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Production of Poly-β-hydroxybutyrate (PHB) by *Azotobacter* sp. Isolated from Different Sources

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THIRTY five bacterial isolates of *Azotobacter* were isolated from different sources, nine samples from clay soil and six samples from irrigation water which collected from different localities in El-Gharebia Governorate (Basyion) and Kafr El-Sheikh Governorate (Sakha) to study their ability for accumulation of biodegradable biopolymer poly-β-hydroxybutyrate (PHB) by screening for presence / absence of PHB using a Nile-red staining approach. Also, to achieve enough biomass from the efficient isolates for the production of PHB various incubation times, different fermentation media, pH, temperature, carbon and nitrogen sources were used. Among all isolates, the amount of PHB in the three efficient isolates were Az14 (2.37 g l^{-1}), Az20 (1.25 g l^{-1}) from clay soil, Az6 (1.66 g l^{-1}) from irrigation water. Also, it can be concluded that the optimal conditions for production of PHB from the most efficient *Azotobacter* sp. (Az14) were at 30 ^{o}C, pH 7, incubation time for 48 h, the best carbon source was starch at (1%) and ammonium sulphate at (0.2%) as nitrogen source. These optimal conditions lead to increase in the amount of PHB from 3.9 g l^{-1} to 5.31 g l^{-1} when using the inexpensive sources. So, these results lead to minimize the cost of production and increase the amount of PHB contribute to solving the environmental pollution problem.

Keywords: PHB; Biopolymer; *Azotobacter*; Renewable resources; Biodegradable plastics.

Introduction

Plastic become materials have an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. They are inexpensive which can readily be moulded into a variety of products that find use in a wide range of applications. The continuous increase of production and consumption of plastic in modern society, are shifting attention to renewable and biodegradable polymers **(Dias et al. 2006).** Nature usually cannot handle plastic waste, since the most of plastics are not degraded by microorganisms**.** The biodegradability of Bioplastics (BPs) in different environments, such as soil and marine/fresh water, is a key that makes their life cycle more eco-friendly compared to the conventional plastics and could be an answer to this serious environmental problem. Despite the environmental benefits of BPs, their actual worldwide production is only about 1 Mt/y (COM, 2018) but their demand is continuously growing and, in accordance with the last market data collected by European Bioplastics, the global production of BP capacity is expected to increase from around 2 Mt/y in 2017 to around 2.4 Mt/y in 2022 (Bioplastics Market Data 2017).

Polyhydroxyalkanoates (PHAs) are a group of polyesters accumulated by many gram positive and gram negative bacteria. These polymers are accumulated intracellular to levels as high as 90% of the cell dry weight in response to unbalanced growth conditions (Rehm 2003; Reddy et al. 2003; Khanna and Srivastava 2005a). PHAs are among the most promising candidates for the production of biodegradable items for different industrial applications (Rydz et al. 2015). They are attracting great attention due to their thermoplastic properties similar to that of polypropylene (PP),

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good mechanical properties and biodegradability in various ecosystems such as fresh water, soil, compost and sea water. Their molecular weight and monomer composition vary depending on the microorganism and growth conditions, which reflect their physical properties (Urtuvia et al. 2014). Based on chain length of PHA monomers, they are classified into three groups: short-chain-length (SCL, 3–5 carbon atoms), medium-chain-length (MCL, 6–14 carbon atoms) and SCL–MCL PHA copolymers (containing SCL as well as MCL monomers) (Phithakrotchanakoon et al. 2013; Pillai and Kumarapillai 2017).

So that the substitution of biodegradable plastics for non-degradable plastics has been one response to problem of environmental pollution and exhaustion non renewable resources have focused interest on biosynthetic materials such as poly-β-hydroxy butyrate (Hopewell et al. 2009). Poly-3-hydroxybutyrate (PHB) the best known member of the (PHAs). It is thermoplastic polymer with various useful properties due to its biodegradability and the current market need for a biodegradable thermoplastic industry. PHB is accumulated intracellularly by many bacterial strains and higher plants as a reserve of carbon and energy (e.g., Halobacteriaceae and Archea) and numerous photosynthetic microorganisms such as purple non-sulfur bacteria or genetically enhanced species of microorganisms (Balaji et al. 2013; Carpine, et al. 2015 and Padovani et al. 2016). PHB is usually produced under un balanced growth conditions in response to a nutrient limitation in the presence of an excess of the carbon sources (Pötter and Steinbüchel 2006; Jendrossek and Pfeiffer 2014; Bresan et al. 2016).). Conditions for the accumulation of PHB in *Azotobacter* spp. have been studied. The aim of this study directed for production of the PHB with the efficient *Azotobacter* spp on different substrates by using renewable resources.

Materials and Methods

Sources of microorganism

Nine samples from clay soil and six samples from irrigation water were used in the present study which collected from different localities in El-Gharebia Governorate (Basyion) and Kafr El-Sheikh Governorate (Sakha).

Isolation of microorganisms

To isolate *Azotobacter* from soil samples by several dilutions of the supernatant were prepared using sterile distilled water under aseptic conditions. Over the surface N_2-free medium plates **(Jensen, 1951)** 0.1 ml samples of the prepared soil dilutions were spread out with a sterilized glass rod. On the other hand water samples were filtrated through sterilized syringe filter 0.2 µm. After filtration, the filters were put over the surface of N_2-free solidified medium then all plates were incubated at 30 °C for 7 days.

Culture media and growth conditions

Stock cultures were grown on sucrose/yeast extract agar (Bormann et al., 1998) composed as follows(g l^{-1}): 5, yeast extract; 20, sucrose; 2.45, KH_2PO_4; 3.13, K_2HPO_4 ; 0.05, $CaCl_2$; 1 ml trace elements; 20 g l^{-1} agar and distilled water to 1 liter, pH 7, were added. The media were generally supplemented by stock solutions of trace elements (g l^{-1}): 71.2, MgSO4.$7H_2O$; 0.44, $ZnSO_4$.$7H_2O$; 0.812, $MnSO_4$.$4H_2O$; 0.785, $CuSO_4$.$5H_2O$; 0.252, Na_2MoO_4.$2H_2O$; 4.98, $FeSO_4$.7H2O and 1.02, H_3BO_3 with 0.05 M H_2SO_4 to 1 liter of distilled H_2O. All components were sterilized for 20 min at 120 ^{0}C. Conical flask (500 ml) containing 100 ml of medium inoculated with 1ml of inoculum containing 10^7 cfu ml^{-1} of different strains. The inoculated flasks were incubated at 30 °C at 150 rpm for 48 h.

Screening of the PHB production

Screening test for the production of the PHB by different isolates using Nile red staining approach (Rehm and Valla 1997). Nile red stock solution [0.25 g/ml DMSO (dimethylsulfoxied)], 20 µl was spread onto sterilized pre-made sucrose/yeast extract agar plates to reach final concentrations of 0.5 µg Nile red /ml medium. After inoculation, the plates were incubated overnight at 30 ^{0}C subsequently; plates were then exposed to ultraviolet light (300 nm) to monitor the accumulation of PHB. The lighted plates were recorded positives, after that these isolates were selected as positive producers for the subsequent experiments.

Extraction of PHB

Cells were collected by centrifugation at 10 000 rpm from 1 to 5 ml samples of the culture suspended in 10 ml of sodium hypochlorite reagent (pH 10.0-10.5 NaOCl content 5.25%-5.5%) according to Williamson and Wilkinson (1958). After 1 h at 37 °C the reaction mixture was centrifuged at 5000 rpm for 10 min and the solid pellet was washed successively with water, alcohol and acetone, the final pellet was dissolved in chloroform, while the insoluble residue was discarded. Finally, the chloroform was evaporated

at room temperature and the polymer was dried for 2 h at 105 °C and then weighed. The filtrate was poured into glass Petri plates and the polymer films obtained were kept open for 1 week in room temperature for complete evaporation of the solvent. Polymer extracted with chloroform from bacterial cells treated with sodium hypochlorite (Shi et al. 1997and Aneesh et al.2016) was kept as control.

Determination of cell dry weight

Culture samples (10 ml) were centrifuged at 10 000 rpm 4 min at 4 °C, the supernatant was refrigerated for further analysis, and the cell pellet was washed twice with deionized water, the cell pellet dried to constant weight at 90 °C for 24 h, cooled in a desiccators, and the dry weight of the pellet was determined as g/l (Kuniko et al., 1988).

Identification of Azotobacter isolates

Identification of the isolates to species level was based on morphological, cultural, physiological and biochemical characteristics. Identification criteria in Bergey's Manual of Systematic Bacteriology (Techan and New, 1984) were followed: gram reaction, motility, acid production from sugars, oxidase test, and sensitivity to antibiotics.

Optimization of culture conditions

Effect of incubation periods

Different incubation periods were applied at 24, 48, and 72 h. PHB was determined by the described method. Three replicates from each treatment were used.

Effect of different fermentation media

The different media yeast extract mannitol agar (YMA) (M1), Sucrose/yeast extract medium (M2), Nutrient broth medium (M3)**,** Bänziger and Tobler 2001 (M4), Synthetic medium (M5) and Burdman et al. 1998 (M6) were used. The inoculated flasks were incubated at 30 °C at 150 rpm for 48 h.

Effect of pH and Temperature

Different pH was applied ranging from 4, 5, 6, 7, 8, 9, 10 and pH11**.** The inoculated flasks were incubated under different temperatures (25, 30, 35, 40, and 45 °C) at 150 rpm for 48 h.

Effect of carbon sources

Different carbon sources included refined sugars such as sucrose, mannitol, glucose, potato extract, and starch, Industrial dairy products (like whey), agricultural residues such as sugar cane, sugar beet (leaves), wheat bran, grind rice straw and molasses were added at different concentrations (1%, 2%, 3 %) (w/v) or (v/v).

Effect of nitrogen sources

Different nitrogen sources included ammonium chloride, ammonium sulphate, yeast extract, peptone and urea at different concentrations of nitrogen (0.2, 0.4, 0.6 % N).

Results and Discussion

In this study concentrate on the isolation of PHB producing *Azotobacter* from different sources and improvement of PHB production abilities. To achieve this target, all isolates subjected to screening by Nile-red staining approach. It was established that the Nile-red stain emitted strongly positive red fluorescence signals only with hydrophobic compounds like PHAs and lipids. Nile-red intended to show any lipid particles inside the cells and it did not react with any tissue constituent except by solution and could be detected by fluorescence spectroscopy or flow cytometry, our results are in agreement with (Degelau et al. 1995; Gorenflo et al. 1999 and Spiekermann et al. 1999).

The results of the present work indicated that 32 of 35 isolates yielded positive results. The results showed that the positive isolates exhibited fluorescence. Whereas the other corresponding isolates showed no signals with the Nile-red test (negative results).

Methods of extraction of PHB from bacterial cells

To extract the PHB, various extraction methods either by chloroform, sodium hypochlorite or sodium hydroxide were performed. The use of chloroform extraction method was time consuming and yielded low purity, which recorded 0.18-50% of dry cell weight of PHB yield. As well as, the results presented clearly showed that the use of sodium hypochlorite extraction method which gave high amount among the others (0.24-66.6%) of dry cell weight of PHB yield, these results were in agreement with (Williamson and Wilkinson, 1958). But when use the NaOH to extract the PHB had some disadvantages such as the unclearly color of the PHB extracts and the content of PHB according to dry cell weight was determined to be 0.21-58.3%.

Selection of efficient isolates for production of PHB

PHB extraction from all 35 isolates was done by sodium hypochlorite digestion method (Arnold et al., 1999). Three isolates of 32 different bacterial

isolates obtained in this study where found to give high PHB yield. Two isolates *Azotobacter* sp. (Az14) and *Azotobacter* sp. (Az20) were isolated from clay soil, while *Azotobacter* sp. (Az6) isolated from irrigation water. The data showed the values of PHB according to cell dry weight. The highest values of PHB in soil were 2.37 g l^{-1} and 1.25 g l^{-1} of *Azotobacter* sp. (Az14) and *Azotobacter* sp. (Az20) respectively while the highest values of PHB in water were 1.66 g l^{-1} of *Azotobacter* sp. (Az6).

Identification of the Azotobacter isolates

Azotobacter was studied for it's morphological, cultural and biochemical characteristics. By using a direct microscopy for pure strains of *Azotobacter*, it was found that microbe is gram negative, large in size, oval or cocci, occur singly or in pairs and motile. Examination of isolated *Azotobacter* on nitrogen-free medium revealed that any of them were aerobic, variant colony forms may arise due to the quantity of extracellular polysaccharides produced, catalase positive according to Bergey's Manual of Systematic Bacteriology (Techan and New, 1984). Therefore these isolates identified into *Azotobacter* sp. (Az6, Az14 and Az20).

Effect of different incubation periods on PHB production

To achieve enough biomass from the efficient isolates for production of PHB, various incubation times were used (24, 48, 72 h) at 30 ^{0}C and estimated PHB in isolates. The data presented in Fig. 1 (A) showed that the highest yields of PHB production were recorded after 48 h at 30^{o}C in chosen strains. All bacteria capable of PHB synthesis accumulate PHB during stationary phase of growth when the cells become limited for an essential nutrient but have excess of carbon source (Page 1989; Anderson and Dawes 1990; Page 1995 and Lee 1996).

It is found that the isolates of *Azotobacter* sp. (Az6, Az14 and Az20) produce high amounts of PHB gL^{-1} of dry cell weight and percentage of PHB yield were 65.8-75.9 and 56.6% respectively. In support of these results; Stevenson and Socolofsky (1966) showed that the polymer content of *Azotobacter vinelandii* which increased rapidly during the 1st day of growth, reached a maximum during the 2nd day, followed by a gradual decline as the culture aged.

Effect of different fermentation media on PHB production

The results presented in Fig.1 (B) showed a maximum yield of PHB on (M2) growth medium. We found that *Azotobacter* sp. (Az6, Az14 and Az20) gave maximum percentage of PHB of the biomass on (M19) medium was 65.3-76.2 and 57 %, respectively while the lowest percentage of PHB of their biomass on (M4) medium were 4.6, 5, 5% respectively. But the growth on (M5) and (M6) there is no growth observed with these strains.

Effect of different (pH) on production of PHB

Under the optimized growth conditions (incubation at 30 ^{0}C for 48 h in mineral salt medium). The influence of initial culture pH on biomass yield and PHB production showed in Fig. **1** (C) an initial pH value 4 gave low production. These results are consistent with (Tavernier et al. 1997) who found a decrease in PHB content in the medium with an acidic pH. The results demonstrated that pH 7 appeared to be optimum which gave the highest production of PHB and high growth rate for all three isolates that showed in Fig. 1 (C). This finding was consistent with that previously reported by (Ryu et al. 1997). The results confirm that the optimum growth and production of PHB at pH 7 where the yield of PHB ranged between 2.47 to 3.86 g l^{-1}. The percentage of PHB in these cells was between 57.4 and 76.1 of dry cell weight.

Effect of different temperature on production of PHB

The effect of temperature on production of PHB for strains was examined. As shown in Fig. 1 (D) the results confirm that the optimum temperature for PHB production was 30 ^{0}C for three isolates *Azotobacter* sp. (Az6, Az14 and Az20) where the yield of PHB ranged between 2.48 to 3.84 g/l. The percentages of PHB in these cells were between 57.8 and 76.8 of dry cell weight. It was observed that they can grow at 40^{o}C and 45^{o}C but produced relatively low PHB, so that over the 40-45^{o}C range the temperature effect was negligible in view of the productivity.

Effect of different carbon sources on production of PHB

Under the optimized growth conditions, the results presented in Tables 1 and 5 showed that the highest PHB yield of Az6 and Az20 among the tested carbon sources was observed with whey (3%) where gave 2.4 - 2.37 PHB g l^{-1} with 68.8 - 64.9 % of dry cell weight respectively. On the other hand 2% sucrose was good carbon source which gave 64.7- 56.1% of dry cell weight, this was followed by glucose at 3% gave 61 – 54.8

% of dry cell weight. Also mannitol and molasses were good carbon sources for accumulation of PHB, while the starch and potato extract was the poorest carbon source gave 38.7 – 28.6% of dry cell weight of two strains of *Azotobacter* sp. (Az6) but with (Az20) gave 37.5 – 27.6 of dry cell weight. The data present in Tables 2 and 6 showed the highest PHB yield with wheat bran (30 g) as wastes for two strains *Azotobacter* sp. (Az6 and Az20).

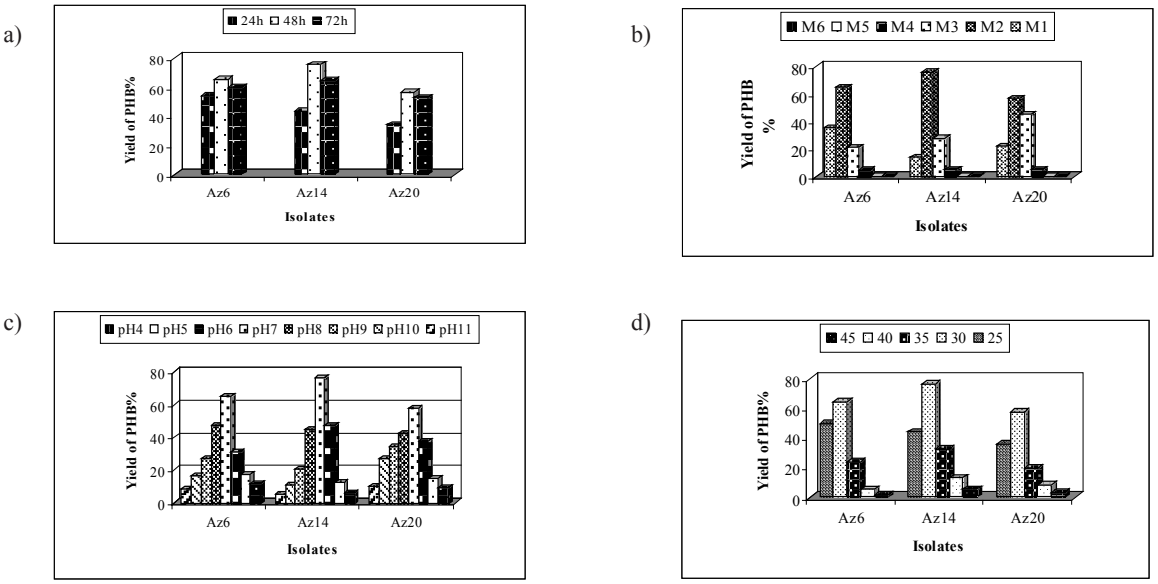

Fig. 1. Optimization of culture conditions, a) Incubation periods, b) Fermentation media, c) Different (pH), d) Different temperature.

TABLE 1. Effect of different concentrations of carbon sources on *Azotobacter* sp. (Az6)

Conc.g/l	1%			2%			3%		
Carbon sources	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %
C1	3.6±0.01	2.2±0.14	61.1	3.83±0.17	2.48±0.08	64.7	3.81±0.01	2.41±0.23	63.2
C2	2.85±0.02	1.5 ±0.01	52.6	2.94±0.025	1.71±0.31	58.2	3±0.08	1.83±0.17	61
C3	3.1±0.14	1.8±0.43	58.1	2.93±0.01	1.65±0.42	56.3	2.87±0.17	1.6±0. 14	55.7
C4	3.1±0.32	1.2±0.11	38.7	2.5±0.08	.91±0.150	36.4	1.84±0.05	0.65±0.34	35.3
C5	3.5±0.17	1.5±0.26	42.8	3.52±0.01	1.52±0.03	43.2	3.57±0.01	1.53±0.08	42.8
C6	1.4±0.03	.4±0.090	28.6	1.45±0.013	0.35±0.2	24	1.47±0.22	0.38±0.02	25.8
C7	2.8±0.18	1.8±0.1	64.3	2.99±0.24	1.99±0.09	66.5	3.5±0.31	2.4±0.01	68.8
Control	0.99±0.02	0.2±0.01	20.2	0.99±0.02	0.2±0.01	20.2	0.99±0.02	0.2±0.01	20.2

DCW: Dry cell weight, Carbon sources **C1**:sucrose, **C2**:glucose, **C3**:mannitol, **C4**:starch, **C5**:molasses, **C6**:potato extract, **C7**:Whey.

TABLE 2. Effect of different concentrations of carbon sources as wastes on *Azotobacter* sp. (Az6)

Conc.g/l	1%		2%		3%	
Carbon sources	Log no. cfu / ml	PHB g\l	Log no. cfu / ml	PHB g\l	Log no. cfu / ml	PHB g\l
Sugar cane	6.8±0.01	1.1±0.03	6.81±0.15	1.17±0.16	6.8±0.23	1.2±0.01
Sugar beet	6.71±0.16	2.77±0.42	6.82±0.04	2.83±0.32	6.84±0.15	2.79±0.11
Wheat bran	7.94±0.12	3.5±0.09	8.1±0.16	3.71±0.26	8±0.09	3.75±0.28
Rice straw	5.8±0.31	0.73±0.24	4.7±0.17	0.81±0.1	4.6±0.01	0.67±0.02
Control	0.99±0.02	0.2±0.01	0.99±0.02	0.2±0.01	0.99±0.02	0.2±0.01

TABLE 3. Effect of different concentrations of carbon sources on *Azotobacter* sp. (Az14).

Conc. g/l	1%			2%			3%		
Carbon sources	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %
C1	4.66±0.22	3.4±0.31	73	5.2±0.02	3.97±0.18	76.3	5.73±0.11	4.2±0.21	73.3
C2	4.3±0.02	3±0.02	69.8	4±0.04	2.65±0.07	66.2	3.91±0.13	2.45±0.32	62.6
C3	3.94±0.11	2.5±0.24	63.4	4.02±0.01	2.73±0.16	67.9	4.16±0.09	2.84±0.12	68.3
C4	5.5±0.13	4.5±0.16	81	5.8±0.06	4.45±0.11	76.7	5.81±0.18	4.42±0.08	76.1
C5	4.1±0.04	2.2±0.1	53.6	4.3±0.00	2.2±0.06	51.2	4.44±0.25	2.21±0.19	49.8
C6	3.1±0.01	1.3±0.11	41.9	3.14±0.00	1.2±0.26	38.2	3.11±0.01	1.11±0.09	35.7
C7	4.2±0.25	3.1±0.09	73.8	4.5±0.00	3.14±0.08	69.8	4.6±0.37	3.21±0.1	69.8
Control	0.99±0.02	0.2±0.01	20.2	0.99±0.02	0.2±0.01	20.2	0.99±0.02	0.2±0.01	20.2

DCW: Dry cell weight, Carbon sources **C1**: sucrose, **C2**:glucose, **C3**:mannitol, **C4**:starch, **C5**:molasses, **C6**:potato extract, **C7**:Whey.

TABLE 4. Effect of different concentrations of carbon sources as wastes on *Azotobacter* sp. (Az14).

Conc. g/l	1%		2%		3%	
Carbon sources	Log no. of cfu /ml	PHB g\l	Log no. cfu /ml	PHB g\l	Log no. cfu /ml	PHB g\l
Sugar cane	8.2±0.19	1.5±0.33	8.1±0.05	1.46±0.03	8.1±0.19	1.48±0.07
Sugar beet	7.97±0.17	2.55±0.12	8.25±0.00	2.9±0.15	8.3±0.33	2.92±0.18
Wheat bran	8.09±0.18	3.1±0.09	8.3±0.07	3.15±0.04	8.5±0.02	3.2±0.34
Rice straw	6.1±0.1	1±0.16	5.86±0.02	1.02±0.02	5.5±0.06	1.06±0.03
Control	0.99±0.02	0.2±0.01	0.99±0.02	0.2±0.01	0.99±0.02	0.2±0.01

TABLE 5. Effect of different concentrations of carbon sources on *Azotobacter* sp. (Az20)

Conc. g/l	1%			2%			3%		
Carbon sources	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %
C1	4.81±0.1	2.49±0.06	51.7	5.04±0.19	2.83±0.25	56.1	5.45±0.13	2.93±0.09	53.8
C2	3.81±0.22	1.99±0.16	52.2	3.85±0.32	2.1±0.02	54.5	4.1±0.02	2.25±0.03	54.8
C3	4.2±0.06	2.1±0.42	50	3.88±0.17	1.81±0.05	46.6	3.76±0.03	1.7±0.11	45.2
C4	2±0.18	0.75±0.03	37.5	1.83±0.05	0.56±0.12	30.6	1.79±0.33	0.65±0.09	36.3
C5	2.47±0.21	1.3±0.01	52.6	2.53±0.06	1.31±0.05	51.8	3±0.41	1.35±0.14	45
C6	2.1±0.09	0.58±0.14	27.6	1.83±0.16	0.41±.0.22	22.4	1.9±0.01	0.32±0.06	16.8
C7	3.5±0.13	2.1±0.16	60	3.6±0.01	2.3±0.01	63.8	3.65±0.09	2.37±0.19	64.9
Control	0.99±0.02	0.2±0.01	20.2	0.99±0.02	0.2±0.01	20.2	0.99±0.02	0.2±0.01	20.2

DCW: Dry cell weight, Carbon sources **C1**:sucrose, **C2**:glucose, **C3**:mannitol, **C4**:starch, **C5**:molasses, **C6**:potato extract, **C7**:Whey.

TABLE 6. Effect of different concentrations of carbon sources as wastes on *Azotobacter* sp. (Az20).

Conc. g/l Carbon sources	1%		2%		3%	
	Log no. cfu / ml	PHB g\l	Log no. cfu / ml	PHB g\l	Log no. cfu/ml	PHB g\l
Sugar cane	7±0.1	1.3±0.04	7.11±0.19	1.25±0.02	7.11±0.23	1.17±0.12
Sugar beet	6.5±0.01	1.5±0.13	6.61±0.04	1.61±0.33	6.65±0.28	1.63±0.03
Wheat bran	7.85±0.16	2.1±0.24	7.9±0.31	2.3±0.01	8.1±0.07	2.54±0.15
Rice straw	5.4±0.09	0.8±0.02	5 ±0.25	0.5±0.21	5.03±0.17	0.45±0.23
Control	0.99±0.02	0.2±0.01	0.99±0.02	0.2±0.01	0.99±0.02	0.2±0.01

But with *Azotobacter* sp. (Az14) the results presented in Tables 3 and 4 showed the highest PHB yield at 1% starch which gave 4.5 PHB g l^{-1} with 81% of dry cell weight. On the other hand 2%sucrose and 1% whey were good carbon sources gave 76.3 – 73.8% of dry cell weight. Also glucose and mannitol were good carbon sources for production of PHB. In the support for these results Kim et al. (1995) reported the ability of *Azotobacter chroococcum* to produce PHB from starch and wheat bran (30 g) as wastes.

It is established that about 40% of the total PHB production cost is due to the raw materials (Choi and Lee 1999). Therefore the use of inexpensive substrates as carbon sources can reduce the high cost required for PHB production. To minimize the cost of PHB production, different industrial wastes were used in the present study. Some researchers have tried to produce PHB from inexpensive carbon sources such as molasses (Solaiman et al. 2006; Albuquerque et al. 2007), Starch (Chen et al. 2006b; Haas et al. 2008), whey (Kim 2000; Koller et al. 2008).

Effect of different nitrogen sources on production of PHB

Under the optimized growth conditions the results shown in Tables 7, 8, and 9 indicated that ammonium sulphate $(NH_4)_2SO_4$ at 0.2% N with Az6, Az14 and Az20 produced highest amount of PHB 2.84, 5.31 and 3.32 g/ l with 72.8%, 87% and 73.8 % of dry cell weight respectively. In confirmation of these results Martinez Toledo et al. (1995) reported that maximum production of polymer by *Azotobacter chroococcum* H23 was obtained in culture cells grown in $NH4^+$culture medium. It appears that under culture conditions the strain H23 takes up source and stores then after conversion to PHB with higher efficiency. This interpretation could be supported by the fact that energy charge of the cells grown on $NH4^+$ medium was lower than that of cells grown in N free medium. Also the ratio of ATP to ADP in cells grown in $NH4^+$ medium was low when compared with cells grown in N-free medium. These results are probably a consequence of a low level of oxidation phosphorylation in cells grown in $NH4^+$ medium, excess un oxidized carbon source could be routed to PHB which is a polymer that serves as an electron and carbon sink (Jackson and Dawes 1976).

TABLE 7. Effect of different concentrations of nitrogen on *Azotobacter* sp. (Az6).

Conc. g/l Nitrogen Sources	0.2%			0.4%			0.6%		
	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %
N1	2.81±0.22	1.75±0.31	62.3	3.00±0.22	1.90±0.18	63.3	3.1±0.14	2.08±0.17	67
N2	2.60±0.12	1.40±0.15	53.8	2.35±0.15	1.22±0.12	51.9	2.18±0.13	1.12±0.09	51.2
N3	3.00±0.22	2.10±0.23	70	2.85±0.16	2.00±0.12	70.2	2.80±0.06	1.96±0.08	70
N4	3.9±0.18	2.84±0.12	72.8	3.5 ±0.16	2.4±0.15	68.6	3±0.08	1.84±0.19	61.3
N5	3.11±0.14	1.74±0.17	55.9	3.33±0.13	1.70±0.19	51	3.41±0.15	1.71±0.18	50.1
Control	1.33±0.02	0.5±0.01	37.6	1.33±0.02	0.5±0.01	37.6	1.33±0.02	0.5±0.01	37.6

DCW: Dry cell weight**,** Nitrogen sources, **N1:** Yeast extract, **N2:** Peptone**, N3:** NH_4Cl**, N4:** $(NH_4)_2SO_4$**, N5:** Urea**.**

TABLE 8. Effect of different concentrations of nitrogen on *Azotobacter* sp. (Az14)

Conc. g/l	0.2%			0.4%			0.6%		
Nitrogen sources	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %
N1	5.5±0.14	4.5±0.18	81	5.75±0.14	4.41±0.41	76.7	5.85±0.18	4.42±0.17	75.5
N2	4.5±0.18	3±0.14	66.6	4.33±0.03	2.75±0.16	63.5	4.18±0.16	2.5±0.22	59.8
N3	3.42±0.08	2.5±0.14	73.1	3.35±0.13	2.3±0.11	71.6	3.31±0.25	2.27±0.09	68.6
N4	6.1±0.07	5.31±0.11	87	6±0.05	5.2±0.31	86.6	5.85±0.23	4.95±0.41	84.6
N5	3.7±0.15	1.63±0.15	44	3.72±0.14	1.6±0.22	43.3	3.77±0.08	1.62±0.33	42.9
Control	1.33±0.02	0.5±0.01	37.6	1.33±0.02	0.5±0.01	37.6	1.33±0.02	0.5±0.01	37.6

DCW: Dry cell weight**,** Nitrogen sources **N1:** Yeast extract, **N2:** Peptone**, N3:** NH_4Cl**, N4:** $(NH_4)_2SO_4$**, N5:** Urea**.**

TABLE 9. Effect of different concentrations of nitrogen on Azotobacter sp. (Az20)

Conc.g/l	0.2%			0.4%			0.6%		
Nitrogen sources	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %	DCW g\l	PHB g\l	yield %
N1	3.51±0.31	2.11±0.11	60.1	3.6±0.33	2.31±0.22	64.2	3.66±0.17	2.4±0.22	65.5
N2	2.8±0.22	1.5±0.14	53.6	2.75±0.15	1.31±0.14	47.6	2.63±0.17	1.19±0.14	45.2
N3	3.11±0.12	1.96±0.16	63	3.09±0.14	1.86±0.08	60	3.02±0.22	1.8±0.13	59.6
N4	4.5±0.11	3.32±0.14	73.8	4.38±0.24	3.1±0.16	70.8	4.23±0.14	2.71±0.31	64.1
N5	3.45±0.09	1.76±0.22	51	3.6±0.22	1.75±0.15	48.6	3.72±0.31	1.73±0.32	46.5
Control	1.33±0.02	0.5±0.01	37.6	1.33±0.02	0.5±0.01	37.6	1.33±0.02	0.5±0.01	37.6

DCW: Dry cell weight**,** Nitrogen sources **N1:** Yeast extract, **N2:** Peptone**, N3:** NH_4Cl**, N4:** $(NH_4)_2SO_4$**, N5:** Urea**.**

Conclusion

From the present study, it can be concluded that the optimal conditions for production of PHB from the most efficient *Azotobacter* sp. (Az14) were at 30 °C, pH 7, incubation time for 48 h , the best carbon source was starch at (1%) and ammonium sulphate at (0.2%) as nitrogen source. These optimal conditions lead to increase in the amount of PHB from 3.9 g l^{-1} to 5.31 g l^{-1} when using the inexpensive sources. So, these results lead to minimize the cost of production and increase the amount of PHB contribute to solving the environmental pollution problem.

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