Isolation and Characterization of a Bacterial Isolate for Polyhydroxyalkanoates Production

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ABSTRACT

Microorganism accumulating polyhydroxyalkanoates (PHA) in its cytosol was isolated from water obtained from InCheon area. This strain was determined to be Bacillus species that was examined by 16S rDNA sequences and phylogenetic tree analysis. The bacteria accumulated up to 20.6% of PHA within 48 hours at 37°C in the presence of 2.0% glucose substrate under aerobic condition. The present work also investigates the influence of substrate on PHA production to find a clue trigger. The PHA accumulation increased to 23.5 and 23.3% containing 2.0% salicylic acid and lactose, respectively. In contrast both glucosamine and xylose have negative effect on PHA production for this bacterial strain. To the best of our knowledge, this report firstly demonstrates the effects of various sugars on PHA production.

Keywords: Polyhydroxyalkanoate, Bacillus, glucosamine, Salicylic acid, 16S rDNA sequences, Phylogenetic tree analysis

INTRODUCTION

Since the invention of plastic, it has brought enormous benefit to mankind. However, conventional plastic can’t be disassembled easily which can cause serious harm to environment. And a lack of crude oil used as raw material for plastics makes it face a huge crisis for plastic industry. For this reason, many industries have been concerned and have attention to find biodegradable sources that can substitute for petroleum-derived plastic. In such situation, polyhydroxyalkanoate (PHA) derived from bacteria is expected to be best suited for these demands and it can be used as a promising eco-friendly plastic in near future. PHA is produced by bacteria as intracellular granules to endure stressful condition which means nutrient depletion (phosphorus, nitrogen, magnesium) with excess carbon. PHA polymer features biodegradable, biocompatible and non-toxic. Because of these versatile characteristics of PHA, it aroused interest in various fields. First of all, PHA can be used as packaging materials like bottles and containers. And in the medical field, when PHA that features biocompatibility is inserted into human’s body, it does not cause any severe immune reaction and also it does not need to be removed again. Medical experts have been trying to apply them as biomaterials such as wound suture, bone plates, vascular implants. Moreover PHA can play a role of drug release carrier (1,2). Previous studies demonstrated that antibiotics including tetracycline and gentamicin were incorporated into PHA and can be secreted under the control (3). In addition, a wide range of applications of PHA still have been studied intensively by industrial experts. So far, numerous PHA producing bacterial genera like Ralstonia, Bacillus, Pseudomonas, Azotobacter (4), Janthinobacterium (5) was isolated and identified. In the present study we have focused on the isolation of PHA-producing bacterial isolates, and identifying the PHA producing ability using different carbon sources.

MATERIALS AND METHODS

Chemicals

Monosaccharides such as N-acetyl glucosamine, N-acetyl Mannosamine, N-acetyl galactosamine and glucosamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloroform and Tween 20 were purchased from Junsei Chemical Co. (Japan). Gram stain kits and reagents were purchased from Becton, Dickinson & Co. (Sparks, Maryland, USA). Yeast extract and Tryptone were obtained from Difco Lab. (Detroit, Michigan, USA). All other reagents were prepared in analytical grade.

Isolation of polyhydroxyalkanoate producing microorganism

The bacterial strain was isolated from tap water from toilet of Gachon University in InCheon-city, South Korea. The microorganism was collected on the bottom of basket and was spread on solid plates containing yeast extract (5 g/L), tryptone (8 g/L), NaCl (2.5 g/L), glucose (20 g/L), (NH₄)₂SO₄ (2 g/L), agar (15 g/L), Na₂HPO₄·12H₂O (13.3 g/L), Mg
SO$_4$$^2$H$_2$O (1.2 g/L), citric acid (1.7 g/L) and trace element solution (10 mL/L). Trace element solution consisted of g/L in 1 M HCl with MnCl$_2$·4H$_2$O (0.86 g/L), ZnSO$_4$·7H$_2$O (0.2 g/L), CuSO$_4$·5H$_2$O (0.25 g/L) was prepared following the earlier study (6). The solid plates were incubated at room temperature for about 3 days. Two single colonies appeared on the plate and re-suspended in liquid medium as described above without agar. Through PHA extraction, one of bacteria was selected finally.

**PHA extraction and quantification**

The growth rate and PHA production rate of bacterial isolates were routinely evaluated for 2 days. The cells with PHA accumulation were centrifuged at 13,500 rpm for 500 sec and the cells were washed with distilled water twice then dried at 100°C to a constant weight and record the dry cell weight (DCW). Subsequently add chloroform and extract the PHA using sonication for 10 min then remove the cell debris and evaporate chloroform at 80°C to estimate the amount of PHA. PHA accumulation is calculated as a percentage of PHA present in the dry cell weight as illustrated below (7).

% PHA accumulation = Dry weight of PHA (g/mL)*100 / Dry cell weight (g/mL)

**Characterization of microorganism**

Gram staining has been proceeded using Gram staining kits and reagents (Crystal violet, Gram Iodine, Gram Decolorizer, Gram safranin) and along with its procedure. Transmission Electron Microscope (TEM) observation was performed by JEM1010 model from Japan. The resolution was 0.14 nm (Lattice Image) and 0.3 nm (Point Image). Bacteria was negatively stained on grids (Ted Pella, Inc.) using freshly prepared uranyl acetate solution. And the bacteria on grids are imaged by using an accelerating voltage of 80kv. Through Transmission Electron Microscope observation, Bacterial shape and the presence of bacterial flagella could be visualized.

**Identification of anti-oxidant activity of polyhydroxyalkanoate originated from bacteria**

DPPH radical scavenging activity was performed. Sample (PHA) was prepared in the state of dissolved in Tween 20 and it was diluted using methanol. And 100 µL of the sample was mixed with 300 µL of 1 mM DPPH ethanolic solution. The mixture was reacted for 20 min in dark condition at room temperature and absorbance was measured under 630 nm. As standard, the solvent, Tween 20 was also carried out in the same way as above.

16S rDNA sequence and phylogenetic tree analysis

In order to identify microbial species of this bacteria, 16S rDNA sequence analysis was conducted and phylogenetic tree was also constructed. With using the Ribosomal Database Project (RDP) software, homology search of determined 16S rDNA sequences of selected bacteria was performed to assign the similarity compared with other gene sequences. Phylogenetic tree construction has been made consequently by using the CLUSTAL W program and the neighbour-joining method (8).

**Effect of sugars on PHA production**

Various sugar substrates were provided into culture medium to assess impact for bacteria producing PHA. Culture medium consisting of 2% glucose was used as control. And 2% each saccharide was provided into culture medium additionally. As saccharide, N-Acetyl glucosamine, N-acetyl-galactosamine, N-acetyl mannosamine, glucosamine, lactose, sucrose, maltose, galactose, mannose, xylose, and salicylic acid were used respectively. Subsequently, in order to confirm concentration-dependent increase in PHA production using salicylic acid, an additional experiment was carried out by using 0.5%, 1%, and 2% salicylic acid, respectively.

**Statistical analysis of data**

All results from the experiments were presented as the means ± S.D., unless representative data were indicated as the means of averages from the experiments. Significant importance of P-values obtained from the experiments was considered less than 0.05 through all experiments.

**RESULTS AND DISCUSSION**

Isolation of polyhydroxyalkanoate producing micro-organism

As described in the introduction, PHA has widely been used to make eco-friendly bioplastics. Thus in order to isolate an effective bacterial strain producing PHA in large scale, we have isolated PHA synthesizing microorganism from on the bottom of basket filled with tap-water used for cleaning toilets in Gachon University, InCheon-city. Among bacterial species, 2 different colonies from solid plate were isolated as potential PHA producers. As the PHA extraction, the result reveals that one of bacterial isolates was finally selected as a PHA producer that is capable of accumulating about 20% PHA. The isolate was used for further study to characterize on the PHA production.

**Characterization of microorganism**

It is important to characterize the bacterial species. Firstly, TEM result shows that this bacterium is rod-shaped and has several flagella extended from its outer membrane. And the size of bacteria is approximately 4 µm (Fig. 1). Gram stain-