Screening of Salt-tolerant Bacteria for PHA Synthesis from food waste fermentation liquid

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Abstract

Synthetic plastics are non-degradable and lead to environmental pollution. Polyhydroxyalkanoates (PHA), a kind of bioplastic, are considered as good alternatives for petroleum derived synthetic plastics because of their similar physical and chemical properties. In the present study, an attempt was made to isolate salt-tolerant and efficient PHA producing bacteria from salty environments. 8 kinds of bacteria with better performance on PHA production were selected. The method of 16S rDNAsequences analysis was used to identify the species. The strain with the highest PHA yield was identified as Bacillus cereus, named Bacillus cereus strain HY-3. This strain was salt tolerant. When the salt content is 5% or less, the salt content has little effect on the yield of PHA. In the range of pH 5 \sim 8, the dry weight has little changes, from 184.4mg/L to 199.4mg/L).At pH of 4, the cell dry weight decreased sharply to 41.7mg/L. The total yield of PHA increased first and then decreased with the increasing of pH, and reached a maximum of 84.2 mg/L at pH 5.The results of infrared spectroscopy showed that the characteristic absorption peak of PHA samples obtained by HY-3 was similar to that of standard samples.

Keywords: polyhydroxyalkanoates (PHA); salt-tolerance; bacteria; food waste; volatile fatty acids (VFA)

1. Introduction

The large-scale use of traditional petrochemical synthetic plastics has accelerated the consumption of non-renewable resources and brought environmental pollution. Therefore, biodegradable plastics have become a hot topic in the world today [1]. Polyhydroxyalkanoate (PHA) is a kind of macromolecular bio-polyester widely found in microbes. It not only has the physical and chemical properties of traditional plastics but also is biodegradable, it is a kind of "environmentally friendly material"[2]. PHA has optical activity, gas separation, piezoelectricity, biocompatibility and other properties, making PHA as an alternatives in medical, electronic and many. The field of replacement for petrochemicals is possible, and the study of PHA is the focus of research by experts and researchers [3]. But at present, compared with the petrochemical plastic, the production of PHAs need more expensive carbon source, resulting in no competitiveness in market. The costs of PHA production mainly refer to the carbon source for the microorganisms, which will account for 30% of the total cost [4]. Excessive production costs hindered the large-scale application of PHA.

PHA can be synthesized by microorganisms with a variety of carbon sources such as organic acids (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, etc.), carbohydrates (glucose, sucrose,

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starch, etc.) (Methanol, ethanol, propanol, isopropanol, etc.), etc. [5, 6]. Food waste is a kind of organic solid waste with high content of organic matter. Volatile fatty acids can be obtained by anaerobic fermentation of food waste. PHA can be synthesized by VFA from hydrolysis and acidification of food waste, which can effectively reduce the PHA production costs. There are many types of PHA according to the monomer structure. Polyhydroxybutyrate (PHB) is the best known PHA, but it is stiffer and more brittle polymer of high crystallinity compared with polystyrene [7], while poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxy valerate] (PHBV) is characterized by increased elongation to break and low melting temperature and more flexible and tougher than PHB [8]. Therefore, PHBV will be more widely used. It has been proved that the PHB was produced from even carbon numbers alkanoates, whereas PHV was produced from odd carbon numbers alkanoates [9].

The food waste of China has a higher salt content, generally 1wt% to 3wt%, and its component is significantly different in regional culture. It is necessary to screen salt-tolerant microbes to synthesize PHA with carbon sources containing a certain salt. Previous study showed that landfill leachate had a high content of salt. In this study, salt-tolerant and high-yielding PHA strains were screened from the different stages of the leachate treatment. The performance of the salt-tolerant bacteria on PHA production was investigated in detail.

2. Materials and methods

2.1. Collection of samples

The water samples of different sections of the municipal solid waste leachate treatment system were selected for the isolation of bacteria, including anaerobic stage, aerobic stage, Meane Biological Reactor (MBR) and concentrated solution pool.

2.2. Medium of isolation of bacteria

Separation medium: beef extract 3g/ L, peptone 10 g/ L, NaCl 5 g/L, agar powder 20 g/L, nylon blue colouring agent (final concentration 30ug/mL), pH 7.0.

Seed medium: peptone 10g/L, beef extract 5g/L, NaCl 10g/L, pH7.0.

Fermentation medium:(NH4)2SO4 1g/L, KH2PO4 1g/L, Na2HPO4 • 12H2O 11.1g/L, 0.2g MgSO4 0.2g/L, trace element 1ml/L, carbon source 15g/L.Volatile fatty acid (VFA) composition: acetic acid, 15.9g; propionic acid 15.8g; isobutyric acid 0.48g; butyric acid 10.12g; isovaleric acid 0.91g; valeric acid 1.79g, pH7.0.

2.3. Screening and identification of PHA bacteria

Screening. The water samples were diluted on three different dilutions of 10-4, 10-5 and 10-6 and then coated on the separation medium. The obtained strains were observed under a UV lamp at 345 nm.

Re-screening. The strains were screened into the fermentation medium, cultured at 30 °C and 160 rpm for 48h, and the yield of PHA was determined by gas chromatography.

Determination of growth curve of strain. The high-yield PHA strain was inoculated into the seed culture medium and incubated at a temperature of 30 °C and a shaking speed of 160rpm for 24 hours. The OD600 value was measured by UV-Vis spectrophotometer Microbial. Growth curve was drew according to the OD600 value against growth time.

Identification of strains. The strains were identified by the analysis of 16S rDNA gene sequence. 27F(5'-AGAGTTTGATCCTGGCTCAG-3 ') and 1492R (5'-GGTTACCTGTTACGT-3') were used as the primer for the PCR amplification. DNA was extracted using the bacterial DNA extraction kit. The 16S rDNA gene sequencing was performed by Chinese National Human Genome Center, Beijing. The sequences were compared

with the known sequences in the GenBank of NCBI website. MAGE5. 0 software was used to construct phylogenetic trees.

2.4. The optimization of fermentation conditions of strain

The effects of salt content and pH on the growth of high-yield PHA strain were investigated. The experiments were conducted at the salt content of 0%, 0.5%, 1.0%, 2.0%, 5.0%, and pH level of 4, 5, 6, 7, 8 in the erlenmeyer flask of 250 mL at 37°C under the conditions of 160 rpm. The strain was harvested after 72 h. Then the dry weight and the amount of PHB and PHV were detected.

2.5. Analysis of PHA content

Cell suspension (90 mL) was centrifuged at 6000 rpm for 20 min in a centrifuge and washed with distillated water. The sediment was dried in the vacuum freeze dryer until the weight remained constant. The stem cells, chloroform and benzoic acid - methanol solution were added in a glass tube. Then the tube was put in an oven at 100 °C for 4h to conduct methyl esterification. After the reaction the tube cooled rapidly to room temperature, 1mL of distilled water was added in the solution shocked for 1min. The organic phase was took for the gas chromatography analysis after delamination. The chromatograph wasoperated with DP-5 capillary column (30 mlength, 0.25 I.D., 0.25 mm film), a splitinjection ratio of 1:25 and nitrogen as the carrier gas (30 mL/min).Aflame ionization detection (FID) unit was operated at 250 °C with an injection temperature of 200 °C. The initial temperature of the oven was 80 °C, and the temperature was raised to 240 °C at 8 °C • min-1 for 1 min.

The chemical construction of PHA was analysed by FTIR spectroscopy. The standard sample and experiment product of 1-2mgPHA were prepared by KBr compression method, and then infrared spectroscopy was carried out by Nicolet Magna-IR 560 (USA) infrared spectroscopy.

3. Results and analysis

3.1. Screening of PHAs strains

The plating medium was put under UV lamp with 345 nm to isolate the colonies with fluorescence, and 8strains with PHAs producing ability were obtained. The above 8 strains of colonies were inoculated into the fermentation medium, and the cell dry weight and PHA content was measured after 48 h. The results are shown in Table 1. Among these bacteria, the performance of the strain named HY-3 was better than others and then it was selected for follow-up study.

Strain	PHAs	Strain	PHAs
number	content/%	number	content/%
HY1-1	5.49	YY2	1.98
HY1-2	2.36	MBR2	1.83
HY2-1	5.94	TJC2	2.82
QY1	2.65	HY3	8.22

 Table 1
 PHAs content of different strains

3.2 Results of the growth curve of the strain

3mL HY-3 bacterial solution was inoculated into 100mL liquid medium, and cultured at 37 $^{\circ}$ C for 180r / min. The OD₆₀₀ value was measured at different time, and the growth curve was drawn according to the OD₆₀₀ value. The results are shown in Fig. 1. It can be seen thahtHY-3 bacteria grow faster, 2h or so into the logarithmic growth period, about 8h into the stable period. Previous literatures show that the PHA synthesis begins gradually at post-exponential phase and reaches the maximum at the early stationary phase.



Fig.1 Growth curve of bacteria with the highest PHA content

3.3 Molecular biology identification of strains

16S rDNA sequence analysis is usually used to identify bacteria. In general, the sequence homology less than 98% is considered as different species, while the sequence homology less than 93-95% is considered as different genera [10]. Strain HY-3was identified 16S rDNA analysis, and a sequence of 1424bpwas obtained. The sequencing information was submitted to GenBank and accession number of CPO12691.1 was obtained. The high homology sequences were selected by BLAST.MEGA5.0 software was used for phylogenetic analysis, and the construction of phylogenetic tree was shown in Fig.2. It can be seen that strain HY-3 is in the same branch as Bacillus cereus strain FORC O24, and the 16S rDNA sequence of strain HY-3D is 100% homologous to Bacillus cereus strain FORC O24. The strain was named Bacillus cereus strain HY-3.



Fig.2 16S rDNA phylogenetic tree of the strain HY-3

3.4 Effects of Fermentation Conditions on PHA Synthesis of PH-3

3.4.1 Effect of Salinity on PHA Production of HY-3

Bacterial cell dry weight, PHA production and PHA% of cell dry weight with salt content changes are shown in Figure 3. The dry weight of cells increased first and then decreased with the increase of salt content. When the salt content was 0.5%, the dry weight of cells reached the maximum value of167.8mg / L. As the growth of bacteria has a maximum tolerance of the culture environment, reached or exceeded the limiting conditions, the bacteria will die. The normal growth of bacteria was seriously inhibited at the salt content of 5%, and the dry weight of cells decreased to the minimum value. The yield of PHA increased with the increase of salt content, and the maximum yield of PHA was 15.6mg / L when the salt content was 0.5%. With the salt content exceeding 0.5%, the yield of PHA tended to

decrease. As the salt content increased, the dry weight of the cells decreased more significantly, while the PHA production decreased more slowly, resulting in the dry weight ratio increased gradually. Therefore, the optimum salt content of PHA was 0.5% by using various organic acids as carbon source. Paula et al. showed that Pandoraea sp. Produced a higher PHA yield by using glycerol containing Na ion as carbon source compared with pure glycerol. [11]A similar result was obtained in the study of other literature. The effects of salt stress on the accumulation of PHA in C. necator DSMZ 545 were studied. The results showed that the addition of a certain amount of salt promoted the bacteria PHA production, when the NaCl concentration of 9g / L, PHA production increased by 30% compared with the control group [12]. According to the results of Natarajan et al., the PHA yield of bacteria under NaCl stress increased obviously because the activity of β -ketothiolase is 5 times higher than that of the control group [13]. The PHB production had a similar trend of PHA, it increased initially and decreased afterwards with the increase of salt content, while the yield of PHV had no evident changes with the increase of salt content.



Fig.3 Influence of salt content on the growth and PHA yield of HY-3



Fig.4 Influence of salt content on the PHB and PHV yield of HY-3

3.4.2 Effect of pH on PHA Production of HY-3

The cell dry weight, PHA production, PHA content with the pH changes are shown in Fig.5.The dry weight of cells reached the lowest value at pH 4, when the pH from5 to 8, the dry weight of cells changed with pH was not significant, and reached a maximum at 199.4 mg / L at pH 7. The yield of PHA increased first and then decreased with the increase of pH. At pH 5, it was obtained as the maximum value of 84.2mg / L. In the process of bacterial

growth, the pH value affects the cell growth and PHA synthesis by changing the metabolic pathway in the bacteria [14]. It can be seen from Fig.5, in the acid condition it is not conducive to the growth of bacteria. At pH 4, the number of bacteria decreased sharply and PHA production was reduced to the lowest. The results were consistent with that reported by Montiel-Jarillo et al. [15]. However, in the results of Montiel-Jarillo's research, the PHA yield was drastically decreased when the pH was adjusted from the weakly alkaline pH to pH 6.5 (the bacteria used in the fermentation system were mixed) because different bacteria had different tolerance to pH. Studies have shown that, at low pH conditions, VFAs is not dissociated, they can rapidly spread into the bacterial cells. The acids in the sells released proton to reduce the intracellular pH, which prevented the PHA synthesis [16]. The trends of PHB and PHV production trends are the same as those of total PHA yield, but PHB production is generally higher than PHV. The PHB % decreased with the increase of pH value, and the trend of PHV % with pH was similar to that of PHA %, increased first and then decreased (Fig. 6).



Fig.5 Influence of pH on the growth and PHA yield of HY-3



Fig.6Influence of pH on the PHB and PHV yield of HY-3

3.5 Analysis of Chemical Construction by FTIR

The infrared spectrum of the PHA standard sample is shown in Fig. 7. The infrared spectra of the standard sample of PHA show that there is a strong absorption peak at 1724.2 cm⁻¹, which indicated that there is a C = O. The significant absorption peak at 1278.4 cm⁻¹ is on behalf of C-O. The peak at 1372.5 cm⁻¹ represents CH₃. The peaks at 1458.3 cm⁻¹ and 2980cm⁻¹ indicated that there are CH₂ and CH. The above five distinct absorption peaks are corresponded to the groups of C = O, C-O, CH₃, CH₂, CH in the PHA molecule structure [17-19].

The spectral spectra of the extracted products produced by HY-3 showed that there were five characteristic

absorption peaks at 1725.1, 1278.8, 1376.6, 1454.2 and 2980cm⁻¹, and the fingerprints were basically the same as that of PHA standard samples, indicating that the extraction products were PHA.



Fig.7 Infrared spectrum of PHA standard sample



Fig.8 Infrared spectroscopy of extraction product of HY-3

4. Conclusion

(1) A strain with high PHA production was screened from the treatment system of leachate municipal solid waste with high salinity. The strain was identified by16S rDNA sequencing analysis, which identified that it is Bacillus cereus, named Bacillus cereus strain HY-3.

(2)With the increase of salt content, the dry weight of bacterial cells decreased significantly, but PHA % increased gradually. Under the stress of salinity, the PHA synthesis ability of bacteria increased, and the PHA yield fluctuated little when the salt content was less than 5%, at the scope of 61.2mg/Lto 57mg/L, which indicated that the bacteria have a certain salt tolerance. When the salt content is 5% or less, the salt content has little effect on the yield of PHA.

(3)In the range of pH 5 ~ 8, the dry weight has little changes, from 184.4mg/L to 199.4mg/L). At pH4, the cell dry weight decreased sharply to 41.7mg/L. The total yield of PHA increased first and then decreased with the increasing of pH, and reached a maximum of 84.2 mg/L at pH 5.

(4) The results of infrared spectroscopy showed that the characteristic absorption peak of PHA samples obtained by HY-3 was similar to that of standard samples, and PHA samples had the same structure as that of standard substance.

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