Production of Polyhydroxyalkanotes from Complex Polysaccharides

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Abstract: - Global energy demand and environmental concerns have stimulated increasing efforts to produce bioenergy and biofuels directly from renewable resources. The production of petroleum alternative compounds like bioplastics from abundantly available renewable materials in nature such as cellulosic and algal biomass through consolidated bioprocessing is an innovative approach. Here we show the production of bioplastics polyhydroxylkanotes (PHA) by consolidated bioprocessing using a bacterium with ability to degrade complex polysaccharides such as agarose and xylan without pre-treatment. The association of the bacterium in co-culture with another bacterium dynamically enhanced agarose degradation and PHA production from xylan. Consolidated bioprocessing of complex polysaccharides to PHA investigated in this study opens up new avenues of employment of pure and co-culture for efficient production of PHA.

Key-Words: - Polyhydroxyaalkanotes, PHA, Complex polysachharides, Consolidated bioprocessing, *Saccharophagus degradans*, *Bacillus cereus*

1 Introduction

Ever-increasing energy costs and global warming concerns have created an international imperative for search of sustainable and renewable transportation fuels. Scientific studies have consistently shown that fuels derived from plant biomass have promise to become one of the best substitutes, provided cost-effective commercial production methods using them are established. Dependency on petroleum has driven an increased research focus to develop strategies for the production of petroleum-alternative compounds such as bioplastics (polyhydroxyalkanoates (PHAs), polylactate, polyglycolate, etc.) from abundantly available and cheaper carbon sources such as plant biomass or marine polysaccharides. PHAs are a class of polyesters that can be produced natively by various microorganisms as a carbon reservoir during growth under stress conditions [1, 2]. Production of PHAs from plant and algal biomass has been reported in a variety of wild type and recombinant microorganisms [3, 4, 5]. However, these systems require hydrolysis pre-treatment of biomass in a step prior to fermentation so that it can be readily taken up by the microorganisms [6]. The pre-treatment processes are often carried out at high temperatures and low pH conditions. These are usually prohibitive for bacterial growth, generate potentially toxic compounds, and hamper downstream fermentative processes resulting in lower yields and increased production costs [7, 8, 9]. Therefore, an approach that couples the hydrolysis and fermentative steps (i.e. consolidated bioprocessing) can be an attractive strategy for the production of PHAs from plant and algae derived carbon sources.

2 Problem Formulation

PHA production has been reported by using mixed culture of microorganisms. However, there are no reports on use of co-cultures for production of PHA using complex carbon sources. The application of co-culture fermentation processes, utilizing agricultural and / or industrial wastes can be very promising in increasing financial attractiveness of PHA production. Recently, we found that the bacterium *Bacillus cereus* grows as a contaminant with *Saccharophagus degradans* 2-40 (*Sde* 2-40) cultures on sea salt minimal media. In the present study, we described (i) the ability of *Sde* 2-40 and *B. cereus* to grow in co-culture on sea salt minimal media, (ii) utilization of agarose and xylan in sea salt minimal media by *Sde* 2-40, *B. cereus*, and co-culture of *Sde* 2-40 and *B. cereus*, and (iii) production of PHA through consolidated bioprocessing of agarose and xylan by *Sde* 2-40, *B. cereus*, and co-culture of *Sde* 2-40 and *B. cereus*.

2.1 Media and cultivation conditions

S. degradans 2-40 (ATCC 43961) and *B. cereus* (KF801505) were grown and maintained on either half-strength marine agar containing 18.7 g /L of Marine Broth (Difco, Detroit, USA) supplemented with 1.5% agar (BD, Franklin Lakes, USA) or minimal medium consisting of 23 g /L of Instant Ocean Sea salts (Aquarium Systems, Mentor, OH), 1 g/L of yeast extract, 50 mM Tris buffer (pH 7.4), and 0.5 g/L of NH_4Cl, supplemented with 20 g/L glucose, xylan (Beechwood; Sigma), or agarose (Amresco 0710) as a carbon source in a shaking incubator at 30°C with speed of 200 rpm.

2.2 Analytical methods

To quantify the PHA contents of the cells, culture samples (10 mL) were centrifuged (15,000 rpm, 15 min) and the cell pellets were washed with deionized water. The pellets were recovered by centrifugation again and freeze dried until reached constant weight. PHA was determined using gas chromatography (GC 6890N, Agilent Inc., HP-5 column, 30 m × 320 μm × 0.25 μm). Two mL of methanol acidified with 3% (v/v) H_2SO_4 and 2 mL of chloroform were added to the freeze dried cells and the mixture was heated at 100°C for 3.5 hours for depolymerisation and methanolysis of polyesters. Benzoic acid was used as an internal standard. After cooling, 1 mL of H_2O was added and the suspension was shaken well for 10 minutes. Two phases were separated by standing; the organic phase was used for the analysis. The operating conditions of GC were as follows: injection volume = 1 μL, initial column temperature = 60°C for 5 minutes, temperature increase rate = 4°C/ minute, final column temperature = 180°C for 5 minutes, carrier gas flow rate = 20 mL/minutes, temperature of injection port = 230°C, and temperature of detection port = 280°C.

3 Problem Solution

The ability of *Sde* 2-40, *B. cereus*, and co-culture of *Sde* 2-40 and *B. cereus* to produce PHA utilizing xylan and agarose was evaluated. Production of PHA using 2% (w/v) xylan and agarose as carbon sources was first attempted by growing the cells on nutrient balanced conditions in minimal media at 30°C. The samples were collected for PHA analyses at 120 hours. The PHA content from agarose for *Sde* 2-40 was 18.1% of the dry cell weight and the PHA concentration was 0.240 g/L. For co-culture, the PHA content from agarose was 19.7% of the dry cell weight and PHA concentration was 0.295 g/L. Control experiment to test efficiency of *B. cereus* to utilize galactose revealed that *B. cereus* can grow well on galactose containing media and produce low amounts of PHA (5.3% of the dry cell weight). This suggests that most of the galactose produced in the medium might be used by *Sde* 2-40 to produce PHA. This may be one of the possible and strong reasons for low PHA content from agarose by co-culture.

PHA content from xylan at 120 hours by *Sde* 2-40 was 22.7% of the dry cell weight, and PHA concentration was 0.198 g/L. In co-culture, the PHA content at 120 hours was 34.5% of the dry cell weight, and PHA concentration was 0.267 g/L. The high amount of PHA production from xylan in co-culture made us to think whether monomeric sugars are produced extracellularly by *Sde* 2-40, which might be serving as carbon source for PHA production by *B. cereus*. If this is correct, *B. cereus* may have ability to grow on xylose, a monomeric sugar produced by *Sde* 2-40 using xylan. Therefore, a control experiment was set to check the growth and PHA production by *B. cereus* from xylose as a carbon source. To our surprise, *B. cereus* was found to grow well and produce 23.8% PHA of the dry cell weight from xylose as a so le carbon source. This suggests that the released sugars in broth may probably be utilized by *B. cereus* in co-culture thereby resulting in high content of PHA from xylan.

4 Conclusion

This study reports PHA production using (i) wild type of bacteria without use of any complex procedure; (ii) xylan and agarose as sole carbon sources, and (iii) relatively inexpensive sea salt minimal media which can reduce cost of industrial production. *Sde* 2-40 alone or in co-culture with *B. cereus* accumulated PHA from xylan and agarose, however accumulation of PHA was observed more in xylan than agarose grown cultures. The observation that *Sde* 2-40 is longer lived in co-culture than in pure culture invites further efforts to determine the nature of this interaction, as well as possible improvements to medium formulation for *Sde* 2-40 culture. Confocal microscopic studies by Nile Blue A staining revealed PHA accumulating cells. The type of PHA produced was indicated to be PHB by GC and FT-IR analyses. The findings of this study demonstrated production of PHA from inexpensive precursors without the need for extensive hydrolysis or other fiber reducing

procedures. Therefore, the observations of this study paves way for use of pure and co-culture of microorganisms for consolidated bioprocessing of different complex polysaccharides to produce commercially valuable metabolites. We believe observations of this study can become groundwork for the researchers in devising innovative microbial technologies for production of value added products like PHA from plant biomass or marine polysaccharides to address the problems of petroleum dependency.

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