Fall 2012 • Volume 11 / Issue 3 • ISSN 1538-8786

BioProcessing JOURNAL

Trends & Developments in BioProcess Technology

A Production of BioProcess Technology Network

BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Mutagenesis of *Azotobacter vinelandii* Strain and Production of Polyβ-Hydroxybutyrate from Distillery Spent Wash

By SUDARSHAN S. LAKHAWAT, AMRENDRA N. PATHAK, M.V. KULKARNI, and GARIKIPATI V. SRIKANTH

Polyß-hydroxybutyrate (PHB) is a heavy, molecular-weight polymer with monomer units of (R)-3-hydroxylbutyric acid (HBA) which is deposited as a storage component in many groups of microbes, and is an eco-friendly thermoplastic. Distillery spent wash (DSW) is an industrial waste which has elevated chemical oxygen demand (COD) levels but consists of valuable minerals that can be used for PHB production. *Azotobacter vinelandii* (ATCC® 12837™ and ATCC 13705™) was used to develop *A. vinelandii* UWD, which is a hyper PHB-producing mutant, by using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. This strain was grown in a fed-batch bioreactor containing diluted DSW at (1–7%) with 5% glucose as the carbon source, and ammonium acetate as the nitrogen source. The transformation efficiency for rifampicin (Rif)-positive colonies was 9×10^{-5} which was good enough to select the hyper PHB-producing mutants after five days of incubation. The amount of PHB produced in different dilutions of spent wash was highest (40 g/L, 92% w/w) with 4% spent wash. The glucose consumption was 89% w/w at 4% DSW, and the dry cell weight was > 23.4 g/L. In addition, elevated COD levels (1100 mg/L) of DSW were decreased to 200 mg/L after fermentation was completed. The spent wash, which is considered to be a production waste and ecological pollutant, can be consequently used for bioplastic production.

Introduction

Characteristics and Significance of Polyß-hydroxybutyrate

Polyhydroxyalkanoate (PHA), like PHB polyoctanoates are deposited as inclusion bodies or PHA granules during elevated carbon feed rates.[1-3] PHA exists in two forms: PHAscl and PHAmcl where PHB is a PHAscl with a four-numbered carbon chain.[4] The PHA is a storage complex deposited in several microbial groups during unstable growth conditions.[5] Of the 140 different PHAs, PHB is the most abundant type and is typically water insoluble, elastomeric, non-toxic, and an enanciomerically-pure, biocompatible bioplastic.[6-8] This class of polymer is completely ecological and can be digested and metabolized by a wide variety of microbes and fungus in animals, soil, etc. Recently, it has been observed that PHB and polyvinyl alcohol (PVA) blends were more ecological and stable as compared to pure plastics.[9] Other types of degradable plastics include starch and polyester-based bioplastics, which are already commercially available under the brand names: Novamont in Italy, Sky Green in Korea, and Bionolle in Japan.[9] Since ester bonds are thermally unstable, polyesters are simply hydrolyzed in hot and damp conditions. The co-polymers PHB/hydroxyvalerate (HV), Biopol/Biomer[10], PHB/ hydroxyhexanoate (HH), and PHB/polyethylene oxide (PEO)[9] were found to be less brittle than homopolymeric forms. Bioplastic tensile power has been studied in up to 400 megapascals (MPa).[11]

ABOUT THE AUTHORS

Sudarshan S. Lakhawat[1], **Amrendra N. Pathak**[1]*, M.V. Kulkarni[2], and Garikipati V. Srikanth[3]

1. Amity Institute of Biotechnology, Amity University Rajasthan, NH-8, Kant Kalwar, Jaipur, Rajasthan, India
2. Division of Biochemistry, Department of Chemistry, University of Pune, Pune, Maharashtra, India
3. Stem Cell Research Facilities, Department of Hematology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Raebareli Road, Lucknow-226014, India

***Corresponding author:** Email: anpathak@jpr.amity.edu; Tel: +91-8829967464; Fax: +91-1426-222836

The progress and growth of the petrochemical industry has decreased the price of oil-derived polymers versus their natural counterparts. Regardless, natural polymers are comprehensively used and offer many rewards over synthetic rivals.[12] The melting point of PHB is 180°C, and in general, PHB properties are comparable with those of polypropylene (PP) (*e.g.*, a similar melting point, crystalline nature, glass/rubber transition temperature).[5, 7, 13] PHB is highly brittle with a low solvent resistance but high ultraviolet weathering resistance. The molecular weight is 183,200 daltons (Da).

Blends of PHB and valerate (3-hydroxypentanoate), such as PHB/polyvinyl butyral (PVB), and PHB/poly-p-dioxanone (PDS) were also studied for similar properties.[14] The blending of PHB decreases its crystalline nature, melting point, and stiffness which are beneficial for increased flexibility as a bioplastic.

PHB also has desirable optical properties since the chiral carbon D(-) configuration rotates the plane of polarized light passing through it and makes it usable in chromatography for optically active sample separation.[13]

It is also a constituent of human blood (D[-] hydroxybutyrate), and can be used as an alternative to glucose[15], where it is truly ecological since it is completely converted to CO_2 and energy by microorganisms like bacteria, fungus, and algae. The blends of PHB include some that are completely ecological and some that are partially biodegradable.[12] Recently nanofibrillar scaffold materials have been prepared from biocompatible and biodegradable PHB polymers with electrospinning and result in promising materials that can be used in biomedical applications.[16] In this study, we were able to produce low-cost and cell-friendly bacterial electrospun PHB polymeric scaffolds by using *Alcaligenes eutrophus* DSM 545 strain.

The rate of degradation depends on several factors such as temperature, biological oxygen demand (BOD), and oxygen supply. The rate of PHB oxidation is insignificant in moist air so its shelf life is higher. All of these attractive properties of PHB make it a good substitute for conventional non-biodegradable polymers like polyethylene and polypropylene.[12]

PHA has been synthesized by many microorganisms such as *Cupriavidus necator*, *Alcaligenes latus*, *Aeromonas hydrophila*, *Pseudomonas putida,* and the *Bacillus* group.[17, 18] However, the preferred organism for PHB production is *Alcaligenes eutrophus* which was first isolated in the soils of northern Germany.

Production of PHB in A. vinelandii

Azotobacteriaceae members have been comprehensively studied for storage of PHB. *Azotobacter vinelandii*, which is an obligate aerobe that's able to fix nitrogen, as well as biosynthesize alginate and polyhydroxybutyrate.[19] The *Azotobacter* group, and other microorganisms, are known for their ability to produce alginate, siderophores, and PHB, where alginate and PHB biosynthesis are inversely correlated. The alginate production will decrease in the absence, or limiting amounts, of nitrogen, oxygen, and phosphate, and results in accumulation of carbon in the form of PHA granules.

On the contrary, alginate production increases with excess carbon addition during favorable conditions. During stress conditions, the *Azotobacter* group will move towards starvation and assimilate internal carbon reservoirs such as PHB, and will form cysts.[20, 21] PHB production is dependent on the NADH pathway, and serves as a PHB precursor along with acetyl-CoA which is formed by the ED pathway. During unbalanced growth, the formation of acetyl-CoA is limited in the tricarboxylic acid (TCA) cycle due to the inhibition of a citrate synthase enzyme that causes elevated levels of NADH. NADH can be oxidized to NAD^+ which eliminates this growth inhibition by the action of acetoacetyl-CoA reductase and the polymerization of acetoacetyl-CoA into PHB. This process also results in the deposited acetyl-CoA being directed for PHB synthesis. PHB production increases (3–10 fold) in mutant *Azotobacter vinelandii* but is not able to synthesize alginate.[22, 23]

PHB gene cluster (PHBBAC) is involved in coding three enzymes for PHB synthesis: PHBA-ketothiolase, PHBB-acetoacetyl-CoA reductase, and PHBC-PHB synthase. The studies of PHB production have also been performed in the most preferred cells (recombinant *E. coli*) in which the PHB-producing genes from the host organism were introduced.[24, 25] In *Azotobacter vinelandii*, the two-component GacS/GacA system is required for synthesis of PHB as well as the production of exopolysaccharide alginate. In *A. vinelandii*, the post-transcriptional RsmA protein represses the expression of the PhbR gene.[26]

There have been very limited studies of PHB production using *Azotobacter vinelandii* at the bioreactor level (both lab and pilot-scale)[19, 27], and there are no reports on the scale-up of *A. vinelandii* in PHB production. Large-scale production of microbial PHA possesses a number of problems such as the cost of fermentation, and the extraction of polymer from the cell. A prospective cost-saving technique is offered by the use of the *A. vinelandii* UWD mutant for PHA production. Since *A. vinelandii* nutritional requirements are much lower, it can be cultured with inexpensive substrates such as beet molasses and swine desecrates.

The spent wash may be used as one of the cheapest nutrient sources for large-scale bioprocessing.

Two hundred ninety-five distilleries in India produce 3.2 billion liters of alcohol per year, and the production of one liter of alcohol generates 15 liters of dark brown, highly-acidic spent wash. This means 4.5 lakh (1×10^5) liters of spent wash is formed and dumped in water bodies causing serious ecological concern.[28] *A. vinelandii* wild-type (OP) has been identified and registered at American Type Culture Collection (ATCC) under accession number ATCC 13705. And then the University of Wisconsin has developed their own strain (UW) of *A. vinelandii* and has registered it as ATCC 12837.

Materials, Methods, and Techniques

Expansion, Proliferation, and Mutagenesis

Azotobacter strain (UW) were subcultured and maintained on modified Burk's medium in appropriate formulations containing (g/L) $MgSO_4$ 0.2, K_2HPO_4 0.8, KH_2PO_4 0.2, $CaSO_4$ 0.13, $FeCl_3$ 0.00145, Na_2MoO_4 0.000253, $C_{12}H_{22}O_{11}$ 10.0, $C_6H_{12}O_6$ 10.0, and agar 10.0 correspondingly at a pH of approximately 7.2, and then poured into six petri plates and incubated at 30°C.[29]

After 24 hours of incubation, the colonies of ATCC 12837 and ATCC 13705 were collected and transferred into two sterile test tubes containing 10 mL of Burk's buffer. After attaining an exponential growth phase of 2×10^6 cells/mL, the culture tube with ATCC 12837 was supplemented with 100 µg/mL of NTG at a pH of 7.2 and then left in the dark for 20–30 minutes without shaking. After incubation, 50 µL of culture were inoculated into a petri plate containing sterile Burk's medium supplemented with 1% glucose, 1.8% agar, and 20 µg/mL rifampicin. These plates were incubated at 30°C for another 24 hours.

Transformation and Transformants Selection

After 24 hours, the colonies were selected as a Rif+ mutant 113 of ATCC 12837 and were transferred into 10 mL of Burk's buffer where they were grown as donor cells. Then DNA was isolated from encapsulated Rif+ ATCC 12837 by a cell lysis technique in 15 mM NaCl/Na Citrate buffer with sodium dodecyl sulfate (SDS) (0.5% freshly prepared) at pH 7.0. Following incubation at 55°C for 45 minutes, the lysate DNA was collected by centrifugation and assayed for purity. Crude lysate DNA was precipitated in 1:3 water and alcohol (95%) v/v, centrifuged, and resuspended in 20 mM saline. The entire 10 mL of the ATCC 13705 culture was aseptically inoculated into 100 mL of Burk's medium and incubated for 24 hours until late exponential growth phase was achieved. Then a cell pellet (100–200 µL) of ATCC 13705 (capsule negative) was collected at (3×10^8 cells/mL) after centrifugation at 3000 rpm for 10 minutes at 4°C. The resulting material was obtained in a sterile Eppendorf flask containing 500 µl of Burk's buffer with 15 mM ammonium acetate (AA), and 8.5 mM $MgSO_4$. Then 2 µg of lysate DNA was added aseptically and the material was incubated at 30°C for 30 minutes to increase transformation efficiency. Pre-incubation of the transformants on non-selective Burk's medium was performed for 12 hours, and followed by transferring colonies onto petri plates having agar and rifampicin (20 µg) supplemented Burk's medium. After incubation for 3–4 days, white colonies were selected, frayed off, and restreaked on modified Burk's medium to produce milky white colonies (UWD).[29] In addition, PHB granules were observed in the cytoplasm under a research microscope.

Shake Flask Culture to Fed-Batch Fermentation

The mutant PHB-producing colonies[29] were sub-cultured and maintained in modified Burk's medium containing 2.0 mg/L $FeCl_3$ and 1.5% agar for 2–3 weeks at 28°C. The colonies of UWD were transferred into 20 mL sterile and modified Burk's medium without agar. After 24 hours, 10 mL of this culture was added aseptically into 100 mL of sterile and modified Burk's medium, and then incubated for 24 hours. Log phase culture of UWD was used as the inoculum in shaker flasks. Ten percent of the starter culture was used as seed culture for a 1.3 L fed-batch fermentor (Eppendorf/New Brunswick Scientific). Cell culture monitoring and control were performed by computer (Advanced Fermentation Software from Eppendorf/New Brunswick Scientific). An external heating jacket and an immersed stainless steel cooling coil were used to maintain a convective temperature at 28°C, and a magnetic drive was used to control the agitator shaft rotations at 250–300 rpm. Three Rushton-style standard impeller blades were mounted on the agitator shaft and used for churning the spent wash medium at pH 7.0 with 1.0 M NaOH. Culture parameters were controlled with a proportional-integral-derivative controller (PID), and an antifoam agent (EX-CELL®

ANTIFOAM, SAFC) was used to control excess foam. The spent wash was provided by Jaipur United Distilleries and was collected in polycarbonyl containers which were properly sealed and stored at 4 °C for further analysis. The physico-chemical properties were analyzed by standard techniques.[30] Then at several dilutions, the spent wash was supplemented with glucose as a growth substrate and evaluated for PHB production in shaker flasks. After finding the optimal effect of the spent wash dilutions, PHB production was investigated in a 1.3 L fermentor. Then spent wash was sterilized, filtered, and centrifuged at 6000 rpm for 20 minutes to remove solid aggregates. At the optimum concentration of spent wash at 4% v/v, and a total volume of 700 mL, the bioreactor received another 350 mL of a 1:1 diluted spent wash with glucose at 50% w/v in distilled water. The bioreactor was fed at a rate of 17.5 mL/h for 15–20 hours, and the sugar concentration was maintained at 5% thereafter. The addition of KH_2PO_4–K_2HPO_4 (0.5 M) was performed at the culture peak of 12 hours, and the PHB concentration was maintained. The opening concentration of AA in the bioreactor was 20 mM, which was maintained at a constant by feeding 0.25 M AA at a rate of 15 mL/h. Airflow of 2 L/min was maintained and the antifoaming agent was added to control the foaming at regular intervals. The repetitive sampling was performed via a sampler (Eppendorf/New Brunswick Scientific).

Selection and Revealing of PHB Inclusions in Azotobacter vinelandii

A loopful of the bioreactor culture was transferred at regular intervals to a glass slide and then heat-fixed and stained with Sudan Black dye (in ethanol solvent) for 10–12 minutes. After the addition of xylene and saffranin, the organisms were observed under oil immersion microscopy. Cells were extracted with commercial bleach containing 5.5% NaClO at 37 °C for one hour.[31] The acetone-insoluble milky white residue remaining at the end of the extraction procedure was dissolved in chloroform and converted to crotonic acid by heating at 90 °C in concentrated H_2SO_4 for one hour. PHB was detected by UV spectrophotometry (λmax 208 nm) and the PHB content was defined as the percentage of PHB weight to dry cell weight.

Results and Discussion

PHB production was carried out in fed-batch fermentation with *A. vinelandii* UWD. Glucose in diluted DSW and AA were employed to provide the desired concentrations of glucose and nitrogen in the spent fermentation medium. The optimum microbial growth and PHB accumulation are dependent on the composition of the fermentation medium. The strain of UWD was developed by transforming recipient strain ATCC 13705 with donor strain ATCC 12837–strain 113. When the transformation of *A. vinelandii* UW/OP ATCC 13705 was carried out using the DNA of strain 113, we observed that ATCC 13705 formed enough Rif+ colonies when grown in Burk's medium. After five days, 10% Rif+ colonies were transformed into milky white colonies. The transformation efficiency of Rif+ colonies was observed to be 9×10^{-5}, and the growth rate of *A. vinelandii* UWD was observed to have a prolonged lag phase followed by log phase at 620 nm. The exponential phase was achieved after 15–17 hours of incubation in spent wash with added glucose and ammonium acetate. The amount of PHB produced was determined at different dilutions of spent wash (Table 1): 68% (w/w) at 1% DSW, 75% (w/w) at 2% DSW, 89% (w/w) at 3% DSW, and 92% (w/w) at 4% DSW, plus other dilutions as shown in Figure 1. The maximum PHB production was observed at the 4% spent wash dilution, and this may be due to two important reasons. First, there was an optimal supply of nutrients required by *A. vinelandii*[31], and second, any further increase in spent wash concentration enhanced COD levels in the diluted spent wash which interfered with the normal growth of *A. vinelandii*. The glucose consumption of mutant *A. vinelandii* was 89% (w/w) at 4% spent wash, which was exceptionally higher as compared to the other dilutions (Figure 1). The maximum dry cell weight at

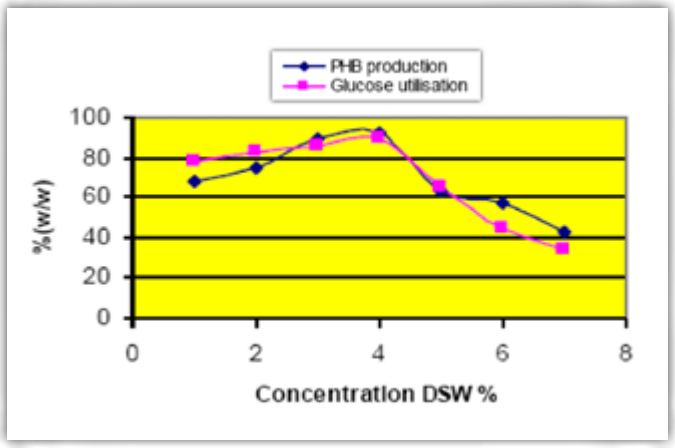

FIGURE 1. The effects of DSW on PHB production and glucose utilisation.

TABLE 1. Components of DSW and concentrations at different dilutions in 5% glucose and 0.15 M ammonium acetate.

Dilution Number	Components of Diluted Spent Wash	Concentration in Undiluted Spent Wash (mg/L)	Concentration After Dilution (mg/L in 5% Glucose Solution w/v and Ammonium Acetate)					
			1%	2%	3%	4%	5%	6%
1	pH	6.23	Maintained at 6.5 units using 1 M potassium hydroxide (KOH)					
2	Total solids	30,300	303	605	910	1,212	1,516	1819
3	Total dissolved solids	22,356	224	448	672	896	1,120	1344
4	Total suspended solids	8,723	87.2	174	262	349	436	523
5	Settleable solids	8,432	84.3	169	253	337	421	506
6	COD	28,693	287	574	861	1,148	1,435	1722
7	BOD	14,982	150	300	450	600	750	900
8	Carbonate	scarce	—	—	—	—	—	—
9	Bicarbonate	11,990	120	240	360	480	600	720
10	Phosphorus	27.43	0.27	0.54	0.81	1.08	1.35	1.62
11	Potassium	5,800	58	116	174	232	290	348
12	Calcium	883	8.83	17.6	26.4	35.3	44.1	53
13	Magnesium	832	8.32	16.6	24.9	33.2	41.6	49.9
14	Sulphate	4,800	48	96	144	192	240	288
15	Sodium	523	5.23	10.4	15.6	20.9	26.1	31.3
16	Chlorides	5,910	59.1	118.2	177.3	236.4	295.5	354.6
17	Iron	8.1	0.081	0.162	0.243	0.324	0.405	0.486
18	Manganese	1,385	13.85	27.7	41.5	55.4	69.2	83.1
19	Zinc	1.09	0.01	0.02	0.03	0.04	0.05	0.06
20	Copper	0.289	0.0028	0.005	0.008	0.011	0.014	0.016
21	Cadmium	0.032	0.0003	0.0006	0.0009	0.0012	0.0015	0.0018
22	Lead	0.14	0.0014	0.0028	0.0042	0.0056	0.0070	0.0084

different dilutions of spent wash was observed to be the highest at 23.4 g/L with a 4% DSW dilution. Additional data show that we achieved 7.5 g/L at 1% DSW, 14.3 g/L at 2% DSW, and 18.5 g/L at 3% DSW (Figure 2). The residual biomass values ranged from 3.4 g/L at 1% dilution and 7.4 g/L at 4% dilution DSW. The initial COD level of 4% DSW was adjusted to 1148 mg/L, and

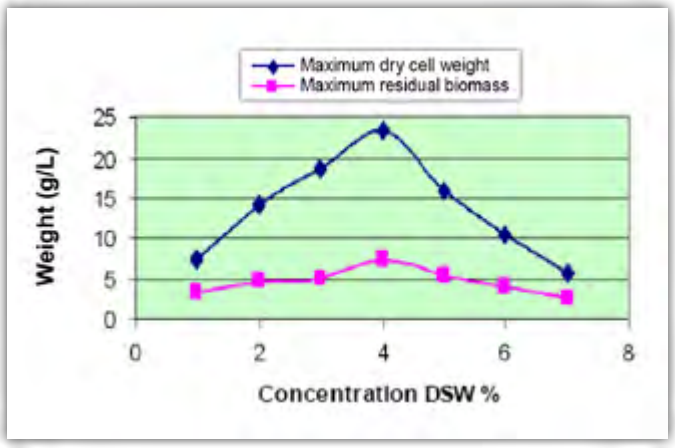

FIGURE 2. The effects of DSW on maximum dry cell weight and maximum residual biomass.

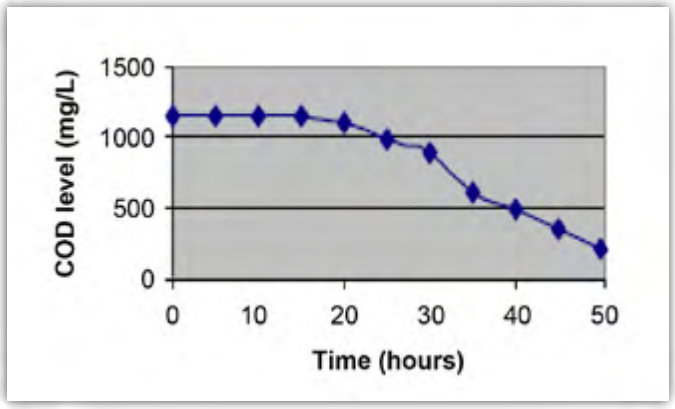

FIGURE 3. Variations in COD levels with time.

this number drastically decreased with incubation time as shown in Figure 3. The COD values remained static during the initial 10–15 hours of incubation, and then decreased exponentially as time passed: 1100 mg/L at 20 h, 980 mg/L at 25 h, 900 mg/L at 30 h, 600 mg/L at 35 h, 500 mg/L at 40 h, 350 mg/L at 45 h, and 200 mg/L at 50 h. These data correspond to those of American Public Health Association (APHA).[32] *Azotobacter vinelandii* will produce PHB in diluted spent wash which has not been previously studied. The reductions in COD levels in spent wash have also been observed previously with *A. eutrophus*.[33] The UWD strain was purified from the sectored colonies that confirmed a pattern of clonal as well as nonclonal growth, as previously observed. The UWD cell growth increased as more cells became homozygous for the chromosome which expresses the mutant NADH oxidase gene.[34] Out of a large population of viable NTG mutants, only limited clones of *A. vinelandii* with mutant chromosomes expressed the white phenotype. However, this mutation is a neutral mutation which is neither valuable nor harmful to *A. vinelandii* cells.[29] In diluted form, the spent wash acted as a good mineral source for growing *A. vinelandii* cells, and the elevated COD levels were adjusted to appropriate levels using glucose. Distillery spent wash has already been used for the production of xylanase enzyme from *Burkholderia* spp.[35] and laccase enzyme from *Aspergillus heteromorphus*.[36]

The maximum enhancement in terms of PHB production has been observed at 4% spent wash which may be due to an optimal dilution which supplied the minerals required by *A. vinelandii*. The glucose served as a carbon and energy source for the recipient microorganisms. The ammonium acetate acted as the best nitrogen source and promoted cell growth and PHB production[29] unlike the ammonium sulphate that resulted in slow PHB accumulation. The staining with Sudan Black showed characteristic black colored PHB granules against a pink background, plus PHB was detected spectrophotometrically.

Conclusion

The experimental results demonstrated here are of great importance for developing an economically efficient PHB production pathway. Cheap carbon sources like molasses, hydrolyzed starch, and cellulose have already been identified, but no one has provided a reliable supply of the minerals which are needed for industrial production. In this study, DSW has been shown to be a base for PHB production, and it gives a regular and optimum supply of minerals when it is supplemented with glucose and ammonium acetate. Since spent wash has elevated mineral content and COD, it was accurately diluted and optimized before being used for PHB production. The DSW served two purposes. First, it provided a good combination of minerals and metal ions to *A. vinelandii* so that the elevated production of PHB was achieved. Second, the elevated COD values of spent wash, which is an environmental pollutant, were decreased to permitted values. Therefore the results of the present study could be valuable in improving the economy of PHB production and reducing ecological pollution caused by DSW. The economy of PHB production may be further improved by the combined use of molasses and DSW as the carbon and mineral source. Molasses, like black strap, beet, and cane are waste generated by the sugar industry, and are a cheap carbon source. Similarly, the DSW is a distillery waste and ecological pollutant. Therefore, the greatest cost of PHB production could be controlled by further study with these two waste products.

REFERENCES

[1] Muller HM, Seebach D. Poly(hydroxyalkanoates): a fifth class of physiologically important organic biopolymers. *Angewandte Chemie*, 1993; 32: 477–502.

[2] Madison LL, Huisman GW. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol Mol Biol Rev*, 1999; 63: 21–53.

[3] Potter M, Steinbüchel A. Biogenesis and structure of polyhydroxyalkanoate granules. In *JM. Shively (ed), Inclusions in prokaryotes*, 2006; 1: 109–136. Springer-Verlag, Berlin, Germany.

[4] Stubbe J, Tian J, He A, Sinskey AJ, Lawrence AG, Liu P. Non-template-dependent polymerization processes: Polyhydroxyalkanoate synthases as a paradigm. *Annu Rev Biochem*, 2005; 74: 433–480.

[5] Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial uses of microbial polyhydroxyalkanoates. *Microbiol Rev*, 1990; 54: 450–472.

[6] Brandl H, Gross RA, Lenz RW, Fuller RC. Plastics from microbe and for microbe: poly(beta-hydroxyalkanoates) as natural, biocompatible, and ecological polyesters. *Adv Biochem Eng Biotechnol*, 1990; 41: 77–93.

[7] Hyoung JC, Jung H, Sung H. Mechanical spectroscopy study on ecological synthetic and biosynthetic aliphatic polyesters. *Park 2 Macromolecular Symposia*, 1997; 119 (1): 149–155.

[8] Sombatmankhong K, Sanchavanakit N, Pavasant P, Supaphol P. Bone scaffolds from electrospun fiber mats of poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-cohydroxy valerate) and their blend. *Polymer,* 2007; 48: 1419–1427.

[9] Maurizio A, Martuscelli A. Poly-d-(-) (3-hydroxy butyrate)/poly (ethylene oxide) blends: phase diagram, thermal and crystallization behaviour. Polymer, 2003: 1731–1737.

[10] Shimamura E, Kasuya K, Kobayashi G, Shiotani T, Shima Y, Doi Y. Physical-properties and biodegradability of microbial poly(3-hydroxybutyrate-co-3-hydroxyhexanoate. *Macromolec*, 1994; 27: 878–880.

[11] Schmack G, Gliesche K, Nitsche M, Werner C. Implantate auf Basis von poly(3-hydroxybuttersäure). *Biomaterialien*, 2002; 3; 21–25.

[12] Barud HS, Souza JL, Santos DB, Crespi MS, Ribeiro CA, Messaddeq Y, Ribeiro SJL. Microbial cellulose/poly(3-hydroxybutyrate) composite membranes. *Carbohydrate Polymers,* 2011; (83): 1279–1284.

[14] Jeong HK, Rooney M, David DJ, McKnight WJ, Karasz FE, Kajiyama T. Miscibility and characterization of the ternary crystalline system: poly(vinyl butyral)/poly(vinyl alcohol)/nylon6. *Polymer*, 2000; 41 (17): 6671–6678.

[15] Pawan GLS, Semple SJG. Effect of 3-hydroxybutyrate in obese subjects on very low energy diets and during therapeutic starvation. *Lancet*, 1983: 15–17.

[16] Karahaliloğlu Z, Demirbilek M, Mesut Ş, Melike ED, Sağlam N, Denkbaş EB. Plasma polymerization modified bacterial polyhydroxybutyrate nano fibrillar scaffolds. *Journal of Applied Polymer Science*, 2012; DOI: 10.1002/app. 38370, in press.

[17] Khanna S, Srivastava AK. Recent advances in microbial polyhydroxyalcanoates. *Proc Biochem*, 2005: 40; 607–619.

[18] Keshavarz T, Roy I. Polyhydroxyalkanoates: bioplastics with a green agenda. *Curr Opin Microbiol*, 2010; 13: 321–326.

[19] Dias M, Cecilia M, Antunes M, Santos AR, Isabel MF. Blends of poly(3-hydroxybutyrate) and poly(p-dioxanone): miscibility, thermal stability and biocompatibility. *Journal of Materials Science: Materials in Medicine*, 2008; 19 (12): 3535–3544.

[20] Stevenson LH, Socolofsky MD. Cyst formation and poly-β-hydroxybutyric acid accumulation in *Azotobacter*. *J Bacteriol*, 1966; 91: 304–310.

[21] Reusch RN, Sadoff HL. Lipid metabolism during encystment of *Azotobacter vinelandii*. *J Bacteriol*, 1981; 145: 889–895.

[22] Martínez P, Guzmán J, Espín G. A mutation impairing alginate production increased accumulation of poly-β-hydroxybutyrate in *Azotobacter vinelandii*. *Biotechnol Lett*, 1997; 19: 909–912.

[23] Segura D, Guzmán J, Espín G. *Azotobacter vinelandii* mutants that overproduce poly-β-hydroxybutyrate or alginate. *Appl Microbiol Biotechnol*, 2003; 63: 159–163.

[24] Lee SY. Poly(3-hydroxybutyrate) production from xylose by recombinant *Escherichia coli*. *Bioprocess Eng*, 1998: 18; 397–399.

[25] Reinecke F, Steinbüchel A. *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. *J Mol Microbiol Biotechnol*, 2009: 16: 91–108.

[26] Hernandez EA, Moreno S, Castellanos M, Castañeda M, Nuñez C, Espín G. RsmA post-transcriptionally controls PhbR expression and polyhydroxybutyrate biosynthesis in *Azotobacter vinelandii. Microbiology,* 2012; 158 (8): 1953–63.

[27] Page W, Cornish A. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly-β-hydroxybutyrate. Appl Environ Microbiol, 1993; 59: 4236–4244.

[28] http://www.nariphaltan.virtualave.net/detox.pdf.

[29] Page W, Knosp O. Hyperproduction of poly hydroxybutyrate during exponential growth of Azotobacter vinelandii UWD. *Appl Environ Microbiol*, 1989; 55: 1334–1339.

[30] APHA and AWWA. *Standard techniques for the examination of water and desecrate water*. 1995, 15th ed, Washington, DC, USA.

[31] Law JH, Slepecky RA. Assay of poly-beta-hydroxybutyric acid. *J Bacteriol*, 1961; Jul; 82:33–36.

[32] American Public Health Association (APHA), http://www.apha.org/programs/environment/.

[33] Kaewkanetra P, Imai T. Biopolymer (polyhydroxyalkanoates: PHAs) production from *Alcaligenes eutrophus* TISTR 1095 and mixed microbial sludge (MMS). *Thai Journal Biotechnology*, 2008; 8 (11):1–4.

[34] Page WJ, Sadoff HL. Physiological factors affecting transformation of *Azotobacter vinelandii*. *J Bacteriol*, 1976; 125: 1080–1087.

[35] Mohana S, Shah A, Divecha J, Madamwar D. Xylanase production by *Burkholderia* spp. DMAX strain under solid strain fermentation using distillery spent wash. *Bioresource Technology*, 2008; 99 (16): 7553–7564.

[36] Singh A, Bajar S, Bishnoi NR, Singh N. Laccase production by *Aspergillus heteromorphus* using distillery spent wash and lignocellulosic biomass. *Journal of Hazardous Materials*, 2010; 176 (1–3): 1079–1082.