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

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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.

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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

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sequences showed that strain is a member of the genus *Bacillus* and is most closely related to *B. aryabhattai* B8W22 (99.01%), *B. megaterium* IAM 13418 (98.86%), *B. flexus* IFO 15715 (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 (anteisoC_{14:0}, iso-C_{15:0}, anteisoC_{16:0}, anteisoC_{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\(^7\) (=KCTC 33686\(^7\)).

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

### 1. INTRODUCTION

Polyhydroxyalkanoates (PHA) are a family of bio polymers 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 polyhydroxyalkanoates (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, biocompatibility 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 octanoate-co-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 *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 -20°C. A strain coded as SKM7\(^1\), which was selected was taken for the study and was deposited in the Korean Collection for Type Cultures (KCTC) as strain KCTC 33686\(^1\).

#### 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 4°C 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, lecinthinase, HCN, oxidation and fermentation test, amylase, arginine hydrolyse, lactose fermenting activity, siderophore production activity, NaCl and pH Tolerance 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'-AGAGTTTGTGAYCCTGCTAG-3') and 1492R (5'-GGCTACCTTGTAGACTT-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 37°C 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. aryabhettai.

3. RESULTS

3.1 Morphological Characteristics and Microscopic Observation
Strain SKM7T (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 15-40°C.

3.2 Biochemical Characteristics
Strain SKM7T 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, lecinthinase. 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).
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)

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

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\textsuperscript{T} and B. aryabhattai

<table>
<thead>
<tr>
<th>Fatty acid %</th>
<th>SKM7\textsuperscript{T}</th>
<th>B. aryabhattai</th>
</tr>
</thead>
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<td>C_{14:0}</td>
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<td>C_{15:0}</td>
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</table>

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-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 to 14 carbons in length and scl-mcl PHA copolymer consists of monomeric subunits 4 to 12 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 water 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 iso-preneoquinone, indicate that the strain belongs to the genus Bacillus, closely related to 99.01% to B. aryabhattai B8W22[13], 98.86% to B. megaterium IAM 13418[17], 97.80% to B. flexus IFO 15715[11]. 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


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