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CHARACTERIZATION AND BIOSYNTHESIS OF SCL-CO-MCL BY *BACILLUS* SP.OU35

P.NAGAMANI*[1], SK.MAHMOOD[1]

Microbiology Lab, Department of Botany, University College of Science, Osmania University, Hyderabad, 500007, India

ABSTRACT

This study aimed to describe an aerobic, gram positive, mesophilic and heterotrophic bacterium, designated OU35 isolated from polluted pond. In shake flask experiments, this bacterium produced a novel scl co mcl PHA, composed of 3-hydroxy butyrate (95 %), 3-hydroxy octanoate (2.5%), and 3-hydroxy decanoate (2.5%), from glucose and rice bran as carbon sources. The yield of the PHA was 1.217g/l and1.530 g/l respectively. Optimal growth occured between 28 and 37^{o}C, at a PH between 6.0 and 7.8 and at an ionic strength of 12gl^{-1} of sea salts. The G+C content of DNA is 45.25%. Major fatty acids were iso-$C_{15,\ 0}$(20.29%), anteiso-$C_{15,\ 0}$(5.31%), iso-$C_{16,\ 0}$(8.35%), anteiso-$C_{15,0}$(5.31%), iso-$C_{16,0}$(8.35%). Strain exhibited maximum homology of 96.28% to *Bacillus aryabhattai* B8W22^{T}, 95.97% to *B.megaterium* IAM 13418^{T}, and 95.282% to *B. flexus* IFO15715^{T} by 16S rDNA sequencing. The 16S rRNA gene sequence is deposited in EMBL/Genbank with accession number FN663629 and has been deposited in the CCTCC as strain CCTCC AB2011468^{T}.

Keywords- ***Bacillus, scl co mcl, PHA, Polyhydroxyakanoates***

P.NAGAMANI
Microbiology Lab, Department of Botany, University College of Science, Osmania University, Hyderabad, 500007, India

Corresponding author

INTRODUCTION

Polyhydroxyalkanoates (PHA) are a family of biopolyesters synthesized by many types of bacteria as carbon and energy reserve materials. PHA combine properties of thermal processibility, biodegradability, biocompatibility and sustainability, they have attracted attention from fermentation, materials and biomedical industries. Their potential use as biodegradable and biocompatible thermoplastics has attracted worldwide attention[1, 2]. Polyhydroxybutyrate (PHB), which occurs most commonly in bacterial cells, does not possess material properties for practical application because it is crystalline and brittle. The copolymer of poly (hydroxybutyrate-co-hydroxyoctanoate-co-hydroxydecanoate) – P (HB-co-HO-co-HD) – is more elastic and flexible compared with PHB. Currently, PHA is produced in an industrial scale using Gram negative bacteria such as *Cupriavidusnecator*[3], *Alcaligeneslatus* and recombinant *Escherichia coli*. However, PHB isolated from Gram-negative organisms contain the outer membrane lipopolysaccharide (LPS) endo toxins, which are pyrogens known to co purify with the PHA. The presence of LPS induces a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHA[4]. Gram-positive bacteria lack LPS and are hence potentially better sources of PHA to be used for biomedical applications[5.] However, only limited work has been carried out on the large-scale production of PHA from the Gram-positive genus *Bacillus*. Gram positive bacteria such as *Bacillus* sp. are ideal candidates for industrial scale PHA production. Members of this genus are known to grow rapidly, possess various hydrolytic enzymes and produce co-polymers from structurally unrelated carbon sources. In this study, we characterized a new bacterium with the capability to synthesize Poly (3-hydroxy butyrate-co-3-hydroxy octanoate-co-3-hydroxy decanoate) with various biomedical applications such as bone tissue engineering, medical implants, drug delivery, protein purification, chiral chemicals and drug development, from cheap carbon sources which significantly reduce the cost of PHA production.

These features of *Bacillus* sp. explore the possibility of utilizing various agricultural raw materials as a carbon source for production of different metabolites. Further, *Bacillus* represents a model system for the heterologous expression of foreign genes associated with PHA production and several fine chemicals too[6].Reports suggesting the use of *Bacillus* for the production of a range of different PHA by utilizing different carbon sources are known. These include incorporation of the terco polymer of 3HB, HV, and 3HHX (3-hydroxy-hexanoate) utilizing b-caprolactone[7], PHB co-PHV in mahua flowers[8], from sucrose[9], sugarcane molasses, and corn steep liquor[10]. The co-polymer synthesis from structurally different carbon sources suggests that *Bacillus* has the potential for production of a new PHA co-polymer using different substrates[11].

The aim of this study was to describe a new PHA - (SCL co MCL) producing strain designated as OU35 which was isolated from a polluted pond. In the presence of simple carbon substrates in excess, the strain was shown to produce a copolymer of biotechnological and biomedical interest. This study investigates the characterization and the ability of strain OU35 *Bacillus* species to accumulate PHA as well as the monomer composition of the PHA accumulated.

MATERIALS AND METHODS

Isolation of bacterial strain

Water samples were collected from different sites of Polluted Pond containing industrial waste water at Hyderabad, India. Stock cultures were grown and maintained at 30^{o} C and 4^{o}C, respectively by periodic transfer on Luria-Bertani (LB) medium. Serially diluted

samples were spread on LB media. Screening was performed in order to isolate PHA producing micro organisms. Selected bacteria were grown in E_2 medium[12] supplemented with $20gl^{-1}$ of glucose and rice bran of 10g/l. Their abilities to synthesize PHA were determined by a viable colony staining method using Nile blue A[13], and bacteria accumulating PHA were isolated. A strain was isolated, purified and coded as OU35 which was maintained on nutrient agar slants over laid with 20% (v/v) glycerol and kept at -20^{o}C.

Scanning electron microscopy

For scanning electron microscopy samples were fixed in 2.5% Gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hrs at 4^{o}C and post fixed 2% in aqueous osmium Tetroxide for 4 hrs, in the same buffer. After the post fixation samples were dehydrated in series of graded alcohols and dried to critical point drying with electron microscopy Science CPD unit. The dried samples were mounted over the stubs with double-sided carbon tape. Applied a thin layer of gold coat over the samples by using an automated sputter coater (JEOL JFC-1600) for 3 min. Then scanned the samples under Scanning Electron Microscope (Model, JOEL_JSM 5600) at various magnifications.

Biochemical characteristics

Biochemical characteristics were checked with the Hi25 Enterobacteriaceae identification kit (KB003) and Hi Carbohydrate kit parts A, B and C (KB009) (both from Hi- Media) according to the manufacturer's protocol. Other tests were performed by growing the culture at 30^{o}C in the appropriate medium. The activities of catalase, oxidase, phosphatase, gelatinase, urease, cellulase, protease, lipase, lecithinase, were determined according to standard methods[14]. Utilization of different sugars, leading to the formation of acid with or without gas production was monitored[15]. Different carbon compounds were used to check the ability of the culture to utilize a carbon compound provided as the sole carbon source[16]. Sensitivity of the culture to nine antibiotics was determined using antibiotic discs(Hi Media), containing, polymixin B (100 IU), penicillin G(10 IU), ampicillin (10 µg), novobiocin (5 µg), tetracycline(50 µg), kanamycin(30 µg), neomycin(50 µg), nitrofurazone(30 µg),and nalidixicacid(50 µg).

Identification of the bacterium

The morphological and physiological properties of isolate OU35 were investigated according to Bergey's manual of determinative Bacteriology. For the phylogenetic analysis the region of 16SrDNA was amplified by PCR using a primer set of 27F (5AGAGTTTGAYCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') and the nucleotide sequence was determined. Phylogenetic analyses were performed using the software package MEGA 4.0[17] after multiple alignments of the sequence data with CLUSTAL_X[18] using the neighbour-joining method[19] against the sequences of against those of the family *Bacillaceae*, which are available in the gene bank .The 16S rRNA gene sequence is deposited in EMBL/Genbank with accession number FN 663629.The G+C content of DNA was determined by HPLC[20].

Production, isolation and extraction of PHA

Cells were grown in duplicate in modified mineral salt medium supplemented with glucose and rice bran carbon source for PHA production[21]. Medium was distributed in 50 ml quantity in 250 ml capacity Erlenmeyer flasks sterilized by autoclaving (15 lb, 20 min) and cooled. They were inoculated with 10% (v/v) inoculum of 24 h grown cultures and incubated at 250 rpm/min for 48 h at 30^{0}C. PHA was extracted from lyophilized cells using chloroform extraction in a Soxhlet apparatus and was subsequently precipitated with 10 vols ethanol. The precipitate was dissolved in chloroform and ethanol-precipitated again in order to obtain highly purified PHA.

Fermentor Studies

PHA production was carried out by using lab level fermentor. Cultures were inoculated into sterilized E_2 minaral media supplemented with 2% glucose and rice hydrolyzed rice bran. Fermentation was carried out by using 3.5 L Bio console ADI (025) Fermentor (Applikon Biotechnology (Holland). Parameters used for operation were pH 7.0, dissolved oxygen content 30%, agitation speed 3000g and temperature 35°C. The pH was maintained mechanically by the addition of 0.5M NaOH and 0.5M HCl. Sesame oil mixed with water (1,1) was used as the anti foaming agent.

Analytical methods

Microbial growth was monitored by measuring the cell density of the culture at 600 nm. Both cell dry weight (CDW) and PHA quantification were determined gravimetrically. The PHA content was defined as the ratio of PHA concentration to cell concentration given as percentage.

Infra red spectra

For FT-IR analysis, the PHB was precipitated from the chloroform using cold ethanol. The precipitated polymer was used to prepare KBr discs (sample, KBr, 1,100). An FT-IR spectrum 1720X spectrometer (Perkin Elmer, USA) was used under the following conditions, spectral range, 4,000–400 cm^{-1}, window material, CsI, 16 scans, resolution 4 cm^{-1}, the detector was a temperature-stabilized, coated FR-DTGS detector.

NMR spectroscopy

Samples of PHAs were dissolved in $CDCl_3$ at 25^o C. NMR measurements were performed on a Bruker 400 DRX spectrometer operating at 400 MHz and deuterated chloroform was used as solvent .For analysis, 10 mg of sample and 1ml of solvent were employed.

Esterification of PHA for GC analysis,

PHA was quantified using a slight modification of the gas chromatographic method of Riis and Mai (1988).Instead of whole cells, pure, extracted PHA was used. Quantitative evaluation was affected by means of the peak areas of hydroxybutyric acid and benzoic acid. PHB content was defined as the ratio of PHB to cell dry weight and expressed as a percentage.

GC-MS analysis

Capillary GC-MS was performed on a Shimadzu GC-MS –G1110/MS data system. Samples were ionized by electron impact (70 eV). Column and temperature were as follows, DB wax column (polar, 30m, 0.32µm, and 0.25µm thickness) was used, temperature of the injector was 250° C, and the initial oven temperature was increased at the rate of 15° C/min from 50 ° C up to 200° C. The helium gas used as carrier gas, with a split less injection (80, 1), 1 µl of sample was injected.

Gel permeation chromatogrsphy

Molecular mass analysis was conducted with purified PHA which was dissolved in chloroform (1mg/ml PHA) and introduced into GPC system equipped with Waters Model 510 pump, Model 486 tunable absorbance detector, and model 730 data module with 500, 10^5 Ultra styragel columns in series. Chloroform was chosen as the eluent at a flow rate of 1.0 ml/min. Sample concentrations of 0.5% (w/v) and injection volumes of 100 ml were used. Polystyrene standards with a low polydispersity were applied to generate a calibration curve.

Cellular fatty acid analysis

Fatty acid analysis was performed by using the MIDI system (Microbial ID), Method, RTSBA6 Sherlock version 6.0B[S/N160291].cells were cultured on LB agar at 37^0C for 24 h. Fatty acid methyl esters were prepared from the esterified lipids in the polar (methanol) fraction by mild acid methanolic transesterification and analyzed by GC[22].Extraction and analysis were performed conforming to the recommendations of the MIDI system. Identification of fatty acids methyl esters was based on comparison of

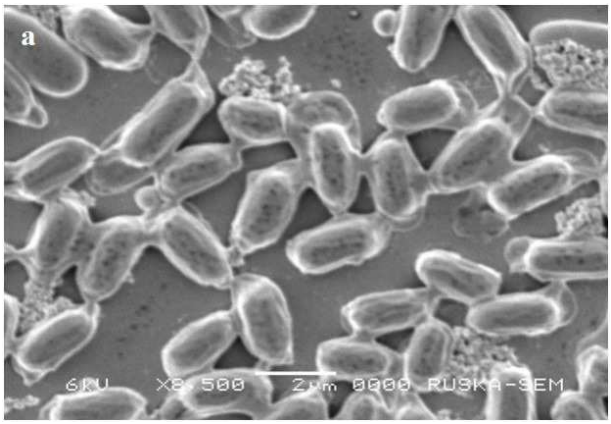
relative retention times and mass spectra of standards.

RESULTS

Morphological characteristics

Strain OU35 (figure 1) was a Gram-positive, motile with peritrichous flagella, endospore-forming rods.1.0-1.5×2.5-3.0µm in size. Ellipsoidal endospores are produced at a central position. Colonies are circular, entire, smooth, creamish yellow and 2 mm in diameter on LB medium after 24 h incubation at 37^0C.Strictly aerobic, moderately halophilic, and growing over a wide range of NaCl. Grows at 25-45^0C (optimally at pH 7.2-8.0).

Figure 1
Morphology



Scanning electron micrograph Morphology of strain OU35 grown in LB medium. Bar represents 2µm.

Biochemical characteristics

Biochemical and Physiological properties of Strain OU35 were compared with its nearest phylogenetically related strain, *B.aryabhattai,*B8W22^T(table1)**.**Strain OU35 utilized and produced acid from L- arabinose, D-glucose, inositol, lactose, D-xylose, raffinose, rhamnose, and trehalose, but not Esculin, adonitol, melibiose, D-cellobiose and galactose.It was able to produce ornithine, lysine decarboxylase, urease, protease, cellulose, amylase, Catalase and Oxidase. Antibiotics susceptibility tests showed that strain OU 35 was sensitivee to Neomycin, novobiocin, nalidixic acid, nitrofurazone and kanamycin. The G+C content of strain OU35 was 45.00 mol. %

Table 1
Physiological and biochemical properties

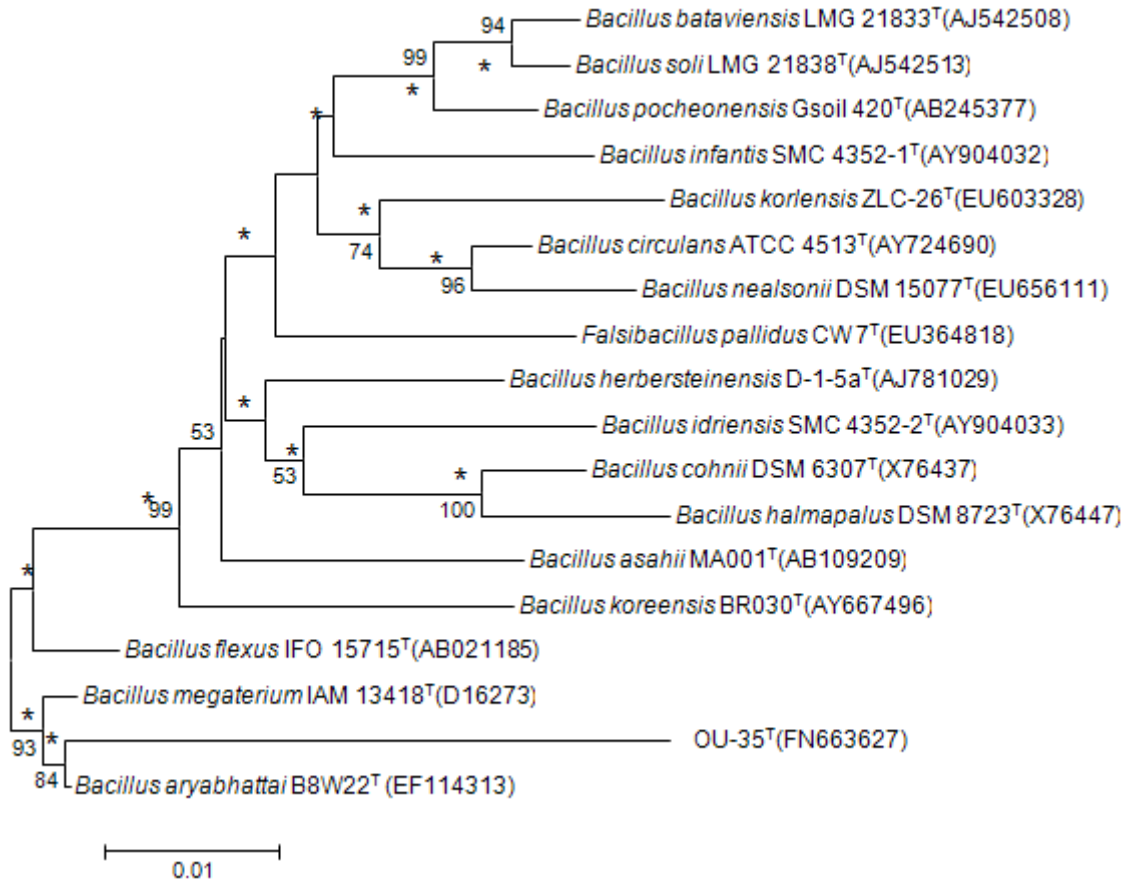
Characteristic	OU35^T	*B.aryabhattai* B8W22^T
Isolation source	Polluted water	Upper atmosphere
Colony diameter(mm)	1-2.5	5-8
Colony colour	Cream	Peach
Spore position	Sub terminal	Central
β- Galactosidase	-	+
Lipase	+	-
Tryptophane deamination	-	+
Voges-Proskauer test	-	+
Nitrate reduction	-	+
PHA production	+	-
Hydrolysis of:		
Gelatine	-	+
Urea	-	+
Cellulose	+	-
Acid from:		
D-Cellobiose	-	+
Galactose	-	+
Rhamnose	+	-
Citrate	+	-
Melanoate	-	+

Physiological properties that differentiate strain OU35^T and its nearest phylogenetic neighbor, Bacillus aryabhattai B8W22^T (data from shivaji et al. 2009). Both strains are Gram-positive, motile, endospore producing, able to grow at 25-45^{0}C, positive for catalase, oxidase, amylase, protease, ornithine decarboxilase, and negative for methyl red test, H_2S production and indole production. Both strains produce acid from D-glucose, D-trehalose, D-xylose, L-arabinose, inositol, D-mannitol, and raffinose.

Phylogenetic analysis

The sequence of the 16S rRNA-encoding gene of OU 35 was determined (1337bp) and deposited in the EMBL sequence database under accession number FN 663627.The culture was identified to be *Bacillus* sp. based on 16SrRNA gene sequencing. A BLAST (EZtaxon server) search using the 16SrRNA gene sequence showed 90% and above homology with 30 known taxa of *Bacillaceae* and maximum homology of 96.28% to *Bacillus aryabhattai* B8W22^T, 95.97% to *B.megaterium* IAM 13418^T, and 95.282% to *B. flexus* IFO15715^T.The evolutionary association of the isolate with selected species of the family *Bacillaceae* was analysed using the Neighbour-joining method (Figure 2).

Figure 2
Phylogenetic tree



Phylogenetic tree showing the relationship between strain OU35and representative 24 taxa of the genus Bacillus. Numbers at nodes indicate the percentage bootstrap support based on a neighbor joining analysis of 1000 sampled datasets. Only bootstrap values of more than 50 % are shown. Asterisks indicate the branches that were also found in the maximum parsimony tree-making algorithm. Scale bar, 0.01 substitutions per nucleotide position.

Fatty acid analysis

The cellular fatty acids were identified as iso-$C_{13,0}$(8.81%), iso-$C_{14,0}$(5.55%), iso-$C_{15,0}$(20.29%), anteiso-$C_{15,0}$(5.31%), iso-$C_{16,0}$(8.35%), $C_{16,0}$(4.56%), anteiso-$C_{15,0}$(5.31%), iso-$C_{16,0}$(8.35%,$C_{17,1}$iso w 10c (4.26) and others at lower concentrations, i.e. iso-$C_{12,0}$(1.09%), anteiso$C_{15,0}$(5.31%), $C_{16,1}$(1.33%), anteiso$C_{17.0}$(1.60%).%).

Characterization of PHA

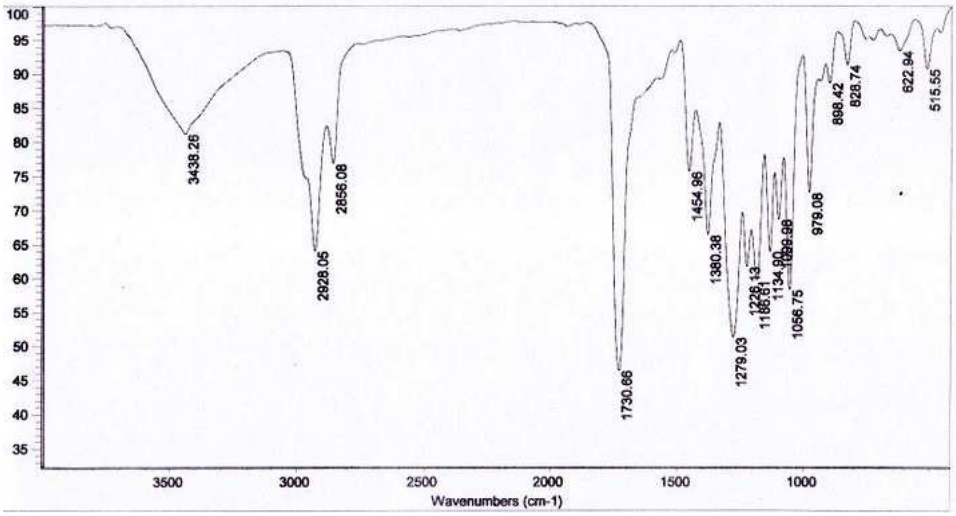
Production of PHA began after 30 h in glucose enriched medium and it was maximum at 48h and yield of PHA produced was 1.217g/l. With hydrolyzed rice bran, PHA was maximum at 44h and yield was1.530 g/l which is higher than glucose.

FTIR

Spectra were recorded for the polymers dissolved in chloroform. Spectra showed two intense absorption bands at 1,730.22 and 1,279.03 cm^{-1}, corresponding to C = O and C–O stretching groups, respectively. Other absorption bands at 1,380, 1,454, 2,928 and 3,435 cm- 1

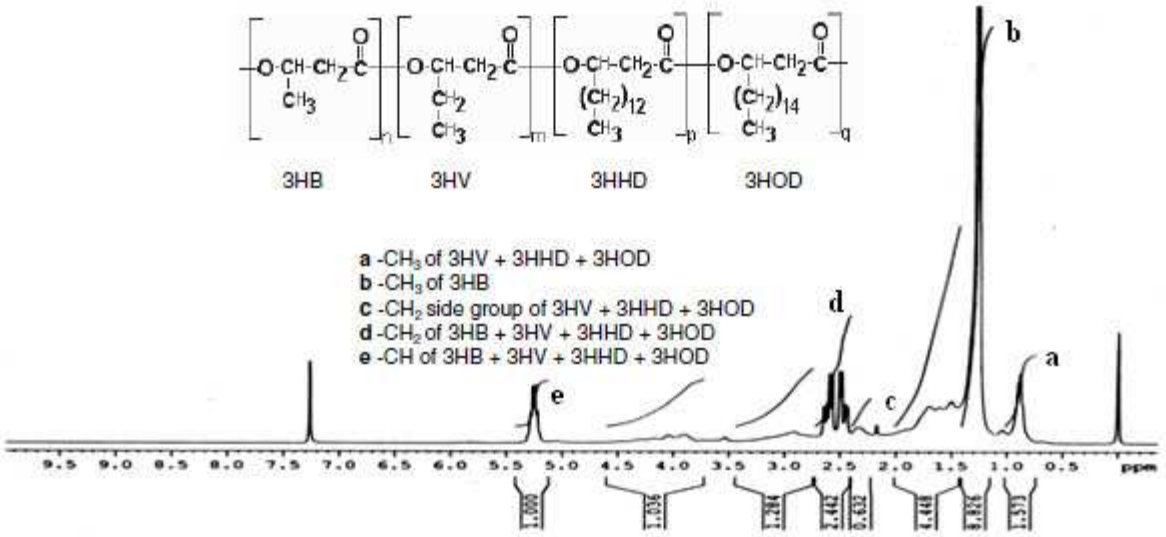
corresponding to -CH3, -CH2, -CH and O–H groups are shown in Figure 3.

Figure 3
IR spectra of isolated polymer when rice bran was used as carbon substrate



^{1}H NMR analysis confirmed the presence of different monomer signals (Figuer 4). The resonance, as observed at 0.880, 1.199, 1.622, 2.518 and 5.251 ppm by ^{1}H-NMR analysis were, respectively, for CH3 (3HV, and 3HOD side group), CH3 (3HB side group), CH2 (3HV, 3HHD and 3HOD side group), CH2 (3HV, 3HB, 3HHD and 3HOD) bulk structures), CH (3HV, 3HB,3HHD and HHD bulk structures) of theCDCl3 -soluble fraction of the polymer confirmed the presence of the copolymer consisting of 3HB, 3HV, 3HHD and 3HOD units.

Figure 4
NMR spectra

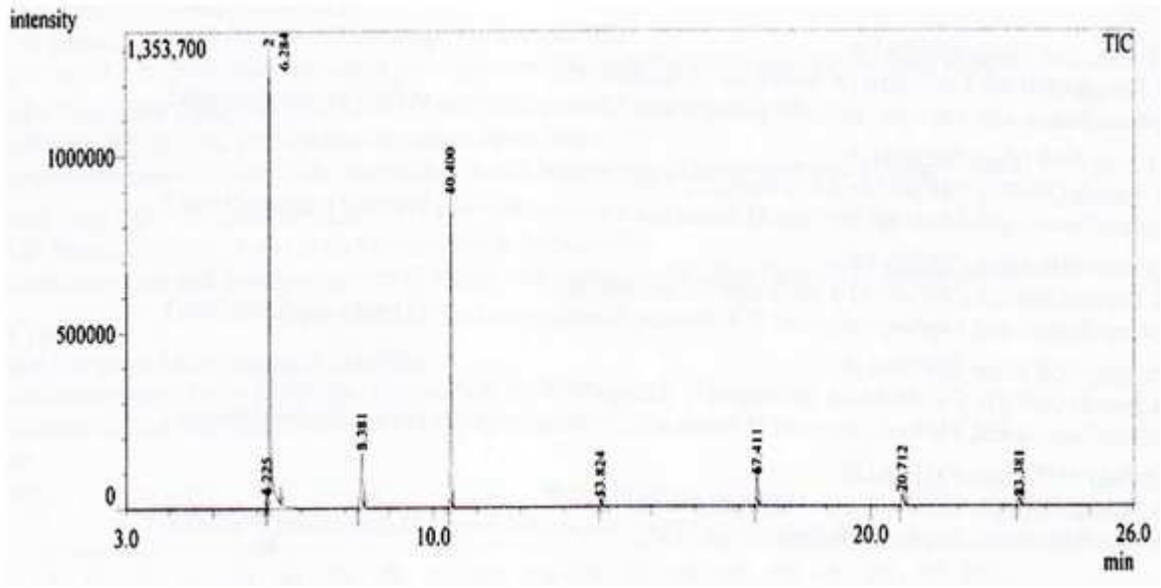


NMR of polymer from isolates OU35 when rice bran used as carbon substrate

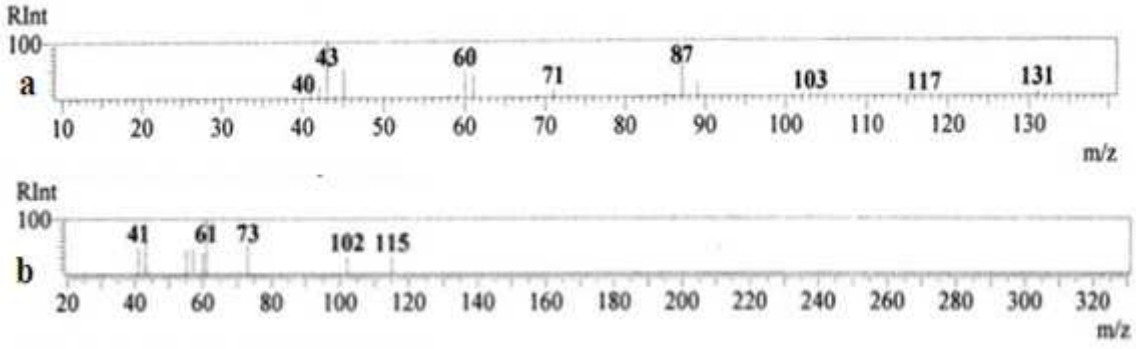
In GC-MS, three major ester peaks were found for the PHA isolated from rice bran grown strain OU35,with retention times of 6.2, 23.3,23.4 min(fig 5).These monomers were identified as, 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) respectively. The

peak at m/z 45 represented the 3-hydroxy butyrate (3HB), the peak at m/z 105.10 represented the standard benzoic acid, and the peak at m/z 61.05 represented the 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD). The composition of PHA synthesized by *Bacillus* OU35 from rice bran was found to consist of 95mol% 3HB, 2.5 mol%3HO and 2.5 mol%3HD. Concerning the molecular mass of the polymer from rice bran, synthesized by OU35 was Mn- Number average Molecular weight 53,846, Mw- Weight average Molecular weight 99,875 with poly dispersity index of, 1.854.

Figure 5
GC-MS spectra



GC-MS spectra showing 6 minutes- 3-hydroxy butyrate, 10minutes - benzoic acid, 23minutes- decanoic acid.



a and b are Mass spectra of the polymer

DISCUSSION

On the basis of morphological, studies, biochemical data, and G+C content, an aerobic, mesophilic, heterotrophic new bacterium isolated from a polluted pond belong to the genus *Bacillus.* Presence of high percentage of ISOs and anti ISOs, [iso-$C_{15,\ 0}$ (20.29%), anteiso-$C_{15,\ 0}$(5.31%)] is indicating that the strain belongs to the genus *Bacillus*. From the phylogenetic analysis, it was clear that, the isolate belonged to the genus *Bacillus* and represented a distinct lineage that could be equated with a separate genomic species. Although it has been demonstrated that 16S rDNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of _97% represents a new species, the meaning of similarity scores of _97% is not as clear[23]. This latter value can represent a new species or, alternatively, indicate clustering within a previously defined taxon. In this study also strain OU35 has showed 96.28% homology to *Bacillus aryabhattai* B8W22^T, 95.97% to *B.megaterium* IAM 13418^T, and 95.282% to *B. flexus* IFO15715^T .Further study is needed to confirm its identity. DNA-DNA hybridization studies have conventionally been essential to provide best answers for this. The type strain OU35^T has been deposited in the China center for type culture Collection (CCTCC) as strain CCTCC AB 2011468^T.

The chemical composition of the PHA accumulated by strain OU35 appeared to be different from those produced by other bacteria from extreme environments. Strain OU35 unlike other *Bacillus sp.* this isolate has the ability to synthesize co-polymers of both scl and mcl PHA polymers by utilizing glucose and rice bran as sole carbon sources with out adding any fatty acid precursors. There are few reports on bacteria, capable of synthesizing both types of monomers and *B. cereus* is one amongst them .*Bacillus cereus* has been reported to utilize caprolactone and biosynthesize 9% (w/w) copolymer containing 3-hydroxybutyrate, 3-hydroxyvalerate and 6-hydroxyhexanoate[7]. Tajima *et al*., isolated and characterized from gas field soil a *Bacillus* sp.INT005 accumulating PHA. Bacillus sp. INT005 produces PHA with various monomer compositions, poly (3HB), poly (3HB-co-3HV), poly (3HBco- 3HHx), poly (3HB-co-4HB-co-3HHx) and poly (3HB-co-6HHx-co-3HHx) when fed with appropriate substrates (butyrate, valerate, hexanoate, octanoate, decanoate, 4HB and e-caprolactone)[24]. A number of *Bacillus* sp. Have been identified to accumulate PHA, but this strain produces a novel P (HB-co-HO-co-HD) having high elasticity with out adding any fatty acid precursors to the media.

CONCLUSION

Strain OU35 could be an interesting candidate for the production of PHA from agricultural wastes, thus resulting in a reduction of the cost production of biopolymers. How ever further studies are needed for large scale production of the PHA and effective utilization of agricultural wastes as carbon source.

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REFERENCES

1. Everest A, Tajalli K, and Ipsita R. Production of polyhydroxyalkanoates the future green materials of choice. J Chem.Technol. Biotechnol., 85, 732–743, (2010).
2. Chen G. Q. Biofunctionalization of Polymers and Their Applications. Adv

Biochem. Engin/Biotechnol., 125, 29-45, (2011).
3. Vandamme P, Coenye T. Taxonomy of the genus *Cupriavidus*, a tale of lost and found. Int. J. Syst .Evo .Microbiol. 54, 2285–2289, (2004).
4. Chen G.Q and Wu Q. The application of polyhydroxy alkanoates as tissue engineering materials. Biomaterials. 26, 6565–6578, (2005).
5. Valappil S.P, Boccaccini A.R, Bucke C, Roy I. Polyhydroxy alkanoatesin Gram-positive bacteria, insights from the genera *Bacillus* and *Streptomyces*. Antonie van Leeuwenhoek. 91, 1–17, (2007a).
6. Law K.H, Cheng Y.C, Leung Y.C. Lo, W.H, Chua H, Yu H.F. Construction of recombinant *Bacillus subtilis* strains for polyhydroxy alkanoates synthesis. BiochemEng J. 16, 203–208, (2003).
7. Labuzek S, Radecka I. Biosynthesis of PHB tercopolymer by *Bacillus cereus* UW85. J. Appl.Microbiol. 90, 53–357, (2001).
8. Anil Kumar P.K, Shamala T.R, Kshama L, Prakash M.H , Joshi G.J, Chandrashekar A, Latha Kumari K.S, Divyashree M.S. Bacterial synthesis of poly (hydroxybutyrate- co-hydroxyvalerate) using carbohydrate-rich mahua (Madhuca sp.) flowers. J Appl. Microbiol. 103, 204-9, (2007).
9. Shamala T.R, Chandrashekar A, Vijayendra S.V, Sharma L. Identification of polyhydroxy alkanoate (PHA) producing *Bacillus* spp. using the polymerase chain reaction (PCR). J. Appl. Microbiol. 94, 369–374, (2003).
10. Gouda M.K, Swellam A.E, Omar S.H. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol. Res., 156, 201–207, (2001).
11. Valappil S.P, Peiris D, Langley G.J, Herniman J.M, Boccaccini A.R, Bucke C, Roy I. Polyhydroxyalkanoate (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus spp*. J Biotechnol . 127, 475–487, (2007b).
12. Lagveen R.G, Huisman G.W, Preustig H, Ketelaar P, Eggnik G, Witholt B. Formation of polyesters by *Pseudomonas oleovorans* , effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyl alkenoates. Appl. Environ. Microbiol. 54, 2924-2932, (1988).
13. Ostle A.G, Holt J.G. Nile blue as a fluorescent stain for PHB. Appl. Environ. Microbiol.44, 238-241, (1982).
14. Holding A. J, Collee J. G. Routine biochemical tests. 6A, 2–32, (1971).
15. Hugh R, Leifson E. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol. 66, 24-6, (1953).
16. Shivaji S, Rao N. S, Saisree L, Sheth V, Reddy G. S. N, Bhargava P. M. Isolation and identification of *Micrococcus roseus* and *Planococcus* sp. from Schirmacher. Oasis.Antarctica. J Biosci. 113, 409-414, (1988).
17. Tamura K, Dudley J, Nei M, and Kumar S. MEGA4, molecular evolutionary genetic analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599, (2007).
18. Thompson J. D, Gibson T. J, Plewniak F, Jeanmougin F, Higgins D. G. The clustal X windows interface, flexible strategies for multiple sequence alignmen aided by quality analysis tools. Nucleic. Acids. Res. 25, 4876–4882, (1997).
19. Saitou N, Nei M. The neighbor-joining method a new method for reconstructing phylogenetic trees, Mol. Biol. Evo. l4, 406–425, (1987).
20. Mesbah M, Premachandran U, and Whitman W. B. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid

chromatography. Int. J. Syst. Bacteriol. 39, 159–167, (1989).
21. Ramsay B.A, Lomaliza K, Chavarie C, Dube B, Bataille P, Ramsay J.A. Production of poly-(beta-hydroxybutyric-co-betahydroxyvaleric) acids. Appl. Environ. Microbiol. 56, 2093-2098, (1990).
22. Sasser M. Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. Newark, DE, MIDI Inc, (1990).
23. Petti C.A. Detection and identification of microorganisms by gene amplification and sequencing. Clin. Infect. Dis. 44, 1108–1114, (2007).
24. Tajima K, Igari T, Nishimura D, Nakamura M, Satoh Y, Munekata M. Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxy alkanoate (PHA) from gas field soil. J Biosci.Bioeng. 95, 77–81, (2003).