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Research Article

Studies on Production and Characterization of Biopolymers Produced by Azotobacter sps.

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ABSTRACT

In the present work the impact of Azotobacter sps. were studied on the garden soil to produce natural polymers. Due to stringent environment protection norms technologies are being directed towards the developments of non polluting biogreen materials. A biologically synthesized plastic polyhydroxyalkanoate has been attracting great interest owing to its properties similar to petroleum based plastics. Microorganism is producers of natural polymers and these microbial polymers are water soluble and have novel physical and chemical properties. Azotobacter sps was isolated from garden soil (IOS) using Ashby`s medium. Polysaccharide production was studied by using Garcia`s minimal medium as production medium in a benchtop bioreactor (HT infors). Harvested biomass was solvent extracted to obtain the polymer film, characterization of polymer was done using Gas chromatography. A clear polymer film was formed after chloroform extraction. Compounds observed in GCMS analysis of dried chloroform extract were Pthalic acid, isobutyl 4octyl ester ($C_{20}H_{30}O_4$) 5.44%, n-Hexadecanoic acid ($C_{16}H_{32}O_2$) 53.51% and Phytol($C_{20}H_{40}O$) 5.28%.

Keywords: *Biopolymers, Azotbactor sps., Biomass, Biodegradable plastic, GC-MS.*

INTRODUCTION

With recent trends in technologies being aimed at amending ways for Environment friendly and sustainability, it compels researchers to develop technologies producing replacement to pollutant and minimizing the pollution with an end to the use of pollutant and making use of available resources to generate product at mass scale, which may or have potential to replace the products causing irreversible damage to the environment. One such pollutant is Petroleum based plastic, which is estimated to be causing pollution at an alarming ever increasing growth rate which should be minimized or controlled for environment to remain healthier for future mankind. The versatility of plastic materials in terms of mechanical properties and durability has been manipulated by mankind to enhance quality of life without realizing they have become increasingly ubiquitous. The world's plastics production was estimated to be 260 million tonnes in 2007 (Lazarevic D., Aoustin E., Buclet N., and Brandt N 2010). It is clear from this figure that the long term deleterious environmental impacts caused by plastics were entirely overlooked and this in turn poses greater difficulties for plastic waste disposal.

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Microbial biopolymer, includes bacterial storage polyesters such as polyhydroxy butyrate (PHB), currently gaining lot of attention because of their biodegradability and may replace petroleum derived polymers such a polyprolene (Steinbuchel &Fuchtenbusch 1998, de Konning &Withold 1997).

Therefore, the development and use of biodegradable plastics is gaining more serious attention. The most extensively studied thermoplastic biopolymers are the polyhydroxyalkanoates (PHA) and polylactic acid (PLA**)** (Chen G-Q 2009). PHA is attractive because of its biodegradability. In addition, because of the diverse types of monomers (about 150 different structures), it is possible to produce PHA copolymers having a wide range of properties. The various PHA monomers can be classified based on the number of carbon atoms as short-chain length PHA (scl-PHA), medium-chain length PHA (mcl-PHA) and long-chain length PHA (lcl-PHA). Scl-PHA refers to PHA comprised of monomers having 5 or less carbon atoms. These include 3-hydroxybutyrate and 3-hydroxyvalerate. The mcl-PHA is comprised of monomers having 6 to 14 carbon atoms. These include 3-hydroxyhexanoate, 3-octanoate and 3-hydroxydecanoate. The lcl-PHA, which is uncommon and least studied, consists of monomers with more than 14 carbon atoms. Recently, it has also been made possible to synthesize a new type of PHA containing lactide as a co-monomer (Taguchi et al, 2008, Yamada et al, 2009, Shozui et al, 2009). All these developments indicate that PHA may become the preferred next generation bioplastic. However, to date the market penetration of PHA is still scarce. This is mainly due to its high production cost. The main reasons behind the economic disadvantages are the costly fermentation and purification technologies. The latter significantly affects the overall process economics. Much work has been carried out to lower the PHA production cost by the use of effective and inexpensive carbon source and genetically engineered microorganisms. Sugars have been shown to be an effective feedstock for PHA production in Brazil, especially when the PHA production is integrated to the sugarcane-processing factory (Koller et al., 2009). However, the real cost associated with PHA would only diminish with the development of a cheaper and environmentally friendly PHA recovery method.

MATERIAL AND METHOD

Bacteria and culture Medium

Azotobacter sps. was isolated from plant rhizosphere of Botanical garden. The Institute of Science, Mumbai. using serial dilution technique. The various culture growth medium (g/L) used are Ashby`s Mannitol agar, Jensen`s and Burk`s medium with various combination of carbon source such as sucrose, glucose and mannitol. For pilot studies experiment were set in triplicate of 100ml medium with 5% (v/v) inoculum, pH 7 $\pm$ 0.1-0.2,inoculated flask were incubated at 28°C at 110 rpm for a period of 24 hrs,36 hrs and 48 hrs ,table no.1 respectively while growth measured using colorimeter at 660 nm. On regular interval for growth pattern of organism. After fermentation the cell broth was concentrated by (Appendorf) centrifugation at 10000 rpm for 10 min at 24°C, washed twice with distilled water and then freeze dried with nitrogen. The resulting powder stored at room temperature until they were needed for analysis.

Inoculum preparation

Using Nutrient Broth a dilution of 0.9 was achieved at 660 nm from overnight cultured medium.

Extraction and determination of Biopolymers: Solvent Extraction with Chloroform

Fresh Biomass : It was treated with 60 times Chloroform, upon adding chloroform biomass turned into a transference suspension of a polymeric film, it was kept for stirring 3-4 hours at 30°C , water was added to keep the film static for 12 hours and a clear polymer was formed and separated. Sample of PHA film dissolved in chloroform subjected to characterizational analytics using, GC/MS.

Polymeric compounds characterization and identification using GC MS.

The GC/MS analyses were carried out on GC Agilent Technic-IIT powai Mumbai (SAIF-Mumbai). GC- System HP 5 column gas chromatography equipped with a Elite-5 capillary column (5% Diphenyl 95% dimethyl poly siloxane) (30nm x 0.25mm ID X 0.25mmdf) and mass detector tubomass gold of the company which was operated in electron ionization impact method (EI) mode. Helium was the carrier's gas at a flow rate of 1 ml/min. the injector was operated at 200^0C and the oven temperature was programmed as follows; 60^0 C for 15min, then gradually increased to 27°C at 3 min Mainlib and replib; library search was used for compound identifications. The Gas chromatography / mass spectrometry (GC / MS) instrument separates chemical mixture (The GC component) and identifies the components at a molecular level (The MS component). It is one of the most accurate tools for analyzing environment samples. The mixture will separate into individual substances when healed. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecule.

RESULT AND DISCUSSIONS

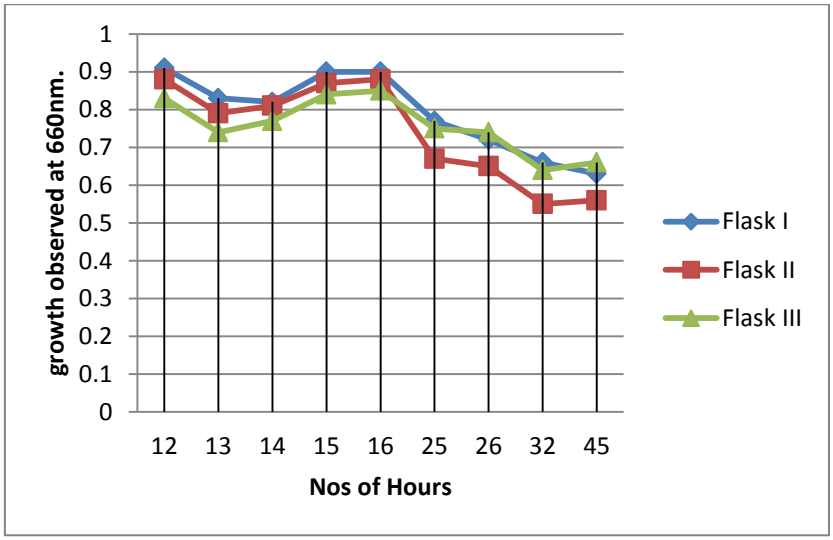
Bacterial growth

Growth was measured by colorimetric analysis, O.D was observed at 660 nm. Using Digital colorimeter -112 at regular intervals.

Table 1: Growth of *Azotobacter sps* at 660nm at different time intervals

Time (hrs)	Flask I	Flask II	Flask III
12	0.91	0.88	0.83
13	0.83	0.79	0.74
14	0.82	0.81	0.77
15	0.90	0.87	0.84
16	0.90	0.88	0.85
25	0.77	0.67	0.75
26	0.72	0.65	0.74
37	0.66	0.55	0.64
42	0.63	0.56	0.66

Observations of growth at interval of an hour showed that there was an initial growth but with passage of time it slowed down and finally was constant after 42 hours (Table 1& Graph 1).



Graph 1: Growth of *Azotobacter sps* at 660nm at different time intervals

Formation of Polymer after Solvent Extraction with Chloroform

Extraction with chloroform turned biomass into into a transference suspension of a polymeric film, addition of water and keeping the film static for 12 hours led a clear polymer formation. The polymer was separated and dissolved in chloroform for further testing

Polymeric compounds characterization and identification using GC MS

GC-MS: Analysis of PHA purity. The purity of PHA was determined by gas chromatography (Braunegg, Sonnieitner, Lattery, 1978).

Percentage of Compound found using Gas chromatography and Mass spectroscopic are as table 2, The chloroform extracts of biodegradable polymer were dried and analyzed by GCMS (Agilient tech).Table 2 shows the result of GCMS analysis, where different biodegradable compounds were found from chloroform extract. The major compounds among the analyzed compounds were n-Hexadecanoic acid (Stearic acid), Phthalic acid, isobutyl 4-octyl ester, n-Hexadecanoic acid and phytol.

Table 2: Compounds reported in Gas chromatography and Mass spectroscopic analysis of dried chloroform extract of biodegradable polymer

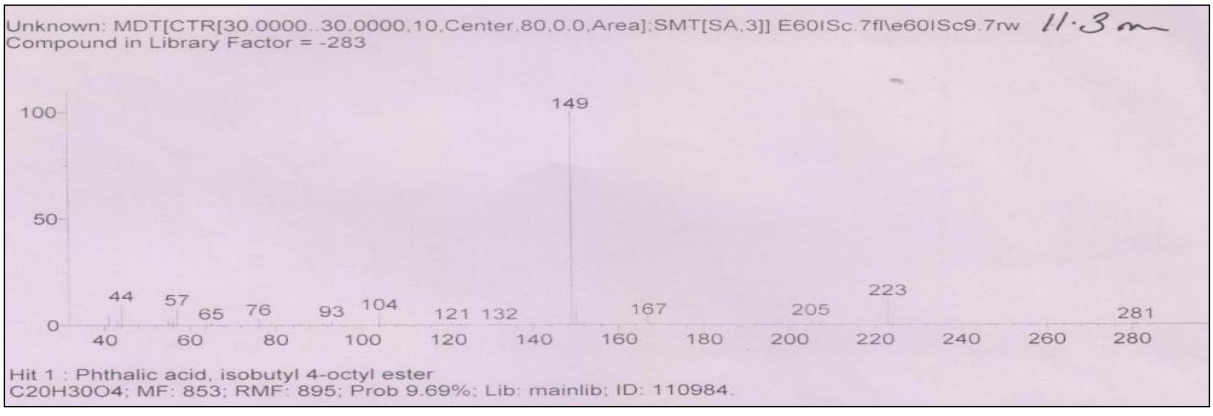
Name of the Compound	Mol str	RT	Mol For	Peak area	Percentage %
Pthalic acid, isobutyl 4octyl ester	$C_{20}H_{30}O_4$	11.3	853	86765.29	5.44
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	12.1	860	853112.72	53.51
Phytol	$C_{20}H_{40}O$	13.6	699	84171.97	5.28

LIBRARY SEARCH OUT PUT OF BIOPOLYMER GC/MS RESULTS

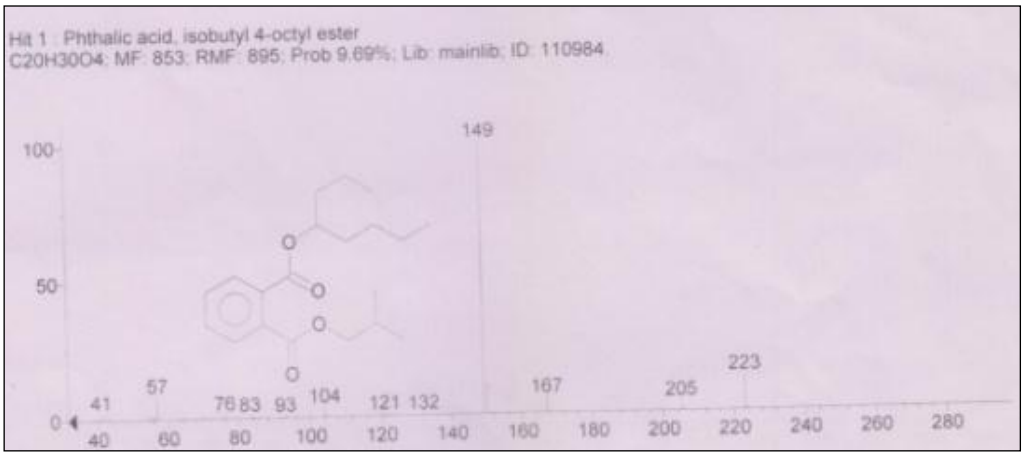
Phthalic Acid

Phthalic acid, also called **1,2-benzenedicarboxylic Acid**, colourless, crystalline organic compound ordinarily produced and sold in the form of its anhydride. The annual production of phthalic anhydride exceeded 1,000,000 metric tons in the late 20th century; most of it was used as an ingredient of polyesters, including alkyd resins (vehicles for paints and enamels), and simple esters used as plasticizers for polyvinyl chloride and other polymers.

Hit 1: Phthalic acid,isobutyl 4-octyl ester



Chromatogram 1 : Mass spectrum of compound Pthalic acid, isobutyl 4- octyl ester ($C_{20}H_{30}O_4$;MF:853;RMF895;Prob9.69%; Lib:Mainlib ;Id110984)

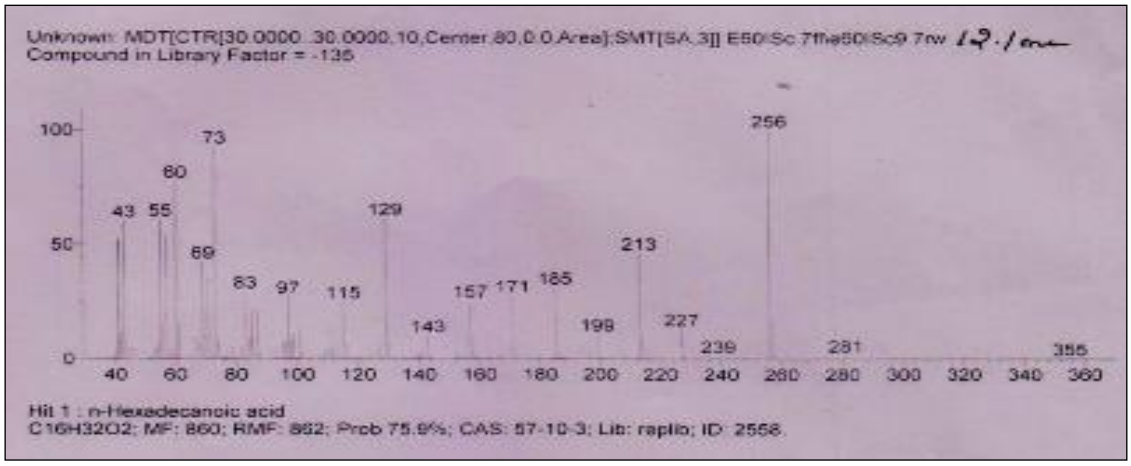


Chromatogram 2: Chromatogram 1 : Mass spectrum of compound Pthalic acid, isobutyl 4- octyl ester with structure ($C_{20}H_{30}O_4$;MF:853;RMF895;Prob9.69%; Lib:Mainlib ;Id110984)

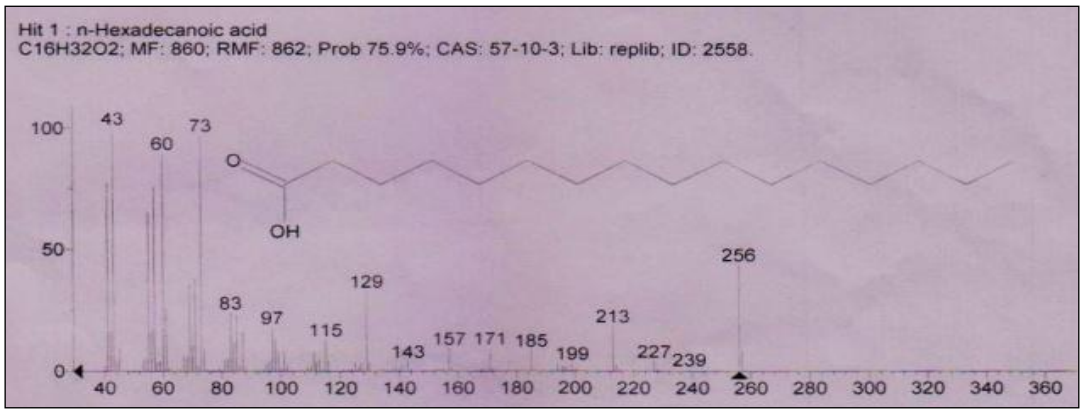
n-Hexadecanoic acid

n-hexadecanoic acid is an aliphatic polymer esters. This aliphatic biodegradable polyester family due to hydrolysable ester bonds was reported by Dawes (1988).

Hit 2 : n-Hexadecanoic acid



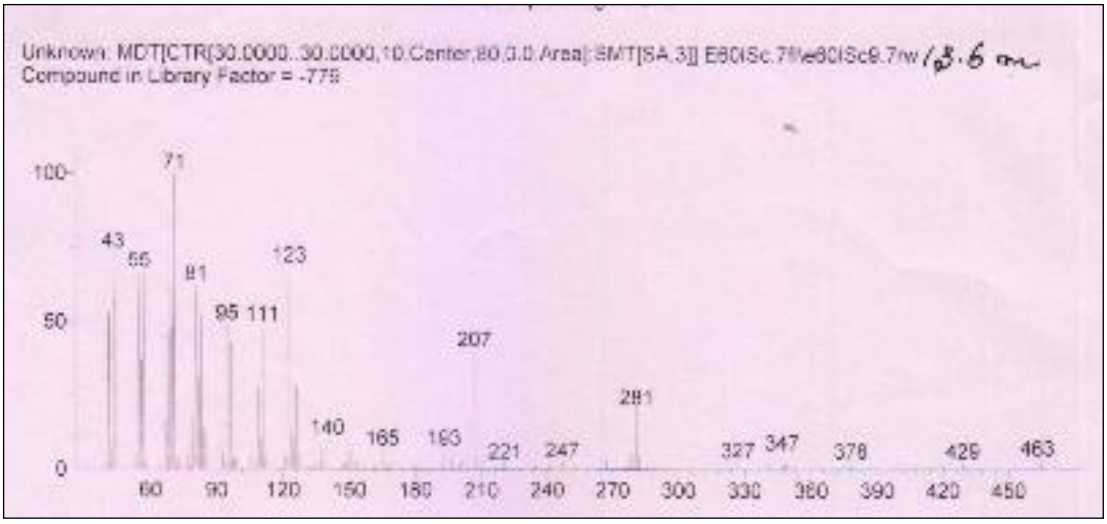
Chromatogram 3: Mass spectrum of compound n-Hexadecanoic acid ($C_{20}H_{30}O_4$;MF:853;RMF895;Prob9.69%; Lib:Mainlib ;Id110984)



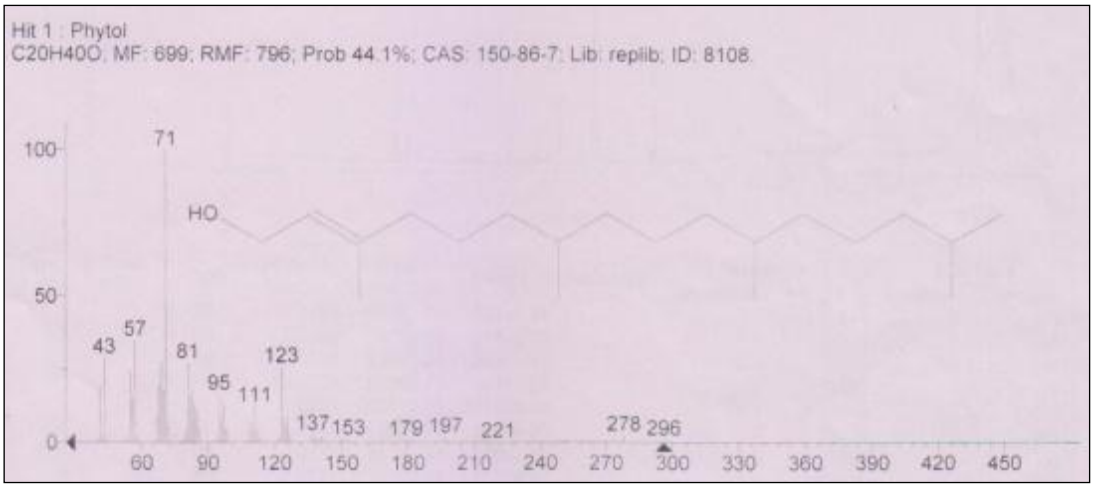
Chromatogram 4: Mass spectrum of compound n-Hexadecanoic acid with structure ($C_{16}H_{32}O_2$;MF:860;RMF8862;Prob75.9%; Lib;replib ;Id:2558)

Phytol

Hit 3: Phytol :



Chromatogram 5: Mass spectrum of compound Phytol ($C_{20}H_{40}O$;MF:699;RMF796;Prob44.1%; CAS:150-86-7;Lib:replib ;ID8108)



Chromatogram 6: Mass spectrum of compound Phytol with structure ($C_{20}H_{40}O$;MF:699;RMF796;Prob44.1%; CAS:150-86-7;Lib:replib ;ID8108)

With respect to GC/MS and production results from the organism growth table it can be concluded that isolated sps of azotobacter holds promising character and ability to synthesizes polyhydroxyalkanote(PHA) ,The Mass Spectroscopic results showing Phthalic acid,isobutyl 4-octyl ester, :n-Hexadecanoic acid, : Phytol with retention time of compound found in the sample along with their peak area, percentage of respective compound,molecular formula and their structure for the reference.

CONCLUSION

The production of biopolymer with respect to isolated organism is significant and Organism respond positively to variation of carbon source, incubation time, pH and temperature, herby it hold advantage over other organism for utilization as raw material of PHA and its Co polymers. While gas charomatographic results showing the presence of polyester compounds of Phthalic acid, isobutyl 4-octyl ester, n-hexadecanoic acid and Phytol which are constituents of polymers.

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REFERENCES

Akiyama, M., Tsuge, T., Doi, Y., Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation. Polymer Degradation and Stability, **80**, 183–194 (2003). DOI: 10.1016/S0141-3910(02)00400-7.

Braunegg, G., Sonnieitner, B., Lattery, R.M., A rapid gas chromatography method for the determination of poly-β-hydroxybutyric acid in microbial biomass biomass *Eur. J. Appl. Microbial. Biotechnol*., 1978, 6, 29.

Chen, G-Q., A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chemical Society Reviews, 38, 2434–2446 (2009). DOI: 10.1039/b812677c

Dawes, E.A., 1988. Polyhydroxybutyrate: an intriguing biopolymer. *Biosci Rep.,* 8: 537–47

de Koning G. and Witholt, B. (1997). A biodegradable rubber from bacteria, poly(hydroxyalkanote) from *Pseudomonads. Mat. Sci. Eng*. C4: 121–124. http://www.britannica.com/EBchecked/topic/458418/phthalic-acid

Lazarevic D., Aoustin E., Buclet N., Brandt N.: Plastic waste management in the context of a European recycling society: Comparing results and uncertainties in a life cycle perspective. Resources, Conservation and Recycling, **55**, 246–259 (2010). DOI: 10.1016/j.resconrec.2010.09.014

Shozui F., Matsumoto K., Nakai T., Yamada M., Taguchi S.: Biosynthesis of novel terpolymers poly (lactate-co-3-hydroxybutyrate-co-3 hydroxyvalerate)s in lactate-overproducing mutant *Escherichia coli* JW0885 by feeding propionate as a precursor of 3-hydroxyvalerate. Applied Microbiology and Biotechnology, 85, 949–954 (2009). DOI: 10.1007/s00253-009-2100-y

Koller M., Hesse P., Kutschera C., Bona R., Nascimento J., Ortega S., Agnelli J. A., Braunegg G.: Sustainable embedding of the bioplastic poly-(3-hydroxybutyrate) into the sugarcane industry: Principles of a future-oriented technology in Brazil. in 'Polymers –Opportunities and risks II: Sustainability, product design and processing' (eds.: Eyerer P., Weller M.,Hübner C.) Springer-Verlag Berlin Heidelberg, New York, Vol 12, 81–96 (2009). DOI: 10.1007/698_2009_11

Loo C-Y., Lee W-H., Tsuge T., Doi Y., Sudesh K.:Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. Biotechnology Letters, 27, 1405–1410 (2005). DOI: 10.1007/s10529-005-0690-8

Lee W-H., Loo C-Y., Nomura C. T., Sudesh K.: Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. Bioresource Technology, 99, 6844–6851 (2008). DOI: 10.1016/j.biortech.2008.01.051

Ng K-S., Ooi W-Y., Goh L-K., Shenbagarathai R., Sudesh K.: Evaluation of jatropha oil to produce poly(3-hydroxybutyrate) by *Cupriavidus necator* H16. Polymer Degradation and Stability, 95, 1365–1369 (2010). DOI: 10.1016/j.polymdegradstab.2010.01.021

Kahar P., Tsuge T., Taguchi K., Doi Y.: High yield production of polyhydroxyalkanoates from soybean

oil by *Ralstonia eutropha* and its recombinant strain. Polymer Degradation and Stability, 83, 79–86 (2004). DOI: 10.1016/S0141-3910(03)00227-1

Sudesh K., Bhubalan K., Chuah J-A., Kek Y-K.,Kamilah H., Sridewi N., Lee Y-F.: Synthesis of polyhydroxyalkanoate from palm oil and some new applications. Applied Microbiology and Biotechnology, 89, 1373–1386 (2010). *Kunasundari and Sudesh – eXPRESS Polymer Letters* Vol.5, No.7 (2011) 620–634 631

Steinbuchel A, Fuchtenbusch B (1998) Bacterial and other biological systems for polyester production. *Trends Biotechnol.* 16: 419–427.

Sudesh K., Abe H., Doi Y.: Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters.Progress in Polymer Science, 25, 1503–1555(2000). DOI: 10.1016/S0079-6700(00)00035-6

Taguchi S., Yamada M., Matsumoto K., Tajima K.,Satoh Y., Munekata M., Ohno K., Kohda K., Shimamura T., Kambe H., Obata S.: A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. Proceedings of the National Academy of Sciences of the United States of America, 105, 17323–17327 (2008). DOI: 10.1073/pnas.0805653105

Yamada M., Matsumoto K., Nakai T., Taguchi S.:Microbial production of lactate-enriched poly[(R)-lactate-co-(R)-3-hydroxybutyrate] with novel thermal properties. Biomacromolecules, 10, 677–681 (2009). DOI: 10.1021 /bm8013846