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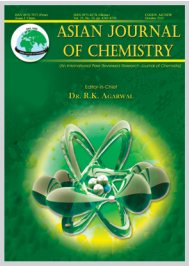

Production of Polyhydroxybutyrate Under Nutrient Limiting Conditions from a Distillery Waste Isolate, *Bacillus pumilus* Dwi

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The biosynthesis of polyhydroxybutyrate (PHB) from distillery waste isolate, *Bacillus pumilus* Dwi was investigated. *Bacillus pumilus* Dwi (Gene Bank Accession No. GQ131871) was shown to accumulate maximum amount of polyhydroxybutyrate in the following nutrient limiting concentrations - 53.4 g/L carbon, 39.8 g/L phosphorus, 85.3 g/L nitrogen and 49.65 g/L sulfur. Furthermore, the recovered polymer was characterized for its chemical structure and thermal properties using NMR, GPC, DSC and its characteristics resemble that of polypropylene. So, our preliminary data supports the possibility of using alcoholic distillery waste water as the carbon source for producing polyhydroxybutyrate. This would be both environmentally and economically significant.

Key Words: Polyhydroxybutyrate, Distillery waste, *Bacillus pumilus* Dwi.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxy alkanoates, which are accumulated as energy/carbon storage materials under unfavourable growth conditions in presence of excess carbon source. Polyhydroxyalkanoates are attracting much attention as substitute for non-degradable petro-chemically derived plastics, because of their bio-degradability and biocompatibility[1]. All bacteria are capable of polyhydroxybutyrate (PHB) synthesis and accumulate polyhydroxybutyrate during the stationary phase of growth when the cells become limited for the essential nutrient but have excess carbon source[2]. First discovery of poly(3-hydroxybutyrate) [P(3HB)] was from the bacterium, *Bacillus megaterium*[3].

Industrial scale production of polyhydroxybutyrate began by using *Alcaligenes eutrophus* and *A. latus*[4]. However, the important factor preventing the industrial and commercial production of polyhydroxybutyrate is its high price compared to synthetic plastic. That is, the cost of the fermentation feedstock contributes significantly to the cost of the product. The viability of microbial large scale production of polyhydroxybutyrate is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to that of petrochemical plastics[5-7].

Earlier works show of using relatively cheap substrates such as methanol[8], beet molasses[9,10], ethanol[11], starch and whey[12,13], cane molasses[14], wheat hydrolysate and fungal extract[7] or soy cake[15] as carbon sources. Interestingly, alcoholic distillery wastewater is a wastewater stream from the manufacture of alcohol in which the biological and chemical oxygen demand are more than 20,000 mg/L[16]. It contains sugar and nitrogenous compounds which makes it a potential feedstock for the fermentation industry, thereby providing a better source of cheap substrate for polyhydroxybutyrate production. Industrial and sewage effluent streams often contain high concentration of organic molecules and can also be used as inexpensive substrates for polyhydroxybutyrate formation.

In this report, we described the characteristics of cell growth and polyhydroxybutyrate production of a distillery waste isolate, *Bacillus pumilus* Dwi. This preliminary data would definitely provide an insight to develop a novel approach to produce polyhydroxybutyrate from waste water.

EXPERIMENTAL

Micro-organisms, medium and growth conditions: Distillery waste isolate, *Bacillus pumilus* Dwi [Gene Bank Accession No. GQ13187] was isolated from Trichy Distillery Unit, Tamil Nadu, India. The strain was grown on a defined glucose medium containing 10 g/L of glucose, 2 g/L NH_4Cl, 4.5 g/L $Na_2HPO_4 \cdot 12H_2O$, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$. The trace element solution contained 60 mg/L of NH_4MoO_4 and 20 mg/L of $CuSO_4 \cdot 4H_2O$, 600 mg/L of H_3BO_3, 400 mg/L of $CoCl_2 \cdot 6H_2O$, 200 mg/L of $ZnSO_4 \cdot 7H_2O$, 60 mg/L $MnCl_2 \cdot 4H_2O$. The organisms were grown at 37 ºC for 24 h on an orbital shaker at

180 rpm. The isolate was assayed for polyhydroxybutyrate quantitatively[17] and qualitatively by nile blue staining[18].

Time course study of polyhydroxybutyrate production on nutrient limitation: Time course of polyhydroxybutyrate production from the distillery isolate under optimal conditions and nutrient limiting conditions was studied. Samples were collected at a time interval of 24 h to determine the wet cell weight and polyhydroxybutyrate production during the 72 h cultivation.

Fermentation: According to the nutrient limiting study, the maximum polyhydroxybutyrate production was observed under nitrogen limiting condition (optimized medium) and the optimized media was used for the scale up process. The cells were grown on 1000 mL Erlenmeyer flask for 24 h at 180 rpm and 37 ºC.

Extraction of polyhydroxybutyrate: The polyhydroxybutyrate produced was extracted by solvent extraction with hot chloroform using Soxhlet apparatus for 12 h. The chloroform solution was filtered to remove any debris, concentrated by evaporation and added to methanol for precipitating polyhydroxybutyrate. The precipitate obtained was dried before its characterization.

Polymer characterization: The polymer was suspended in spectrochem grade deuterochloroform ($CDCl_3$). The ^1H NMR spectra of sample was obtained at 800 MHZ (Bruker Biospin AG, Switzerland). The ^{13}C NMR spectral analysis was performed at 75 MHz. Samples were dissolved in deuterated chloroform (1 mg mL^{-1} solvent) that was employed for each analysis. The chemical shift scale was in parts per million (ppm)[19]. The thermal property of polyhydroxybutyrate was examined by differential scanning calorimeter measurement using a DSC-TA Instruments, Q20 series, USA. The temperature was scanned from 0 ºC to 80 ºC at a heating rate of 10 ºC/min. For the precise determination of T_g and T_m values, the samples were first melted at 80 ºC and immediately quenched in liquid nitrogen before the measurement[20,21]. The apparent average molecular weight and the polydispersity index were determined by gel permeation chromatography by using GPC-HP1090, Agilent Technologies, USA. Polysterene standards with a low polydispersity were used to generate a calibration curve and THF was used as an eluent at a flow rate of 1 mL/min and at 35 ºC. The sample concentration and injection volume were 1.67 wt % and 100 µL.

RESULTS AND DISCUSSION

To determine the effect of nutrient limitation on polyhydroxybutyrate production, the distillery waste isolate *Bacillus pumilus* Dwi was cultured in media in which specific nutrient was eliminated (Table-1). Our data clearly indicates that nitrogen limitation positively influences the cell growth and polyhydroxybutyrate production that correlates with the earlier work[22]. It shows that N, P and certain other elements greatly increase the polyhydroxybutyrate accumulation rate in most polyhydroxybutyrate accumulating bacteria isolated from distillery waste. The course of polyhydroxybutyrate concentration over time for various nutrient limitations and the specific polyhydroxybutyrate formation rate are shown in Figs. 1-4. Although, among various limiting conditions,

TABLE-1
EFFECT OF DEFICIENT-NUTRIENTS ON CELL MASS AND POLYHYDROXYBUTYRATE (PHB) PRODUCTION OF DISTILLERY WASTE ISOLATE (DWi)

Deficient nutrients	Dry cell weight (g/L)[a]	PHB weight (g/L)	PHB content (wt. %)[b]
NH_4^+	3.517 ± 0.0623	15.883 ± 0.0623	85.300 ± 0.0816
PO_4^{3+}	2.517 ± 0.0623	7.450 ± 0.0408	39.800 ± 0.0816
SO_4^{2-}	5.050 ± 0.1080	9.233 ± 0.1247	49.643 ± 0.0334
C	1.800 ± 0.0816	10.100 ± 0.0816	55.060 ± 0.0245
Control	2.767 ± 0.0623	9.900 ± 0.0816	53.467 ± 0.0213

[a]Initial concentration of resuspended cells was adjusted to 1.5 g/L and the culture was carried out of 24 h.
[b]Indicated as the percentage per dry cell weight.

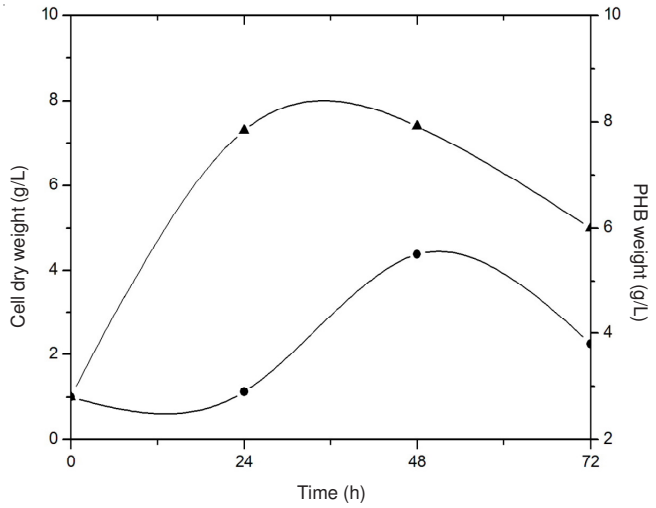

Fig. 1. Time course of cell growth and polyhydroxybutyrate accumulation during batch culture of *Bacillus pumilus* Dwi on glucose media in sulphur limitation. –●– cell dry weight in g/L and –▲– polyhydroxybutyrate weight in g/L

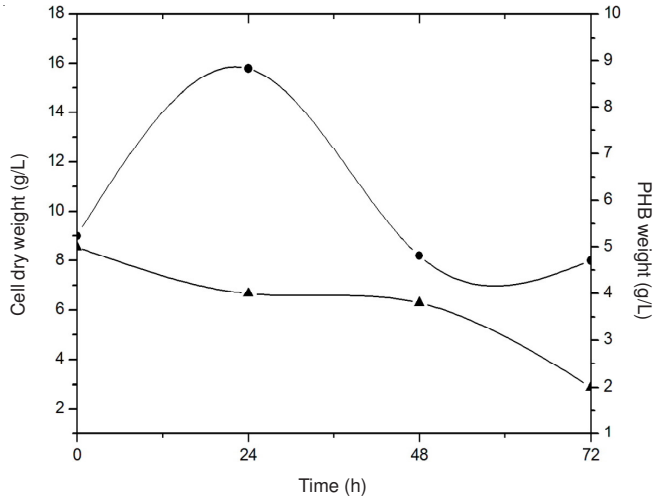

Fig. 2. Time course of cell growth and polyhydroxybutyrate accumulation during batch culture of *Bacillus pumilus* Dwi on glucose media in nitrogen limitation. –●– cell dry weight in g/L and –▲– polyhydroxybutyrate weight in g/L

nitrogen limitation (Fig. 2) obtained higher specific polyhydroxybutyrate formation rate at the initial fermentation period, polyhydroxybutyrate concentration was lower at the late exponential phase. These data are in concurrence with the earlier data obtained by Page[23] where the polyhydroxybutyrate production in a variety of commercially available complex

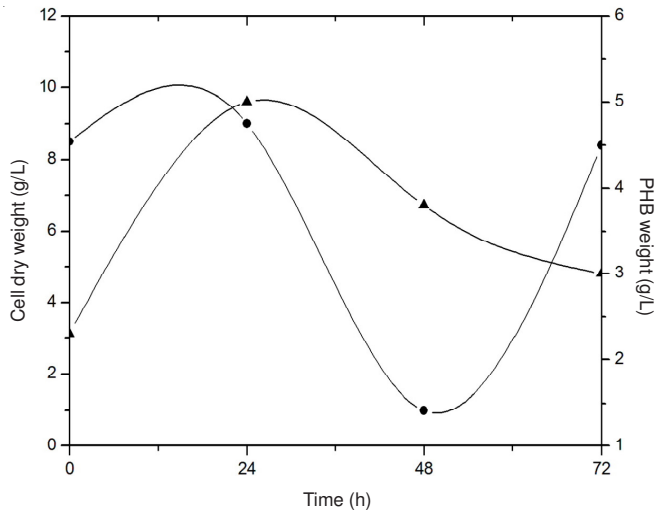

Fig. 3. Time course of cell growth and polyhydroxybutyrate accumulation during batch culture of *Bacillus pumilus* Dwi on glucose media in carbon limitation. –●– cell dry weight in g/L and –▲– polyhydroxybutyrate weight in g/L

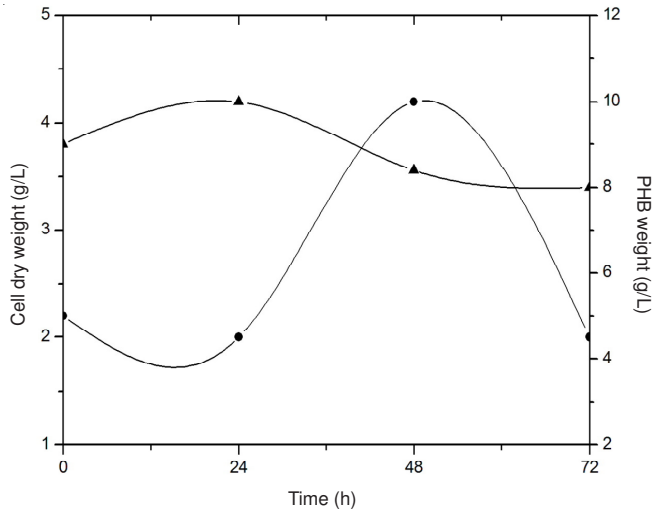

Fig. 4. Time course of cell growth and polyhydroxybutyrate accumulation during batch culture of *Bacillus pumilus* Dwi on glucose media in phosphorus limitation. –●– cell dry weight in g/L and –▲– polyhydroxybutyrate weight in g/L

nitrogen sources (fish, peptone, protease peptone, yeast extract, casitone, phytone and tryptone) was studied. It was found that complex nitrogen sources increased the yield of polyhydroxybutyrate produced by *Azobacter vinelandii* UWD strain. This also clearly shows that polyhydroxybutyrate being a metabolite which is expressed in a smaller quantity in a normally metabolizing strain can be produced in a larger amount by providing certain nutrient limitation or excess condition according to the metabolic pathways required for polyhydroxybutyrate production.

Our data obtained on polyhydroxybutyrate (13240 g/mol) characterization using ^{1}H NMR, the spectra indicate the presence of saturated hydrocarbon attached to aliphatic carbon atoms. Furthermore, the signal obtained at 0.8 δ and 1.2 δ clearly shows that it is due to hydrogen attached to aliphatic carbon atoms [-CH_2-CH_3]. The melting temperature of the polymer is 47 ºC which coincides with the earlier studies[24]. The work shows that highly crystalline polymers are usually stiff and brittle resulting in very poor mechanical properties with low extension at break and they have low resistance to thermal degradation.

In conclusion, distillery waste water can be utilized for a dual purpose of producing of polyhydroxybutyrate and reducing the amount of waste water to be treated. The accumulation of polyhydroxybutyrate has been reported to be triggered by limitation of nutrient[25]. But, polyhydroxybutyrate production by *Bacillus pumilus* Dwi was not so dependent on nutrient limitation, because it shows polyhydroxybutyrate production on glucose media without any nutrient limitation. Distillery waste water could be a suitable substrate for polyhydroxybutyrate production. This observation could lead to a considerable saving in the cost of the fermentation feed stock, if the polyhydroxybutyrate yield from this substrate could be improved. Studies concerning the production of polyhydroxybutyrate from modified distillery waste as media so as to make the organism to accumulate large amount of polyhydroxybutyrate are in progress.

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