British Biotechnology Journal
8(3): 1-9, 2015, Article no.BBJ.17999
ISSN: 2231–2927

SCIENCEDOMAIN *international*
www.sciencedomain.org

Isolation, Molecular Characterisation of Polyhydroxyalkanoate Producing Novel *Bacillus* sp., skm7^T from a Polluted Pond Water

K. Chaitanya[1*], S. K. Mahmood[2] and M. Mohammed Idris[3]

[1]Jawaharlal Nehru Technological University, Hyderabad, India.
[2]Department of Botany, Nizam College, Osmania University, India.
[3]Centre for Cellular and Molecular Biology, Hyderabad, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author SKM designed the study. Author KC performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author MMI managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2015/17999
Editor(s):
(1) P. Mary Anupama, Department of Chemical Engineering and Biotechnology, Anil Neerukonda Institute of Technology and Sciences, India.
(2) Kuo-Kau Lee, Department of Aquaculture, National Taiwan Ocean University, Taiwan.
Reviewers:
(1) Ilham Zahir, Department of Biology, University Sidi Mohamed Ben Abdellah, Morocco.
(2) Anonymous, Zhejiang University, China.
(3) P. Vijayaraghavan, Centre for Marine Science and Technology, M. S. University, India.
(4) Anonymous, Universiti Sains Malaysia, Malaysia.
Complete Peer review History: http://sciencedomain.org/review-history/10108

Original Research Article

Received 31st March 2015
Accepted 17th June 2015
Published 8th July 2015

ABSTRACT

Bio-plastics are natural biopolymers that are synthesized and catabolised by various organisms and these materials do not cause toxic effects in the host and have certain advantages over petroleum-derived plastics. The current emphasis on sustainability, eco-efficiency and green chemistry has led to intensive search for renewable and environmentally friendly resources. Thus, sustainable development is recognized to be essential for the growth of the economy and industrial productivity. A polyhydroxyalkanoate (PHA) producing Gram-positive, rod-shaped, motile bacterium was isolated from the polluted pond water. Strain SKM7^T grew at 15–40°C and pH 5.0–8.5 and in the presence of 0–1.5% (w/v) NaCl. The strain was catalase-positive and oxidase-positive. Antimicrobial activities were studied. The DNA G+C content was 53 mol%. Phylogenetic analysis based on 16S rRNA gene

**Corresponding author: Email: kchaitanya03@gmail.com;*

sequences showed that strain is a member of the genus *Bacillus* and is most closely related to *B. aryabhattai B8W22*$^{(T)}$ (99.01%), *B. megaterium IAM 13418*$^{(T)}$ (98.86%), *B. flexus IFO 15715*$^{(T)}$ (97.80%). The sequence of the 16S rDNA gene of strain SKM-7 was determined as 1554 bp and deposited in the EMBL under accession no. LM655314. The major isoprenoid quinine was MK-7 and an unidentified glycolipid was identified. The major fatty acid are dominated by saturated iso and anteiso (anteiso$C_{14:0,}$ iso-$C_{15:0}$, anteiso$C_{15:0}$, anteiso$C_{16::0}$). The phenotypic and genotypic properties clearly indicate that strain represents a novel species of the genus *Bacillus*. The strain produced PHA in mineral medium consisting of glucose and nitrogenous substances. The type strain is SKM7^{T} (=KCTC 33686^{T}).

Keywords: Bacillus sp.; polluted pond water; PHA; polyhydroxyalkanoates; 16S rRNA.

1. INTRODUCTION

Polyhydroxyalkanoates (PHA) are a family of bio polyesters synthesized by many types of bacteria as carbon and energy reserve materials [1]. PHA can be divided into three classes depending on the number of carbon atoms in their monomer units; short chain-length (SCL), medium-chain-length (MCL) and long-chain-length poly-hydroxyalkanoates (LCLPHAs), composed by hydroxyacids with 3–5, 6–14 or more than 14 carbon atoms respectively. About more than 100 different monomer units reported so far, none of them contain more than 14 carbon atoms as constituents of PHA [2]. Polyhydroxyalkanoates (PHAs) represent a large family of intracellular bacterial storage polyesters with wide range of material properties permitting applications as biodegradable and biocompatible thermoplastics and elastomers [3]. PHA combine properties of thermal processibility, biodegradability, bio-compatibility and sustainability, they have attracted attention from fermentation, materials and biomedical industries. 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 copolymers 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 octanoateco-3-hydroxydecanoate) 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 [4].

The aim of this study was to describe a new PHA - (SCL co MCL) producing strain 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 SKM7^{T} *Bacillus sp*., to accumulate PHA.

2. MATERIALS AND METHODS

2.1 Isolation of Bacterial Strain

A water sample was collected at 17.40, 78.47 GPS location of Hussain Sagar Pond. Screening was performed serial dilution technique in order to isolate PHA producing microorganisms. The bacterium is grown in E2 medium [5] supplemented with 20 g/l of glucose and rice bran of 10 g/l. Their abilities to synthesize PHA were determined by a viable colony staining method using Nile blue “A” [6], and bacteria accumulating PHA were isolated. Selected strains were maintained on nutrient agar slants and 50% and 80% glycerol stocks and kept at -20ºC. A strain coded as SKM7^{T}, which was selected was taken for the study and was deposited in the Korean Collection for Type Cultures (KCTC) as strain KCTC 33686^{T}

2.2 Morphological Characteristics and Microscopic Observation

The selected bacterial isolates were examined for their morphological features. The morphological characteristics were examined on LB agar plates. The pure cultures from the slants were placed on the agar plates. After the growth of colonies morphological characters of the colonies were recorded. Gram staining, motility and endospore formation was observed [7].

2.3 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 h at 4ºC and post fixed 2% in aqueous osmium Tetroxide for 4 h, 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. A thin layer of gold coat is applied over the samples by using an automated sputter coater (JEOL JFC-1600) for 3 min. And then the samples are subjected to scanning using Scanning Electron Microscope (Model, JOEL_JSM 5600) at various magnifications.

2.4 Biochemical Characteristics

The activities of catalase, oxidase, gelatinase, cellulase, protease, lipase, lecithinase, HCN, oxidation and fermentation test, amylase, arginine hydrolyase, lactose fermenting activity, siderophore production activity, NaCl and pH Tolerence were determined according to standard methods with respective media [8,9]. Some of the Biochemical characteristics were checked with the Hi25 biochemical identification kit (KB003) and Hi Carbohydrate kit parts A, B and C (KB009) (both from Hi- Media) according to the manufacturer's protocol.

2.5 Antibiotic Assay

Antibiotic sensitivity of the strain was tested using antibiotic discs (HiMedia Laboratories) containing the following antibiotics (ug): pencillin G (10), cephalothin (30), clindamycin (2), co-trimoxazole (25), erythromycin (15), gentamicin (10), ofloxacin(1), vancomycin (30). Effects of the all antibiotics on cell growth were assessed from the zone of inhibition and compared according to the instructions of the manufacturer for the susceptibility testing [10].

2.6 Identification of the Bacterium

The phylogenetic analysis the region of 16SrDNA was amplified by PCR using two primers, 27F (5'-AGAGTTTGAYCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') and the nucleotide sequence was determined. Identification of phylogenetic neighbors and the calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server [11]. The phylogenetic trees of 16S rRNA sequences were constructed using the MUSCLE algorithm of MEGA version 6.0 [12] and the distance was calculated by using default parameters, Jukes-Cantor method [13] and the neighbor-joining (NJ) algorithm pairwise deletion procedure.

2.7 Phospholipid and Cellular Fatty Acid Analysis

Isoprenoid quinines were extracted and purified according to Collins [14], polar lipids were examined by 2D TLC and identified using the method of minnikin [15]. 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 37ºC 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 [16]. 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 and compared with most closely related sp., *B. aryabhattai.*

3. RESULTS

3.1 Morphological Characteristics and Microscopic Observation

Strain SKM7^T (Fig.1) was a Gram-positive, motile, Endospore-forming rods (Fig. 2). Colonies are circular, entire, matt, convex, cream colour and 2 nm in diameter under Scanning Electron Microscope (Model, JOEL_JSM 5600) (Fig. 3). Strictly aerobic, grows at 15-40ºC.

3.2 Biochemical Characteristics

Strain SKM7^T was positive for catalase (Fig. 4.), oxidase, protease, gelatinase, ammonia, O-F (oxidation and fermentation) test (Fig. 5) and negative for arginine, starch, HCN, lactose production, lipase, lecthinase. It produced acid from esculin hydrolysis, arabinose, xylose, melibose, saccharose, raffinose, trehalose, glucose, lactose (Fig. 6). The bacterium was also positive for ONPG, lysine utilization, ornithine utilization, urea, H2S production, citrate utilization (Fig. 7).

Fig. 1. SKM7^T on LB medium

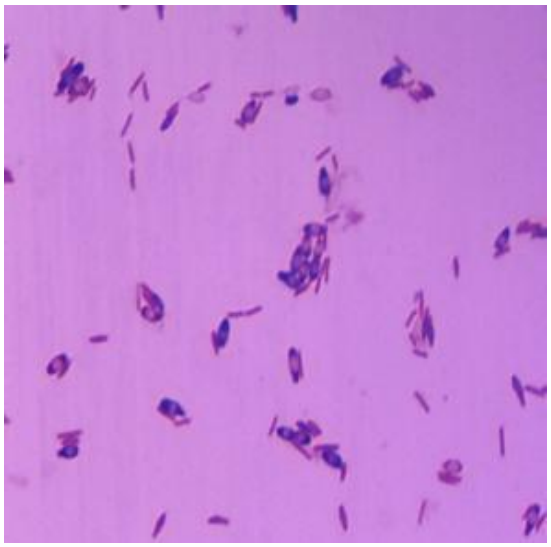

Fig. 2. Endospore stain

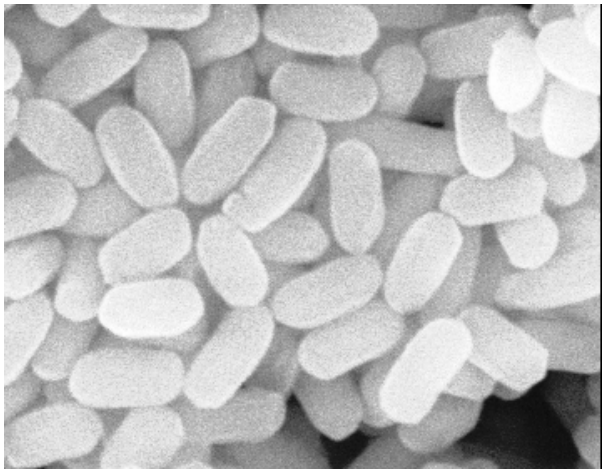

Fig. 3. Scanning electron microscope picture
Bacillus having round end rods, 2 nm in diameter (Model, JOEL_JSM 5600)

3.3 Antibiotic Assay

Effects of the all antibiotics on cell growth were assessed for the zone of inhibition. Strains were sensitive to all antibiotics on the disc clindamycin, cephalothin, clindamycin, co-trimoxazole, erythromycin, gentamicin, ofloxacin, vancomycin and pencillin G. (Fig. 8).

3.4 Identification of the Bacterium

The sequence of the 16S rRNA-encoding gene of SKM7^T was determined 1554bp and deposited in the EMBL sequence database under accession number LM655314. The culture was identified to be *Bacillus* sp. Based on 16SrRNA gene sequencing. A BLAST (EZtaxon server) search using the 16SrRNA gene sequence showed 95% and above homology with 20 known taxa of *Bacillaceae* and maximum homology of 99.01% to *Bacillus aryabhattai B8W22*$^{(T)}$, 98.86% to *Bacillus megaterium IAM 13418*$^{(T)}$, 97.80% to *Bacillus flexus IFO 15715*$^{(T)}$. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.40539397 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [17]. The tree is drawn to scale, with branch lengths in the same units as those of

the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1568 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 [18].

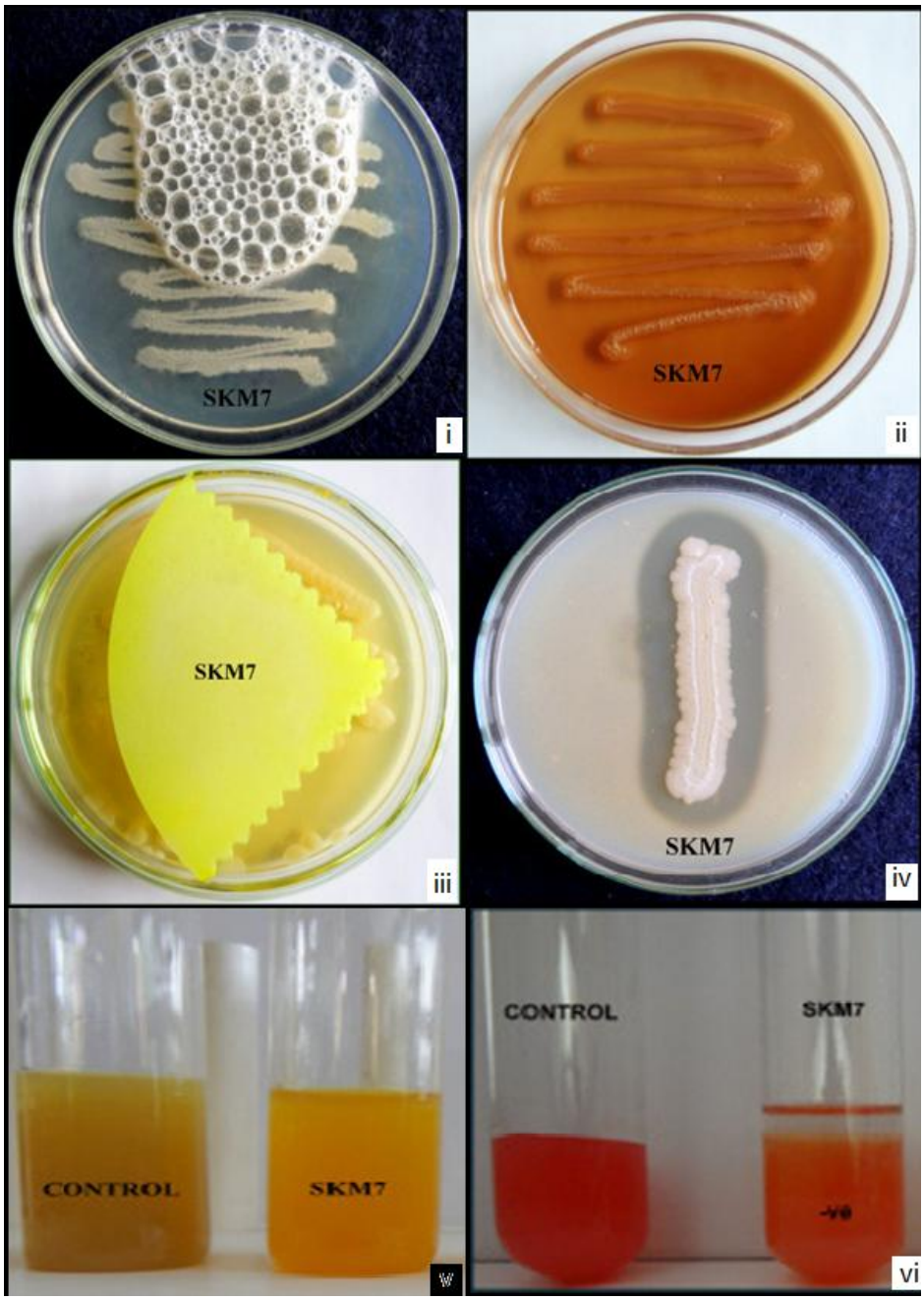

Fig. 4. Biochemical assay showing i. catalase +ve, ii. lactose fermentation –ve, iii. HCN production -ve, iv. Protease +ve, v. ammonia production +ve, control was seen dark, vi. arginine hydrolyse –ve, control doesn't change the colour

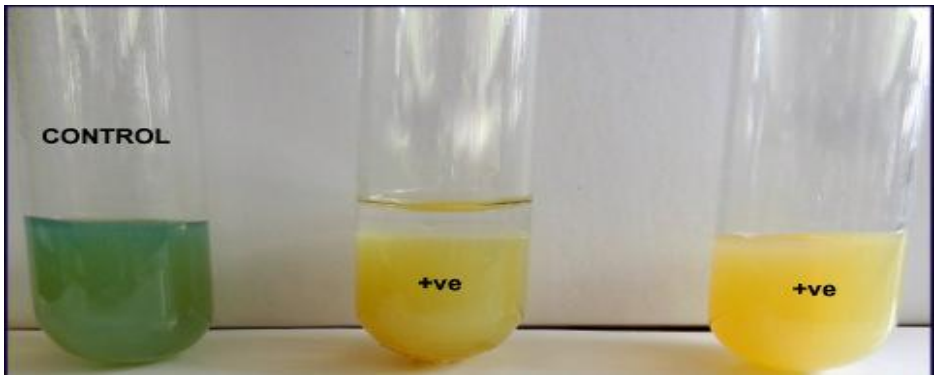

Fig. 5. Oxidation-Fermentation test: fermentation +ve (tube covered with liquid paraffin) and oxidation +ve (tube with no liquid paraffin)

Fig. 6. Carbohydrate utilization tests (Positive-Yellow; Negative-Pink)

1. Esculine; 2.Arabinose; 3.Xylose; 4.Adonitol; 5. Rhamnose; 6.Cellobiose; 7.Melibiose; 8. Saccharose; 9.Raffinose; 10.Trehalose; 11.Glucose; 12.Lactose

Fig. 7. Biochemical tests(+ve:positive, -ve:negative)

1. ONPG; β galactosidase +ve; 2. Lysine decarboxylase +ve; 3. Ornithine decarboxylase +ve; 4.Urease +ve; 5. Phenylalanine deamination -ve; 6. Nitrate reduction-ve; 7. H_2S production +ve; 8. Citrate utilization +ve; 9.Voges Proskauer's-ve; 10. Methyl red -ve; 11.Indole -ve; 12.Malonate -ve.

Fig. 8. Strain showing sensitive to all antibiotics

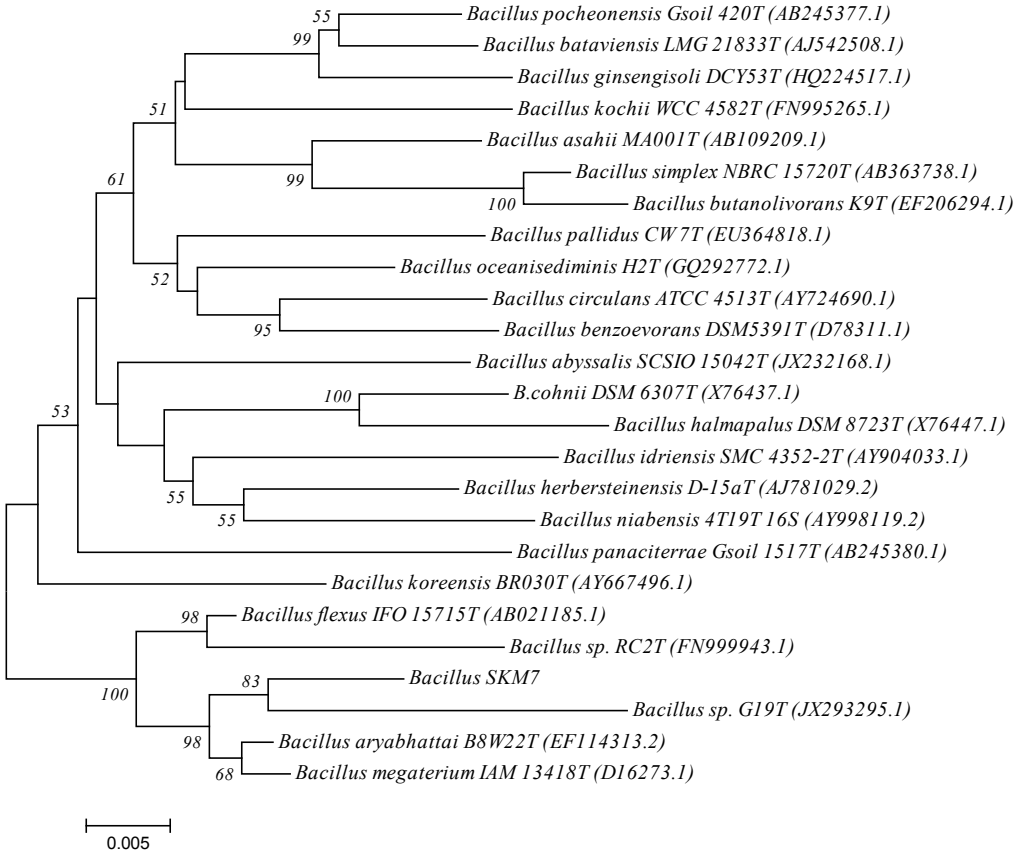

Fig. 9. Evolutionary-distance dendrogram depicting the phylogenetic relationships of strain KCTC 33686T within the family bacillus, determined using 16S rRNA gene sequence analysis. The Phylogenetic tree was constructed by using the neighbour joining (NJ) method. Numbers on branch nodes are percentage bootstrap support values (1,000 replicates) greater than 50% are given at nodes. GenBank accession numbers are shown in parentheses. Bar 0.005 nucleotide substitutions per position

Table 1. Fatty analysis of SKM7^T and *B. aryabhattai*

Fatty acid %	SKM7^T	*B. aryabhattai*
$C_{14:0}$	0	4.2
$C_{15:0}$	51.5	46.2
$C_{18:0}$	0	2.8
$isoC_{14:0}$	0	0
$anteisoC_{14:0}$	0.3	0.0
$iso\text{-}C_{15:0}$	1.3	3.2
$anteisoC_{15:0}$	33.2	35.3
$isoC_{16:0}$	1.9	3.0
$anteisoC_{16:0}$	5.4	5.7

3.5 Phospholipids and Cellular Fatty Acid Analysis

MK-7 identified as major isoprenoid and an unidentified glycolipid was seen. The cellular fatty acids were identified as major group. The major isoprenoid quinine was MK-7 and an unidentified glycolipid was identified. The major fatty acid are dominated by saturated iso and anteiso ($anteisoC_{14:0,}$ $iso\text{-}C_{15:0}$, $anteisoC_{15:0}$, $anteisoC_{16::0}$).

4. DISCUSSION

Use of biopolymers is assuming importance because of the pollution problems associated with synthetic plastics, which are produced from non-biodegradable. As an alternative to this, PHA's are gaining much attention world over. Bacteria synthesize a wide range of PHA and approximately 150 different constituents of PHA have been identified [19]. PHA produced by bacteria consist of three main types : (i) polymers composed of short-chain- length (scl) monomers (ii) polymers composed of medium-chain-length (mcl) monomers and (iii) polymers composed of

scl-mcl monomers. Scl-PHA consists of monomeric subunits 3 to 5 carbons in length, while mcl PHA consists of monomers 6 to14 carbons in length and scl-mcl PHA copolymer consists of monomeric subunits 4 to12 carbons in length [20]. There are only a very few reports on bacteria that are capable of synthesizing both types of monomer units [21,22]. In the current study morphological and biochemical data, phylogenetic analysis, show that the strain is 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(44.7%), anteiso-C (6.8%)] and MK-7 as major isoprenoidquinone, indicate that the strain belongs to the genus *Bacillus, closely related to* 99.01% to *B. aryabhattai B8W22*$^{(T)}$, 98.86% to *B. megaterium IAM 13418*$^{(T)}$, 97.80% to *B. flexus IFO 15715*$^{(T)}$. Further study is needed to confirm its identity. PHA-producing bacteria are classified into three groups based on PHA synthases present in them. But recently it has been shown that *B. megaterium* possesses enzymes that are distinctively different from all known PHA synthases in sequence and arrangement and hence may form a separate class by itself [23]. In addition to *B. megaterium*, *B. cereus* is also reported to produce PHA [24]. Strain SKM7^{T} unlike other *Bacillus sp.* has the ability to synthesize PHA by utilizing glucose as sole carbon sources without adding any fatty acid precursors. A novel *Bacillus* sp., have been identified to accumulate PHA, from waste water pond.

5. CONCLUSION

The results demonstrate that the bacterium, which is isolated from a polluted water pond, was identified as *Bacillus sp* SKM7^{T}, could be an interesting bacterium for production of PHA from glucose. However, use of inexpensive substrates such as starch could contribute to reduce the PHA production cost. Further studies are needed for large scale production of the PHA.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial use of bacterial polyhydroxyalkanoates. Microbiol Rev. 1990;54:450-72.
2. Reddy CSK, Ghai R Rashmi, Kalia VC. Polyhydroxyalkanoates: An overview. Bioresour Technol. 2003;87:137-46.
3. Chen GQ. Biofunctionalization of polymers and their applications. Adv Biochem Engin/Biotechnol. 2011;125:29-45.
4. Nagamani P, Mahmood SK. Characterization and biosynthesis of scl-co-mcl by Bacillussp.OU35T. Int J Pharm Bio Sci. 2012;3(4):(B)695-706.
5. Lagveen RG, Huisman GW, Preustig H, Ketelaar P, Eggnik G, Witholt B. Formation of polyesters by *Pseudomonas oleovorans*, effect of substrates on formation and composition of poly-(R)-3hydroxyalkanoates and poly-(R)-3-hydroxylalkenoates. Appl Environ Microbiol. 1988;54:2924-32.
6. Ostle AG, Holt JG. Nile blue as a fluorescent stain for PHB. Appl Environ Microbiol. 1982;44:238-41.
7. Smibert RM, Krieg NR, Phenotypic characterization. Methods for general and molecular bacteriology. Am Soc Microbiol, Washington DC. 1994;607-654.
8. Holding AJ, Collee JG. Routine biochemical tests. Methods Microbiol. 1971;6A:2-32.
9. Hugh R, Leifson E. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J Bacteriol. 1953;66:24-6.
10. Vaddavalli R, Peddi S, Kothagauni SY, Linga VR. *Nocardia bhagyanesis* sp. nov., a novel actinomycete isolated from the rhizosphere of *Callistemon citrinus* (Curtis), India. Antonie Van Leeuwenhoek. 2014;106(2):413.
11. Valappil SP, Boccaccini AR, Bucke C, Roy I. Polyhydroxy alkanoates in Gram positive bacteria, insights from the genera *Bacillus* and *Streptomyces*. Antonie van Leeuwenhoek. 2007a;91:1-17.
12. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Bio Evoln. 1987; 4:406-25.
13. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, editor. Mammalian Protein Metabolism, New York: Academic Press. 1969;21-132.
14. Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V. A ribosomal RNA gene probe differentiates member species of the *Anopheles*

gambiae complex. Am J Trop Med Hyg. 1987;37:37-41.

15. Minnikin DE, O'Donnell AG, Goodfellow M, Alderson G, Athalye M, Schaal K, Parlett JH. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. J Microbiol Methods. 1984;2:233-41.
16. Sasser M. Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. Newark, DE: MIDI Inc; 1990.
17. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985;39:783-91.
18. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Bio and Evol. 2013;30:2725-9.
19. Steinbüchel A, Valentin HE. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 1995;128:219-28.
20. Ojumu TV, Yu J, Solomon BO. Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. Afr J Biotechnol. 2004;3:18-24.
21. Nakamura S, Kunioka M, Doi Y. Biosynthesis and characterization of bacterial poly (3-hydroxybutyrate-co-3-hydroxypropionate). Macromol Rep. 1991; 28,15-24.
22. Fukui T, Doi Y. Cloning and analysis of the poly (3-hydroxybutyrate-co-3-hydroxy hexanoate) biosynthesis genes of *Aeromonas caviae*. J. Bacteriol. 1997;179: 4621-30.
23. McCool GJ, Cannon MC. PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. J Bacteriol. 2001;183(14):4235-43.
24. Labuzek S, Radecka I. Biosynthesis of PHB tercopolymer by *Bacillus cereus* UW85. J Appl Microbiol. 2001;90,353–7.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/10108