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A N I N T E R N A T I O N A L J O U R N A L

Production of Polyhydroxybutyrate by *Bacillus axaraqunsis* BIPC01 using Petrochemical Wastewater as Carbon Source

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ABSTRACT

The aim of this study was to use petrochemical wastewater as the source of carbon for the production of polyhydroxyalkanoates (PHA) in an effort to decrease its cost of production. For this purpose, PHA producing bacteria were isolated from the petrochemical wastewater of Bandar Imam, Iran. The purified colonies were screened for PHA by Sudan Black B and Nile Blue A staining. Among positively stained bacteria, the best PHA producer was selected on the basis of cell growth, PHA content and the monomer composition of PHA. The phenotypic and genotypic identification this isolate showed it to be Bacillus axaraqunsis. The PHA was produced at a cell density of about 9.46 g/l of maximum concentration of 6.33g/l $_{l}$, corresponding to 66% of cell dry weight. These results showed that B. axaraqunsis BIPC01 could be a potent PHA producer using wastewater for industrial purpose and simultaneously reducing the environmental pollution.

Key words: polyhydroxyalkanoate, petrochemical wastewater, *Bacillus axaraqunsis*, biodegradable polymer, bioplastic

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polymers with biodegradable and biocompatible properties, and therefore, are considered as good substitutes for petrochemical plastics (Luengo et al. 2003). These bioplastics are produced as intracellular storage compounds by a large numbers of bacteria under imbalanced growth conditions, when there is an excess in carbon source but limitations in nitrogen, phosphorus, magnesium, oxygen or sulfur concentration (Lee et al. 1999; Liu 2009). PHAs are thermoplastic, water insoluble, non-toxic and moldable biopolymer (Andreessen et al. 2010; Arshad et al. 2010; Aarthi et al. 2011). They can be used to make various products, including films, bottles, packing materials, etc and have biomedical applications such as tissue engineering. Also, they are used as carriers for the drugs and hormones (Choi 1997; Zinn et al. 2001; Rehm 2007; Wu et al. 2009). These polymers can vary in the compositions, number and size of granules and physicochemical properties depending on the microorganism employed for their production (Fernandez et al. 2005). Commercial production of PHA is currently performed using a pure culture of bacteria such as *Ralestonia eutropha* on a synthetically derived carbon substrate, such as glucose. However, the high cost of this process has restricted the application of PHA (Lopez-Cuellar et al. 2011). Therefore, the use of inexpensive substrates (such as crude wastewater) for reducing the costs has been proposed (Chen et al. 2001).

The purpose of this study was to isolate and identify PHA-producing strains from the petrochemical wastewater and use this culture

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medium for production of PHA on the same wastewater.

MATERIAL AND METHODS

Isolation and Screening of PHA- producing Bacteria

Liquid samples of the wastewater were collected from Bandar Imam Petrochemical Company (Mahshahr, Iran). The concentrations of C, N and P in these samples were measured. Primary isolation of wastewater inhabiting bacteria was done by culturing 200 µL of serially diluted (two-fold dilution) wastewater with sterile distilled water on nutrient agar medium. The plates were incubated at 30ºC for 48 h. Various colonies with different morphologies (color, shape and edge appearance) were individually selected and sub-cultured on nutrient agar plates till a pure culture was obtained. Stock cultures were grown and maintained at 4ºC. All the isolates were screened for their PHA-producing ability. For this purpose, these isolates were grown on PHA-Detection Agar (PDA) for 72 h and stained with Sudan Black B (Merck, Germany) and Nile blue A (Merck, Germany) and visualized by fluorescence microscope. A potent isolate was selected based on Sudan Black B and Nile blue A staining methods (Ostle and Holt 1982; Baltz et al. 2010).

Identification

Both phenotypic (biochemical) and genotypic (16S rRNA sequencing) methods were used for the identification of the isolates. For the biochemical characterization, bacteria were grown on nutrient agar plates for 24 h and then inoculated into differential media. The genomic DNA was extracted from 1.0 mL of broth culture using DNA extraction kit (Bioneer, Korea). The partial 16S rRNA gene was amplified according to Weisburg et al. (1991). The sequences of the partial 16S rRNA were compared with the 16S rRNA sequence data available at the public nucleotide data base of National Center for Biotechnology Information (NCBI) using the BLAST (basic local alignment search tool) algorithm (Weisburg et al. 1991).

PHA production and Extraction

PHA production was carried out in a two-step batch cultivation process. The isolates were grown in 50 mL nutrient broth for 20-22 h and then simultaneously an inoculum (1:10) was transferred into PHA detection broth and also to petrochemical wastewater as N-free medium. These were incubated at 27ºC and 150 rpm for five days. The cells were harvested by centrifugation (2016 g, 20 min) and washed with sterile distilled water. The biomass was kept overnight at -20ºC and then freeze-dried. PHA extraction was done using sodium dodecylsulfate (SDS) digestion method by taking 30 g/l of freeze-dried biomass and pretreated by different concentrations of SDS (Merck, Germany) (4, 6, 8, 10 and 12 $^{g}/_{l}$) at 55ºC and different concentrations of NaClO (Merck, Germany) (10, 20 and 30%) at 30ºC (Jacquel et al. 2008; Ceyhan et al. 2011).

Bacterial growth curve

Two culture media (PDB and wastewater) were inoculated with10% seed culture and incubated at 27ºC with continuous shaking at 150 rpm for 48 h. Cell growth was monitored by measuring the optical density of the broth at 600 nm. Three replicates were prepared for each culture medium (Zhaolin 2000; Hahn et al. 2005).

Polymer production curve

In order to determine the rate of polymer production, polymer production curve were plotted. The isolate was cultured and the PHA content of cells was extracted and measured as previously described at 72 h intervals and plotted against bacterial growth. In this manner, the polymer production curve was obtained (Baltz et al. 2010).

Characteristics of PHA

PHA content and composition in the lyophilized cells were determined using FTIR spectrum and nuclear magnetic resonance analysis. For FTIR and NMR analysis, PHA was extracted from the lyophilized cells using 5% sodium hypochlorite (Merck, Germany) solution. After 15-30 min at 37ºC, the PHA granules were collected and then washed with water, methanol (Merck, Germany) and acetone (Merck, Germany). The pellet was then dissolved in chloroform (Merck, Germany). After complete solvent evaporation, FTIR spectra was recorded and scanned in the range of 600 to 4000 wave number (cm^{-1}). For NMR analysis, the PHA was extracted from the freeze-dried cells. Purified PHA was dissolved in deuterated chloroform ($CDCl_3$) and subjected to the H-NMR analysis (Amirul et al. 2008; Ataei et al. 2008; Xu et al. 2010).

UV-visible absorption spectroscopy
The sample containing polymer was digested in the concentrated sulfuric acid (Merck, Germany) and the solution was heated in a water bath for 20 min. In this method, PHB was converted to crotonic acid that has a maximal absorption at 210 nm. Therefore, the absorbance of the solution was measured at 210 nm against H_2SO_4 as blank (Khanna et al. 2005).

Optimization of petrochemical wastewater as production medium
According to the concentration of nitrogen in the wastewater, different sources of nitrogen, including urea (Merck, Germany), $NaNO_3$ (Merck, Germany) and NH_4NO_3 (Merck, Germany) with different ratio of C/N including 30/1, 25/1, 20/1, 15/1, 10/1 and 5/1 were added to the petrochemical wastewater in order to increase biomass production. After incubation at 27ºC and 150 rpm, the growth of the isolate was monitored as previously mentioned and compared with growth on PDB (Ryu et al. 2008, Chai et al. 2009).

RESULTS AND DISCUSSION

Screening of PHA- producing Bacteria
Different bacterial strains were isolated and identified as possible PHA producer based on Sudan Black B and Nile blue A staining methods, which emitted orange fluorescence in the presence of PHA under UV exposure (Fig. 1A, B). Based on the cell growth and PHA content, BIPC01 isolate with high PHA contents was selected for further studies.

Identification and characteristic of BIPC01
Based on the descriptions in Bergey`s manual of systematic bacteriology for the macroscopic, microscopic and biochemical characters of the selected PHA-producing strain, the strain was identified as a member of the genus *Bacillus* sp. and was designated as BIPC01 (Table 1). Further characterization was confirmed with 16S rRNA sequencing and its comparison with available data in the NCBI databases. It was closely related to *Bacillus axaraqunsis* (98% homology). Thus, the bacterium was identified as *Bacillus axaraqunsis* BIPC01. The Gram-positive bacteria have a good potential for using raw materials and PHA production. *Bacillus* spp. are known for their rapid growth on simple nutrients.

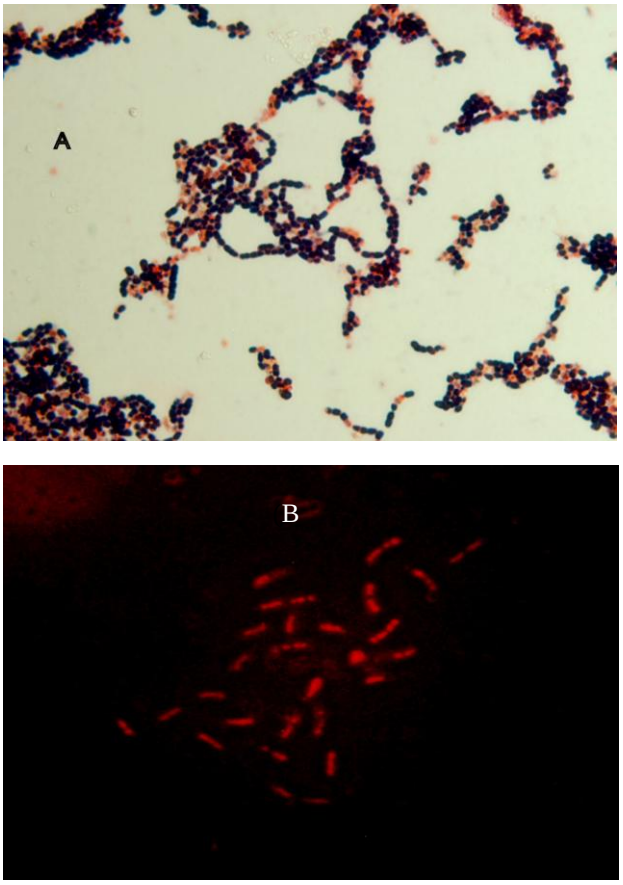

Figure 1 - Sudan Black B, (A) and Nile Blue A, (B) staining of *Bacillus axaraqunsis* BIPC01. Stained PHA granules with Nile blue A were observed under fluorescent light microscope.

Table 1 - Phenotypic characteristics of BIPC01 strain.

Cell Shape	Rod	**Nitrate Reduction**	+	**Urease**	-
Gram Reaction	+	**TSI**	Alk/ Alk	**Xylose**	-
Snot	-	**O-F Test**	O^+/ F^+	**Citrate Utilization**	-
Catalase	+	**MR**	+	**D-glucose**	+
Oxidase	-	**VP**	+	**Indol production**	-
Motility	-	**Gelatin hydrolysis**	-	**Growth at 10% NaCl**	+
Spore	+	**Growth at 50ºC**	+		

PHA production and Extraction from *B. axaraqunsis* BIPC01
Two culture media, including PHA detection broth and petrochemical wastewater as N-free medium were used for determining the PHA production by *B. axaraqunsis* through growing at 27ºC and

continuous shaking conditions for five days. This isolate was initially grown in NB for 20 h in the first stage to produce sufficient amount of cell biomass and in the second stage, the cells were transferred to the wastewater as an N-free medium for PHA production. The extraction of PHB from freeze-dried cells of both the media was carried out by sodium hypochlorite-SDS digestion. For this, different concentrations of SDS and NaClO were used for the treatment and subsequently polymers with different purity were extracted. The result is shown in (Table 2). The best concentration of SDS and NaClO for this isolate was 4 g/L and 10%, respectively. The yields of PHA in PHA detection broth and wastewater were also 75.4 and 79.96%, respectively.

The growth curve of the organism in both culture media reached to the first log phase at the 12th h and log phase was until 62nd h. The rate of bacterial growth in PDB was higher than wastewater (Fig. 2).

Table 2 - Effect of SDS and NaClO concentration on purity of PHA .

SDS g/L % NaClO	4g/L	6g/L	8 g/L	10g/L	12 g/L
10 %	75.4	63.3	66.6	70	56.6
20%	64.4	73.3	56.6	66.6	40
30%	72.18	65.55	57.74	69.98	53.3

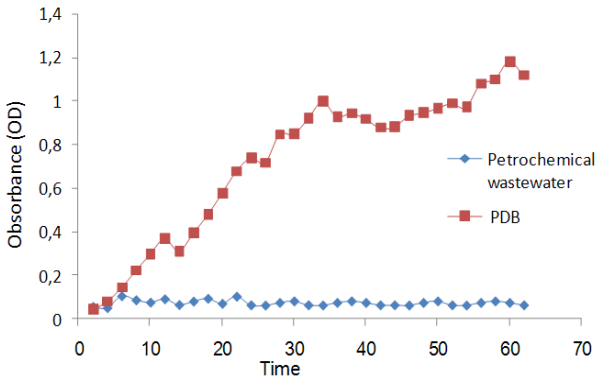

Figure 2 - Growth curve of *B. axaraqunsis* BIPC01 on the PHA detection broth and wastewater as N-free medium.

Polymer production curve

The production curve for the PHA is shown in Figure 3. Polymer synthesis began at the 12th h and the maximum yield of polymer (66%) was obtained at 48th .

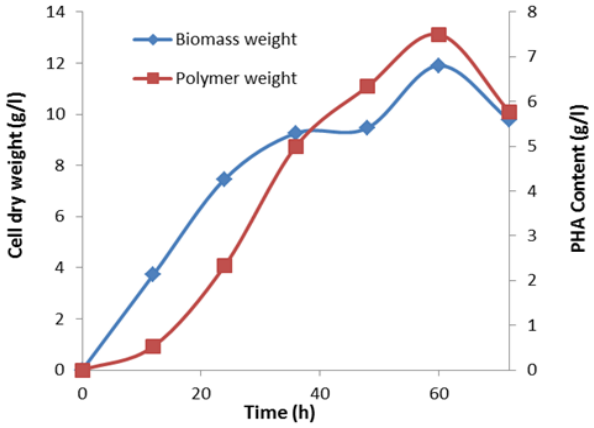

Figure 3 - Growth curve and PHA content of *B. axaraqunsis* BIPC01 on the PHA detection broth.

Composition of PHA

The polyhydroxyalkanoate content and its composition in bacteria are influenced mainly by the strain of microorganism as well as the type and concentration of carbon substrate (Vande velde et al. 2002). In the present study, a new strain of *B. axraquensis* and an alternate medium for PHA production were used. The FT-IR spectrum showed the practically identical structure of the biopolymers produced by the culture (Fig. 4), with characteristic peaks for PHB. The peaks found at 1426 cm^{-1} corresponded to the asymmetrical deformation of the CH_2 groups, while the one found at 1363 cm^{-1} was the equivalent for CH_3 groups. The peaks found at 1711 cm^{-1} corresponded to the stretching of the C= O bonds and the peaks located at 1215 cm^{-1} corresponded to the stretching of the C-O bond of the ester group (Taran 2011).

H-NMR study was conducted to reconfirm the structure of PHB. The NMR spectrums of polymers in $CDCl_3$ solution were obtained. The methyl protons (-CH3) appeared to have a double resonance at 2.520 ppm, methylene protons (-CH2) appeared to have a multiplet resonance at 1.274 ppm and methin proton (-CH) of bacterial polyhydroxybutyrate also had multiplet resonance at 5.2 ppm. The H-NMR spectrum of PHB showed each compound of the polymer as well as additional peaks resulting from the PHB random chain degradation (Fig. 5).

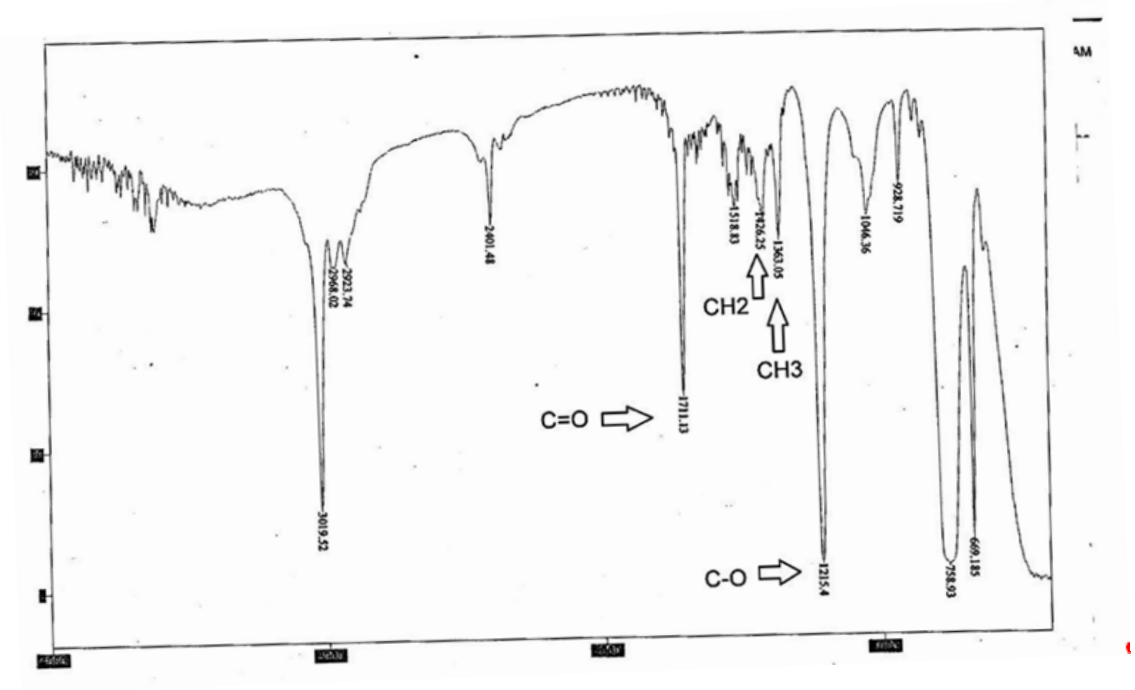

Figure 4 - FTIR spectrophotometer of the PHA extracted from *Bacillus axaraqunsis* BIPC01.

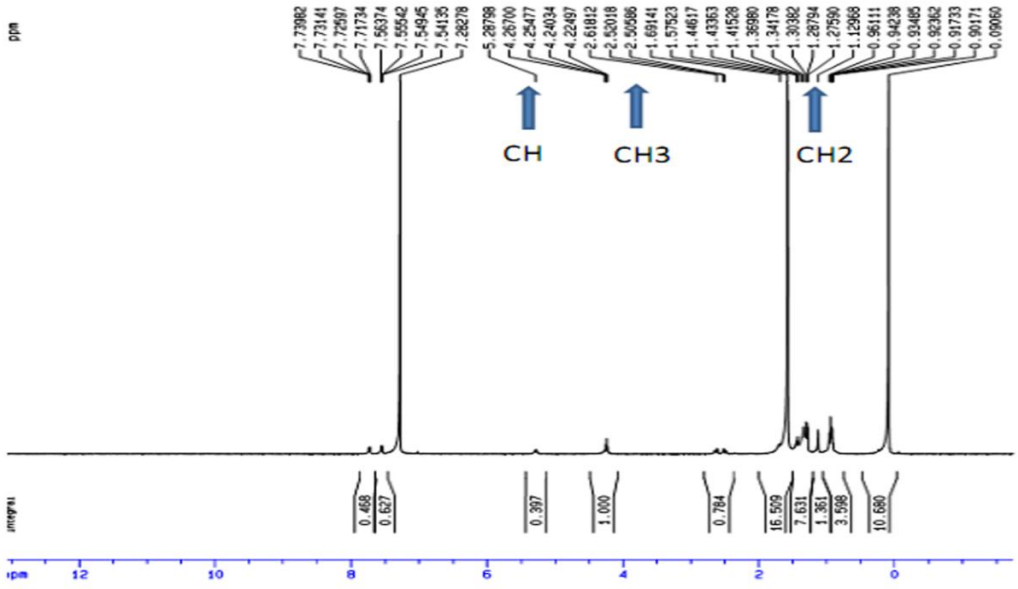

Figure 5 - H-NMR spectrum of PHA purified from *Bacillus axaraqunsis* BIPC01.

UV-visible absorption spectroscopy

When digested by sulfuric acid, PHB was converted to crotonic acid, which had a maximal absorption at 210 nm (Fig. 6). Concentrated H_2SO_4 destroys cell membrane, and the fragments of cell have no absorption on at 210 nm.

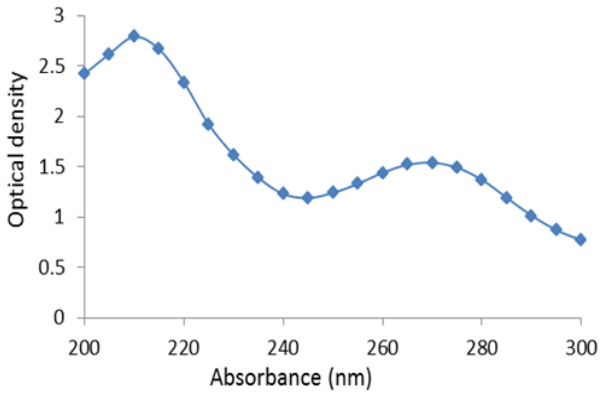

Figure 6 - Ultraviolet absorption spectra of PHB following depolymerization and dehydration in concentrated sulfuric acid.

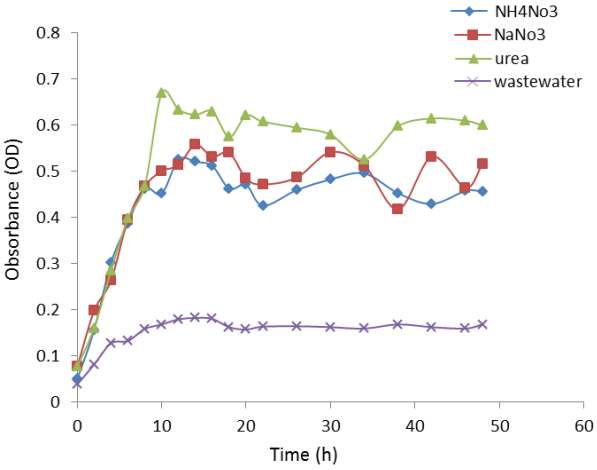

Figure 7 - Growth curve of *B. axaraqunsis* BIPC01 on the Optimized petrochemical wastewater.

Optimization of petrochemical wastewater as culture medium

For the optimizations of nitrogen source, different sources of nitrogen were added to wastewater. As shown in Figure 7, high growth of biomass was achieved in the case of urea. When N-free wastewater was used as culture medium, after about 10 h, the isolate reached to the end of log phase and showed a prolong stationary phase while in N supplemented wastewater, the isolate reached to maximum growth by multitude greater than crude wastewater in less than 10 h. Among other N sources, urea showed highest efficiency for the cell growth. The culture could utilize petrochemical wastewater as a sole carbon source for PHA production. As stated in other investigations, use of inexpensive renewable carbon sources such as industrial wastewater would be essential to reduce the production cost of PHA (Arun et al. 2006). Ceyhan et al. (2011) used domestic wastewater directly as a sole carbon source for PHB synthesis. In this study, the highest PHB yield was up to 96.25%. In other study, the PHB production in various industrial wastes was analyzed by Arun et al. (2006) and the highest yield of PHB production from *Alcaligenes eutrophus* was 40%. In the present study, 75%PHB yield was obtained by *B. axraquensis* BIPC01.Taran et al. (2011) optimized Arak petrochemical wastewater with regard to carbon and nitrogen sources using *Haloarclua* sp. IRU1, a halophilic isolate, and reported the maximum production of PHA as 46.6%. In different studies, the efficiency of polymer production was from 10 to 97%.

While nitrogen limitation can cause decrease in bacterial growth, but is the most favorable condition for polymer production by bacteria, especially by *Bacillus* sp. The carbon content in the petrochemical wastewater was approximately half of carbon content in PHA synthetic medium whereas C/N and C/P ratio in the petrochemical wastewater were four times higher than PHA synthetic medium. With regard to the primary nitrogen concentration and high ratio of C/N in the wastewater, this stressful environment could be a good choice for PHA production but due to the over reduction in nitrogen concentration, bacterial growth was very low in this medium. Based on the results of this study it could be suggested that it was possible to stimulate the cell growth by supplementation of wastewater with a cheap nitrogen source. So, it will be practical that in two-phasic cultures, wastewater with a suitable nitrogen source such as urea could be used instead of nutrient broth.

CONCLUSION

It was concluded that petrochemical wastewater could be used as a valuable carbon and energy source for PHA production, which could reduce the cost of PHA production. This also would prevent environmental pollution due to petrochemical plastics. *B. axraquensis* BIPC01 became the first isolate of BIPC wastewaters that showed good potential for PHB production and could be a good choice for industrial purpose.

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