

The Screening of PHB Secretion Strain of PHB Synthesis

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Abstract. PHB is similar with non degradable plastic which is widely used in most properties. It is supposed to be an excellent substitute of traditional petrochemical plastics because of its biodegradable character. However, the production cost of PHB is much higher than that of traditional plastic. The substitution has not really realized and the production method which can reduce the production cost is to be a research hotspot. In this study, in order to reduce the difficulty of producing process and co-production high added value product, a high yield PHB synthesizing strain was screened for decreasing the production cost among from high concentration ectoine synthesizing halophilic bacteria strains screened from saltern pond and those commercially available halophilic bacteria.

Introduction

PHB was first isolated from *Bacillus megaterium* by Lemoigne in 1926. Because of its superior and biodegradable “Environment friendly” physicochemical character, PHB is supposed to be an excellent substitute of traditional petrochemical plastics and attract a lot of researchers carried out extensive research on it^[1]. The distribution of PHB synthesizing strains is very extensive, so far, more than 300 kinds (65 genus) microorganisms were found to accumulate PHB which concluded light and chemical energy bacteria, autotrophic and heterotrophic bacteria.

The Growth rate of *Alcaligenes eutrophus* was fast, so that, under certain conditions, the maximal synthetic quantity of PHB could reach 80% of cell dry weight. A relatively mature technology in PHB production was formed with this bacteria and applied in PHB and PHBV industrial production with small scale. However, its production process was using pure culture to ferment and needed maintain sterility that made the operation complex. Furthermore, the production cost was much higher than that of traditional plastic with petroleum and chemical material synthesis. So industrial production and application of bioplastics was limited and the production method which could reduce the cost of production was to be the research hotspot. *Halophilic bacteria* can prevent non halophilic organisms growth without additional operating and realize of pure fermentation due to the salt in its growth environment. Moreover, the majority of moderately halophilic bacteria were able to synthesize ectoine, an osmotic pressure compensation solute, with high total synthesis of concentration and without the limit of intracellular concentration threshold. If it can synthesize PHB, high added value product synthesized together will undoubtedly greatly reduce the production cost.

In order to obtain high fermentation yield strain of ectoine, some ectoine-synthesis-strains were compared with each other about their production level of PHB. One of them is halophilic bacteria which are screened from saltern ponds.

Materials

Strains: *Halomanas salina* DSM 5928, *Halomonas venusta* DSM 4743, *Halomanas elongata* DSM 2581. H09 was isolated from salt lake sediment.

Sample source: Experimental samples collected from saltern ponds.

Medium: MG-2 medium contained 60g/L glucose, 2g/L yeast extract, 3g/L KH_2PO_4 , 11.79g/L K_2HPO_4 , 0.4g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 60g/L NaCl, pH 7.0. The medium was sterilized at 121 °C for 15 min. HM-2 medium contained 0.4g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g/L NaBr, 0.75g/L KCl, 0.13g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2g/L peptone, 1g/L yeast extract, 0.2g/L KH_2PO_4 , 8g/L sodium acetate, 8mL butyric acid. The concentration of NaCl was determined by the experimental conditions.

Methods

Separation Method of Ectoines Synthesis Strain

The rDNA 16S of H09 was analyzed and identified by PCR which was performed with TaKaRa 16S rDNA Bacterial Identification PCR Kit. The PCR product was sequenced by TAKARA BIOTECHNOLOGY (Dalian) Co., Ltd. The comparison of nucleic acid similarity was analyzed between the sequence of 16S rDNA and that in GenBank database.

Shake-flask Culture

The strain of *H. salina* was activated in medium A at 30 °C for 24h at 120rpm. A shake flask (300mL) containing 30mL of medium was inoculated with 1% cultures and grown at 30 °C and 120rpm in a rotary shaker.

Batch Fermentations

The working volume of the fermentor (Baoxing, Shanghai, China) was 10L. The fermentor was filled with 6L of fermentation medium which was set according to experimental conditions and inoculated with 300mL *H. salina* shake flask cultures.

The automatic temperature control was set at 30 °C and the pH was 7.0. The dissolved oxygen level was never less than 40%. Batch fermentation (Phase A, 0-20h) was performed first; the fed-batch fermentation (phase B, 20-48 h) was started after the batch growth phase.

Cell Dry Weight

Fermentation broth was centrifuged at 4 °C and 16,000×g for 15 min and the pellets were then washed with KH_2PO_4 - K_2HPO_4 buffer (KPi buffer, 100 mmol/L, pH 7.2) containing NaCl at the same concentration as that in the medium. After centrifugation, the pellets were dried at 105 °C until no further change in weight occurred, and were then weighed. Cell density was defined as cell dry weight (CDW) per liter fermentation broth (g/L).

Synthesis of Ectoine

The strain was incubated and activated in the broth culture medium at 30 °C for 24 hours. Then 1% cultures were inoculated to 30ml fresh MG-1 medium to synthesize ectoine. The new cultures were cultivated at 30 °C, 120 rpm for 48 hours.

Extraction of Ectoine

The intracellular ectoines were extracted by alcohol. After centrifugation (4 °C, 12,000 r/min, 15 min, the same as following centrifugation), cells were collected from 1 mL fermentation broth. The bacterial precipitation was washed with 100mmol/L KH_2PO_4 - K_2HPO_4 buffer (contained the same NaCl concentration as that in the fermentation medium). The precipitate was resuspended in 1 ml ethanol (80%, v/v), followed by room temperature incubation overnight and centrifugation. Ectoine

concentration was detected by using supernatant and showed as intracellular ectoines quality which was contained in per liter fermentation broth.

Hydroxyectoine Analysis by ^1H -NMR

Hydroxyectoine was extracted by alcohol and purified as method “Extraction of ectoine”. Then purified sample was vacuum distilled, evaporated and crystallized, then dissolved with distilled water and used to be determined by ^1H -NMR (Varian INOVA 400 M NMR, Varian, and American) at 400MHz, room temperature. Tetramethylsilane was used as internal standard substance.

Intracellular Ectoine Measurement

Cells were collected by the centrifugation method described above and the pellets were washed with 100mmol/L KPi buffer (pH 7.2) containing NaCl at the same concentration as that in the medium. After centrifugation, pellets were extracted with 1 mL ethanol (80%, v/v), resuspended, and then kept at room temperature overnight. The suspension was centrifuged again and the supernatant was then used for HPLC analysis. Here, the ectoine concentration by HPLC analysis was defined as the concentration of intracellular ectoine (mg intracellular ectoine per liter fermentation broth, mg/L or mg ectoine per gram CDW, mg (g CDW)/L).

Extracellular Ectoine Measurement

Cells were separated from media by the centrifugation (4 °C) or filtration (30 °C) methods described above then the supernatant or filtrate, respectively, were diluted tenfold with distilled water before HPLC measurement. Here, the ectoine concentration by HPLC analysis was defined as the concentration of extracellular ectoine (mg extracellular ectoine per liter fermentation broth, mg/L or mg ectoine per gram CDW, mg (g CDW)/L).

The total concentration of ectoine was the sum of the concentrations of intracellular and extracellular ectoine. Excretion rate (%) was expressed as the percentage of the concentration of extracellular ectoine divided by the total concentration of ectoine.

Sultan Black Staining of PHB

Solution A: 0.3g Sultan black was dissolved in 100mL 70% (v/v) alcohol, followed by mixing well and by room temperature incubation overnight. Fadeometer: xylene. Solution B: 5% eosine. The sample was conventionally stained to make smear by using solution A for 5 minutes. Then solution A was removed and solution B was used to stained sample for 1 minute followed by washing, drying and microscopic examination.

^1H -NMR Analysis of PHB^[2]

The extraction method of PHB: A 100mL sample of fermentation broth was centrifuged at 4 °C and 14,000 ×g for 15 min to collect polymer-containing *H. salina* cells. Fifty mL of sodium hypochlorite solution (pH 9.8) was added to the cells and left at 60 °C for 1h. The pellets were washed with deionised water and then washed with absolute ethyl alcohol (with centrifugation conditions as noted above). Fifty mL of chloroform was added at 60 °C and extracted for 1h. The sample of PHB was obtained after the chloroform extract liquor was transferred and evaporated at 70 °C. The extract was investigated with a Varian INOVA 400 M NMR (Varian, America) at room temperature and 400 MHz using tetramethylsilane as an internal standard.

Quantitative PHB Assay^[3]

The PHB concentration was determined by using a colorimetric method. A 1mL sample of fermentation broth was centrifuged at 4 °C and 14,000×g for 15 min. One mL of sodium hypochlorite solution (pH 9.8) was then added to the cells at 60 °C for 1h. The pellets were washed with deionised water and then washed with absolute ethyl alcohol (the centrifugation conditions are listed above). One mL of chloroform was added at 60 °C and extracted for 1h. After adding volatilised chloroform, 0.5mL of the chloroform extract liquor was transferred to a colorimetric tube. The chloroform was evaporated at 100 °C and 10mL of concentrated sulphuric acid was added

and heated for 10 min at 100 °C in a water bath. The solution was cooled and its absorbance was determined at 235nm. A PHB standard curve was determined in the same way.

Results

Screening of Ectoine Synthesis Strain

The 16S rDNA of H09 has the highest similarity with *Halomonas* sp. QY113 (100%). It was named with *Halomonas* sp. H09.

Ectoine Analysis by ¹H-NMR

Osmotic pressure compensatory solute ectoine was synthesized by *Halomonas* sa. A1 and determined by HPLC. The results are shown in Fig. 4. The sample presents the resonance absorption peak. It is consistent with the ectoine resonance absorption peak that reported in references^[4]. It is considered that ectoine can be synthesized under the induce of NaCl.

Similarly, most of high yield strains of ectoine belonged to *Halomonas*. So some ectoine synthesis *Halomonas* strains were purchased for saving the experimental cost and the time, such as *Halomonas salina* DSM 5928, *Halomonas venusta* DSM 4743 and *Halomonas elongata* DSM 2581. However, ectoine synthesis yield of *Halomonas* strains were higher than those we separated (The result was shown as Table 1). The target strain was screened from *Halomonas* strains which we purchased.

Tab.1 Ectoines Synthesis of Several Bacterias

strain ^a	Intracellular ectoine concentration (mg/L) ^b	Extracellular ectoine concentration (mg/L) ^c	
		Centrifugation (4 °C)	Filtration (30 °C)
<i>H. salina</i> DSM 5928	824.6±4.4	98.8±4.3	99.3±4.9
<i>H. elongata</i> DSM 2581 ^T	810.7±19.2	—	—
<i>H. venusta</i> DSM 4743	963.8±9.3	122±3.2	328±5.6
H09	678.1±10.2	—	—

^aStrain was incubated in the MM63 medium. The new cultures were collected at logarithmic growth phase.

^bIntracellular ectoine concentration (mg/L): The ectoine concentration by HPLC analysis was defined as the concentration of intracellular ectoine (mg intracellular ectoine per liter fermentation broth, mg/L).

^cExtracellular ectoine concentration(mg/L): The bacterial precipitation was used for HPLC analysis. The concentration was defined as the concentration of intracellular ectoine (mg intracellular ectoine per liter fermentation broth, mg/L).

The Evidence of Ectoine-excreting Strain *H. Salina* Producing PHB

In order to examine ectoine-excreting strain in moderate halophilic bacteria whether producing PHB, *H. salina* was cultivated in HM-2 by method “Shake-flask culture” in shake-flasks at 30 °C and 120 rpm.The fermented liquid was dyed by Sudan black B after 48 h.The results through the microscope were shown in figure 5, more than half of the bacteria in dark blue. These results proved that *H. salina* DSM 5928 could synthesize PHB.

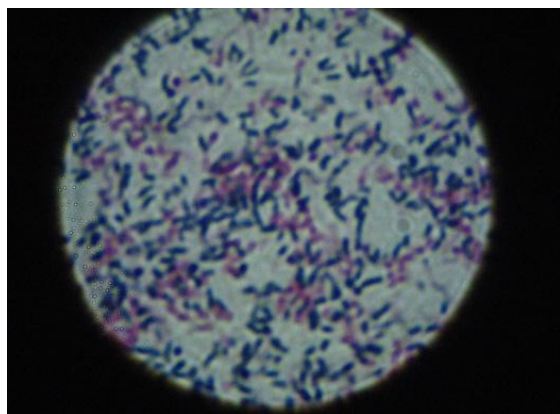


Fig.5 *H. Salina* DSM 5928 Sudan Black Staining

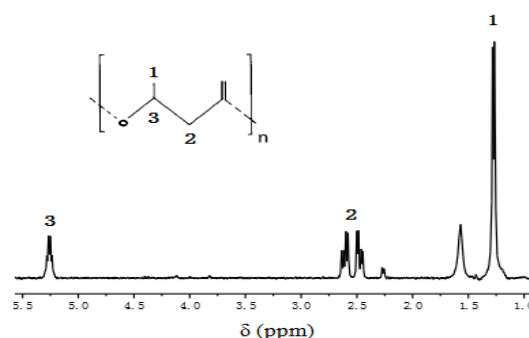


Fig.6 ^1H -NMR Spectrum of PHB from *H. Salina* Cells

The ^1H -NMR spectra were recorded after 48h using method “ ^1H -NMR analysis of PHB”, the results are depicted in Fig.6. As indicated by the PHB structure, there are three resonance absorption peaks at δ 1.3, 2.4–2.6 and 5.3ppm, which matched the report in reference^[5]. This finding established that PHB could be synthesised by *H. salina*.

The Evidence of Ectoine-excreting Strain *H. Venusta* Producing PHB

In order to examine ectoine-excreting strain in moderate halophilic bacteria whether producing PHB, *H. venusta* was cultivated in HM-2 by method “Shake-flask culture” in shake-flasks at 30 °C and 120rpm. The fermented liquid was dyed by Sudan black B after 48h. The results through the microscope were shown in Fig.7, more than half of the bacteria in dark blue. These results proved that *H. venusta* DSM 4743 could synthesize PHB.

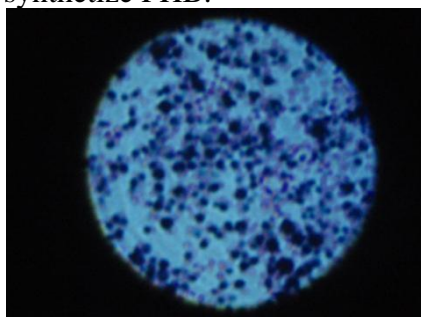


Fig.7 *H. Venusta* DSM 4743 Sudan Black Staining

The Comparison of the Capacity PHB in *H. Salina* and *H. Venusta*

H. venusta and *H. salina* was cultivated in 1 M NaCl concentration of MG-2 by method “Shake-flask culture” in shake-flasks at 30 °C and 120 rpm respectively. The PHB concentrations and the cell dry weight were measured after 48 h of fermentation. The results as shown in table 2.

Tab.2 *H. Salina* and *H. Venusta* in the Same Conditions for Synthesis of PHB

Bacterial strain	Cell dry weight (g/L)	PHB concentrations (g/L)
<i>H.salina</i> DSM 5928	6.07±0.23	2.96±0.15
<i>H. venusta</i> DSM 4743	5.02±0.35	2.84±0.27

The results are averages \pm SD from three independent experiments. The results by above knowable, *H. venusta* DSM 4743 was cultivated in MG-2 for 48h, then the cell dry weight was 5.02g/L, the amount of PHB synthesized by unit cells was 56.6%.

Discussion

The Selection of Ectoine High Synthetic Strains in the Salt Water of Salt Pond

Extremely halophilic bacteria because of its special in the extreme environment of adaptability caused widespread interest of people. And it becomes the hot spots in the field of microbial research. Compared moderate halophilic bacteria and extremely halophilic bacteria, the former with a wider salt adaptation range, it has the best feature in the medium containing 3% ~ 15% salt^[6]. We diluted the sample with Physiological saline, then cultivated it in the medium culture with 5% ~ 20% NaCl, make the most of the halophilic bacteria growth. Selected the single colony which growing faster on flat, and analyzing their Ectoine synthetic quantity.

The Selection of Ectoine-excreting Strain in *Halomonas*

At present, the research of *Halomonas* mainly concentrated in synthesis Ectoine and degradation of dye wastewater. Thomas et al. used *H. elongata* did a detailed study, then made the research of bacteria milking process^[7]. In the bacteria milking process, the strain could release Ectoine under down-shock. We bought a few *Halomonas* strains, selected the high-yield ectoine-excreting strain from these and the existing species. As result, *H. salina* DSM 5928 and *H. venusta* DSM 4743 were selected.

The Selection of PHB Synthesized Strains in Ectoine-excreting Strains

In order to achieve the goal of reducing PHB production cost, we judged the synthesis of PHB in ectoine-excreting strains. Identified that *H. salina* and *H. venusta* could produce PHB all by the method ¹H-NMR. In the same condition, *H. venusta* DSM 4743 had the more production of PHB. But the mechanism of poly-β-hydroxybutyrate/ectoine co-production are still unclear, further experimental studies will be needed.

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