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Production of Biopolymer – Poly-B-Hydroxybutrate (PHB) By *Azotobacter* Species Using Agro-Industrial Wastes

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A B S T R A C T

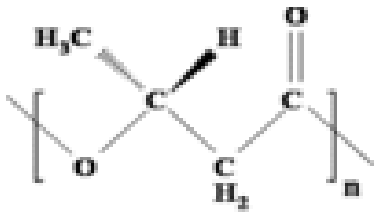
Due to the growing industrial interest of Poly-β-hydroxybutrate and its high production cost, the present work has been undertaken for the production of PHB by *Azotobacter* sp. The biosynthesis of PHB by *Azotobacter* grown on agro – industrial wastes such as rice chaff, sugarcane molasses and sesame oil cake as growth substrates was investigated. PHB producing strains were screened by Sudan Black method and Thin Layer Chromatography.12 strains were isolated from the soil samples. Of the 3 strains (S2, S7, and S10), which were positive for cyst staining only S2 indicated the presence of PHB accumulation in the cells. The PHB production in various agro – industrial wastes based medium was studied by crotonic acid method It was found that strain S2 showed a higher absorbance value of 1.73±0.03 than the strain MTCC 2641 which was 1.691.69 ±0.02 in Mineral Salts Medium (MSM). In Rice Chaff (RCM) and Sugarcane Molasses Medium, MTCC 2641 showed a higher absorbance value than the strain S2. The biomass production in strain MTCC 2641 strain showed highest biomass of 25 g/l in RCM followed by 18 g/l in SOCM. The soil isolate S2 produced 20 g/l in SOCM and 19 g/l in RCM. Highest PHB production was obtained from MTCC 2641 in RCM which was 2.6 ±0.2g/l, while the strain S2 produced 0.8 ±0.1g/l in RCM and 0.8 ±0.2 in SOCM. The highest value of absorbance for PHB was observed in the strain MTCC 2641, which was 458 ±2.6 in SOCM followed by 390 ±2.5 µg/ml in MSM. Strain S2 showed high values for PHB in 395 ±2.1 in SOCM and 192 ±1.5 in SMM. The pure form of PHB was collected and qualitatively analyzed by nuclear magnetic resonance (NMR). Among the various agro - industrial wastes based medium, highest yield was obtained with sesame oil cake waste as growth substrate.

Introduction

Biodegradable plastics

Biodegradable plastics are biopolymers, which undergo degradation particularly by enzymes into CO_2 and H_2O under aerobic conditions and into methane and inorganic compounds under anaerobic conditions. Biodegradable plastics currently being developed are polyhydroxyalkanoates (PHA), polyglycolic acid, polylactic acid, poly (ethylene oxide) and blends of starch and polypropylene. Of the above mentioned, only PHAs are 100% biodegradable. (Sporleder *et al*., 2011). These are polyesters of various hydroxyalkanoic acids which are synthesized by numerous microorganisms as energy reserve materials when an essential nutrient such as nitrogen or phosphorus is limited in presence of excess carbon source. They are non – toxic and moisture resistant (Khanna and Srivastava, 2007; Kirithika *et al*., 2011). The presence of these lipids like inclusions, which were soluble in chloroform, was first observed in *Azotobacter chroococcum* and the chemical composition of similar inclusions in *Bacillus megaterium* identified them as Polyhydroxybutyrate (PHB) (Barnard and Sanders, 1989).

Poly-3-hydroxybutyric acid (PHB)



PHB

PHB material was first described in 1926 by Maurice Lemoigne (Stephanie DeMarco, 2005). PHB is a very common and widespread storage material in many micro-organisms. PHB has been found to be a very basic polymer of variety of chemically similar polymers, the polyhydroxyalkonates.

Poly-3-hydroxybutyric acid (PHB) is the most well known member of the family of Polyhydroxyalkanoates (PHA). PHB is similar to polypropylene (Aremu *et al*., 2011, Guoqiang *et al.*, 2001) in its physical properties but has the advantage of being biodegradable. PHB has been shown to be biodegradable by bacteria into water and carbon dioxide (and methane under anaerobic conditions) in natural environments including water, soil and compost.

More than 300 different microorganisms are known to synthesize and intracellularly accumulate PHAs. PHAs are accumulated (Porter *et al*., 2011, Singh and Parmer, 2011) intracellularly (as amorphous mobile polymers) in granules of different sizes. PHB accumulation has been found in many organisms, such as representatives of gram positive and gram negative bacteria and also archaebacteria, as insoluble inclusions in cytoplasm (Luengo *et al.*, 2003; Jendrossek, 2009). This occurs mainly if the cells are cultivated in the presence of an excess carbon source and growth is impaired or restricted by the lack of another nutrient, such as nitrogen, phosphorus and sulphur, or also dissolved oxygen in the presence of a key enzyme known as PHA synthase and stored in the form of water insoluble inclusions in the cell cytoplasm (Jain *et al.*,

2011; Rehm, 2003; Pettinari *et al.*, 2001). When the supply of the limiting nutrient is restored, PHA can be degraded by intracellular depolymerases and subsequently metabolized as a carbon and energy source (Thakor *et al*., 2006; Ojumu *et al.*, 2004; Amara, 2008).

The molecular mass of PHAs varies per PHA producer but is generally on the order of 50,000 to 1,000,000 Da. Bacterially produced P (3HB) and other PHAs have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics such as polypropylene (Van de Velde and Kiekens, 2001). Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. In nature, a vast consortium of microorganisms is able to degrade PHAs by using secreted PHA hydrolases and PHA depolymerases (Verlinden *et al.*, 2007). Biodegradable plastics opened the way for new waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities. Most of the plastics on the market, claimed to be biodegradable, are based on synthetic and microbial polyesters (Belal, 2013).

There are important limitations in the bulk production of bioplastics, the special growth conditions required for the synthesis of these compounds (usually unbalanced nutrient conditions that cause slow growth), the difficulty involved in synthesizing them from inexpensive precursors, and the high cost of their recovery (Choi and Lee, 1999; Lee *et al.*, 2000). However, current knowledge about their biosynthetic pathways and regulation has allowed the construction of recombinant organisms (other microbes, yeasts and plants) able to synthesize bioplastics from inexpensive carbon sources (e.g. molasses, sucrose, lactose, glycerol, oils and methane) (Tian *et al.*, 2009; Madison and Huisman, 1999).

Agriculture and its related industries produce large quantities of feedstocks and coproducts that can be used as inexpensive substrates for fermentative processes. One potential concept is the production of poly (hydroxyalkanoate) (PHA) polymers, a family of microbial biopolyesters with a myriad of possible monomeric compositions and performance properties. The economics for the fermentative production of PHA could benefit from the use of low-cost agricultural feed stocks and coproducts. From an ecological point of view, they are renewable and from an economic point-of view, many of the co products being studied are derived from surplus or low-cost processing streams.

The present study was aimed to design an efficient strategy for the production of the biopolymer poly – β - hydroxybutrate (PHB), by *Azotobacter* sp using various agro – industrial wastes.

Materials and Methods

Isolation and identification

Different garden soil samples were collected from the rhizosphere of different plants. Samples were withdrawn at a depth of 10-15 cm below the surface and collected into sterile vials, sieved through a 4-mm mesh sieve and stored at field moisture content at 4°C. *Azotobacter sps* was isolated by means of the soil paste – plate technique which was described by Aquilanti *et al.*, 2004. The isolates were identified by biochemical tests and were screened for the presence of PHB producing bacteria.

Screening for PHB producing isolates

The strains were stained with India ink / Nigrosin and the presence of cyst and with Sudan Black B for the presence of PHB granules / intracellular lipid granules. 50µL sample was loaded on the TLC plate impregnated with silica gel and allowed to run in the solvent system consisting of ethyl acetate and benzene (1:1), mixture for 40 minutes (Senthil Kumar and Prabakaran, 2006**)**. For staining, 50 ml of iodine solution was vaporized in water bath at 80 - 100°C. TLC plate was kept over the beaker containing iodine solution for 5-10 minutes to get saturated with iodine vapour. After 10 minutes green-black colour spots on the plate indicated the presence of PHB. The Rf value was measured and compared with the standard chart (Rawte and Mavinkurve, 2002).

Production of PHB in Mineral Salts Medium and Agro-Industrial Wastes

Azotobacter beijerinckii MTCC 2641 was obtained from the Microbial Type Culture Collection (MTCC) and Gene Bank of Institute of Microbial Technology, Chandigarh to study the production of PHB.

Mineral salts medium was prepared and autoclaved. Three different agro – industrial wastes such as sugarcane molasses, rice chaff and sesame oil cake were collected and used for the PHB production. 50 % of the filtrate of agro industrial wastes was added to 50% mineral salts medium and autoclaved.

The mineral salts medium and the three agro industrial waste medium were inoculated with *Azotobcter beijerinckii* MTCC 2641 and the soil isolate and incubated at 37°C for 72 hours. PHB staining was done every 24 hours to observe the presence of the intracellular lipid. Growth was determined spectrophotometrically at 400 nm every 24 hours.

Biomass determination

The total biomass was determined after 72 hours of incubation. Cells from 50 ml of culture broth were pelleted by centrifugation at 5000 rpm for 10 minutes, washed twice with sterile distilled water, dried for 24 hours at 100°C and used for total cell dry weight determination **(**Khanna and Srivastava, 2007).

PHB Extraction and Estimation

PHB is extracted and the amount of PHB in the sample is determined spectrophotometrically at 235 nm by Law and Slepecky, 1961 method.

H^1 NMR spectral analysis

The pure form of PHB was collected and qualitatively analyzed by Proton H^1 NMR method.

Results and Discussion

Soil samples were collected from different garden and processed for the isolation of *Azotobacter sp*. A total of 12 strains were isolated from the soil samples. The colonies produced were slimy and glistening, turning brown with aging. The isolates showed that the cells were oval, negative to gram stain, motile, capsulated, catalase positive, oxidase positive, indole negative, M.R. positive, V.P. positive hydrolyzing gelatin but not starch, acid produced from dextrose, mannitol and sucrose. Acid not produced from lactose. Only strain S2, S7 and S12 were found to produce cyst. Organism with cyst appeared as a hollow area surrounded by the cyst. Of the 3 strains (S2, S7, and

S10), which were positive for cyst staining only S2 indicated the presence of PHB accumulation in the cells. Lipid inclusion granules were blue-black or blue-grey while the bacterial cytoplasm was stained pink. The S2 strain was then run on a thin layer chromatogram to screen for PHB. Greenish black spot was observed on the plates which indicated the presence of PHB. The retention factor, Rf was measured and values were compared with standard.

It was found that strain S2 showed a higher absorbance value of 1.73±0.03 than the strain MTCC 2641 which was 1.691.69 ±0.02 in Mineral Salts Medium (MSM). In Rice Chaff (RCM) and Sugarcane Molasses Medium, MTCC 2641 showed a higher absorbance value than the strain S2. The biomass production in strain MTCC 2641 strain showed highest biomass of 25 g/l in RCM followed by 18 g/l in SOCM. The soil isolate S2 produced 20 g/l in SOCM and 19 g/l in RCM.

Highest PHB production was obtained from MTCC 2641 in RCM which was 2.6 ±0.2g/l, while the strain S2 produced 0.8 ±0.1g/l in RCM and 0.8 ±0.2 in SOCM.

The highest value of absorbance for PHB was observed in the strain MTCC 2641, which was 458 ±2.6 in SOCM followed by 390 ±2.5 µg/ml in MSM. Strain S2 showed high values for PHB in 395 ±2.1 in SOCM and 192 ±1.5 in SMM.

The presence of PHB was qualitatively estimated using an ECA 500 MHz Joel Japan Int. High Resolution liquid state NMR Spectrometer and the results were recorded. The H^1 NMR spectrum obtained from the strains MTCC 2641 and soil isolate S2 had the characteristic peaks at 1.2, 2.4-2.6 and5.2 ppm found in the PHB standard.

The most widely produced microbial plastics are polyhydroxyalkanoates (PHAs) and their derivatives (Pozo *et al*., 2002; Naik *et al.*, 2008). PHB containing only 3-hydroxybutrate (HB) as is constituents is one of the most extensively studied PHAs (Khanna and Srivastava, 2007). PHB is a bioplastic or biobased polymer that is synthesized and catabolized by numerous micro-organisms (Verlinden *et al.*, 2007). This polymer is primarily a product of carbon assimilation and is employed by micro-organisms as a form of energy storage material accumulated intracellularly to be metabolized when common energy sources are not available (Porter and Yu, 2011).

They can be used as biodegradable carriers for long-term dosage of drugs (Sharma *et al*., 2011) and as surgical devices such as sutures, staples, fixation rods, pins and swabs (Khanna and Srivastava., 2007). They can also be used for the manufacture of disposable items, such as razors, utensils, diapers, feminine hygiene products, cosmetic containers—shampoo bottles and cups (Hazer, 2011; Ceyhan and Ozdemir, 2011).

Several factors affect the production cost of PHB, such as PHB productivity, content, yield and the cost of the carbon substrate. The major restriction in the commercialization of bioplastic is their high production cost (Tsuge *et al.*, 2002). The use of readily available cheap agro – industrial residues as carbon sources may reduce the higher cost (Aremu *et al.*, 2011). Thus finding a less expensive substrate is therefore, a major need for wide commercialization of PHB. A production process based on waste carbon sources is the requirement of the day instead of noble ones.

Azotobacter sps was isolated by means of the soil paste – plate technique which was found as an effective method by Aquilanti *et al.,* 2004**.** The smooth and glistening colonies that appeared upon the smoothed soil paste was selected and utilized for *Azotobacter* isolation onto Ashby's Mannitol Agar. The isolates were identified by biochemical tests which showed similar results of El-Sawah *et al.,* 2008 with the exception that the isolates were positive for gelatin hydrolysis.

The isolates were screened for the presence of PHB producing bacteria. The strains were stained with India ink / Nigrosin and the presence of cyst was found in the isolates S2, S7 and S12.

The strains positive for cyst were then stained with Sudan Black B for the presence of PHB granules / intracellular lipid granules which was used by Wei *et al.,* 2011 for screening of PHB producing strains.

The strain S2 was screened for the presence of PHB by thin layer chromatography, which showed greenish black spot on the chromatogram with a Rf value of 0.72. A distinction of this method was that it showed a Rf value (0.72) which were similar to the results reported by Kumar and Prabakaran, (2006) and Rawte and Mavinkurve, 2002**.**

Production of PHB in Mineral Salts Medium (MSM), has shown that the soil isolate S2 had better absorbance value than the MTCC 2641 strain. After 72 hours, the absorbance for S2 was 1.73± 0.03 while it was 1.69± 0.02 for MTCC 2641.

It was found that the absorbance value for the production of PHB in Rice Chaff Medium (RCM) in the strain MTCC 2641 is higher with a value of 2.75± 0.02, when compared to the soil isolate S2 which was only 2.35± 0.01.

PHB production was found to be influenced by the agro – industrial waste that was used as substrate. When Sugarcane Molasses Medium (SMM) was used as a substrate, the absorbance for the production was higher in MTCC 2641 strain with 1.82± 0.01 and for S2 it was 1.74± 0.02. Also, results of Arun *et al.,* 2006 makes it clear that sugarcane molasses shows only 42% of PHB production when compared with other agro – industrial waste substrates. Chaijamrus and Udpuay, 2008 has showed the effective production of PHB from molasses by *Bacillus megaterium* ATCC 6748. Sucrose content of dry sugarbeet powders can reach up to 80% and it contains nutrients such as N, P, K and Na which makes it highly suitable for microbial cultivation The downstream products of beet sugar industry such as molasses and pulpswere investigated for PHB production using *Azotobacter vinelandii* strain(Wang *et al*., 2013)

Sesame Oil Cake Medium (SOCM), has shown high absorbance value in both the MTCC 2641 and S2 strains, which was 3.00± 0.02 and 3.08± 0.03. It was found that the production of PHB was gradually increasing up to 48 hours but remained constant after that. Sesame Oil Cake seems to be a substrate of considerable interest. Results of Ramdass *et al.,* 2009**,** has showed that sesame oil cake was a poor substrate for PHB production for which enzymatic hydrolysis of the substrate could be a reason. Whereas, Arun *et al.,* 2006 has shown that Sesame Oil Cake Medium (SOCM) at varying concentrations was the highest producer both in aerobic and semi – aerobic conditions.

Table.1 Biomass Determination

STRAIN	CELL DRY WEIGHT g/l			
	MSM	RCM	SMM	SOCM
MTCC2641	10 ±1	25 ±3	14 ±1	18 ±2
S2	14 ±2	19 ±1	12 ±2	20 ±3

Table Note: Mean value of triplicates ± SD.
MTCC 2641 – *Azotobacter beijerinckii*

Table.2 Extraction of PHB

STRAIN	DRY WEIGHT OF PHB g/l			
	MSM	RCM	SMM	SOCM
MTCC2641	0.2 ±0.1	2.6 ±0.2	0.4 ±0.2	0.6 ±0.1
S2	0.4 ±0.1	0.8 ±0.1	0.4 ±0.1	0.8 ±0.2

Table Note: Mean value of triplicates ± SD.
MTCC 2641 – *Azotobacter beijerinckii*
S2 - Soil Isolate
MSM - Mineral Salts Medium
RCM - Rice Chaff Medium
SMM - Sugarcane Molasses Medium
SOCM - Sesame Oil Cake Medium

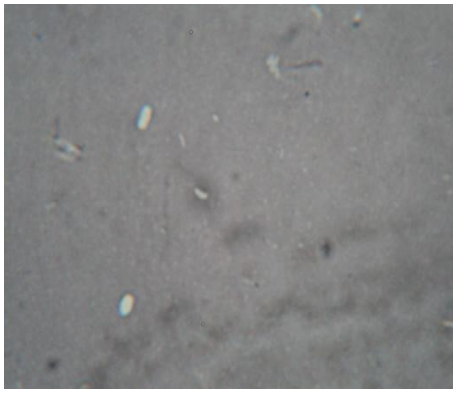
Table.3 Estimation of PHB

STRAIN	PRODUCTION MEDIUM							
	MSM		RCM		SMM		SOCM	
	OD VALUE*	PHB CONC#	OD VALUE*	PHB CONC#	OD VALUE*	PHB CONC#	OD VALUE*	PHB CONC#
MTCC 2641	1.397 ±0.02	390 ±2.5	0.915 ±0.01	205 ±2.2	0.926 ±0.03	210 ±2	1.998 ±0.03	458 ±2.6
S2	0.233 ±0.01	58 ±1.4	0.628 ±0.01	156 ±1	0.828 ±0.02	192 ±1.5	1.399 ±0.02	395 ±2.1

* - OD VALUE AT 235 nm. #- CONCENTRATION OF PHB µg/ml; Table Note: Mean value of triplicates ± SD.

MTCC 2641 - *Azotobacter beijerinckii*
S2 - Soil Isolate
MSM - Mineral Salts Medium
RCM - Rice Chaff Medium
SMM - Sugarcane Molasses Medium
SOCM - Sesame Oil Cake Medium

Plate.1 and Plate 2 Cyst Staining and PHB Staining



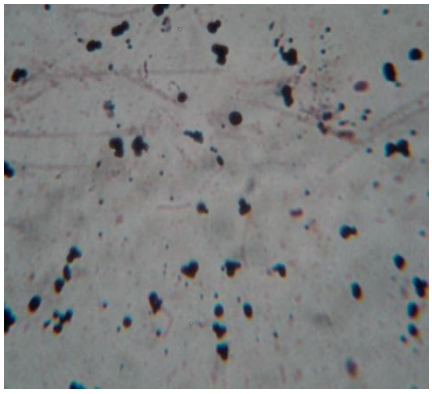


Plate.3 Production of PHB in sesame oil cake medium

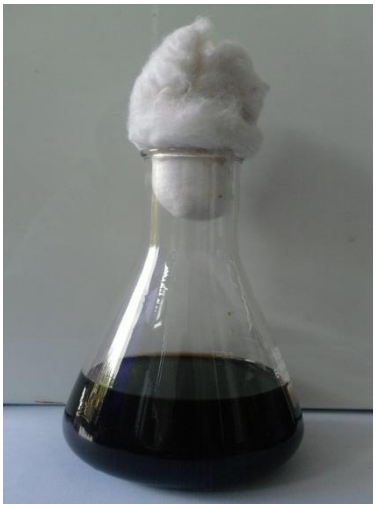
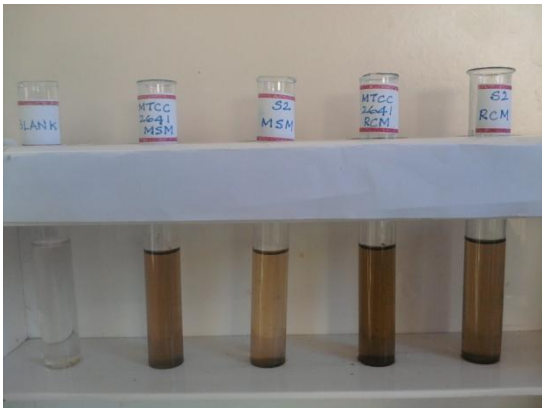
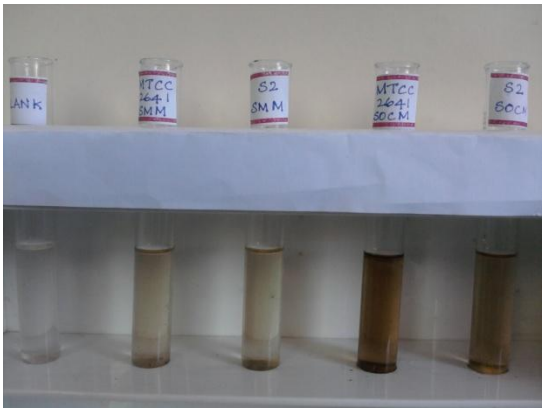


Plate.4 Estimation of PHB by law and speckley method





The dry cell weight was measured after centrifugation and drying by the method adopted by Xin *et al*., 2011**.** The cell dry weight / biomass production after 72 hours

of growth in agro – industrial wastes was found to be high in Rice Chaff Medium (RCM) for MTCC 2641 strain with 25± 3 g/l. this was followed by Sesame Oil Cake Medium (SOCM) with 18± 2 g/l. For the S2 strain highest biomass production was in Sesame Oil Cake Medium (SOCM) with 20± 3 g/l and Rice Chaff Medium (RCM) WITH 19± 1 g/l. Sugarcane Molasses Medium (SMM) gave the least dry cell weight of 14± 1 g/l for MTCC 2641 and 12± 2 g/l for S2 strain. Chen and Page, 1997**,** has showed that *Azotobacter* produced 11 mg/ml of cell dry weight upon fermentation.

The dry weight of PHB was highest in Rice Chaff Medium (RCM) with 2.6± 0.2 g/l for MTCC 2641 and 0.8± 0.1 g/l for S2 strain. This was followed by Sesame Oil Cake Medium (SOCM) with 0.8± 0.2 g/l in S2 and 0.6± 0.1 g/l in MTCC 2641. According to Sri Kumanlaningish *et al.,* 2011, dry weight of PHB was found to range from 1.03 g/l to 2.5 g/l in liquid bean curd waste by *Alcaligenes latus*. The concentration of PHB was estimated in accordance with Law and Slepecky's 1960 method shows highest PHB concentration in Sesame Oil Cake Medium (SOCM) with 458± 2.6 µg/ml in MTCC 2641 strain and 395± 2.1 µg/ml in S2.

H^1 NMR spectral analysis of the PHB polymer from the strains MTCC 2641 and S2 has revealed the presence of protons at the chemical shift 1.2, 2.4-2.6 and 5.2 ppm respectively. H^1 NMR spectrum obtained for PHB produced by Lopez *et al.,* 1996 and Ab-El-haleem *et al.,* 2007 also have shown the presence of similar peaks in other micro-organisms such as *Azotobacter chroococcum, Alcaligenes eutrophus* and genetically modified yeast.

Readily available low-price carbon sources may have high amount of nutrients, such as amino acids and peptides, which contributes to improved cell growth and metabolite biosynthesis and pave the way for resourceful and cost-effective production of PHB.(Priyanka and Bajaj,2015) PHB being the reserved food polymer produced during the time of starvation is degraded to provide carbon and energy when external carbon source is exhausted Sugar industry waste water with nutritive adjustment has been used for PHB production from *Bacillus subtilis* NG220 (Singh *et al*., 2013)

Conclusion

It could be expected that many other bioplastics with different structures, properties and applications could be obtained if the appropriate organism were selected and genetically modified. The use of low cost agro–industrial wastes as fermentative substrates could improve the economics of microbial PHA production. An added attractiveness for using these substrates is that they are renewable resources making the production process ecologically sound and geopolitically independent. Because of their special characteristics and broad biotechnological applications, bioplastics are compounds with an extremely promising future.

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