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# PRODUCTION OF POLY (3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE) BY A NOVEL *BACILLUS* OU40$^{T}$ FROM INEXPENSIVE CARBON SOURCES

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## ABSTRACT

The goals of this work are to characterize and evaluate the ability of strain OU40$^{T}$ to produce poly (3-hydroxybutyrate-co-3-hydroxyvalerate)(PHBV) using various agricultural wastes, isolated from polluted water.Growth of the isolate OU40$^{T}$ was assessed in mineral media containing glucose, starch, bagasse, whey and rice bran without adding any precursors. Biopolymers produced by strain OU40$^{T}$ had an average molecular weight 1,304 kDa to 2,332 kDa with the melting point 155-160 $T_m$(°C) and percentage of crystallinity 40- 50%. Based on phenotypical, biochemical and genotypic investigations, strain OU40$^{T}$ is assigned to the genus *Bacillus*. The DNA-DNA relatedness between OU40$^{T}$ and *Bacillus cereus* ATCC14579$^{T}$ (AE016877) was found 60% of mean similarity, 4.24 SD value, ΔTm value of 6% and G+C content was 46.5%.These result suggests that the strain could be a novel genomic species.The 16S rRNA gene sequence is deposited in EMBL/Genbank with accession number FN663629.The type strain is OU40$^{T}$ (=JCM17287$^{T}$=CCM7835$^{T}$ =DSM24141$^{T}$).

**KEYWORDS : Poly (3-hydroxybutyrate-co-3-hydroxyvalerate); PHBV; *Bacillus cereus*; Polyhydroxyalkanoates.**

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# INTRODUCTION

Polyhydroxyalkanoates (PHA) are accumulated as a carbon and energy storage material by various microorganisms[1]. PHA are environmentally benign and can be produced by fermentation of renewable feed stocks[2]. They are considered as attractive "green" substitutes for petroleum-derived polymers in many applications such as medicine, drug delivery agents, agriculture, horticulture, fibers, and other consumer products[3]. The cost of the raw materials, mainly the carbon source, accounts up to 50% of the overall production cost of PHA.Therefore the use of inexpensive renewable agricultural and industrial co products as feedstock could be tremendous advantage to the economics of PHA polymer production. It is essential to explore an alternate substrate for bacterial growth and copolymer production. Cheaper raw materials such as whey, wastewater from olive mills, molasses, corn steep liquor, starchy wastewater, palm oil mill effluent,industrial oil effluent, have been used as nutrient supplements for bacterial PHA production[4,5,6].

The genera *Bacillus* being identified as one of the first Gram-positive bacteria capable of PHA production, offers several advantages of PHA fermentation studies. These include chemo organotropic features, of *Bacillus* sp. explore the possibility of utilizing various agricultural raw materials as a carbon source for production of different metabolites. Bacteria synthesize different types of polyesters composed of various kinds of monomers depending on the fermentation conditions and the carbon source supplied[7]. The co- polymer synthesis from structurally unrelated carbon sources suggests that *Bacillus* has the potential for production of new PHA co-polymer using different substrates[8]. This work deals with the production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate)(PHBV) from whey, the major byproduct from cheese industry, waste starch collected from flour mills and also available abundantly from plant sources, bagasse a cheap source of hemicellulose, xylose and rice bran. Till now,limited research is conducted on PHBV production by Gram positive bacteria such as *Bacillus* sp., using agro industrial wastes. This paper focuses on biological characterization of a PHA accumulating bacterium isolated from polluted water and also deals with the physicochemical characterization of polymer produced from cheap carbon sources.

# MATERIALS AND METHODS

### *(i). Bacterial strain and culture media*

The bacteria $OU40^T$ was isolated from industrially polluted water pond in Hyderabad city, India.Stock cultures were grown and maintained at 30±2 by periodic transfer on LB agar slants by overlaying the slants with 20% (v /v) glycerol. The first stage of the two stage cultivation was a cell-growth stage using a nutrient rich medium consisting of 10g/L Tryptone , 5g/L east extract , 5g/L NaCl in a 500-mL shaker flask at $30^o$ C and shaken at 150 rpm for 24h. The second stage was a nutrient-deficient copolymer accumulation stage. Cells from the first stage were transferred into nitrogen-deficient $E_2$ mineral medium[9].Sugars and mineral salt solutions were autoclaved seperately at $121^oC$ for 15min. Flasks were incubated at 30± $2^oC$ for 48h on a rotatory shaker at 150 rpm. $E_2$ mineral medium was supplemented with starch, rice bran, bagasse, whey and glucose independently with out hydrolysis.

### *(ii). Characterization of the isolated bacteria*

In order to characterize the strain OU40, standard phenotypic tests were performed. Cell morphology was examined by light microscopy (Olympus) and scanning electron microscopy using cells from exponentially growing cultures. Gram staining was performed by the Burke method[10].Motility was assessed on 0.4% nutrient agar plates and also by hanging drop method[11]. Spore staining was done using Schaeffer & Fulton's spore-staining kit (K006-1KT; HiMedia) according to the manufacturer's protocol. Growth and

biochemical characteristics, carbon assimilation and sensitivity of cultures to different antibiotics were determined by previously described methods[12]. Biochemical characteristics were also checked with the Hi25 Enterobacteriaceae identification kit (KB003) and HiCarbohydrate kit parts A, B and C (KB009) (both from HiMedia) according to the manufacturer's protocol. Sensitivity of the culture to nine antibiotics was determined using antibiotic discs(Hi Media), containing polymyxin B(100 IU $ml^{-1}$), penicillin G(10 IU $ml^{-1}$), ampicillin (10 µg $ml^{-1}$), novobiocin (5 µg $ml^{-1}$), tetracycline(50 µg $ml^{-1}$), kanamycin(30 µg $ml^{-1}$), neomycin(50 µg $ml^{-1}$), nitrofurazone(30 µg $ml^{-1}$),and nalidixicacid(50 µg $ml^{-1}$).

For cellular fatty acid analysis, cell mass of strain OU40 was harvested from LB plates after incubation for 24 hours at 30$^0$C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System[13](Sasser, 1990). The resulting profles were identified with the Microbial Identifcation software (MIDI) using the RTSBA database (version 6.0) (Microbial ID, Newark, DE, USA).The DNA G+C content was determined by the method of Tamaoka and Komagata[14] with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-PHAe HPLC. Universal primer set of 27F (5´-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5´-GGCTACCTTGTTACGACTT-3´)[15] (Weisburg 1991) were used for amplification of 16S rRNA of the isolate. The sequence of the strain was compared with similar sequences of the bacterial strains was aligned and clustered against those of the family *Bacillaceae*, which are available in the gene bank using clustal W analysis.

Levels of DNA-DNA relatedness between OU40$^T$ and reference strain *Bacillus cereus* ATCC 14579$^T$ (AE016877) was performed by using dot-blot hybridization method[16] and simple fluorimetric method for the estimation of DNA relatedness by thermal denaturation temperatures[17]. Genomic DNA was isolated from the test strain, along with the reference strain, according to the method of Tripathi and Rawal[18]. The DNA was quantified using UV spectrophotometer at A260 nm (Nanodrop Technologies) and checked for integrity in the agarose gel. The 16S rRNA gene sequence was deposited in EMBL/Genbank with accession number FN 663629.

### *(iii). Analytical methods*

Growth of the organism was determined spectro-photometrically by measuring the attenuance of the culture at 620 nm using an AMIL Photochem5 photoelectric colorimeter. For determination of total dry mass, cells were harvested by centrifugation at 10,000 rpm in a Hitachi SCR 20B centrifuge, washed thoroughly, transferred to pre-weighed aluminium cups and dried to a constant mass at 80$^o$C. The residual mass was defined as total dry cell weight (DCW) minus PHA weight; PHA% was defined as the percentage of the ratio of PHA to DCW. Qualitative and quantitative estimation of PHA was carried out by GC (Shimadzu GC 17-A) analysis using lyophilized cells[19]. P (HB-co-HV), containing 5 mol% of hydroxyvalerate (Sigma Aldrich, USA) was used as standard. For $^1$H NMR spectroscopic analysis, the purified polymer was dissolved in analytical grade deutero chloroform ($CDCl_3$) and chemical shifts were recorded using a Bruker AMX 300 NMR Spectrophotometer with 5 mm multinucleate probe head. $^{13}$C NMR Analysis were performed at 75.4 MHz on a Varian Unity Inova spectrophotometer. The FTIR spectrum was recorded at 400 - 4000 $cm^{-1}$ on Shimadzu (FT-IR) spectrophotometer. The monomeric composition of the polymer was determined by GC-MS, using a Turbo Mass Gold Mass Spectrometer (Perkin Elmer Instruments)[20]. Benzoic acid was used as internal standard and the concentration of each peak was determined by using total area. Molecular mass analysis was conducted with purified PHB 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. Polystyrene standards with a low

polydispersity were applied to generate a calibration curve. The X-ray diffraction patterns of the samples were recorded at 25$^{o}$C in the range 2Ø = 0 to 40 degree at scan speed of 2$^{o}$C/min. Thermal analysis was performed on a Mettler TA 4000 system instrument.

## RESULTS

The microbiological and biochemical properties of strain are revealed in table 1.

### 1. *Characterization of Bacillus sp.OU40$^{T}$*

This strain was isolated from the polluted water, is a gram positive, rod shaped cell (1.5x2.5x49µm)(fig 1) and was motile, could grow and accumulate PHA between 35-45$^{0}$C, with an optimal growth temperature of 37$^{0}$C and is an obligate aerobe. Transmission electron microscope observations of strain OU40$^{T}$ revealed the presence of inclusion granules, identified as polymer granules (Fig.2). The cells were characterized by the presence of iso-C15: 0, iso-C17: 0 and C13:0 as the predominant fatty acids.

**Table 1**
***Taxonomic characteristics of strain Bacillus sp. OU40$^{T}$***

| Characteristics | *Bacillus* OU40$^{T}$ | Metabolic activity | *Bacillus* OU40$^{T}$ |
|---|---|---|---|
| Shape | Rod | Ammonia | + |
| Size (µ m) | 1.5 x2.5 - 4.0 | HCN production | _ |
| Motility | + | Citrate | + |
| Gram's character | + | Indole formation | _ |
| Endospore | + | $H_2S$ formation | _ |
| Catalase | + | Methyle red | _ |
| Oxidase | _ | Arabinose | _ |
| Lipase | + | Xylose | + |
| Lecithinase | _ | Adonitol | _ |
| Cellulase | + | Cellobiose | + |
| Amylase | + | Trehalose | + |
| Urease | + | Glucose | + |
| Protease | + | Lactose | + |

The DNA G+C content was 46.5%. Based on the above characteristics, strain OU40$^{T}$ was assigned to the genus *Bacillus*. The placement of the strain within the genus *Bacillus* was further supported by phylogenetic analysis based on the 16S rRNA gene sequence, wherein it clustered with the type strain of *Bacillus cereus* ATCC 14579T (AE016877). In the phylogenetic tree based on the neighbor joining algorithm, strain OU40$^{T}$ fell within the cluster comprising *Bacillus* species, (Fig.3) and exhibited 16s RNA gene sequence similarity values of 99%. The DNA-DNA relatedness between OU40$^{T}$ and *Bacillus cereus* ATCC 14579$^{T}$ (AE016877) was found 60% of mean similarity, 4.24 SD value and ΔTm value of 6%. The above result suggests that the strain should be a novel genomic species, but needs further study to confirm its identity because isolate was shown >98% identity with more than one type strains.

**Morphology of OU40$^T$**

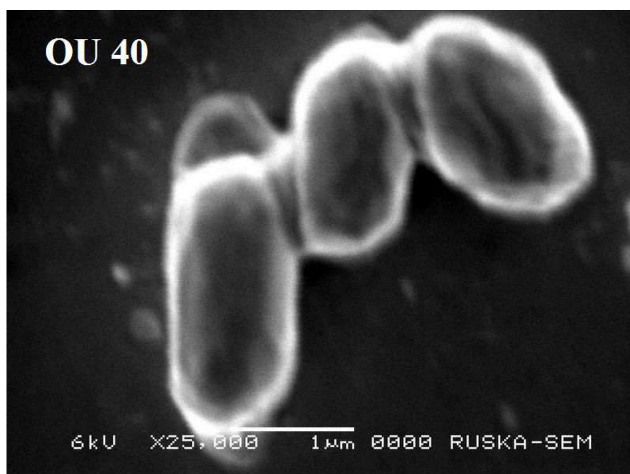


**Figure 1**
***Scanning electron micrograph showing morphology of strain OU40$^T$***

**Transmission electron microscopy of OU40$^T$**

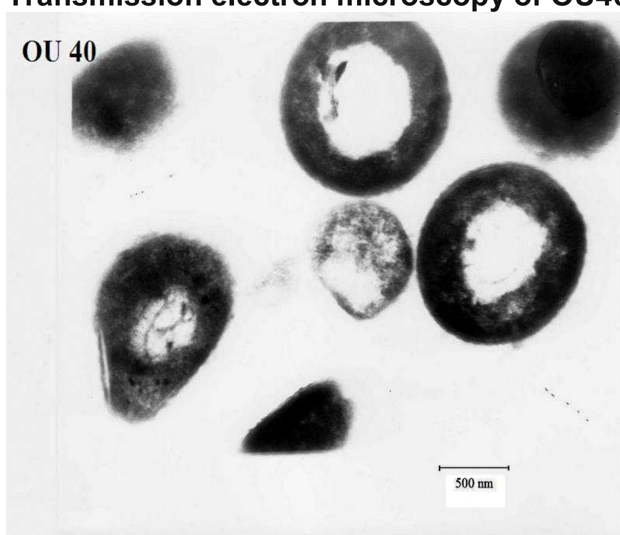


**Figure.2**
***Transmission electron microscopy photomicrograph thin section of strain OU40$^T$ showing inclusion granules after 48 h growth on glucose as sole carbon source. Bar represents 0. 5 µ m.***

**Phylogenetic analysis of OU40$^T$**

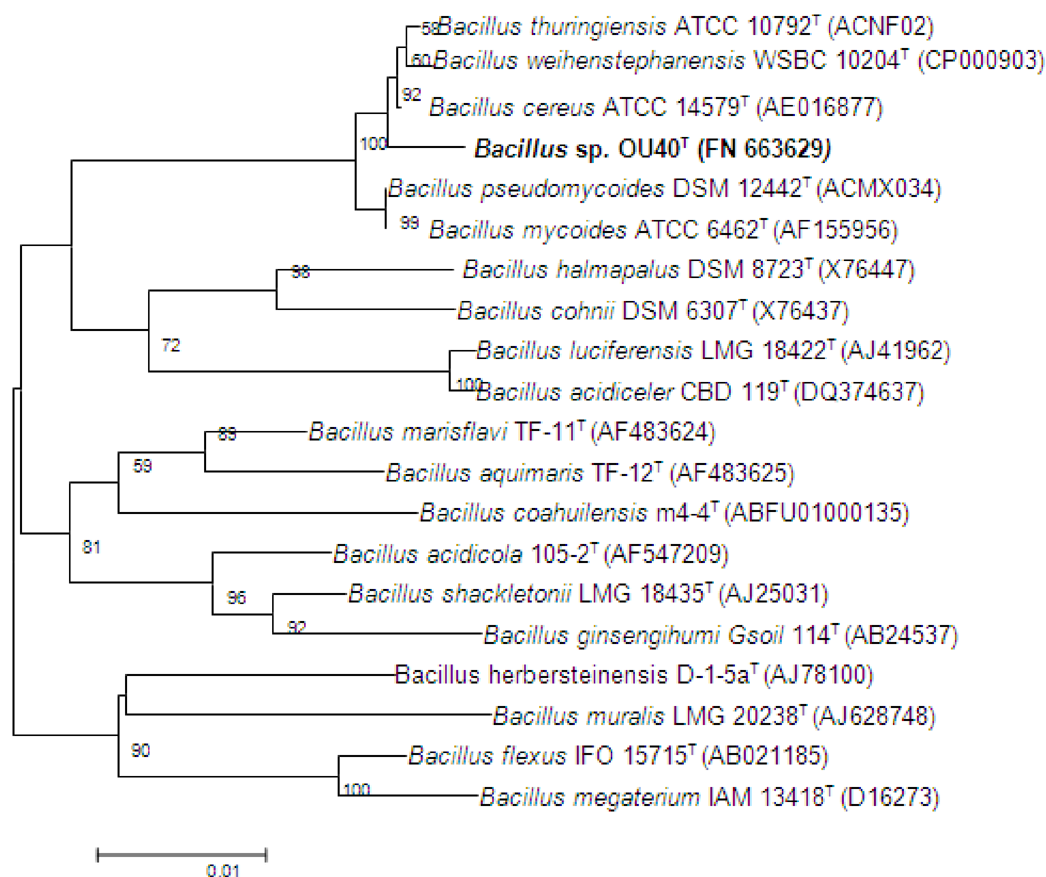


**Figure.3**

***Phylogenetic tree based on 16S r DNA sequences of members of the genus Bacillus, showing the location of strain OU40$^T$.Gene bank accession numbers are provided in parenthesis. Scale bar represents 0.01 substitutions in nucleotide sequence.***

***2. Biosynthesis of P (3HB-co-3HV) from various carbon sources***

$E_2$ medium was supplemented with 2% (w/v) glucose. Polymer production was observed from 36h to 60h incubation period. Maximum growth was at 50h and polymer content was 45% (w/v) of CDW (table 2) and, having 92.73% HB units and 7.27% HV units and bio mass 3.12 gm/l, without using valeric acid as precursor. With sugar cane bagasse, polymer production was maximum at 52h incubation period, maximum growth was occurred at 48h. The bacterium utilized cellulose, hemicelluloses and produced PHBV content up to 57.29 % of CDW, with 94.39% HB units and 5.71% HV units and biomass 3.20 %( g/l). When waste starch 2% (w/w) was supplemented to $E_2$ mineral medium, an increase in cell growth was observed up to 3.50(g/l) from 40h to 50h. PHBV content was observed as 64.85% CDW; with 95.06% HB units and 4.94% HV units. 2% Whey was added as carbon source to the $E_2$ Mineral medium. *Bacillus* sp. OU40 completely utilized whey and produced biomass 3.0% (g/l). The yield of PHBV was 60.09% CDW with 85.74% HB units and 14.26% HV units. Polymer production was maximum at 48h incubation period. It was observed that biomass production was fastest between 30h to 38h of growth. $E_2$ Mineral medium was supplemented by 1% crude rice bran as carbon source to promote PHA accumulation. Our strain utilized rice bran completely and biomass was noticed up to 3.012 % ( g/l) (fig.4), and PHBV content was reported as 71.97% CDW with 84.40% HB units and 15.60%HV units. Till now no

report is there to produce PHBV copolymer with 15.60% HV units by utilizing rice bran as carbon source by *Bacillus* sp.OU40$^T$.

Table 2 summarizes the results of copolymer production by *Bacillus* sp. OU40$^T$ from different agro industrial wastes including glucose, starch, whey, rice bran and bagasse. The composition of polymers was affected by the option of carbon source. The polymer content in dried cells ranges 45% to 71.98% depending on the contents present in carbon source. The maximal PHBV 71.98% was produced when rice bran was used and 64.85% PHBV was derived when starch was used.

**Table 2**
***PHA content and composition of polymer accumulated by Bacillus sp. OU 40$^T$ grown in different carbon sources***

| Carbon source | ODat 620 nm | CDW(gL,W/V) | Monomer composition | | PHA% |
|---|---|---|---|---|---|
| | | | HB | HV% | |
| Glucose | 1.61 | 3.12±0.005 | 92.73 | 7.27 | 45.00 |
| Bagasse | 2.21 | 3.201±0.004 | 94.39 | 5.27 | 57.29 |
| Whey | 2.14 | 3.00±0.101 | 85.74 | 14.26 | 60.09 |
| Starch | 2.20 | 3.50±0.003 | 95.06 | 4.94 | 64.85 |
| Rice bran | 2.43 | 3.012±0.141 | 84.40 | 15.60 | 71.90 |

CDW- cell dry weight
PHA% CDW was estimated by GC analysis

**Table 3**
***Physical properties of the polymers extracted from Bacillus sp. OU 40 when grown on glucose and rice bran***

| Carbon source | Numberaverage Molecular weight ($M_n$) | Weight average Molecular weight ($M_w$) | Poly dispersity index ($M_w/M_n$) | Melting Endotherm $T_m$ ($^oC$) | %Crystallinity |
|---|---|---|---|---|---|
| Glucose | 2, 04 | 2,332 | 1.14 | 155 | 50.06 |
| Rice Bran | 737 | 1,304 | 1.76 | 160 | 40.4 |

## GRAPH

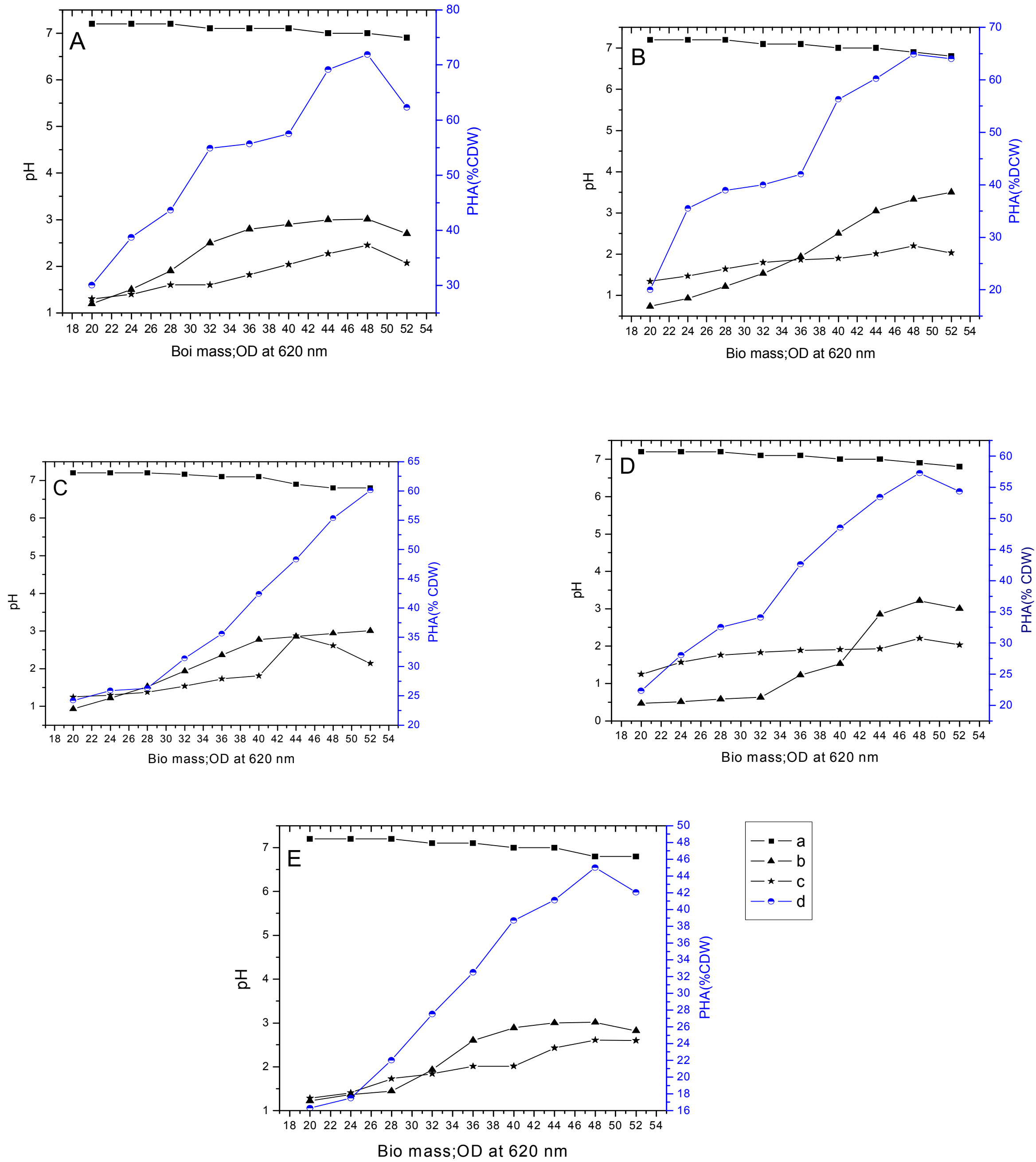


**Figure.4**

***a. pH, b.Cell density, c.OD at 620 nm , and d.PHA content (% CDW) of Bacillus sp. OU 40$^T$ when (A)Rice bran(B)Starch(C)Whey(D) Bagasse and (E) Glucose was used as carbon source with $E_2$ mineral medium.***

***3. Biochemical characterization of the polymer***

Both $^1$H and $^{13}$C NMR spectra analysis of purified PHA polymer from the *Bacillus* sp. OU 40$^T$ confirm the structure of the co-polymer, poly (3-hydroxy butyrate-CO-3-hydroxy Valerate)(Data not shown). Figure.5 reveals the absorption bands at 1720 cm$^{-1}$ corresponding to the Easter carbonyl group and at 1280 cm$^{-1}$ corresponding to the –CH group characteristic of PHA. The methyne (CH) group gave a strong band in the range of 1379-1450 cm$^{-1}$ and 2925-3450 cm$^{-1}$ for -$CH_3$, -$CH_2$, -CH, C=O and O-H groups respectively.

**IR spectrum**

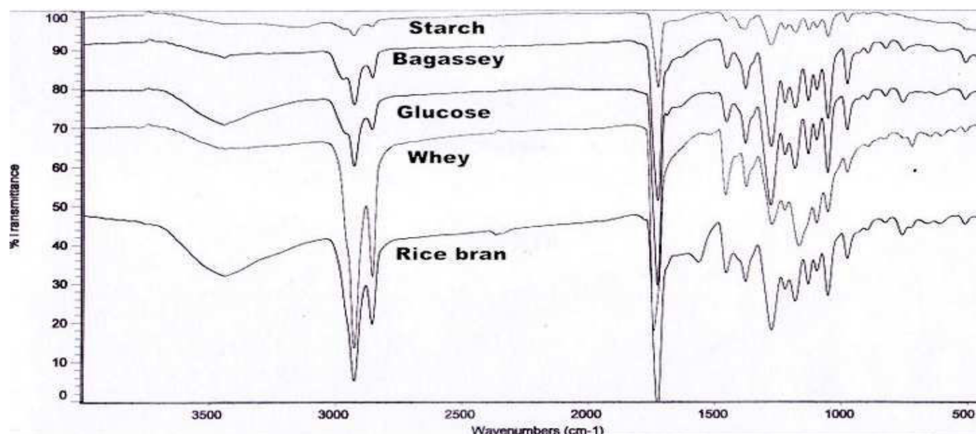


**Figure.5**
***IR spectrum of the polymer produced by Bacillus OU 40$^T$ showing spectra with various carbon sources.***

In the GC-MS analysis (Figure.6) of the PHA produced by *Bacillus* sp. OU40$^T$ indicated that the polymer was composed by P (HB-co-HV) units. The peak area at m/z 287 corresponding to PHBV, was rationalized to the area of the (M-17)$^+$ ion at m/z of 105 of the benzoic acid. The concentration of each peak was determined by using total area. Concerning the molecular mass of PHBV from rice bran, synthesized by *Bacillus* sp. OU40$^T$ was 737kDa with poly dispersity index of 1.769, whereas with glucose it produced PHB with molecular mass 2,040 kDa with poly dispersity index of 1.143. . Regarding the thermal properties of the polymer, the value for $T_m$ ($^o$C) was onset melting point 145.2$^o$C, End set $T_m$ 175.0$^o$ C and $T_m$ value obtained was 168.0$^o$C. This indicates that the formation of co-polymers with HV units increased with increasing melting point. The percentage of crystallinity calculated from diffracted intensity data according to Vonks method was presented in table (3). According to the data, the presence of more HV units determines less crystallinity.

**Mass spectrum**

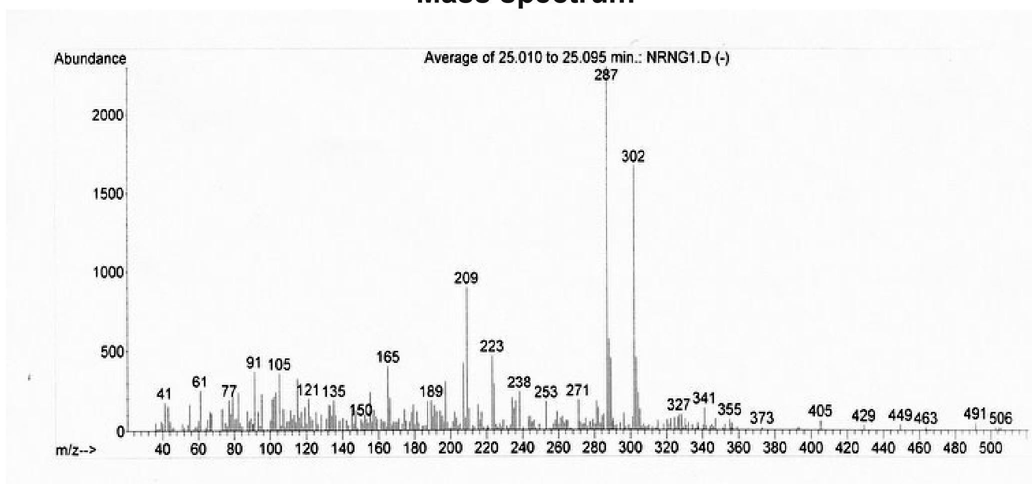


**Figure.6**
***Mass spectra of methyl esters of the polymer produced by Bacillus OU 40$^T$ when rice bran was supplemented with $E_2$ mineral media.***

# DISCUSSION

Agro industrial wastes are desirable feedstock for PHA production because they are relatively inexpensive as compared to most sugars. On the other hand, agricultural wastes are predicted to produce higher PHA yield because it contains higher carbon content on weight basis than simple sugars[21]. Here *Bacillus* sp. OU40$^T$ was able to utilize a wide variety of carbon sources. It was observed that more than 2% glucose was inhibitory effect on the strain. 1% sugarcane bagasse was used as carbon source with $E_2$ medium to produce PHBV. About 70% of the dry mass in lignocellulosic biomass consists of cellulose and hemicellulose. The hemicellulose was completely hydrolysed to D-Xylose and L-Arabinose and cellulose were converted to glucose (not shown in data) detected by HPLC. Rice bran contains hemicelluloses 7.9-15.4% cellulose 8.9%-12.3% and sugars (glucose, fructose, sucrose, and raffinose) 3to8%.The growth of the culture at high temperature in combination with its starch utilization ability and PHBV production can be a better choice than the two steps of enzymatic hydrolysis of starch followed by PHA production[22]. Previously the strain of *Haloferax Mediterranean* and *Azotobacter chroococcum* were employed to produce PHA in a starch medium[23] where hydrolysis of starch was carried out separately. The strain *Bacillus sp.* OU40$^T$ has desirable properties of tolerance to extreme conditions of $p^H$ and temperature. The ability to ferment various carbon sources without hydrolysis enables PHBV production in different economical substrates by recycling agro industrial wastes.

Biochemical characterization of the polymer indicated the structure as poly (3-hydroxybutyrate-co-3-hydroxyvalerate**.** The $^{13}C$ NMR analysis (result not shown) supported the results from the proton NMR analysis. The melting temperatures and enthalpies of fusion of the PHA samples obtained were determined using DSC. The thermal properties of the polymer such as the glass transition temperature (Tg) and the melting temperature (Tm) are crucial for polymer processing. Here the melting point obtained was in agreement with literature[24]. PHA molecular mass is an intrinsic aspect for each given strain. For example, *Azotobacter* strains accumulate PHA whose molecular masses range from 800 to 2,000 kDa, *Pseudomonas* sp. from 50 to 60kDa and *Methylobacterium* sp. from 250 to 300kDa[25]. In the case of biopolymer produced by *Bacillus* sp. OU40$^T$, the molecular mass obtained was in the range of the above mentioned bacteria.It is also significant that

the molecular mass of PHA is inclined by the extraction technique employed.Indeed, neutral solvents extraction yields higher values than alkaline hypochlorite treatment[26]. Since the neutal solvvents extraction was used in this study the extraction procedure may distress the molecular mass. GPC analysis showed that (table 2) the PHA extracted using the hypochlorite-chloroform extraction technique was of quite high weight average molecular weight (MW = 1.7×106) and number average molecular weight (MN = 737). Hence, the low polydispersity index of 1.76 obtained for the PHA produced in this study compared to what is reported for most other PHA producing micro-organisms[27] can further extend the use of this PHA in controlled drug delivery applications.
In Conclusio*n,* the *Bacillus* sp. OU40$^T$ which was newly characterized possessed the unique capability of producing P (3HB-co-3HV) copolymer from renewable agro industrial wastes without adding any precursors. In this study glucose was replaced by whey, starch, bagasse and rice bran to enhance the PHBV production. These carbon substrates not only reduced the cost of material, but also increased the cell concentration and co-polymer accumulation. This is the first report where in a *Bacillus* sp*.* is producing PHBV copolymer with above mentioned feed stocks without supplying any precursors. Thus the identified isolate *Bacillus* sp. OU40$^T$ capable of accumulating P-3(HB-co-HV) depending on the carbon substrate used, may act as a robust strain for industrial application. The type strain is OU40$^T$ (=JCM 17287$^T$=CCM 7835$^T$ =DSM 24141$^T$).

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