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ISOLATION, MOLECULAR CHARACTERIZATION AND PHA PRODUCTION BY A NOVEL *BACILLUS SP.*, SKM155 FROM POLLUTED WATER

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ABSTRACT

A polyhydroxyalkonate (PHA) producing Gram-positive, rod-shaped, motile bacterium was isolated from the polluted water pond. Strain SKM-155 grew at 15–45 °C and pH 5.0–9.0 and in the presence of 0–2 % (w/v) NaCl. The strain was catalase-positive and oxidase-positive. Antimicrobial activities were studied. The DNA G+C content was 55.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain is a member of the genus *Bacillus* and is most closely related to *Bacillus invictus Bi.FFUP1*^{T} (98.94%), *Bacillus altitudinis 41KF2b*^{T} (98.34%), *Bacillus aerophilius 28K*^{T} (98.34%). The sequence of the 16S rDNA gene of strain SKM-155 was determined (1574 bp) and deposited in the EMBL under accession no. LM655321. The major isoprenoid quinine was MK-7 and the polar lipid consisted of diphosphotidylglycerol and an unidentified glycolipid was identified. The major fatty acid are dominated by saturated iso and anteiso (iso-$C_{15:0}$, anteiso$C_{15:0}$, anteiso$C_{17:0}$, iso$C_{17:0}$). 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. Optimum conditions for polymer production were determined, extracted and confirmed through Spectroscopic and IR analysis.

KEYWORDS: ***Bacillus*** **sp., PHA, Polyhydroxyalkonates.**

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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; shortchain-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 a 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 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 designated as SKM155 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 SKM155 *Bacillus sp.*, 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. Screening was performed in order to isolate PHA producing micro organisms. Selected bacteria were grown in E 2 medium[5] supplemented with 20gl 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[6], and bacteria accumulating PHA were isolated. Selected strains were maintained on nutrient agar slants and 50% and 80% glycerol stocks and kept at -20o C. A strain coded as SKM155, which was selected was taken for the study.

Morphological and microscopic observation

The selected bacterial isolates were examined for their morphological features 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 staning, motility and endospore formation were obsereved.

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, salt and pH tolerence were determined according to standard methods with respective media[7, 8]. 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.

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.

Identification of the bacterium

The morphological and physiological properties of isolate SKM155 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. Identification of phylogenetic neighbors and the calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server[9]. The phylogenetic trees of 16S rRNA sequences were constructed using the MUSCLE algorithm of MEGA version 6.0[10] and the distance was calculated default parameters, Jukes-Cantor method[11] the neighbor-joining (NJ) algorithm pairwise deletion procedure.

Phospholipid and Cellular fatty acid analysis

Isoprenoid quinines were extracted and purified according to Collins[12], polar lipids were examined by 2D TLC and identified using the method of minnikin[13]. 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 370C 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[14] .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 *Bacillus invictus.*

Production, isolation and extraction of PHA

Cells were grown in duplicate in modified mineral salt medium supplemented with glucose as sole source of carbon for PHA production[15]. 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 30^0C. PHA was extracted from lyophilized cells using sodium hypoclorite method of extraction[16].

Infra red spectra

For FT-IR analysis, the PHA was precipitated from the chloroform using cold ethanol. The precipitated polymer was analysed under FT-IR spectrum 1720X spectrometer (Perkin Elmer, USA) and used under the following conditions, spectral range, 4,000–500 cm–1, window material, CsI, 16 scans, resolution 4 cm–1 , the detector was a temperature-stabilized, coated FR-DTGS detector.

RESULTS

Morphological characteristics and microscopic observation

Strain SKM155 (figure 1.1) was a Gram-positive (figure1.2), motile, endospore- forming rods(figure1.3). Colonies were circular, entire, matt, covex, white and 2 mm in diameter on LB medium after 48h incubation at 37^0C.Strictly aerobic, Grows at 15-45^0C (optimally at pH5.0-9.0).

Figure 1.1
SKM155 on LB Medium

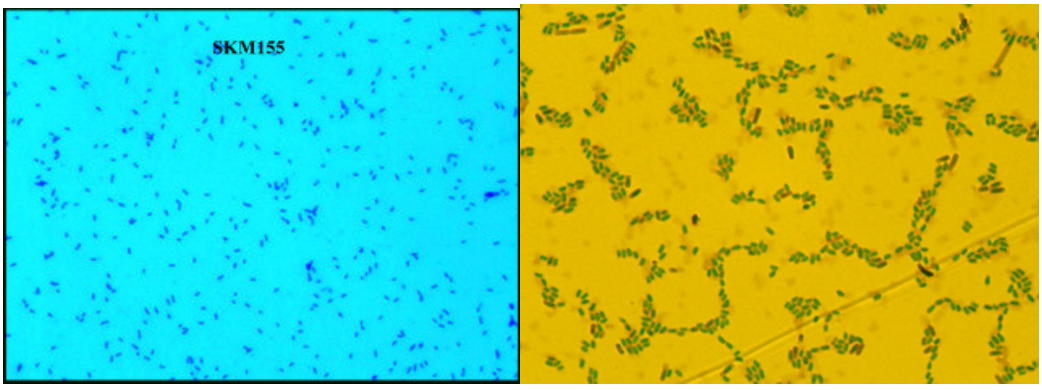

Figure 1.2 Gram stain **Figure1.3 Endospore stain**

Biochemical characteristics

Strain SKM155 was positive for catalase(figure 2.1), oxidase, lipase, lecthinase, starch, gelatinase, HCN, O-F test(figure 2.2) and produced acid from cellobiose, saccharose, trehalose, D-glucose, It was able to utilise ornithine, lysine decarboxylase, citrate, malonate and esculin hydrolysis (figure 2.3).

Table1
physiological and biochemical characteristics

Gram character	**+**	**Lecthinase**	**+**
Endospore	+	Lysine utilisation	+
Arginine hydrolysis	+	Nitrate reduction	-
Amylase	+	O-F test	+
Catalase	+	Oxidase	+
Cellulose	-	Protease	+
Citrate utilisation	+	Rhmanose	-
Gelatinase	+	Raffinose	-
H2S production	-	Urease	-
Lipase	+	Voges proskauer's	-

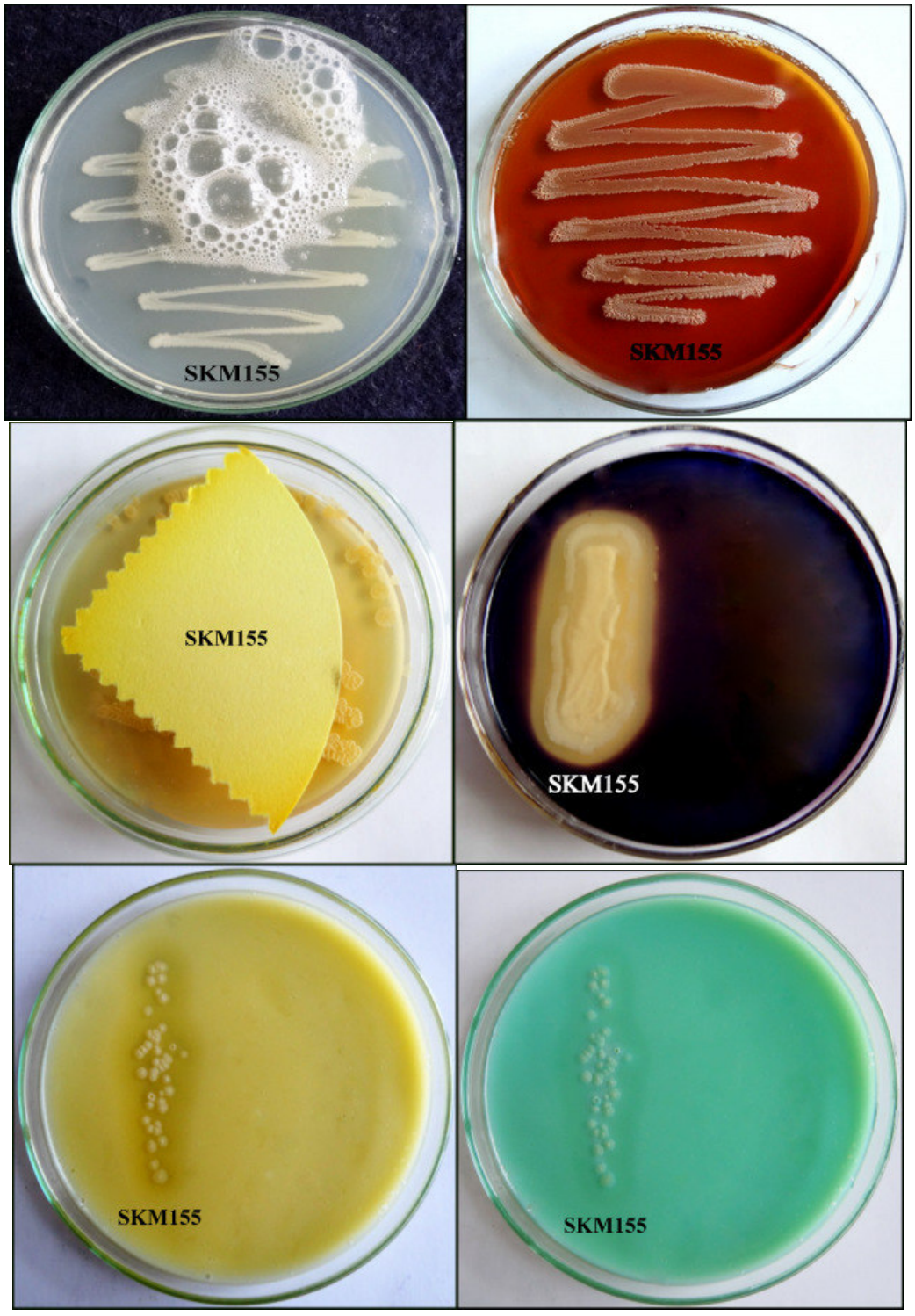

Figure 2.1
biochemical assay showing catalase +ve, lactose fermentation –ve, HCN production slightly, Starch hydrolysis +ve, Lipase and lecthinas +ve.

Fig2.2
oxidation +ve and fermentation +ve

Figure 2.3
biochemical and carbohydrate test kit

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.(Figure 3).

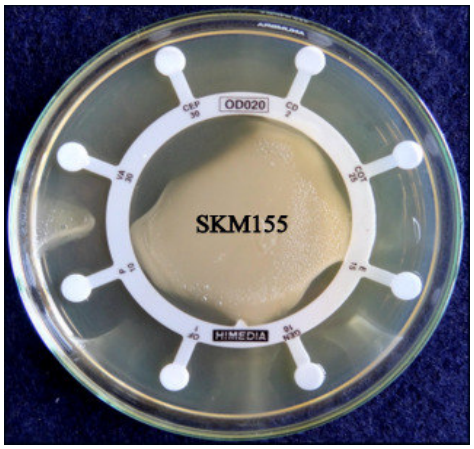

Figure 3
strain showing sensitive to all antibiotics

Identification of the bacterium

The sequence of the 16S rRNA-encoding gene of SKM155 was determined (1574bp) and deposited in the EMBL sequence database under accession number LM655321.1. 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 10 known taxa of *Bacillaceae* and maximum homology of 98.94% to *Bacillus invictus Bi.FFUP1*^{T} , 98.34% to *B. altitudinis 41KF2b*^{T}, and 98.34% to *B. aerophilius 28K*^{T}. The

evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.45616789 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are 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 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1619 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[18].

Phospholipids and Cellular fatty acid analysis

MK-7 identified as major isoprenoid and the polar lipid consisted of diphosphotidylglycerol and an unidentified glycolipid was seen. The cellular fatty acids were identified as major group iso-$C_{15:0}$(42.7%), anteiso$C_{15:0}$(33.2%), anteiso$C_{17:0}$(6.3%), iso$C_{17:0}$(5.2%) and as minor iso$C_{14:0}$(0.9%), iso$C_{16:0}$(1.9%), anteiso$C_{16:0}$ (1.8%)

Table2
Fatty analysis of SKM155 and B.invictae

Fatty acid %	SKM155	*B.invictae*
isoC$_{14:0}$	0.9	1.0
anteisoC$_{14:0}$	0.3	0.0
iso-C$_{15:0}$	42.7	41.3
anteisoC$_{15:0}$	33.2	35.3
isoC$_{16:0}$	1.9	3.0
anteisoC$_{16:0}$	1.8	2.0
isoC$_{17:0}$	5.2	4.9
anteisoC$_{17:0}$	6.3	6.6

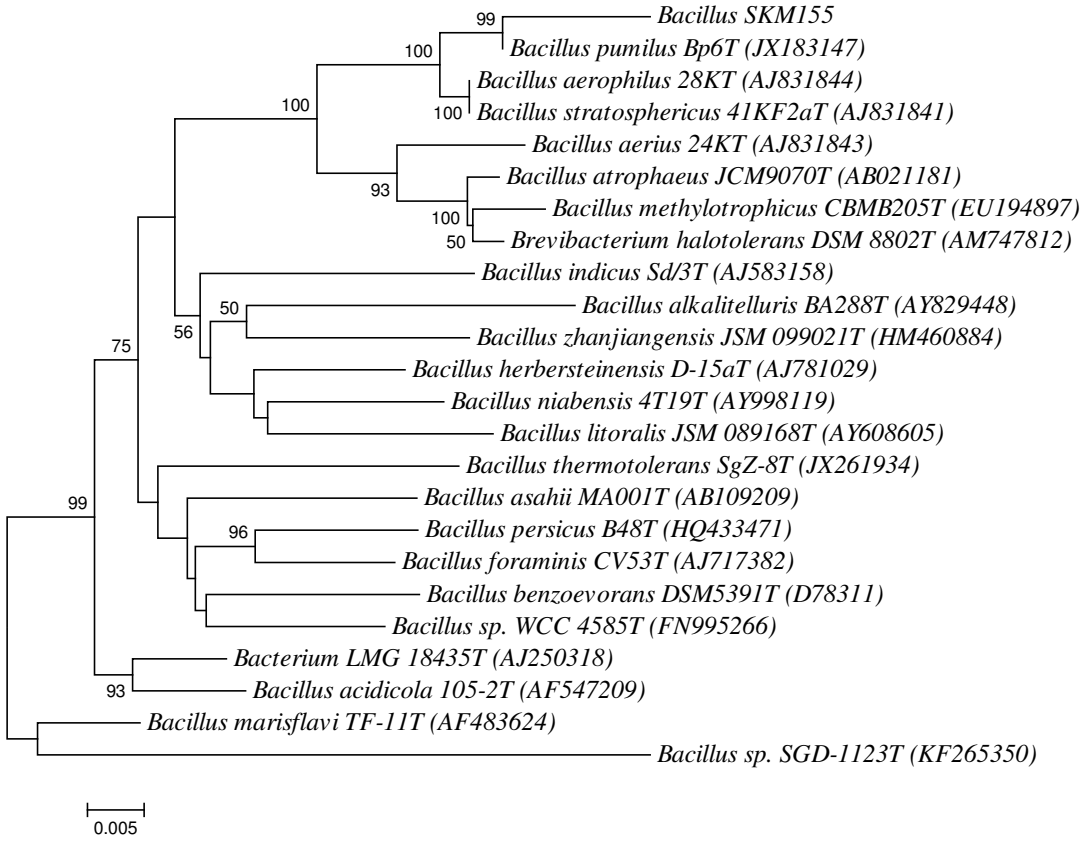

Figure 4
phylogentic tree constructed based on Neighbor-Joining method

Production, isolation and extraction of PHA

Production of PHA began after 30 h in glucose enriched medium and it was maximum at 48h and yield of PHA produced was1.347g/l. using Hypochlorite extraction method.

Figure 4
polymer extracted from the strain

Infra red spectra

Spectra were recorded for the polymers dissolved in chloroform. Spectra showed two intense absorption bands at 1,699.78 and 1,2839.07 cm-1, corresponding to C = O and C–O stretching groups, respectively. Other absorption bands were present.

DISCUSSION

Morphological and biochemical data, phylogenetic analysis, shows 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(42.7%), anteiso-C (6.3%)] and MK-7 as major isoprenoidquinone, polar lipid as diphosphotidlyglycerol is indicating that the strain belongs to the genus *Bacillus* . In this study also strain SKM155 has 98.94% to *Bacillus invictus Bi.FFUP1*^{T} , 98.34% to *B. altitudinis 41KF2b*^{T}, and 98.34% to *B. aerophilius 28K*^{T}. Further study is needed to confirm its identity. DNA-DNA hybridization studies have conventionally been essential to provide best answers for this. The chemical composition of the PHA accumulated by strain SKM155 appeared to be different from those produced by other bacteria from extreme environments. Strain SKM155 unlike other *Bacillus sp.* this isolate has the ability to synthesize PHA produces polymers by utilizing glucose as sole carbon sources without adding any fatty acid precursors. A noval *Bacillus* sp., have been identified to accumulate PHA, from waste water pond.

CONCLUSION

The results shown that the bacterium which was isolated from polluted water, identified as *bacillus sp.,* SKM155, could be an interesting bacterial sp., for production of PHA from glucose. However, use of inexpensive substrates such as starch could contribute to reducing the PHA production cost. Further studies are needed for large scale production of the PHA.

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