International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 05-09-2021, Accepted: 22-09-2021, Published: 06-10-2021
Volume 6, Issue 5, 2021, Page No. 1021-1026

In vitro studies on characterization of polyhydroxybutyrate

R Rameshwari, P Ilamthy, R Uma Maheswari, M Keerthiga
Assistant Professor, Department of Biotechnology, Cauvery College for Women (Autonomous), Affiliated to Bharathidasan University, Trichy, Tamil Nadu, India

Abstract
The bacterial polyesters may be considered as "Green Plastics", because of their biodegradable nature. These polyesters can be employed for packaging and coating materials/as biodegradable carriers and applied in biomedical field. Twelve bacterial isolates were isolated from dairy industry effluent sample. Screening for PHB was done by Sudan black staining. PHB extraction was carried out by chloroform digestion method. Biochemical and 16s rRNA analysis showed that PHB producing bacteria belong to *Bacillus* genera with Maximum production of PHB was analyzed by U.V spectrophotometer and finally it was characterized by FTIR, NMR and GC MS.

Keywords: biopolymer, poly hydroxyl butyrate (PHB), dairy industry effluent

Introduction
Industrialization not only responsible for economic development, but the release of hazardous substances, heavily affect the environment. The health of an ecosystem, flora, fauna is largely disturbed by industrial pollutants. In recent days, people are more interested in the treatment of industrial waste, if left untreated harmfully affect the soil and water ecosystem (Chhonkar *et al.,* 2000) [1]. Nowadays the number of physicochemical techniques is extensively studied by a number of researchers (Rodrigues *et al.,* 2008) [2]. Treatment of effluent through the use of microorganismslargely remove the sludge particles, the removal of sludge is more through the aerobic system than anaerobic system (Verheigen *et al.,* 1996) it also reduces the COD value. The ecosystem is greatly affected by the discharge of dairy wastewater with a high pollution load (Ganapathy *et al.,* 2011) [4]. Due to the presence of large amounts of nutrients indairy waste water, cause eutrophication in receiving water bodies (Kushwaha *et al.,* 2011) [5, 29]. Bioremediation process converts the complex toxic substances into simple organic substances, with the commonly available indigenous microbiota (Perelo *et al.,* 2010) [6]. The treatment of effluent using biological organisms is the commonly accepted method (Porwal *et al.,* 2015) [7]. The efficiency or rate of biodegradation can be increased by the use of consortium of microorganism (Semrany *et al.,* 2012; Karamalidis *et al.,* 2010) [8, 9]. The need for plastic is increasing day by day due to population explosion, and the petrochemical plastic is used in many applications, but at the same time itreleases more toxic gases during the degradation. Plastic Posses more advantages due to its easy availability and durability. One of the major drawbacks in synthetic plastic was the nonbiodegradability (Atlas, 1998; Mueller, 2006; Tokiwa and Calabia, 2004) [10, 11, 12]. Polyhydorxybutyarte is a homopolymer of polyhydroxyalkanoate, which is synthesized by bacteria under nutrient limiting conditions. Biolpolymers are biodegradable in nature which are completely oxidized into CO_2 and H_2O by PHA hydrolases and PHA depolymerases (Jendrossek, 2007; Chen *et al.,* 2009) [13, 14]. Renewable sources are the precursor for biopolymer production (Braunegg *et al.,* 2007; Mohanty *et al.,* 2002; Braunegg *et al.,* 1998) [15, 16, 17]. This research work mainly studied the PHB producing strains, extraction and chacterization of PHB.

Materials Required
The chemicals used for preparation of reagent, solutions and microbiological growth media were purchased from Hi-media Laboratories Pvt Ltd, Mumbai, India. Solvents used in the studies were of AR grade and were purchased from Merck Pvt Ltd. *Bacillus cereus* showing appreciable PHB production was isolated from dairy industry effluent sample. The bacterium was identified based on their biochemical and molecular characterization. The PCR amplification and DNA sequencing of the 16S rRNA gene fragment of the bacterial strains were carried out by isolating genomic DNA from the pure culture pellet using 27F forward primer and 1492 reverse primer, the gene fragment was amplified using MJ Research Peltier thermal cycler. The PCR product was sequenced bidirectionally using the 785 forward and 907 reverse primer. The sequence data was aligned and analysed to identify the bacterium and its closest neighbours (Woese CR, 1987; Garrity G, 2005) [18, 19].
Bacillus cereus colonies initially maintained in nutrient agar medium and were selected and sub cultured on minimal agar medium for PHB production (Schut *et al*., 1993) [20]. Sudan black B staining was used to screen the bacterial isolates for PHB production (Arun *et al.,* 2009) [21]. For quantitative screening, PHB was extracted from bacterial isolate by solvent extraction method (Ibrahim and Steinbuchel, 2009) and the study was proceeded upto 4 days to quantify the production of PHB. The produced PHB was weighed and the percentage was determined by Hungund 2013) [23].

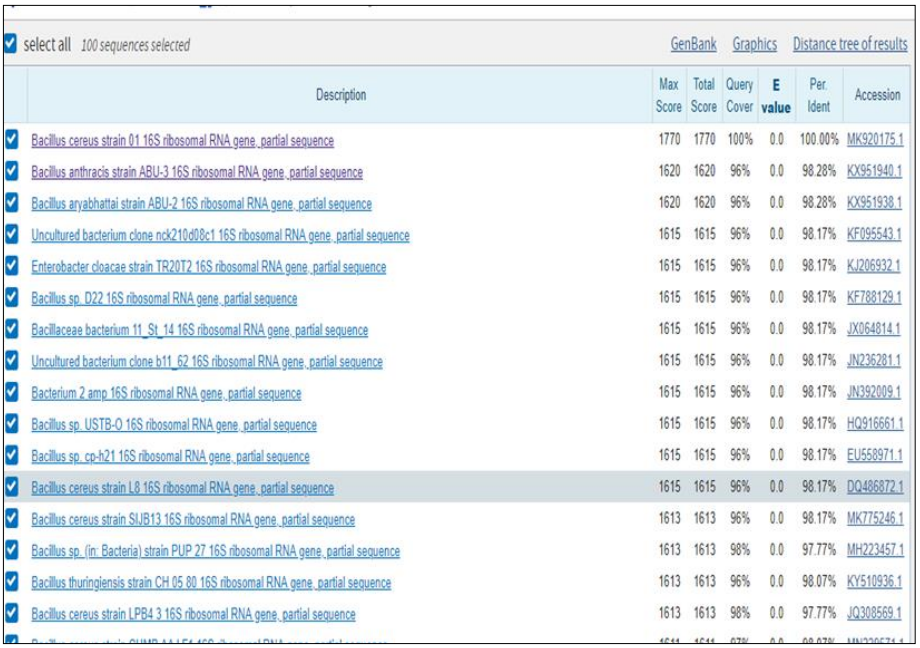
The PHB extracted from the bacterial isolate *Bacillus cereus* by sodium hypochlorite method and was analysed by FT IR spectroscopy. The FT IR spectrum of the PHB was obtained under the spectral range 400 – 4000cm^{-1}. ^{1}H NMR spectra was acquired by dissolving the primer in deuteron chloroform ($CDCl_3$) as a concentration of 32mg/ml and analysed on AV300 spectrometer at 300K with 9.65 ms pulse width, 2 sec pulse repetition. H^{1} NMR spectrum was recorded at 6172.8 HZ spectral width at 24^{0}c using Brucker spectrometer. GC MS analysis of the sample was carried out after methanolysis of PHB (Lee and Choi, 1997) [25]. For methanolysis of PHB, polymer sample was suspended in 1 ml chloroform and 1 ml methanol containing 2.8 M H_2SO_4 in a screw capped tube, and then incubated at 100^{0}c for 2 hours. After cooling, 0.5 ml demineralized water was added, and then the organic phase containing the resulting methyl esters of 4-hydroxyalkanoic acids were analysed by using GC- MS QP 2010 MS spectrometer. The column used was VF-5 ms, 30 m x 0.250mm dia with the film thickness of 0.25 µm and the column oven was programmed between 70 and 300^{0}c at the rate of 10^{0}c/min with the injection temperature of 240^{0}C. Mass spectra was recorded under scan mode in the range of 40 – 1000m/z. Compounds were identified using NIST 11 library.

Results and Discussion

The bacterium showing appreciable amount of PHB production was isolated from dairy industry effluent sample. The bacterial isolate was initially characterized using various microbiological and biochemical tests and was identified to be gram positive *Bacillus* sp. Analysis of 16S rRNA gene sequence of *Bacillus cereus* was performed using NCBI BLAST.

The complete sequence was aligned to the homologous sequence available for *Bacillus cereus* was performed using NCBI – BLAST.

The complete sequence was aligned to the homologous sequence available for *Bacillus* strains. The test organism sequence showed 100% similarity in BLAST sequence of *Bacillus cereus*. The nucleotide sequence of bacterial isolate determined in this study have been deposited in Gen Bank database under accession number MK920175.



select all 100 sequences selected GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus cereus strain 01 16S ribosomal RNA gene, partial sequence	1770	1770	100%	0.0	100.00%	MK920175.1
Bacillus anthracis strain ABU-3 16S ribosomal RNA gene, partial sequence	1620	1620	96%	0.0	98.28%	KX951940.1
Bacillus aryabhattai strain ABU-2 16S ribosomal RNA gene, partial sequence	1620	1620	96%	0.0	98.28%	KX951938.1
Uncultured bacterium clone nck210d08c1 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	KF095543.1
Enterobacter cloacae strain TR20T2 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	KJ206932.1
Bacillus sp. D22 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	KF788129.1
Bacillaceae bacterium 11_St_14 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	JX064814.1
Uncultured bacterium clone b11_62 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	JN236281.1
Bacterium 2 amp 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	JN392009.1
Bacillus sp. USTB-O 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	HQ916661.1
Bacillus sp. cp-h21 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	EU558971.1
Bacillus cereus strain L8 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	DQ486872.1
Bacillus cereus strain SIJB13 16S ribosomal RNA gene, partial sequence	1613	1613	96%	0.0	98.17%	MK775246.1
Bacillus sp. (in: Bacteria) strain PUP 27 16S ribosomal RNA gene, partial sequence	1613	1613	98%	0.0	97.77%	MH223457.1
Bacillus thuringiensis strain CH 05 80 16S ribosomal RNA gene, partial sequence	1613	1613	96%	0.0	98.07%	KY510936.1
Bacillus cereus strain LPB4 3 16S ribosomal RNA gene, partial sequence	1613	1613	98%	0.0	97.77%	JQ308569.1

Fig 1: Sequences producing significant alignments with *Bacillus cereus*

Optimization of Parameters for PHB production

The bacterial growth and PHB production by *Bacillus cereus* was followed simultaneously and the results are given in Fig 2. The highest amount of PHB was observed at P^{H} 7 by the isolate after 48 hours incubation. The amount of PHB was observed is found to be 14.63 ± 1.20 mg/L with the % of 64.03 ± 0.003.

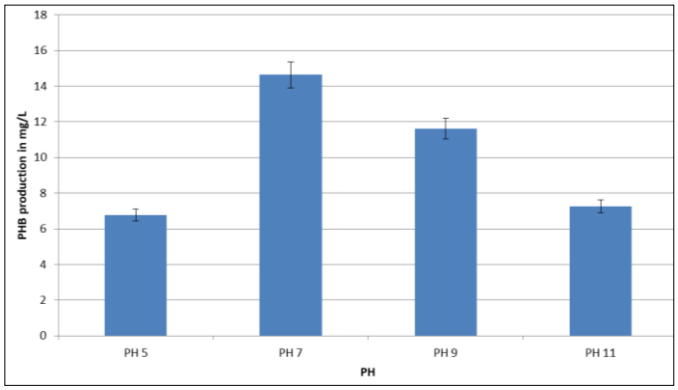


Fig 2: Optimization of P^{H} for PHB production

The optimum temperature for PHB production was found to be 35^0c, the amount of PHB produced was 15.5 ± 1.95 mg/L with the percentage of 62.46 ± 4.52 (Fig. 3).

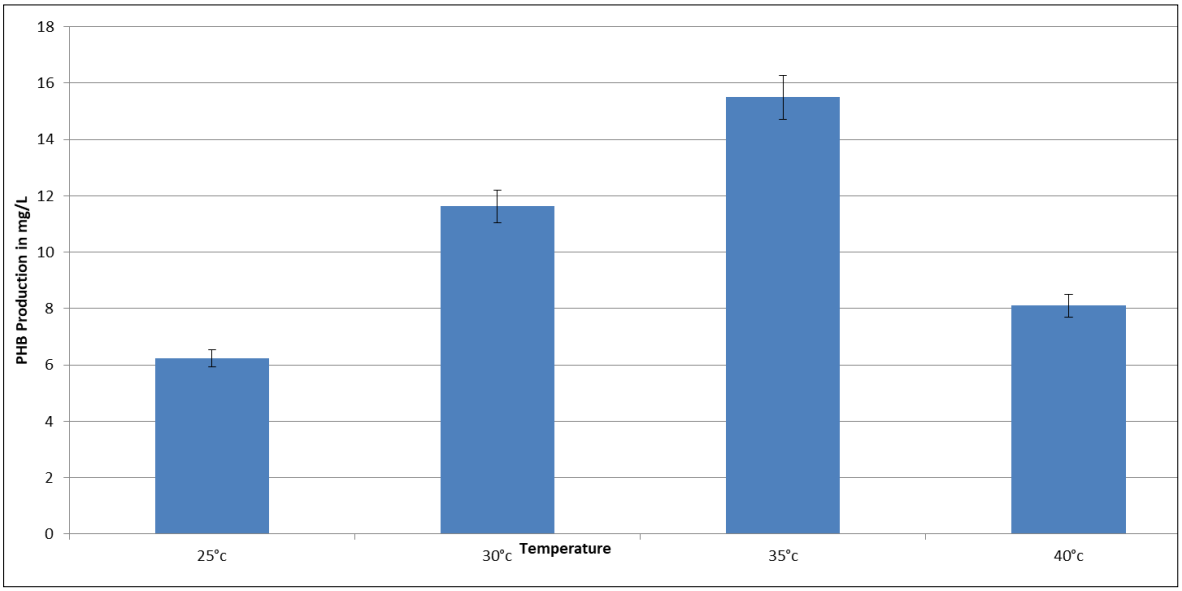


Fig 3: Optimization of temperature for PHB production

The production of PHB from different carbon sources (Glucose, fructose, maltose, mannitol, sucrose and succinate at 0.2 % to 2% were studied. *Bacillus cereus* exhibited a maximum PHB production in 2% sucrose supplementation medium and the optimization of nitrogen source for the isolate *Bacillus cereus* and the results were given in Fig 4 and 5.

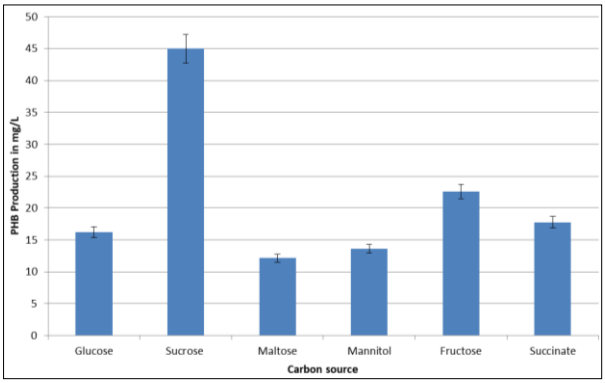


Fig 4: Optimization of carbon source for PHB production

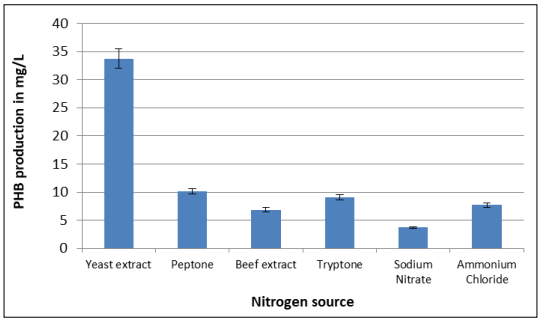


Fig 5: Optimization of nitrogen source for PHB Production

PHB Characterization

Ftir Analysis of Polymer

The FTIR spectra of PHB from isolate DIB1 was given in Fig 6. The IR spectra of PHB from *Bacillus cereus* shows peaks at 1725.5 cm^{-1} represent the esterfunctional of PHB and fatty acids. The absorption band at 2957.61 cm^{-1} represents CH_2 Stretch. The peak at 1636.81cm^{-1} resemble to amide carbonyl stretch. The peak at 1565.96 cm^{-1} represent the NH bending.

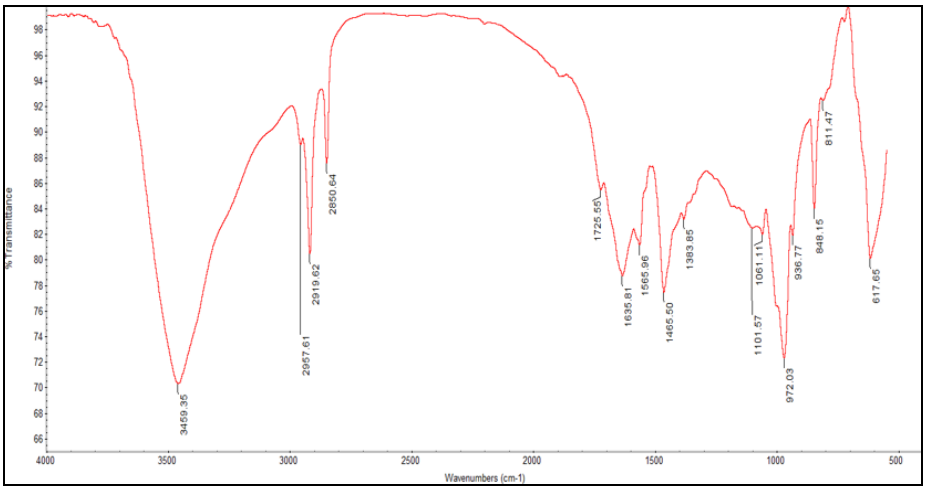


Fig 6: FT–IR Analysis of polymer from *Bacillus cereus*

NMR analysis of Biopolymer

The NMR characterization of PHB from *Bacillus cereus* was presented in Fig. 7. The structures of polyesters was analysed by ^{1}H-NMR. The structures of polyesters was analysed by ^{1}H-NMR. The ^{1}H-NMR spectra of the PHB extracted from *Bacillus cereus* shows the following resonance signals (Fig. 6)CH_2O COOH bond at 1.880ppm, a high signal at 0.8ppm belong to the hydrogen of methylene in saturated lateral chain of terminal CH_3 group.

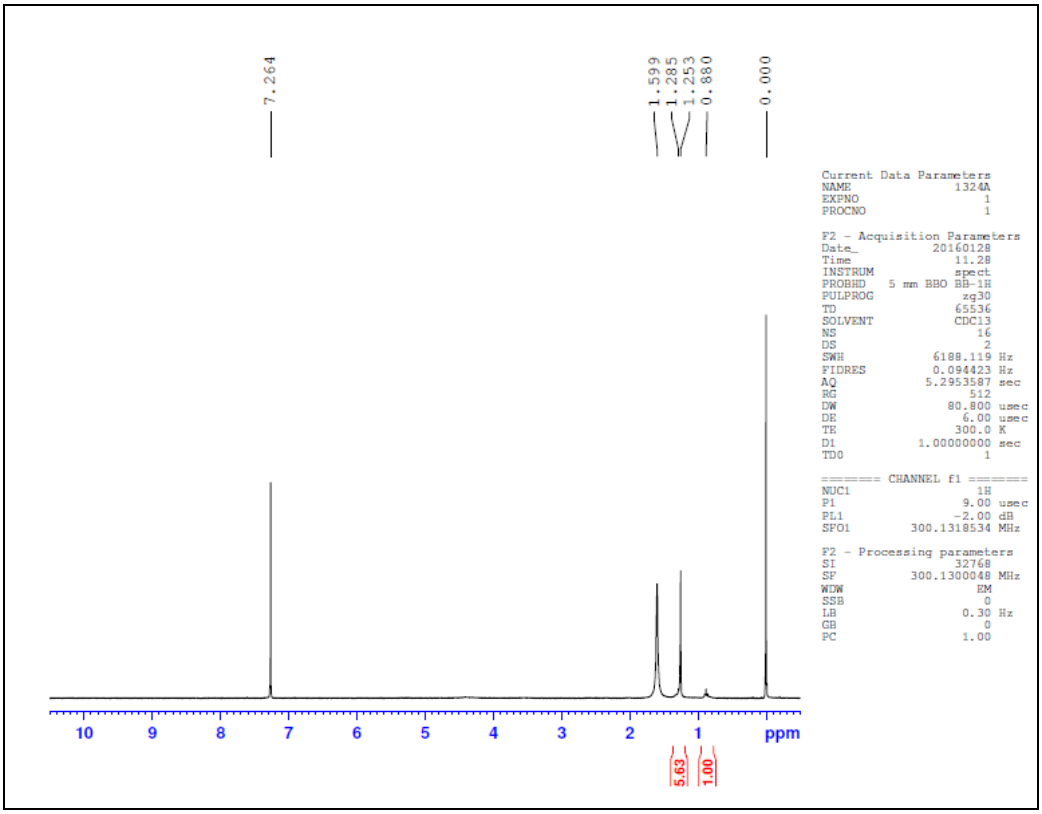


Fig 7: NMR Analysis of polymer from *Bacillus cereus*

GC MS analysis of polymer

The methanolic extracts of PHB from *Bacillus cereus* polymer were dried and analysed by GC MS Perkin Elmer Clarus 500 make. Table 1 shows the result of GC MS analysis, where twelve different biodegradable compounds were found from chloroform extract.

The major among the analysed compounds were n-hexadecanoic acid, octadecanoic acid and benzene propionic acid. The n-hexadecanoic acid is an aliphatic polymer esters. This aliphatic biodegradable polyester family due to hydrolysable ester bonds was reported by Dawes (1988) [26] and others (Anonymous 2002) [27].

Table 1: Chemical Composition of the Biodegradable Polymer of *B.cereus* as revealed by GCMS analysis.

S. No	Compound Name	Retention time	Molecular weight	Peak area	% of peak area
1	Octanol	6.33	128	243994	0.8822
2	2-Nananone	8.28	142	514842	1.8615
3	1-methyldodecylamine	10.93	199	180857	0.6539
4	Phenol-2,4-bis 1,1- dimethylethyl	19.00	206	1116896	4.0384
5	Tridecanoic acid methyl ester	24.24	228	1146749	4.1463
6	E9- Tetradecanoic acid	28.96	226	428696	1.5500
7	Hexadecanic acid methyl ester	29.50	270	8188739	29.60181
8	Benzenepropanoic acid 3,5- bis(1,1 – dimethyl – 4-hydroxy methyl ester	30.11	292	3051825	11.0345
9	n-Hexadecanoic acid	31.14	256	7514607	27.1706
10	15- octadecanoic acid methyl ester	33.74	296	661562	2.3920
11	Octadecanoic methyl ester	34.35	298	4469045	16.1588
12	E-2 Octadecadecen-1-ol	35.83	268	139284	0.5036

Discussion

The dairy industry is one of the important agricultural industry, found all over the country but the product production varies from place to place (Verheijen *et al.*, 1996) [28]. Dairy industry effluent contains large amount of pollutants, which may be organic or inorganic form (Kushwaha *et al.*, 2011; Briao and Tavares, 2007) [29, 30]. In India, the dairy industry is one of the prime industry (Chawla *et al.*, 2009; Sharma and Gulati, 2003) [31, 32]. Dairy industry consumes huge volume of water for their processing, hence it release more amount of effluent, so more number of treatment methods are necessary (Kolhe and Pawar, 2011) [33]. A number of researchers have been extensively studied the biological treatment of dairy industry effluent (Garrido *et al.*, 2001) [38]. Treatment of waster waters through microorganisms is one of the

commonly used mechanism, because of its low cost and easy maintenance (Zamora and Lit, 1995) [39]. So the bioremediation is one of the widely accepted method for the treatment of effluent, because of its low cost, less manpower and easy techniques (Ojo 2006) [40]. People are taking an intrest in searching biodegradable plastic, an alternative to synthetic plastic. Due to the elastic and biodegradable nature bioplastic can be used for a variety of applications such as packaging material. The extensively studied biopolymer is the polyhydroxybutyrate (PHB), which act as a green alternative to synthetic plastics. A lot of interest has been created for production and large scale cultivation of biopolymer producing microorganisms, but the major limitation was the production cost and time for PHB production by bacteria (Grothe *et al.*, 1999; Goff *et al.*, 2007; Cesario *et al.*, 2014; Haas *et al.*, 2015) [41, 42]. Low cost and effective PHB production method are studied by many researchers. Utilization of effluents and low cost substrates such as corn starch, whey for biopolymer production are taking intereset.

Conclusion

This study concluded that, PHB has been considered to be good candidate for biodegradable material. 12 bacterial isolates were isolated from dairy industry effluent samples, best PHB producing strains and identified by morphological and biochemical techniques using taxonomic scheme of Bergey's Manual of Bacteriology. Optimization of physical and chemical parameters was theP^H 7, 35^{o}C, and 48 hrs incubation and the optimum inoculum size for PHB production was 30% DAIE, optimum carbon and nitrogen source was the 2.0% sucrose and 1% yeast extract. The *Bacillus cereus* regarding to PHB production was confirmed by gene sequencing studies. FT-IR, NMR and GC MS analysis of PHB from *Bacillus cereus* shows peaks similar to standard PHB.

Acknowledgement

The authors would like to thank Department of Physics (FIST Sponsored Laboratory), Cauvery College for Women (Autonomous), Tiruchirappalli for providing facility to carry out GC MS analysis.

References

1. Chhonkar PK, Datta SP, Joshi HC, Pathak H. Impact of industrial effluents on soil health and agriculture-Indian experience: Part I-Distillery and paper mill effluents, 2000.
2. Rodrigues MAS, Amado FDR, Xavier JLN, Streit KF, Bernardes AM, Ferreira JZ. Application of photoelectrochemical–electrodialysis treatment for the recovery and reuse of water from tannery effluents. Journal of Cleaner Production,2008:16(5):605-611.
3. Verheijen LAHM, Wiersema D, Pol LH, De Wit J. Management of waste from animal product processing. International Agriculture Center, Wageningen, The Netherlands, 1996.
4. Ganapathy SG, Baskaran R, Mohan PM. Microbial diversity and bioremediation of distilleries effluent. J Res Biol,2011:3:153-162.
5. Kushwaha JP, Srivastava VC, Mall ID. An overview of various technologies for the treatment of dairy wastewaters. Critical reviews in food science and nutrition,2011:51(5):442-452.
6. Perelo LW. In situ and bioremediation of organic pollutants in aquatic sediments. Journal of Hazardous Materials,2010:177(1-3):81-89.
7. Porwal HJ, Mane AV, Velhal SG. Biodegradation of dairy effluent by using microbial isolates obtained from activated sludge. Water Resources and Industry,2015:9:1-15.
8. Semrany S, Favier L, Djelal H, Taha S, Amrane A. Bioaugmentation: possible solution in the treatment of bio-refractory organic compounds (Bio-ROCs). Biochemical Engineering Journal,2012:69:75-86.
9. Karamalidis AK, Evangelou AC, Karabika E, Koukkou AI, Drainas C, Voudrias EA. Laboratory scale bioremediation of petroleum-contaminated soil by indigenous microorganisms and added Pseudomonasaeruginosa strain Spet. Bioresource Technology,2010:101(16):6545-6552.
10. Atlas RM. Microbial ecology: fundamentals and applications. Pearson Education India, 1998.
11. Mueller RJ. Biological degradation of synthetic polyesters—Enzymes as potential catalysts for polyester recycling. Process Biochemistry,2006:41(10):2124-2128.
12. Tokiwa Y, Calabia BP. Review degradation of microbial polyesters. Biotechnology letters,2004:26(15):1181-1189.
13. Jendrossek D. Peculiarities of PHA granules preparation and PHA depolymerase activity determination. Applied Microbiology and Biotechnology,2007:74(6):1186.
14. Chen GQ. A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. Chemical Society Reviews,2009:38(8):2434-2446.
15. Braunegg G, Koller M, Varila P, Kutschera C, Bona R, Hermann C, Pereira L. .Production of plastics from waste derived from agrofood industry CRC Press, Taylor&Francis Group, Boca Raton, Florida, USA, 2007, 119-135.
16. Mohanty AK, Misra M, Drzal LT. Sustainable bio-composites from renewable resources: opportunities and challenges in the green materials world. Journal of Polymers and the Environment,2002:10(1-2):19-26.
17. Braunegg G, Lefebvre G, Genser KF. Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. Journal of Biotechnology,1998:65(2-3):127-161.
18. Woese CR. Bacterial evolution. Microbial Revolution,1987:57:221-71.
19. Garrity G. Bergeys Mannual of systemic bacteriology. London. Springer, 2005.
20. Schut F, de Vries EJ, Gottschal JC, Robertson BR, Harder W, Prins RA, Button DC. Isolation of typical marine bacteria by dilution culture, groth, maintenance and characteristics of isolates under laboratory conditions. Applied Environmental Microbiology,1993:59(7):2150-2160.
21. Arun A, Arthi R, Shanmugabalaji V, Eyini M. Microbial production of poly-β-hydroxybutyrate by marine microbes isolated from various marine environments. Bioresource technology,2009:100(7):2320-2323.
22. Ibrahim MH, Steinbüchel A. Poly (3-hydroxybutyrate) production from glycerol by Zobellella denitrificans MW1 via high-cell-density fed-batch fermentation and

simplified solvent extraction.Applied Environmental Microbiology,2009:75(19):6222-6231.
23. Hungund B, Shyama VS, Patwardhan P, Saleh AM. Production of polyhydroxyalkanoate from Paenibacillus durus BV-1 isolated from oil mill soil. Journal of Microbiology, Biochemistry Technology,2013:5:013-017.
24. Balaji S, Gopi K, Muthuvelan B. A review on production of poly β hydroxybutyrates from cyanobacteria for the production of bio plastics. Algal Research,2013:2(3):278-285.
25. Lee EY, Choi CY. Structural identification of polyhydroxyalkanoic acid (PHA) containing 4-hydroxyalkanoic acids by gas chromatography – mass spectrometry (GC-MS) and its applications to bacteria screening, Biotechnology Research,1997:11:167-171.
26. Dawes EA. Polyhydroxybutyrate: an intriguing biopolymer. Bioscience Report,1988:8:537-547.
27. Anonymous. Biodegradable plastics, Developments and Environmental impacts. Nolan – ITU – Pvt Ltd, prepared in association with Excelplas Australia, 2002.
28. Verheijen LAHM, Wiersema D, Pol LH, De Wit J. Management of waste from animal product processing. International Agriculture Center, Wageningen, The Netherlands, 1996.
29. Kushwaha JP, Srivastava VC, Mall ID. An overview of various technologies for the treatment of dairy wastewaters. Critical reviews in food science and nutrition,2011:51(5):442-452.
30. Brião VB, Tavares CR. Effluent generation by the dairy industry: preventive attitudes and opportunities. Brazilian Journal of Chemical Engineering,2007:24(4):487-497.
31. Chawla A, Chawla N, Pant Y, Kandhari P. Milk and dairy products in India–production, consumption and exports. Hindustan Studies and Services Limited, Bhopal, India, 2009.
32. Sharma VP, Gulati A. Trade liberalization, market reforms and competitiveness of Indian dairy sector (No. 596-2016-40027), 2003.
33. Kolhe AS, Pawar VP. Physico-chemical analysis of effluents from dairy industry. Recent Research in Science and Technology, 2011, 3(5).
34. Munavalli GR, Saler PS. Treatment of dairy wastewater by water hyacinth. Water Science and Technology,2009:59(4):713-722.
35. Kolhe AS, Ingale SR, Bhole RV. Effluent of dairy technology. Shodh Samiksha aur Mulyankan (International Research Journal),2009:2:459-461.
36. Macoon B, Woodard KR, Sollenberger LE, French EC, Portier KM, Graetz DA, Van Horn HH. Dairy effluent effects on herbage yield and nutritive value of forage cropping systems. Agronomy Journal,2002:94(5):1043-1049.
37. Nagda GK, Diwan AM, Ghole VS. Seed germination bioassays to assess toxicity of molasses fermentation based bulk drug industry effluent. Electronic Journal of Environmental, Agricultural and Food Chemistry,2006:5(6):1598-1603.
38. Garrido JM, Omil F, Arrojo B, Mendez R, Lema JM. Carbon and nitrogen removal from a wastewater of an industrial dairy laboratory with a coupled anaerobic filter-sequencing batch reactor system. Water Science and Technology,2001:43(3):249-256.
39. Zamora AF, LitM AL. Biodegradation of Effluents from Fish Canning Factory and Dairy Products Processing Plant. In Environmental Biotechnology. Springer, Dordrecht, 1995, 481-490.
40. Ojo OA. Petroleum-hydrocarbon utilization by native bacterial population from a wastewater canal Southwest Nigeria. African Journal of Biotechnology,2006:5(4):333-337.
41. Grothe E, Moo-Young M, Chisti Y. Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme and Microbial Technology,1999:25(1-2):132-141.
42. Goff M, Ward PG, O'Connor KE. Improvement of the conversion of polystyrene to polyhydroxyalkanoate through the manipulation of the microbial aspect of the process: a nitrogen feeding strategy for bacterial cells in a stirred tank reactor. Journal of Biotechnology,2007:132(3):283-286.
43. Cesário MT, Raposo RS, de Almeida MCM, van Keulen F, Ferreira BS, da Fonseca MMR. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnology,2014:31(1):104-113.
44. Haas R, Jin B, Zepf FT. Production of poly (3-hydroxybutyrate) from waste potato starch. Bioscience, Biotechnology, and Biochemistry, 2008. 0712210692-0712210692.2Q