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Bacterial Strain Isolated from High-Salt Environments Can Produce Large Amounts of New Polyhydroxyalkanoate (PHA)

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Abstract

Since the major problem connected to the industrial production of Polyhydroxyalkanoates (PHAs) is their high production price, this study was performed to inspect the new potential bacterial species for industrial PHA production. The bacterial samples were collected during a screening program from Pink Salt Lake as an extreme environment in the south of Iran, Fars province. The PHA-producing bacteria were isolated. Then further studies on different morphological, cultural, and physiological characteristics of isolates were performed. Among the isolated microorganisms in this study, 18 of 143 bacteria were selected as PHA-producer microorganisms to be studied for analysis along with a partial sequence of the 16S rRNA gene. This study introduces two bacteria; *Bacillus endophyticus* BCCS 011 and *Lysobacter* sp. BCCS 052 as new potential PHA producer that has not been reported previously. They could be an ideal option for cheaper PHAs production.

Keywords: Polyhydroxyalkanoate (PHA), *Bacillus, Lysobacter*, 16S rRNA, Extreme environment

1 Introduction

Polyhydroxyalkanoates (PHA), a family of biopolymers with diverse structures, are polyoxoesters of hydroxy alkanoic acids which are synthesized by various bacteria to overcome environmental stress (1). Originally, they are accumulated as carbon and energy reserves by a variety of bacterial species under nutrient (Phosphorus, Nitrogen, or Sulfur) depleted circumstances with excess carbon (2). Based on the monomer structures, PHA are divided into short-chain-length (SCL) PHA commonly consisting of 3-hydroxypropionate (3HP), 3-hydroxybutyrate (3HB) and 3 hydroxyvalerate (3HV); medium-chain-length (MCL) PHA containing 3-hydroxyhexanoate (3HHx), 3 hydroxyheptanoate (3HHp) to 3-hydroxytetradecanoate (3HTD) (3, 4). Many bacteria are capable of producing PHAs in activated sludge, in high seas, and extreme environments (5). PHAs are biocompatible, biodegradable, and environmentally friendly thermoplastics as compared to petroleum-based plastics that are harmful wastes and take several years to degrade completely (6, 7). Because of increasing global environmental concerns associated with discarded petrochemical-based plastics (8-12), several studies have been conducted on the development of an appropriate eco-friendly material that can substitute at least some of the commodity plastics (13-17). PHAs properties like conventional plastics (especially polypropylene) have versatile plasticable properties and are produced as high molecular mass polymers in bacteria (18). Therefore, they have a wide range of applications, such as in the packaging industry, pharmacy, medicine, agriculture, and food industry (19). As a result, the fabrication of biodegradable polymers such as PHAs from renewable sources is the need of the today, in the face of these environmental facts.

Production and marketing of PHAs have been restricted in two ways. The first cope with the ability of bacteria in accumulation of the polymer, while more than 300 bacterial species have been found in PHA accumulation, but accumulation levels in many of them is very low (20). On the other hand, species such as *Alcaligenes latus, Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*), *Pseudomonas putida*, *Pseudomonas oleovorans*, recombinant *Escherichia coli* and *Azotobacter vinelandii* have been extensively investigated (21, 22). The second aspect, which has restricted PHA production and marketing, is associated with the high costs of the substrate (mainly carbon source) as compared to those of petrochemical origin (23). New strains, using economic substrates along with having a high accumulation proportion, must thus be isolated to solve these problems mentioned above. Interest has been a focus on Gram-positive bacteria such as the genera *Bacillus*, where this bacterium has chemoorganotrophic features(24-28), secretion of a large number of amylases and proteases (29) and lack lipopolysaccharide (LPS) (30). These properties of *Bacillus*

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spp. are of interest for exploring the possibility of utilizing different agricultural raw materials as a carbon source for the fabrication of various metabolites (24). Furthermore, the genera of *Bacillus* are suitable model systems for the heterologous expression of foreign genes related to PHA manufacturing and numerous fine chemicals (31). In the present study, PHB producing bacteria from a salty lake (Pink Lake, Shiraz, Iran) were isolated, identified, and characterized using morphological, biochemical, and molecular methods. To our best knowledge, this is the first report of *Bacillus endophyticus* along with *Lysobacter* sp., and *Pantoea* sp. as PHA producers that can be industrially exploited for bioplastics fabrication.

1 Materials and Methods

1.1 Sample collection

Bacterial isolates were obtained from a salty lake "Pink Lake" Fars province, in the south of Iran. Briefly, liquid samples were collected and transferred in sterile tubes at the ice. The samples were then serially diluted with sterile distilled water, and then 200 µL of the dilution was spread on a sterile Nutrient Agar (NA) plates. The plates were incubated at pH=7 and 30 °C for two days. Various colonies of different morphology, including color, form, and edge appearance, were individually picked and subcultured 3-4 times on nutrient agar plates. Pure bacterial isolates were achieved by subculturing individual colonies several times on a fresh NA medium to gain single colonies. Agar slants of these colonies were kept at 4 °C for one month.

1.2 Screening the PHA-producing bacteria

All the bacterial isolates were qualitatively examined for PHA production by Sudan Black B dye to detect the existence of lipid granules in the bacteria (32). A positively-stained isolate was considered a potential PHA producer and cultured in the modified E2 medium, a nitrogen-limiting medium containing 2% (w/v) glucose (20 g/l of Glucose), 0.9 gr/l of NH_4Cl, 0.9 gr/l of NaCl, 5.22 gr/l of K_2HPO_4, 3.7 gr/l of KH_2PO_4, 0.246 gr/l of $MgSO_4.7H_2O$, and 1ml of MT microelement (1 liter of MT stock contains 2.78 gr of $FeSO_4.7H_2O$, 1.98 gr of $MnCl_2.4H_2O$, 2.81 gr of $CoCl_2.6H_2O$, 1.47 gr of $CaCl_2$, 0.17 gr of $CuSO_4.5H_2O$ and 0.29 gr of $ZnSO_4.7H_2O$) at 37°C for 72h. Then all the isolates were stained by Nile blue a dye to confirm the PHA production.

1.3 Identification of Bacterial isolates

The PHA-positive bacterial isolates were identified according to conventional biochemical tests and by partially sequencing the ribosomal 16s RNA gene. Total genomic DNA was extracted according to Gholami *et al.* and was used as a Chromosomal DNA template for the amplification of the 16s RNA gene (33). The following oligonucleotide sequences 5'-ACGGGCGGTGTGTAC -3' were used as forward, and 5'-CAGCCGCGGTAATAC-3' reverse primers. The PCR products were purified and then sequenced by CinnaGen Company (Tehran, Iran). The resulting 16S rRNA gene sequences were aligned and compared to the nucleotide sequences of some known microorganisms in the GenBank database of the National Center for Biotechnology Information by using the Basic Local Alignment Search Tool (BLAST). The nucleotide sequences of 16S rRNA genes were published to GenBank under the accession numbers, as shown in table1. Then the PHA extracted from all the PHA positive isolates were analyzed by an spectroscopic methods, FT-IR.

1.4 FT-IR spectroscopy

The infrared (IR) spectrum was recorded using Bruker, Vertex 70, FTIR spectrometer (34). The extracted PHA was dissolved in chloroform, and the unfiltered solution was cast onto NaCl crystal.

2 Results and discussion

In this study, a total of 143 bacterial isolates were examined for isolation of PHA-producing bacteria from a salty lake (Pink Lake). This seasonal lake also locally known as Maharloo is located in the 27.0 km southeast of Shiraz, Iran, which is full of potassium and other salts and looks pink (Fig. 1).

Of these isolates, 21 of them were positive for PHA using Sudan Black B and then confirmed by Nile Blue A. Sudan Black B was used as the first-line screening for PHA-accumulating bacteria when they were cultured in an unbalanced growth medium. It was assumed that the negatively stained of isolates with Sudan Black B did not form lipid granules and thus also did not produce PHA due to the lipidic feature of polyester. Sudan black B stains PHA non-specifically as well as for other lipid bodies. In contrast, Nile Blue A is more specific than Sudan black B for PHA detection. Therefore, Nile Blue A was used for confirmation of PHA-accumulating bacteria. The bacterial flora is categorized into two groups, according to their Gram's reaction. In general, the Gram-positive bacteria tended to dominate the salty lake, nearly 66.67% of the total 21 PHA-producing isolates showed Gram-positive character. The result of PCR blasted with other sequenced bacteria in NCBI showed a similarity of more than 95% to the 16S rRNA of other bacteria. Various microbiological and biochemical tests were carried out as a means for the identification of native strains (Table 1). Among 21 of PHA-producing isolates, 13 isolates were belonged to *Bacillus* and one isolate to *Halobacterium* genera along with other Gram-negative bacteria (Table 2). These isolates (*Bacillus* spp. and *Halobacterium*) showed strong growth in 10% (w/v) salt concentration suggesting that they could tolerate relatively salt concentration, which is consistent with the natural condition of this salty lake. These bacteria tend to accumulate PHA, which is commonly consumed by the bacterium itself when the growth circumstances are unfavorable. The PHA-positive isolates opted after Nile blue A staining and then grown in an E2 broth medium containing 2% (w/v) glucose in 100-ml flasks, and were employed to extract PHA after two days of incubation on a rotary shaker. The PHA from the isolates was extracted by the chloroform method, developed by Vizcaino-Caston *et al*. (35). The concentration of PHA in the E2 medium and PHA% of cell dry weight along with cell dry weight achieved for various positively stained isolates are depicted in Table 2. These isolates produced PHA from 0.031 to 0.34 g/l, amounting to about 2.16–23.13% PHA of cell dry weight (Table 2).

Figure 1: Satellite image of salty Pink Lake

Table 1: The morphological and biochemical traits used for classification of 21 selected PHA-producer isolates

Morphology	Gelatinase production
Cell shape	Catalase production
Cell size	Oxidase production
Motility	Lipase production
Gram staining	Hippurate hydrolysis
Endospore	Esculin hydrolysis
Spore shape	**Acid production from**
Spore position	Glucose
Cultural characteristics	Galactose
Colony shape	Fructose
Optimum pH	Mannitol
Optimum temperature	Maltose
Growth on nutrient agar	Sucrose
Growth on McConkey agar	**Utilization of**
Growth on Eosin methylene blue agar	Succinate
Growth at 5, 20 and 50 °C	Citrate
Growth in NaCl 2.5–7.0%	**Resistance to antibiotics**
Biochemical properties	Erythromycin
Urease production	Neomycin
Nitrate reduction	Novabiocin
Voges-Proskauer	Tetracycline
Arginine hydrolysis	Kanamycin
Casein hydrolysis	Chloramphenicol
Lecithinase production	Ampicillin
HCN production	Tetracycline

Table 2: The amount (mg/ml) and the weight yield (w %) of PHA of production, the accession numbers and the concentration of obtained from the screened bacteria which were isolated from Maharlu Lake.

Bacteria	Dry cell weight (gr/l)	PHA.con (gr/l)	% PHA (CDW)
Bacillus endophyticus BCCS 011	1.36	0.24	17.64
Bacillus sp. BCCS 060	0.786	0.066	8.39
Bacillus subtilis BCCS 028	0.926	0.08	8.63
Bacillus subtilis BCCS 033	3.24	0.126	3.9
Bacillus sp. BCCS 036	0.926	0.128	13.82
Halobacterium sp. BCCS 030	0.973	0.09	9.4
Bacillus subtilis BCCS 031	1.4	0.033	2.3
Bacillus endophyticus BCCS 024	1.36	0.034	2.5
Bacillus pumilus BCCS 002	0.686	0.10	15.4
Bacillus subtilis BCCS 005	1.03	0.04	4
Bacillus subtilis BCCS 012	0.66	0.085	13
Pantoea sp. BCCS 053	0.613	0.05	8.4
Escherichia coli BCCS 054	1.14	0.045	3.93
Escherichia coli BCCS 055	0.933	0.106	11.4
Aeromonas sp. BCCS 056	2.166	0.038	1.8
Bacillus sp. BCCS 057	0.793	0.05	6.4
Aeromonas sp. BCCS 058	1.866	0.07	4
Bacillus sp. BCCS 059	1.286	0.113	8.8
Bacillus sp. BCCS 061	1.46	0.026	1.8
Lysobacter sp. BCCS 052	1.466	0.046	3.2

FTIR spectroscopy of the polymer producing bacteria was investigated along with Poly(*R*)-3-hydroxybutyric acid (PHB) prepared from Sigma-Aldrich (cat no: 363502). The polymer extracted illustrated the intense absorption characteristic for ester carbonyl (C=O) stretching groups at 1720, 2325 and 2985 cm^{-1} corresponding to the –CH group in comparison with the PHB (Fig. 2).

An important finding was that all *Bacillus* spp could able to produce a high amount of PHA in comparison to other bacterial species. To our knowledge, this study introduces a new species of *Bacillus* named *Bacillus endophyticus* that has a high ability to produce PHA (23.13% of CDW) as compared to other isolates. The *Bacillus* spp. are critical industrial bacteria for the production of such enzymes and also for PHAs production. The properties, including lack of lipopolysaccharides (LPS) and the ability to consume a wide range of cheap carbon sources, made this bacterium a suitable candidate for the production of PHAs, especially for medical implant purposes (33). Moreover, recovery of approximately 70 to 90% of bacterial dry biomass as PHA production is potentially enough for determining an economically feasible process (2).Most interestingly, this research also identifies a novel bacterium *Lysobacter*, which has not been reported as a PHA producer so far. This bacterium is considered a rich source for the fabrication of novel antibiotics, such as macrocyclic lactams, β-lactams containing substituted side chains and macrocyclic peptides (36). The ease of genetic manipulation and able to occupy a wide range of ecological niches, including a broad range of extreme environments, provides this bacterium an ideal candidate for PHA production (37). Therefore, such studies are needed for the development of these new potential strains for commercial PHA production.

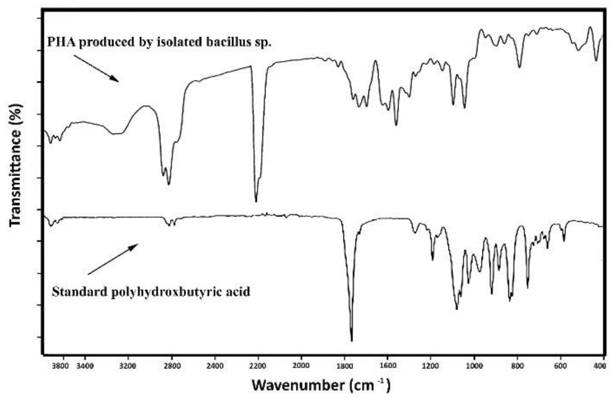

Figure 2: FT-IR spectra of standard PHB and PHA produced by isolated bacterial strain

3 Conclusion

In conclusion, different bacterial strains were isolated from an extremely salty lake and screened for polyhydroxyalkanoate production, and bacterial isolates were identified and characterized using morphological, biochemical, and molecular methods in this study. Within this extremely halophilic environment, most PHA-producing strains were from genus bacillus. Besides, other strains such as Pantoea sp., Halobacterium sp., and Lysobacter sp. have a high potential for the conversion of carbohydrates into PHB. Among the isolated strains, Bacillus endophyticus was able to produce the highest amount of polyhydroxy butyrate (23.13% of the cell dry mass, which is very important from an industrial point of view. Presently, the selected microorganisms are further considered to enhance the production of polyhydroxyalkanoates by the optimization of the process factors. In order for the industrial production of this environmentally valuable product, it is critical to produce more cost-effectively and to achieve greater competitiveness with similar petroleum products that severely pollute the environment.

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Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing.

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