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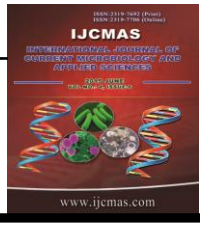

Original Research Article

Polyhydroxyalkanoate Producing Novel *Bacillus* sp., SKM11 isolated from Polluted Pond Water

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A B S T R A C T

A polyhydroxyalkanoate (PHA) producing Gram-positive, rod-shaped, motile bacterium was isolated from the polluted pond water. Strain SKM-11 grew at 10–40^{o}C and pH 5.0–8.0 and in the presence of 0–3 % (w/v) NaCl. Antimicrobial activities were studied. The DNA G+C content was 55mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain is a member of the genus *Bacillus* and is most closely related to *B. aryabhattai B8W22*^{T} (99.66%), *B. megaterium IAMB418*^{T} (99.25%), *B. flexus IF015715*^{T} (98.34%). The sequence of the 16SrDNA gene of strain SKM-11 was determined was 1466bp and deposited in the EMBL under accession no. LM655313. 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. Starin SKM11= KCTC3368^{T}.

Keywords

Bacillus sp., PHA, Polyhydroxy alkanoates

Introduction

Polyhydroxyalkanoates (PHA) are a family of bio polyesters synthesized by many types of bacteria as carbon and energy reserve materials, Anderson AJ et al (1990). 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.

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, Guo-Qiang Chen (2011). 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 P.Nagamani (2012).

The aim of this study was to describe a new PHA producing strain designated as SKM11 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.

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 supplemented with 20g/l of glucose and rice bran of 10g/l, Lagveen R.G et al (1988). Their abilities to synthesize PHA were determined by a viable colony staining method using Nile blue A by Ostle A.G et al (1982) and bacteria accumulating PHA were isolated. Selected strains were maintained on nutrient agar slants and glycerol stocks and kept at -20^{o} C.

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 staning, motility and endospore formation was 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, Hugh R et al (1953). 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 SKM11 were investigated according to Bergey's manual of determinative Bacteriology. For the phylogenetic analysis the region of 16SrDNA was amplified by PCR using a two 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, Valappil S.P et al (2007a). The phylogenetic trees of 16S rRNA sequences were constructed using the MUSCLE algorithm of MEGA version 6.0 by Saitou N and Nei M. (1987) and the distance was calculated default parameters, Jukes-Cantor method the neighbor-joining (NJ) algorithm pairwise deletion procedure Jukes T.H. and Cantor C.R. (1969).

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, Lagveen R.G et al (1988). 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, Rawte T and Mavinkurve S (2002).

Results and Discussion

Morphological characteristics and microscopic observation

Strain SKM11 (figure 1.1) was a Gram-positive, motile, endospore- forming rods. Colonies are circular, entire, matt, convex, white and 2 mm in diameter on LB medium after 48h incubation at 37^0C.Strictly aerobic, Grows at 10-40^0C (optimally at pH5.0-8.0).

Biochemical characteristics

Strain SKM11 was positive for catalase(figure 2.1), starch, gelatinase, 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 (Table 1).

Antibiotic assay

Effects of the all antibiotics on cell growth were assessed for the zone of inhibition. Strains were sensitive to clindamycin, cephalothin, clindamycin, co-trimoxazole, erythromycin , gentamicin, ofloxacin, vancomycin and resistant pencillin G.(Figure 3)

Identification of the bacterium

The sequence of the 16S rRNA-encoding gene of SKM11 was determined 1574bp and deposited in the EMBL sequence database under accession number LM655313. The culture was identified to be *Bacillus* sp. Based on 16SrRNA gene sequencing. A BLAST (EZtaxon server) search using the 16SrRNA gene sequence showed 96% and above homology with 10 known taxa of *Bacillaceae* and maximum homology of 99.66% to *B. aryabhattai B8W22*^{T} , 99.25% to *B. megaterium IAMB418*^{T}, and 98.50% to *B. flexus IF015715*^{T}.

Table.1 Physiological and biochemical characteristics

Gram character	+	Malonate utilisation	+
Endospore	+	Lysine utilisation	+
Arginine hydrolysis	+	Nitrate reduction	-
Amylase	+	O-F test	+
Catalase	+	Oxidase	-
Cellulose	-	Protease	+
Ornithine utilisation	+	Melibiose	+
Gelatinase	+	Raffinose	+
H2S production	-	Saccharose	+
Indole	+	Trehalose	+

Fig.1 SKM11 on LB Medium

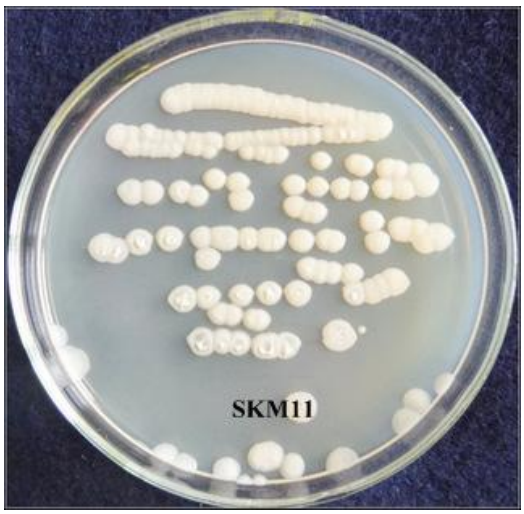
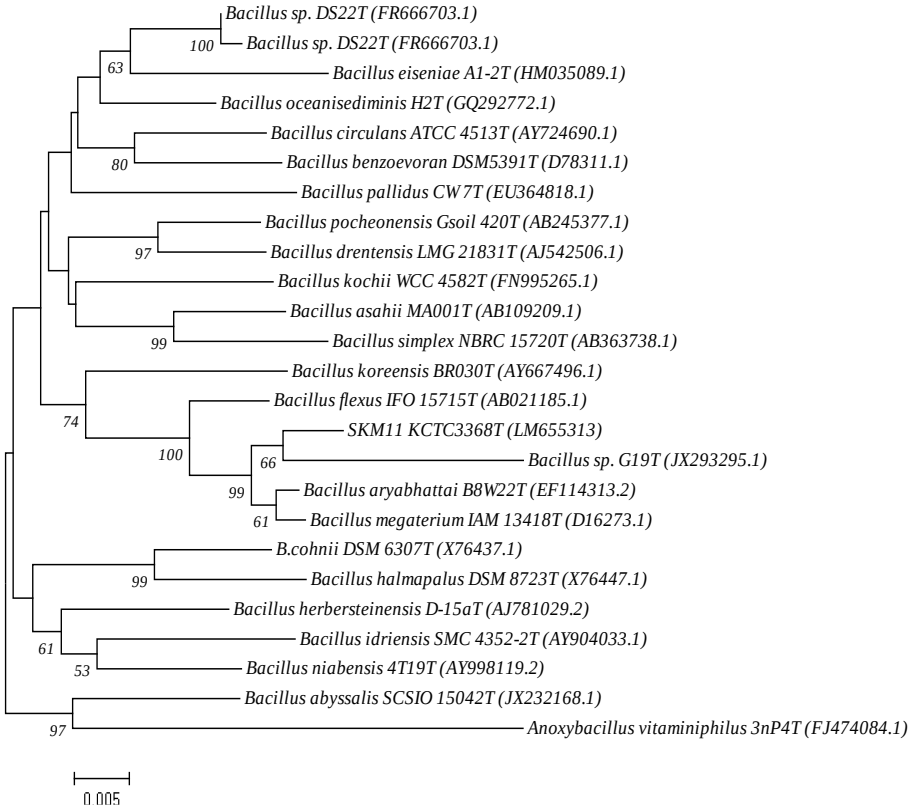


Fig.2.1 Biochemical assay showing catalase +ve, protease +ve.

Fig.2.2 Oxidation-Fermentation test: fermentation +ve and oxidation +ve

Fig.3 Strain showing resistant penicillin antibiotic

Fig.4.1 phylogentic tree constructed based on Neighbor-Joining method



The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.41317798 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, Felsenstein J. (1985). 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 1564 positions in the final dataset. Evolutionary analyses were conducted in MEGA6, Tamura K et al (2013).

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.

Morphological and biochemical data, phylogenetic analysis, shows that the strain is an aerobic, mesophilic, heterotrophic new bacterium isolated from polluted pond water, belong to the genus *Bacillus.* In this study also strain SKM11 has 99.66% to *B. aryabhattai B8W22*^{T} , 99.25% to *B. megaterium IAMB418*^{T}, and 98.50% to *B. flexus IF015715*^{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 SKM11 appeared to be different from those produced by other bacteria from extreme environments. Strain SKM11 unlike other *Bacillus sp.* 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.

The results shown demonstrates that the bacterium, which was isolated from polluted water, identified as *bacillus sp.,* SKM11, 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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