



Bioconversion of sugarcane molasses to poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by endophytic *Bacillus cereus* RCL 02

Rituparna Das¹, Arundhati Pal², Amal Kanti Paul^{1*}

¹Microbiology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India.

²Post Graduate Department of Botany, Serampore College, 9, William Carey Road, Serampore, Hooghly 712201, West Bengal, India.

ARTICLE INFO

Article history:

Received on: May 30, 2018

Accepted on: June 27, 2018

Available online: March 15, 2019

Key words:

Bacillus cereus, endophytic bacteria, polyhydroxyalkanoates, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), sugarcane molasses.

ABSTRACT

Sugarcane molasses, the by-product of sugar industry are rich in nutrients, growth factors and minerals and are efficiently utilized by the microorganisms for growth and production of primary as well as secondary metabolites of commercial importance. In this communication, we report the utilization of sugarcane molasses as the sole source of carbon for the production of copolymers of polyhydroxyalkanoates (PHAs) with improved material properties. The endophytic bacterium *Bacillus cereus* RCL 02 (MCC 3436) produced 7.8 g/L of PHA when grown in MS medium with 4% (w/v) sugarcane molasses under batch cultivation. Proton nuclear magnetic resonance (¹H NMR) analysis revealed that the copolymer so produced contain 12.4 mol% 3-hydroxyvalerate (3HV) along with 87.6 mol% 3-hydroxybutyrate (3HB). The copolyester, P(3HB-co-12.4 mol%-3HV) has been isolated and purified following standard solvent extraction method and partially characterized by Fourier-transform infrared (FTIR), X-ray diffraction (XRD), thermogravimetric (TG) and differential scanning calorimetric (DSC) analysis. The material and thermal properties of the copolyester so produced indicated its potential for industrial application.

1. INTRODUCTION

Production of copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate [P(3HB-co-3HV)], one of the most common representatives of polyhydroxyalkanoates (PHAs) by a wide diversity of microorganisms have attracted significant attention for its improved material properties. These microbial polyester exhibits low crystallinity, increased impact strength and flexibility in addition to its biodegradability and biocompatibility [1]. However, despite its huge potential for eco-benign applications, it could not gain substantial consideration, so far, due to its low yield and expensive production cost. To circumvent these limitations, efforts are being made to find newer high-yielding microbial strains, relatively cheaper substrates for production and development of low-cost as well as easier down-stream processing strategies. Since, the carbon feedstock used in fermentation accounts for more than 50% of production cost [2], development of an optimized up-stream bioprocessing utilizing inexpensive and renewable carbon sources has become an important issue. Exploitation of agro-industrial residues and industrial wastes are beneficial options from ecological as well as economic perspectives [3].

Sugarcane molasses, the nutrient and mineral rich by-product of the sugar industry is inexpensive, available in abundance and has been exploited for the production of wide variety of industrial products including the biopolyester PHAs [4,5]. Beside sucrose, the molasses also contain other sugars, vitamins, and variety of minerals which promote microbial growth. Utilization of this sugar refinery by-product as a substrate for PHA production was initially reported by Beaulieu *et al.* [4] with the strain *Alcaligenes eutrophus*. Following this, a variety of microbial species such as *Bacillus* sp. [6], *Bacillus megaterium* [5,7], *Bacillus cereus* [8], *Pseudomonas aeruginosa* [9] and recombinant *Escherichia coli* [10] have been reported to produce homopolymers of 3-hydroxybutyrate [P(3HB)] from molasses. A mixed microbial culture also produced PHA containing 3-hydroxybutyrate (3HB), 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxyvalerate (3HV), 3-hydroxy-2-methylvalerate (3H2MV) and 3-hydroxyhexanoate (3HHx) when sugarcane molasses was supplemented with synthetic feeds containing volatile fatty acids (VFAs) in the fermentation media [11]. Recently, Zafar *et al.* [12] has documented P(3HB-co-3HV) accumulation by *Azohydromonas lata* in propionic acid supplemented molasses medium. These reports focussed primarily on the process optimization for PHA production using sugarcane molasses with very little emphasis on the material properties of the accumulated biopolyesters.

Bacillus cereus RCL 02, an endophytic bacterium of oleaginous plant *Ricinus communis* L. has already been reported to accumulate P(3HB)

*Corresponding Author

Amal Kanti Paul, Microbiology Laboratory, Department of Botany, University of Calcutta, Kolkata 700 019.

E-mail: amalk_paul@yahoo.co.in

in mineral salts medium containing glucose as the sole source of carbon [13]. The present study demonstrates the bioprocessing as well as partial characterization of P(3HB-co-3HV) copolyesters produced by the endophytic isolate RCL 02 following bioconversion of sugarcane molasses as the sole source of carbon in the growth medium.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Maintenance

Bacillus cereus RCL 02 (MCC 3436; GenBank accession no. KX458035), a leaf endophyte of oil-yielding plant *R. communis* L. was isolated in this laboratory and used throughout this study. The culture was grown on slopes of tryptic soy agar by regular sub-culturing.

2.2. Growth and Polymer Production

Microbial growth and polymer production by the endophytic isolate RCL 02 were evaluated in modified mineral salts (MS) medium [14] under batch cultivation. The MS medium contained (g/L) $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 6.7; KH_2PO_4 , 2.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 (pH-7.2). The commercially available sugarcane molasses was separately sterilized and added to the medium as the sole source of carbon. The medium (25 per 100 mL Erlenmeyer flask) was inoculated with a freshly prepared inoculum of *B. cereus* RCL 02 and incubated at 32 °C on a rotary shaker at 120 rpm.

2.3. Measurement of Growth

Growth was determined by measuring the dry weight of the biomass. Cells were harvested by centrifugation (10,000 rpm × 10 min) after a definite period of incubation, washed thoroughly with deionized water as well as acetone and the biomass was determined after drying to a constant weight at 80 °C.

2.4. Extraction and Purification of the Polymer

The polymer accumulated in the dried cell mass was extracted thrice in warm chloroform (45-50 °C), filtered through glass wool, concentrated and precipitated with double volumes of pre-chilled diethyl ether [14]. The precipitated polymer was recovered by centrifugation (12,000 rpm × 12 min) and dried to obtain the powdered polymer.

2.5. Assay of the Polymer

The extracted polymer was converted to crotonic acid following treatment with concentrated H_2SO_4 in a boiling water bath for 10 min [15], cooled to room temperature and the absorbance was recorded at 235 nm in a UV-VIS spectrophotometer (Jenway, Model 6505) using authentic P(3HB) from Sigma, USA as standard. The total PHA content of the dried cell mass was quantified gravimetrically.

2.6. Preparation of the Polymer Films

Films of PHA were prepared by casting 2% (w/v) chloroform solution of the polymer onto a glass Petriplate, following slow evaporation of the solvent under regular humidity at 50 °C. The remaining solvent was evaporated for two days.

2.7. Characterization of the Polymer

2.7.1. ^1H NMR spectroscopic analysis

The monomer composition of the purified polymer was elucidated from proton nuclear magnetic resonance (^1H NMR) spectroscopic analysis. The polymer dissolved in deuterated chloroform (CDCl_3)

were analyzed in a Bruker AV300 Supercon NMR (at 300 MHz) spectrophotometer. A multinucleate probe head at 30-degree flip angle was used. The chemical shift-scale was in parts per million and tetramethylsilane (Me_4Si) was used as the internal standard.

2.7.2. Fourier-transform infrared spectroscopic analysis

For FTIR spectroscopy, the pellet was prepared by mixing the purified polymer and KBr and scanned in Bruker FTIR spectrophotometer in the range of 4000 to 400 cm^{-1} .

2.7.3 X-ray diffraction analysis

The wide angle X-ray diffractograms of the purified polymer sheets were recorded in X'pert PRO PANalytical X-ray diffractometer. The nickel filtered $\text{CuK}\alpha$ radiation ($\lambda = 0.154$ nm, 40 kV, 30 mA) was used at room temperature. The scan speed was $2\theta = 5^\circ/\text{min}$.

2.7.4. Thermogravimetric and differential scanning calorimetric analysis

Thermal characterization was done with TA STDQ 600 thermogravimetric analyzer and Perkin-Elmer Diamond DSC thermal analyzer operating with nitrogen flow of 100 cc/min and a heating rate of 10 °C/min. While the PHA sample (10 mg) was heated from room temperature to 500 °C in an alumina crucible under dinitrogen atmosphere in TGA analysis, the scanning temperature in DSC ranged from -50 to 200 °C.

3. RESULTS AND DISCUSSION

3.1. Growth Kinetics and Accumulation of PHA

The endophytic bacterium *B. cereus* RCL 02 accumulated PHA during growth in molasses medium and maximum intracellular polyester accumulation [85.4% of cell dry weight (CDW)] occurred after 48 h of growth under shake-flask condition. Compositional analysis of the PHAs isolated from *B. cereus* RCL 02 following gravimetric as well as proton nuclear magnetic resonance (^1H NMR) spectral analysis revealed that the addition of sugarcane molasses (2%, w/v) in the growth medium resulted in production of copolymers of 3HB and 3HV [P(3HB-co-3HV)]. It was also evident that during the early stages of growth and polyester accumulation, there was no incorporation of 3HV monomers in the polymer, but the 3HV monomer fraction in PHA increased to 5.1 mol% at the highest polyester accumulation phase. Biosynthesis of P(3HB-co-3HV) was associated with a decline in pH of the growth medium (pH 5.2 after 96 h) which may possibly be due to the production of several metabolic intermediates during growth [16]. In addition, cells of *B. cereus* RCL 02 started sporulating in late stationary phase and about 78% of cells showed matured endospores at the end of 96 h of incubation (Figure 1). Such delayed sporogenesis, therefore, could not interfere in the biosynthesis and accumulation of the intracellular polymer during the exponential to early stationary phases of growth. Concomitant with the increase in the frequency of sporulation, there was an initiation of decline in the PHA content of the cells from the late stationary phase of growth. Such decline in intracellular PHA may be due to the possible utilization of the accumulated polyesters as carbon and energy sources for the process of sporogenesis.

The present study clearly indicated that the efficiency of utilizing sugarcane molasses for PHA production by the endophytic bacterium *B. cereus* RCL 02 was much better as compared to the purified sugars viz. glucose, sucrose, maltose etc. [13]. It may also be presumed that

molasses served not only as a potential source of carbon but also provided vitamins and other essential growth factors for effective growth and sustainable production of the biopolyester by the isolate RCL 02. Survey of literature pertaining to the PHA production utilizing sugarcane molasses clearly indicated that most of the bacterial strains were explored mainly for synthesis and accumulation of the homopolyester, P(3HB) [4-10,17]. While, supplementation of different volatile fatty acids [11] and propionic acid [12] along with sugarcane molasses have led to the production of PHA copolymers, here we report the accumulation of P(3HB-co-3HV) utilizing molasses as the sole source of carbon by the endophytic bacterium *B. cereus* RCL 02. Production of intermediate acids during growth and polymer accumulation by RCL 02 in molasses medium might contribute to the incorporation of 3HV monomers in the polymer chain.

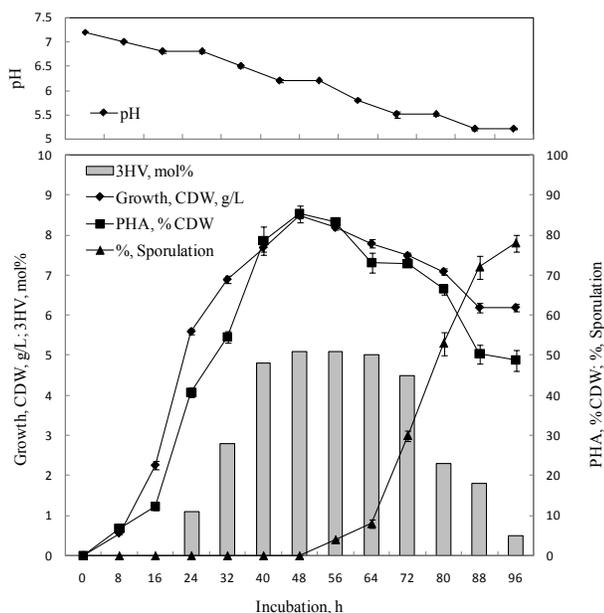


Figure 1: Time course of growth, PHA production, sporulation, changes in pH of the medium and 3HV monomer incorporation in the copolymer produced by the endophytic isolate *B. cereus* RCL 02.

3.2. Effect of Molasses Concentration

Influence of different concentration of sugarcane molasses on growth, production of the copolymer and 3HV monomer (mol%) incorporation was elucidated. Addition of molasses at 4% (w/v) level in the growth medium showed maximum production of P(3HB-co-3HV) (7.8 g/L) with the incorporation of 12.4 mol% 3HV monomers (Table 1). However, the PHA content of the cell mass (83.5%, CDW) was lower than those grown in 3% (w/v) molasses. Further increase in molasses concentration showed a negative effect on biomass formation, polymer accumulation as well as 3HV monomer incorporation in the copolymer and corroborated well with the findings of Zafar *et al.* [12].

3.3. Characterization of the Polymer

The copolymer so produced with 12.4 mol% 3HV was isolated and purified from the cell mass and partially characterized.

3.3.1. ¹H NMR spectroscopic analysis

The ¹H NMR spectrum (at 300 MHz) (Figure 2) of the copolyester displayed chemical shifts (δ) at 1.26 and 0.89 ppm indicating the

presence of methyl (CH₃) group of 3HB (87.6 mol%) and 3HV (12.4 mol%) monomer units, respectively. Moreover, the characteristic resonance absorption at 2.4-2.6 and 5.2 ppm were assignable to the methylene group (CH₂) and methyne group (CH), respectively. The characteristic signals were consistent with the previous findings of Masood *et al.* [18].

Table 1: Effect of different concentrations of sugarcane molasses on growth and production of P(3HB-co-3HV) copolyester by the endophytic *B. cereus* RCL 02.

Sugarcane molasses, % (w/v)	Growth, CDW, g/L	PHA, % CDW	PHA, g/L	PHA composition, mol%	
				3HB	3HV
1	7.7 ± 0.03	50.6 ± 1.4	3.9 ± 0.01	98.8	1.2
2	8.5 ± 0.02	85.4 ± 0.4	7.3 ± 0.02	94.9	5.1
3	8.5 ± 0.04	86.5 ± 0.5	7.4 ± 0.01	91.1	8.9
4	9.4 ± 0.02	83.5 ± 1.5	7.8 ± 0.02	87.6	12.4
5	8.9 ± 0.09	71.4 ± 1.2	6.4 ± 0.03	88.2	11.8
6	8.4 ± 0.04	60.4 ± 3.1	5.0 ± 0.05	90.7	9.3

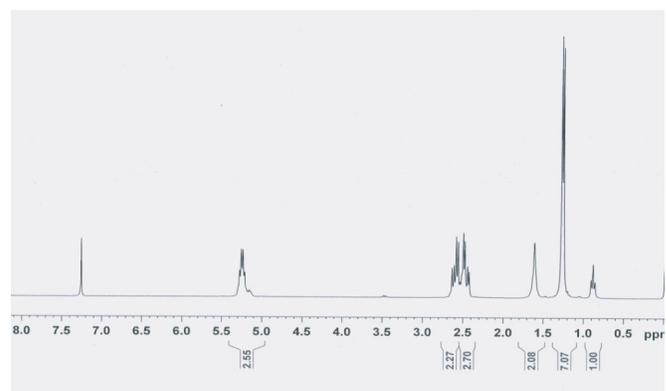


Figure 2: ¹H NMR spectra of purified copolyester P(3HB-co-3HV) produced by the endophytic isolate *B. cereus* RCL 02.

3.3.2. Fourier-transform infrared spectroscopic analysis

The FTIR spectra (Figure 3) of the copolyester showed absorption bands at 1727 cm⁻¹ and 1278 cm⁻¹ corresponding to ester C=O and C-O groups, respectively. The other characteristic bands at 3439 cm⁻¹, 2933-2979 cm⁻¹ and 1225-1382 cm⁻¹ represented the -OH bending, C-H stretching and -CH group, respectively. These absorption peaks showed close proximity with the peaks of P(3HB-co-10 mol% 3HV) from non-endophytic *B. cereus* SPV [19] and provided supportive evidence in favor of the accumulation of similar copolyester by the endophyte *B. cereus* RCL 02.

3.3.3. X-ray diffraction analysis

The wide angle X-ray diffractograms of the purified copolymer were represented as peak positions at 2 θ and X-ray counts (intensity) in a xy plot (Figure 4). The sharp and intense peak at 13° was attributed to high crystallinity. The XRD pattern of the copolymer was consistent when compared with previous crystallographic studies of P(3HB-co-3HV) [20].

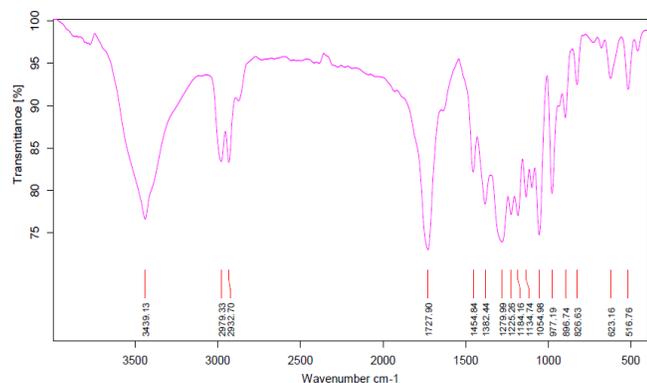


Figure 3: FTIR spectra of purified copolyester P(3HB-co-3HV) produced by the endophytic isolate *B. cereus* RCL 02.

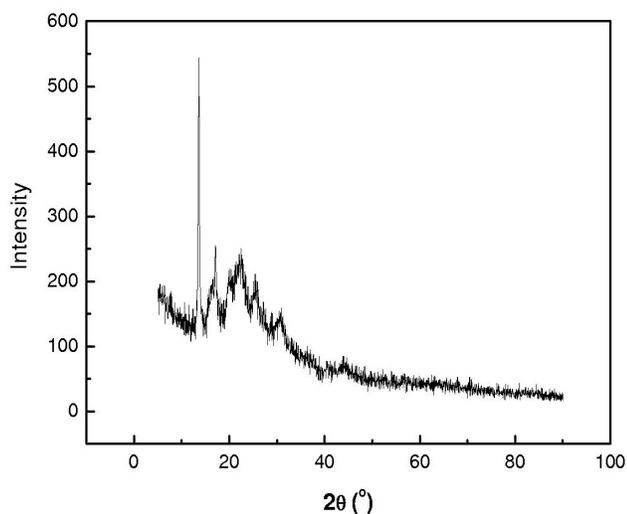


Figure 4: X-ray diffraction pattern of purified copolyester P(3HB-co-3HV) produced by the endophytic isolate *B. cereus* RCL 02.

3.3.4. Thermogravimetric analysis

Thermogravimetric analysis (TGA) of P(3HB-co-3HV) from *B. cereus* RCL 02 showed that the major weight loss occurred between 230 to 300 °C as a single exotherm (Figure 5). The weight loss at the temperature <150 °C observed in the thermogram was due to loss of components like water, low molecular weight solvents or gas desorption [8]. The degradation temperature of the copolymer (in terms of 50% weight loss) was recorded at 263.14 °C and thus indicated its thermostability. The degradation was probably associated with ester cleavage of the copolyester chain by the β -elimination reaction.

3.3.5. Differential scanning calorimetry

Determination of melting behavior of the polymer is crucial for its processing. Differential scanning calorimetry (DSC) study of the isolated and purified P(3HB-co-3HV) showed a melting temperature (T_m) of 169.72 °C and enthalpy of fusion (ΔH_m) of 63.98 J/g (Figure 6). The values of T_m , as well as ΔH_m , were consistent with findings of You *et al.* [21]. Incorporation of 3HV monomers into the 3HB polymer chain usually decreases the T_m as well as ΔH_m of the copolymer matrix and thus reduces its crystallinity [22].

4. CONCLUSION

Novel bacterial strains with high production efficiency and cost-effective substrates are always essential for potential industrial usage to enhance process economy. The present study revealed that sugarcane molasses, an industrial by-product of sugar industries were efficiently utilized by the endophytic bacterial isolate *B. cereus* RCL 02 for the production of copolyesters of 3HB and 3HV. Supplementation of 4% (w/v) molasses as the sole carbon source in MS medium incorporated 12.4 mol% 3HV in the P(3HB-co-3HV) copolyester chain indicating its potential industrial application.

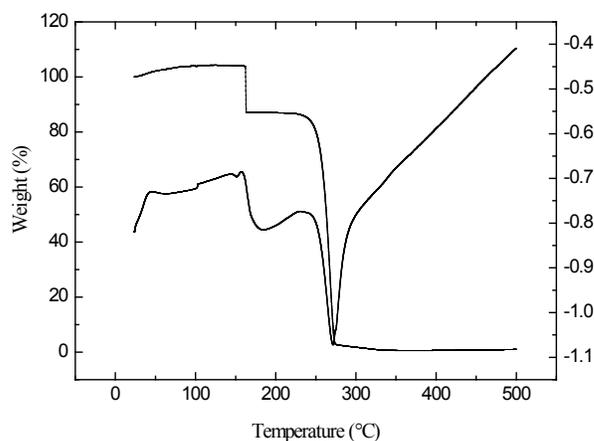


Figure 5: Thermogravimetric analysis of purified copolyester P(3HB-co-3HV) produced by the endophytic isolate *B. cereus* RCL 02.

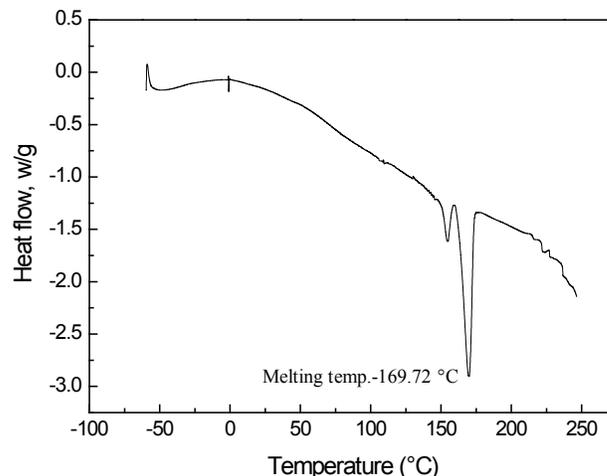


Figure 6: DSC curve showing melting temperature (T_m) of purified copolyester P(3HB-co-3HV) produced by the endophytic isolate *B. cereus* RCL 02.

5. ACKNOWLEDGMENTS

Financial support from Department of Science and Technology, New Delhi, India in the form of INSPIRE Fellowship (Sanction No. DST/INSPIRE Fellowship/REL5/3013/C1) to Rituparna Das is duly acknowledged.

6. REFERENCES

1. Anjum A, Zuber M, Zia KM, Noreen A, Anjum MN, Tabasum S.

- Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of recent advancements. *International Journal of Biological Macromolecules* 2016; 89:161-174.
2. Sindhu R, Silviya N, Binod P, Pandey A. Pentose-rich hydrolysate from acid pretreated rice straw as a carbon source for the production of poly-3-hydroxybutyrate. *Biochemical Engineering Journal* 2013; 78:67-72.
 3. Koller M, Atlıć A, Dias M, Reiterer A, Braunegg G. Microbial PHA production from waste raw materials. In: *Microbiology monographs*, 2010, p. 85-119.
 4. Beaulieu M, Beaulieu Y, Melinard J, Pandian S, Goulet J. Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. *Applied and Environmental Microbiology* 1995; 61(1):165-169.
 5. Gouda MK, Swellam AE, Omar SH. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. *Microbiological Research* 2001; 156(3):201-207.
 6. Wu Q, Huang H, Hu G, Chen J, Ho KP, Chen GQ. Production of poly-3-hydroxybutyrate by *Bacillus* sp. JMa5 cultivated in molasses media. *Antonie van Leeuwenhoek* 2001; 80(2):111-118.
 7. Chaijamrus S, Udpuay N. Production and characterization of polyhydroxybutyrate from molasses and corn steep liquor produced by *Bacillus megaterium* ATCC 6748. *Agricultural Engineering International: CIGR Ejournal*. Manuscript FP 07 030, May, 2008, Vol. X: 1-12.
 8. Sharma P, Bajaj BK. Production and characterization of poly-3-hydroxybutyrate from *Bacillus cereus* PS 10. *International Journal of Biological Macromolecules* 2015; 81:241-248.
 9. Tripathi AD, Yadav A, Jha A, Srivastava SK. Utilizing of sugar refinery waste (cane molasses) for production of bio-plastic under submerged fermentation process. *Journal of Polymers and the Environment* 2012; 20(2):446-453.
 10. Liu F, Li W, Ridgway D, Gu T, Shen Z. Production of poly-β-hydroxybutyrate on molasses by recombinant *Escherichia coli*. *Biotechnology Letters* 1998; 20(4):345-348.
 11. Bengtsson S, Pisco AR, Johansson P, Lemos PC, Reis MAM. Molecular weight and thermal properties of polyhydroxyalkanoates produced from fermented sugar molasses by open mixed cultures. *Journal of Biotechnology* 2010; 147(3-4):172-179.
 12. Zafar M, Kumar S, Dhiman AK. Artificial intelligence based modeling and optimization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production process by using *Azohydromonas lata* MTCC 2311 from cane molasses supplemented with volatile fatty acids: a genetic algorithm paradigm. *Bioresource Technology* 2012; 104:631-641.
 13. Das R, Pal A, Paul AK. Production of biopolyester poly(3-hydroxybutyrate) by *Bacillus cereus* RCL 02, a leaf endophyte of *Ricinus communis* L. *Journal of Microbiology and Biotechnology Research* 2017; 7(4):32-41.
 14. Ramsay BA, Lomaliza K, Chavarie C, Dube B, Bataille P, Ramsay JA. Production of poly-(β-hydroxybutyric-co-β-valeric) acids. *Applied and Environmental Microbiology* 1990; 56(7):2093-2098.
 15. Law JH, Slepecky RA. Assay of polyhydroxybutyric acid. *Journal of Bacteriology* 1961; 82(1):32-36.
 16. Benoit TG, Wilson GR, Baugh CL. Fermentation during growth and sporulation of *Bacillus thuringiensis* HD-1. *Letters in Applied Microbiology* 1990; 10(1):15-18.
 17. Kumar BS, Prabhakaran G. Production of PHB (bioplastic) using bio-effluent as substrate by *Alcaligenes eutrophus*. *Indian Journal of Biotechnology* 2006; 5:76-79.
 18. Masood F, Hasan F, Ahmed S, Chen P, Hameed A. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from *Bacillus cereus* S10. *Journal of Polymers and the Environment* 2012; 20(3):865-871.
 19. Valappil SP, Rai R, Bucke C, Roy I. Polyhydroxyalkanoate biosynthesis in *Bacillus cereus* SPV under varied limiting conditions and an insight into the biosynthetic genes involved. *Journal of Applied Microbiology* 2008; 104(6):1624-1635.
 20. Sato H, Ando Y, Mitomo H, Ozaki Y. Infrared spectroscopy and X-ray diffraction studies of thermal behavior and lamella structures of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(HB-co-HV)) with PHB-type crystal structure and PHV-type crystal structure. *Macromolecules* 2011; 44(8):2829-2837.
 21. You JW, Chiu HJ, Shu WJ, Don TM. Influence of hydroxyvalerate content on the crystallization kinetics of poly(hydroxybutyrate-co-hydroxyvalerate). *Journal of Polymer Research* 2003; 10(1):47-54.
 22. Chen LJ, Wang M. Production and evaluation of biodegradable composites based on PHB-PHV copolymer. *Biomaterials* 2002; 23(13):2631-2639.

How to cite this article:

Das R, Pal A, Paul AK. Bioconversion of sugarcane molasses to poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by endophytic *Bacillus cereus* RCL 02. *J App Biol Biotech*. 2019;7(2)20-24.