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Optimisation of the use of products from the cane sugar industry for poly(3-hydroxybutyrate) production by *Azohydromonas lata* DSM 1123 in fed-batch cultivation

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ABSTRACT

Thailand is the second largest cane sugar exporter in the world. The potential use of Thai cane sugar industrial products, including cane juice, 60-Brix syrup, raw cane sugar, refined cane sugar and cane molasses, as carbon and nutrient sources for the production of polyhydroxybutyrate (PHB) by *Azohydromonas lata* DSM 1123 was investigated in this study. The aim of this study was to investigate the use of the less-expensive raw materials to attain a competitive advantage based on a partial cost analysis of the raw material cost. Process optimisation led to a PHB production of 16.9 ± 0.2 g/L with 83.9 ± 2.5% PHB content in the cell dry mass, a $Y_{P/S}$ value of 0.40 g PHB/g sucrose and a productivity of 0.234 g PHB/(L h) using 60-Brix syrup and a fed-batch cultivation process. Finally, using 60-Brix syrup and fed-batch cultivation yielded a 77% cost reduction compared with using refined cane sugar and batch cultivation.

1. Introduction

Plastic waste is a major cause of environmental problems because plastics are commonly used in everyday life due to their versatility, low density, modern design and low cost. By the 1970s, rapid developments in polymer science and cutting-edge technology facilitated the production of petroleum-derived materials to such a large extent so as to allow their incorporation into every aspect of human activity. Recently, national policies and social sentiment have called for a continuous reduction in the usage of petrochemical-based plastics. One of the proposed solutions is replacing petroleum-based plastics with bio-based materials. Recent developments also have raised the hope that natural sources could be a major resource for industrial production. Therefore, many scientists have recently focussed their efforts on the synthesis of bio-based and biodegradable plastics and composites [1].

Polyhydroxyalkanoates (PHAs) are microbial polyesters that are synthesised and accumulated in a wide variety of microorganisms as a stored energy source. The mode of PHAs formation in term of the relationship with the growth of microorganisms can be found in all three classes of Gaden's classification [2]. In general, PHAs are degraded naturally in aerobic or anaerobic environments through thermal degradation or enzymatic hydrolysis. In the soil, saprophytic microorganisms secrete extracellular enzymes that depolymerise or degrade PHAs to carbon dioxide and water [3]. Thus, PHAs can be considered as green materials throughout their life cycle; they are produced and degraded by living organisms, and their residual substances are recycled as a function of living organisms without harmful products being left in the environment [4]. Notably, the PHA manufacturing process can be considered as white biotechnology because it relies on the use of whole cells or enzymes as catalysts [5].

Among the PHAs, polyhydroxybutyrate (PHB) has been the most extensively studied since its discovery [6]. However, the remaining bottleneck in the PHB-manufacturing process is achieving cost competitiveness for this material compared with petroleum-based

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Nomenclature

C/N	molar ratio of carbon to nitrogen (−)
F	feed rate of alcohol (L/h)
S	sugar concentration in the culture medium (g/L)
S_f	sugar concentration in the feed medium (g/L)
t_f	final cultivation time (h)
V	volume of the culture medium (L)
$Y_{P/S}$	yield coefficient of PHB produced from the consumed sucrose (g PHB/g sucrose)
$Y_{X/S}$	yield coefficient of the residual cell mass produced from the consumed sucrose (g PHB/g sucrose)
$Y_{P/X}$	yield coefficient of PHB produced/g residual cell mass (g PHB/g residual cell mass)
% yield	% PHB produced from the total amount of sucrose supplied to the culture (Table 4)

Greek letters

μ	specific growth rate (1/h)
π	specific production rate of polymer (g PHB/g dry cell/h)

plastics. The major reasons for the high production cost of PHB are the expenses of the carbon source and the downstream processes. PHAs are normally produced using various commercially available carbon sources, such as carbohydrates, alcohols, alkanes, vegetable oils and short-chain to long-chain fatty acids [1,7]. The current trend in PHA production involves utilising inexpensive carbon sources, such as industrial and agricultural by-products that contain carbohydrates or fatty acids. In some cases, pre-treatment is required, but this step should be avoided to maximise cost effectiveness.

The United States Department of Agriculture (USDA) Foreign Agricultural Service reported that the Thai cane sugar industry has been ranked the second largest cane sugar exporter in the world and the fourth largest cane sugar producer [8]. In 2015, Thailand's cane sugar production is estimated to be 11.4 million tons [8,9]. Exports are expected to increase to a record of 8.3 million tons based on higher yields and stocks drawn down to meet increased Asian demand, particularly from China and Malaysia. Consumption continues to trend higher, driven by rising household and industrial use [9,10]. In addition to refined sugar, the final product of the cane sugar manufacturing process, several products with different sugar compositions and operating costs are generated. To attain sustainable development as well as sustainable economic growth in Thailand, in this study, some of the products that are obtained during the upstream, middle stream, and downstream steps of cane sugar processing were selected as potential candidates to apply in PHB production. Herein, the results of efforts to optimise the use of cane sugar industrial products, such as cane juice, 60-Brix syrup, raw sugar, refined sugar and cane molasses, for PHB production using *Azohydromonas lata* DSM 1123 are reported. The efficiency of PHB production using batch and fed-batch cultivation was evaluated. Finally, the cost competitiveness was analysed based on the amount of PHB produced relative to the total amount of substrate supplied and the retail price at the factory site of the raw materials.

2. Materials and methods

2.1. Microorganisms

A. lata DSM 1123 (JCM 20734, Japan Collection of Microorganisms, RIKEN Bio Resource Centre, Ibaraki, Japan) was used as the PHB-producing bacteria in this study. The stock cultures were maintained at −80 °C in a 15% (v/v) glycerol solution.

2.2. Carbon sources

Cane juice, 60-Brix syrup, cane molasses, raw sugar and refined sugar produced during cane sugar processing between January 2010 and August 2011 were provided by the Kaset-Thai International Sugar Corporation Public Company Ltd. (Nong Pho District, Amphur Taklee, Nakhonsawan province, Thailand).

The cane sugar milling process is illustrated in Fig. 1, demonstrating that cane juice is obtained from a juice extraction process in which the cane plants are chopped and shredded in roller mills to extract the juice. Next, the cane juice, a viscous liquid of a brownish to deep-green colour, is concentrated using multiple heaters and clarified by adding gases such as carbon dioxide obtained from combustion of bagasse. The concentrated clear juice is evaporated in triple or quadruple evaporators to obtain 60-Brix syrup, where one degree Brix corresponds to one gram of sucrose in 100 g of solution. The raw cane sugar is obtained via crystallisation, centrifugation and drying. The raw cane sugar contains 96.5–98.6% sucrose and impurities. Finally, the refined cane sugar is obtained via a refining process involving affinated centrifugation, clarification, crystallisation, centrifugation and drying. Molasses is the end product from the centrifugation of the final refined sugar in a raw sugar refinery. Normally, molasses still contains considerable amounts of sucrose but does not crystallise spontaneously [11].

2.3. Culture conditions

The shake flask experiments were performed using 500-mL Erlenmeyer flasks containing 100 mL of medium. Various types of locally manufactured cane sugar products, including cane juice, 60-Brix syrup, raw cane sugar, refined cane sugar and cane molasses, were used separately as the sole carbon source. Ammonium sulphate was used as the nitrogen source. The effects of the total carbon sources were investigated at concentrations of 20, 30 and 40 g/L. Carbon and nitrogen sources were added at 12-h intervals, and the molar ratio of carbon to nitrogen (C/N) was maintained at an appropriate value throughout the cultivation using an intermittently fed-batch culturing technique. The effect of the C/N ratio on the relationship between the specific growth rate and the specific production rate was investigated in detail for C/N values of 4 and 200.

For the batch experiments, 500 mL of seed culture was prepared in flasks and grown on a rotary shaker at 30 °C at a 200-rpm shaking speed for 24 h. The cells were harvested by centrifugation, washed to remove the nitrogen source and re-suspended in 100 mL of 0.85% sodium chloride. The cells then were inoculated into a synthetic medium in a 5-L bioreactor (MBF-500ME, EYELA, Tokyo Rikakikai Co., Tokyo, Japan) that was interfaced with an EPC control box (EPC-1000, EYELA, Tokyo Rikakikai Co., Tokyo, Japan). The working volume of the batch cultures was 3 L. The synthetic production medium was a mineral salt medium consisting of 4.5 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L Fe(III)(NH_4) citrate (17% of Fe), 0.02 g/L $CaCl_2{\cdot}2H_2O$ and 1 mL of trace element solution [0.3 g/L H_3BO_4, 0.2 g/L $CoCl_2{\cdot}6H_2O$, 0.01 g/L $ZnSO_4{\cdot}7H_2O$, 0.04 g/L $MnCl_2{\cdot}4H_2O$, 0.03 g/L (NH_4) $6MoO_4H_2O$, 0.02 g/L NiCl2·$6H_2O$, 0.01 g/L $CuSO_4{\cdot}5H_2O$]. The fermentation temperature was 30 °C, and the pH value was maintained at 7.0 throughout the experiment. The values for these parameters were monitored and recorded using the online TK97 Data Record (version 2.04 program; EYLA Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The aeration rates evaluated were 0.25, 0.5 and 1.0 vvm. The agitation speeds tested were 200, 400, 500 and 600 rpm.