transferred to 500 mL of nutrient broth in a wide-necked 1 L culture flask, incubated at $30^0$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 at $4^0$C 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 $KH_2PO_4$, 0.25 g of $K_2HPO_4$, 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 $MnCl_2.4H_2O$, 0.020 g of $Na_2MoO_4$, 0.020 $NiCl_2.6H_2O$, 0.01 of $CuCl_2.2H_2O$, 0.010g 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 $30^0$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 $60^0$C until constant weight and weight was measured.

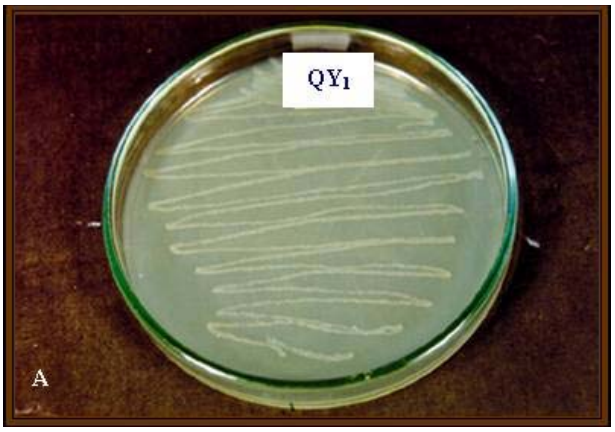
**Biodegradable polymer extraction.** Allumina was used to crush the cells with mortar and pestle. From the white powdery mass of the crushed cells, the polymer was extracted with chloroform ($CHCl_3$). The chloroform mixtures were vigorously shaken, left for 1 h; the clear solution was carefully decanted 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.e., coated with DB-5, 0.25 µm film thickness; column temperature 100-$280^0$C at the rate of $4^0$C/min., injection port temperature $250^0$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.

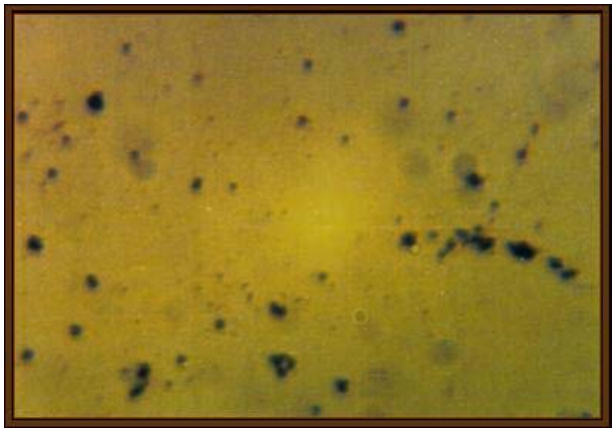
## RESULTS AND DISCUSSION

Among the fifteen isolates, ten showed PHB positive reaction after staining with Sudan Black B stain. Out of 10 positive isolates, $QY_1$ was finally selected for further studies. Isolate $QY_1$ was then studied for morphological, cultural and biochemical characteristics (Fig. 1: A, B) and compared with the standard description of the Bergey`s Manual of Determinative Bacteriology, 8th Ed. (Buchanan & Gibbonns, 1974). The bacterial isolate $QY_1$ was found to belong to the genus *Pseudomonas* and was provisionally identified as *Pseudomonas pseudomallei* (Whitemore) Haynes (1957). Many workers reported the ability of biodegradable polymer production by various members of the genera *Pseudomonas* (Anthony, 1976; Smet *et al*., 1983; Suzuki *et al.,* 1986; Taylor *et al.,* 1989).

**Fig. 1. Photographs showing (A) Colonies on NA plate (B) Sudan Black staining of the isolate $QY_1$ .**




**A**



**B**

**Medium composition and culture conditions of the culture.** Table I shows that maximum biomass and biodegradable polymers were produced when fructose was used as the carbon source and yeast extract used as the nitrogen source. Anderson and Dawes (1990) and Braunegg *et al.* (1978) 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 Fukui (1976), Nishimura *et al.* (1978) and Fernandez-Castillo *et al.* (1986). No considerable effect of vitamins on biomass and biodegradable polymer production was observed.

**Biodegradable polymer production.** An attempt was made for the production, isolation and purification of biodegradable polymer of $QY_1$. Biodegradable polymer was extracted from the crushed cells with chloroform, then dried and measured. The ratio of the biodegradable polymer was determined and recorded as 1.91% in case of the isolate $QY_1$ (Table Π). Similarly, biodegradable polymer extraction with chloroform from bacterial biomass was also reported by many other researchers (Schlegel *et al.*, 1961; Wallen & Rohwedder, 1974; Braunegg, 1978; Findlay & White, 1983;

**Table I. Effect of carbon, nitrogen and vitamin on the Biomass and Biodegradable Polyester production of isolate $QY_1$ *(Pseudomonas pseudomallei)***

| Carbon sources | | Nitrogen sources | | | |
|---|---|---|---|---|---|
| | | Ammonium sulfate | Asparagine | Yeast extract | Yeast extract + Ammonium sulfate |
| Sodium acetate+ Sodium malate+ | Vit + | 0.02 | 0.03 | 0.03 | 0.00 |
| Sodium pyruvate + Sodium succinate | Vit - | 0.03 | 0.00 | 0.01 | 0.05 |
| Glucose | Vit + | 0.19 | 0.22 | 0.29 | 0.60 |
| | Vit - | 0.19 | 0.08 | 0.29 | 0.58 |
| Fructose | Vit + | 0.10 | 0.42 | 0.85 | 0.25 |
| | Vit - | 0.17 | 0.41 | 0.85 | 0.28 |
| Methanol | Vit + | 0.00 | 0.09 | 0.22 | 0.15 |
| | Vit – | 0.01 | 0.09 | 0.14 | 0.13 |

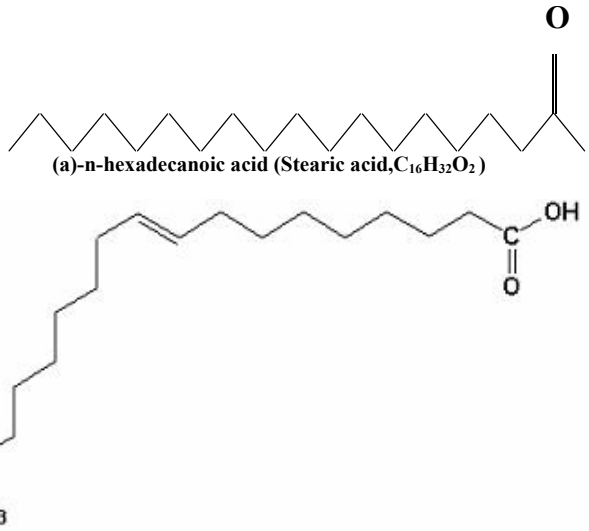
**Table II. Cell dry weight and Biodegradable Polymer content (%) of the isolate $QY_1$. *(Pseudomonas pseudomallei)***

| Total cell dry weight (g) | Biodegradable polyester weight (g) | Biodegradable polyester content (%) |
|---|---|---|
| 1.5657 | 0.03 | 1.91 |

Fernandez-Castillo *et al.*, 1985; Suzuki *et al.*, 1986; Anderson & Dawes, 1990; Anwar & Hakim, 2002).

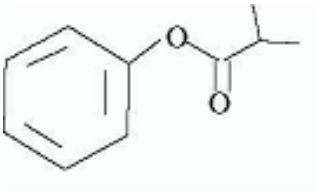
**Analysis.** The chloroform extracts of biodegradable polymer were dried and analyzed by GCMS (Qp5050A). Table III shows the result of GCMS analysis, where twenty eight different biodegradable compounds were found from chloroform extract. The major compounds among the analyzed compounds were n-Hexadecanoic acid (Stearic acid), Oleic acid and Phenyl isobutyrate (Fig. 2). The n-hexadecanoic acid is an aliphatic polymer esters. This aliphatic biodegradable polyester family due to hydrolysable ester bonds was reported by Dawes (1988) and others (Anonymous, 2002).

**Fig. 2. The structure of (a)-n-hexadecanoic acid (Stearic acid), (b)-Oleic acid and (c)- Phenyl isobutyrate recovered from chloform extraction of biodegradable polymer**




**(a)-n-hexadecanoic acid (Stearic acid,$C_{16}H_{32}O_2$ )**

**(b)-Oleic acid ($C_{18}H_{34}O_2$ )**




**(c) Phenyl isobutyrate ($C_{10}H_{12}O_2$)**

## REFERENCES


Anderson, A.J. and E.A. Dawes, 1990. Occurance, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbial Rev.,* 54: 450–72

Anonymous, 2002. *Biodegradable plastics– Developments and Environmental Impacts*. Nolan–ITU Pty Ltd. Prepared in association with ExcelPlas Australia

Anwar, M.N. and M.A. Hakim, 2002. Isolation and characterization of poly–β–hydroxybutyrate producing bacteria. *Bangladesh J. Microbiol.,* 19: 91–3

Braunegg, G., B. Sonnleitner and R.M. Lafferty, 1978. A rapid gas chromatographic method for the determination of poly–β–hydroxybutyric acid in microbial biomass. *European J. Appl. Microbiol. Biotechnol.,* 6: 29–37

Buchanan, R.E. and N.E. Gibbonns, 1974. *Bergey's Manual of Determinative Bacteriology,* 8th ed., p. 1268. The Williams and Wilkinson Company, Baltimore

Carr, N.G., 1996. The occurance of poly–beta– hydroxybutyrate in the blue–green alga *Chlorogloea fritschii. Biochim. Biophys. Acta.,* 120: 308–10

Dawes, E.A., 1988. Polyhydroxybutyrate: an intriguing biopolymer. *Biosci Rep.,* 8: 537–47

Dawes, E.A. and P.J. Senior, 1973. The role and regulation of energy reserve polymers in microorganisms, *Adv. Microb. Physiol.,* 10: 135–266

Fernandez–Castillo, R., V.F. Rodriguez, R.J. Gonzalez and B.F. Ruiz, 1986. Accumulation of poly (β– Hydroxybutyrate) by Halobacteria. *Appl. Environ. Microbiol.,* 51: 214–6

Findlay, R.H. and D.C. White, 1983. Polymeric Betahydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Appl. Environ. Microbiol.,* 45: 71–8

Fukui, T., A. Yoshimoto, M. Matsumoto, S. Hosokawa, T. Satio, H. Nishikawa and K. Tomita, 1976. Enzymatic synthesis of poly–β–hydroxyburtyrate in *Zoogloea ramigera. Arch. Microbiol.,* 110: 149–56

Haynes, 1957, cited from R.E. Buchanan and N.E. Gibbonns, 1974. *Bergey's Manual of Determinative Bacteriology,* p. 1268 8th edition. The Williams and Wilkinson Company, Baltimore

**Table III. Percentage of different components with MW and MF present in Chloroform extract of biodegradable polymer-of isolate $QY_1$ (*Pseudomonas pseudomallei)***

| Sl. No. | Retention Time | % Total | Name of Compound | Molecular Formulae | Molecular Weight |
|---|---|---|---|---|---|
| 1 | 14.205 | 0.17 | n-Cetyl alcohol/Aldol | $C_{16}H_{34}O$ | 242 |
| 2 | 14.814 | 0.40 | Tetradecanoic acid, | $C_{14}H_{28}O_2$ | 228 |
| 3 | 15.395 | 1.46 | Tetradecanoic acid, | $C_{14}H_{28}O_2$ | 228 |
| 4 | 16.494 | 2.38 | Pentadecanoic acid | $C_{15}H_{30}O_2$ | 242 |
| 5 | 16.629 | 1.00 | Pentadecanoic acid | $C_{15}H_{30}O_2$ | 242 |
| 6 | 17.139 | 0.47 | Hexadecanoic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284 |
| 7 | 17.233 | 0.30 | 1,2-Benzenedicarboxylic acid/ Butyl Octylster | $C_{20}H_{30}O_4$ | 334 |
| 8 | 19.067 | 3.04 | Oleic acid 9-ctadecanoic acid (2).delta | $C_{18}H_{34}O_2$ | 282 |
| 9 | 19.620 | 59.68 | n-Hexadecanoic acid(Stearic acid) | $C_{16}H_{32}O_2$ | 256 |
| 10 | 20.055 | 2.64 | Hexadecanoic acid/ ethyl ester/Palmitic acid | $C_{18}H_{36}O_2$ | 284 |
| 11 | 20.473 | 0.19 | Dodecanoic acid | $C_{12}H_{24}O_3$ | 216 |
| 12 | 20.796 | 7.46 | Oleic acid | $C_{18}H_{34}O_2$ | 282 |
| 13 | 20.942 | 0.39 | 2-t-Butyl-6-Chloroquino l-4-yl-2-Pyridyl Ketone | $C_{19}H_{17}$. $CIN_2O$ | 324 |
| 14 | 21.149 | 0.25 | Ethyl Oleate | $C_{20}H_{38}O_2$ | 310 |
| 15 | 21.733 | 1.16 | Oleic acid 9-ctadecanoic acid(2).delta | $C_{18}H_{34}O_2$ | 282 |
| 16 | 21.793 | 4.82 | Oleic acid 9-ctadecanoic acid(2).delta | $C_{18}H_{34}O_2$ | 282 |
| 17 | 21.909 | 1.11 | Oleic acid 9-ctadecanoic acid (2).delta | $C_{18}H_{34}O_2$ | 282 |
| 18 | 21.961 | 3.08 | Octadecanoic acid | $C_{18}H_{36}O_2$ | 284 |
| 19 | 22.067 | 0.45 | Oleic acid | $C_{18}H_{34}O_2$ | 282 |
| 20 | 22.108 | 0.21 | Oleic acid | $C_{18}H_{34}O_2$ | 282 |
| 21 | 22.191 | 0.58 | Oleic acid | $C_{18}H_{34}O_2$ | 282 |
| 22 | 22.256 | 0.67 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |
| 23 | 22.652 | 0.61 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |
| 24 | 23.860 | 1.17 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |
| 25 | 24.631 | 0.50 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |
| 26 | 24.802 | 4.59 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |
| 27 | 26.961 | 0.34 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |
| 28 | 27.595 | 0.91 | Phenyl isobutyrate | $C_{10}H_{12}O_2$ | 164 |

Haywood, G.W., A.J. Anderson, L. Chu and E.A. Dawes, 1989. Characterization of two – 3 – Ketothiolases in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. *FEMs Microbiol. Lett.,* 52: 91–6

Kannan, L.V. and Z. Rehacek, 1970. Formation of Poly–Beta–hydroxybutyrate by Actinomycetes. *Indian J. Biochem.,* 7: 126–9

Maeda, I., 2000 *Isolation of PHB Producing Purple Non–Sulfer Bacteria.* Personal communication (i–maeda@phs.osaka–u.ac.jp).

Nishimura, T., T. Saito and K. Tomita, 1978. Purification and properties of β–Ketothiolase from *Zoogloea ramigera. Arch. Microbiol.,* 116: 21–7

Pringsheim, E.G. and W. Wiessner, 1963. Minimum requirements for heterotrophic growth and reserve substance in *Beggiatoa. Nature,* 197: 102

Schlegel, H.G., G. Gottschalk and V. Bartha, 1961. Formation and Utilization of Poly–β–hydroxybutyric acid by Knallgas Bacteria (Hydrogenomonas). *Nature,* 191: 463–5

Smet, D., M.J. Eggiink, K.K.H Withlt and H.J. Wynberg, 1983. Characterization of intracelluar inclusions formed by *Pseudomonas oleovorans* growth on octane. *J. Bacteriol.,* 154: 870–8

Smibert, R.M. and N.R. Krieg, 1981. General characterization, In Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg and G.B. Phillips (eds.), *Manual of methods for general bacteriology*. pp. 409–43. American Society for Microbiology, Washington, D.C.

Suzuki, T., T. Yamane and S. Shimizu, 1986. Mass production of poly–β–hydroxybutyric acid by fully automatic fed–batch culture of metylotroph. *Appl. Microbiol. Biotechnol.,* 23: 322–9

Taylor, I.J. and C. Anthony, 1976. Acetyl–CoA production and utilization during growth of the facultative methylotroph *Pseudomonus* AM I on ethanol, malonate 3–hydroxybutyrate. *J. Gen. Microbiol.,* 95: 134–43

Wallen, L.L. and W.K. Rohwedder, 1974. Poly–β–hydroxyalkanoates from Activated Sludge. *Environ. Sci. Tech.,* 8: 576–9

**(Received 10 September 2004; Accepted 10 November 2004)**