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Preparation of *A. succinogenes* immobilized microfiber membrane for repeated production of succinic acid

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ABSTRACT

A new applicability of cell-immobilized system for biological production of target chemical was reported in this work. *A. succinogenes* CCTCC M2012036 was immobilized on positively charged polypropylene microfiber membrane by physical interaction and were used for converting glucose into succinic acid. Glucose consumption and succinic acid production kinetics were investigated for optimizing the operational parameters. The cell-immobilized membrane presented good reuse stability, and six cycles of fermentation without activity loss were realized with an average succinic acid yield of 0.83 g/g. Importantly, a biofilm was formed which favored the production of succinic acid. A microfiber membrane bioreactor was further constructed with the cell-immobilized membrane to perform fermentation in a larger scale, and the yield and productivity of succinic acid were 0.82 g/g and 1.04 g L^{-1} h^{-1} using a fed-batch strategy. By combining mesoporous support with biotechnological techniques, this work offered a prospect of adopting reusable cells feasible for industry.

1. Introduction

Succinic acid, also known as butanedioic acid or amber acid, is a natural metabolite in plants, animals and microorganisms [1]. It has attracted widespread attention as an important C4 platform chemical participating in the synthesis of 1,4-butanediol, tetrahydrofuran, γ-butyrolacetone, polybutylene succinate, polyamides and other bulk chemicals, thus is widely used in the agricultural, food and pharmaceutical industries [2–4]. Succinic acid has been identified as one of the top 12 building block chemical by the U.S. Department of Energy [5]. Its production has reached 30,333–35,000 in the year 2010 with a market value of US $225 million and is continuing to increase gradually [6]. Currently, succinic acid is produced mainly by hydrogenation of petroleum-derived maleic anhydride, which inevitably results in many pollutants. In the latest decade, fermentative production of succinic acid by anaerobic microorganisms has become more appealing as an alternative to the non-sustainable petrochemical route [7]. Moreover, in this fermentation process, CO_2, a primary greenhouse gas, will be consumed, providing further incentive for production by microorganisms [8].

Recent studies have shown that many different microorganisms, including *Actinocacillus succinogenes* (*A. succinogenes*), *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens* and recombinant *Escherichia coli* are all capable in the production of bio-based succinic acid [9–13]. Among them, *A. succinogenes* is able to produce an unusually large amount of succinic acid from a broad range of carbon sources [14]. It has an adequate tolerance to inhibitors and sufficient fermentation efficiency even with crude renewable resources, thus is considered to be one of the most promising microorganisms for industrial succinic acid production [15,16].

Although *A. succinogenes* has been intensified studied due to its high succinic acid yield and the obvious environmental benefits may bring, the practical usage of *A. succinogenes* in fermentation processes always faces some problems. First, the percentage of substrate used to generate cell is always very small compared with the percentage used to generate product. Also, the slow growth rate of the *A. succinogenes* brings obstacle in improving overall production rates since cell concentration is the rate-limiting factor in anaerobic fermentations [17,18]. Second, the cost in cultivating cells is relatively high, but the cells are always discarded after fermentation process. To overcome these limitations, cell immobilization methodology was put forward. According to this methodology, a high biomass concentration in fermentation system can be retained, which is favorable to improve succinic acid productivity and yield [19]. Moreover, immobilized cells can be easily separated from products in solution without losing activity, and cells can be re-utilized as well as more physically stable [20,21].

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In order to give a beneficial environment for cell immobilization, the support must be carefully chosen. Up to now, *A. succinogenes* has been reported to be efficiently entrapped in chitosan, alginate and carrageenan beads, and attached on Poraver®, zeolite, activated carbon, plastic composite support, and polypropylene tube [18,22–25]. In recent years, nano- and micro- scale materials have attracted much attention due to their large surface area to volume ratios for high cell loading, and their fine porous structure allows ready accessibility of substrate to cells as well as the low diffusion resistance necessary for high reaction rates and conversion [26]. In addition, compared with particulates, they are easily recoverable from reaction media, showing great promise for continuous operations [27].

In this paper, an effective and convenient protocol to enable cell immobilization using polypropylene microfiber membrane is described. The membrane was positively charged by corona discharge, which favored the immobilization of the negatively charged *A. succinogenes* by physical attachment. The effect of operating variables (namely, adsorption time, membrane usage, initial glucose concentration, and membrane charge properties) on cell growth and succinic acid production was evaluated. Furthermore, these variables are related to produce performance metric of reusability in repeated batch fermentation, and a microfiber membrane bioreactor was even constructed with the succinic acid producing capability tested. It is expected that this work gives inspiration to a further application of this efficient and economically viable cell-immobilization system in producing bio-based succinic acid.

2. Materials and Methods

2.1. Materials

Pristine polypropylene microfiber membrane, with a weight of 18.2 g/m^2, was kindly supplied by Dr. Xiangyu Ye in Zhejiang Textile Testing Research Institute. The average fiber diameter of this membrane was 2.0 ± 0.5 μm. Glucose was bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Tryptic soy broth, yeast extract and corn steep powder were obtained from Yixing Yongxin Biological Co., Ltd (Jiangsu, China), Angel Yeast Co., Ltd (Hubei, China) and Henghui Starch Sugar Co., Ltd (Jiangsu, China), respectively. All other chemicals were of analytical grade and used without further purification.

2.2. Cell and growth conditions

A. succinogenes CCTCC M2012036, obtained in our group and stored at the China Center for Type Culture Collection was used in this work [28]. The primary seed culture was prepared by transfering1 mL of *A. succinogenes* CCTCC M2012036 glycerol stock to 25 mL of tryptic soy broth medium containing tryptic soy broth (3.23 wt.%) and yeast extract (0.50 wt.%), and then incubating under CO_2 phase at 38 °C. The secondary seed culture was prepared by inoculating 4 mL of primary seed culture into 100 mL of medium containing yeast extract (1.0 wt.%), glucose (1.0 wt.%), $NaH_2PO4{\cdot}2H_2O$ (1.0 wt.%) and $K_2HPO4{\cdot}3H_2O$ (2.0 wt.%) and cultivating under CO_2 phase at 38 °C [28]. The mediums were both sterilized at 115 °C for 20 min in advance and the medium pH was adjusted to 6.5 by 1.0 M of $NaHCO_3$ solution filtered through a 200-μm membrane before cell inoculation.

2.3. Preparation of charged polypropylene microfiber membrane

The pristine polypropylene microfiber membrane was positively charged by a corona discharge device (152A, Coronatrol, Monroc, USA) at 45 kV with a treatment distance of 3–5 cm. The charged properties of the polypropylene microfiber membrane were detected by an electrostatic voltmeter (Model 244A, Monroe Electronics, New York, USA).

2.4. Preparation of cell immobilized polypropylene microfiber membrane

Both the pristine and charged polypropylene microfiber membranes were sterilized at 115 °C for 20 min in advance. In the cell immobilization process, polypropylene microfiber membrane was immersed in 50 mL of secondary seed culture at 38 °C for 30 min, with CO_2 as the environmental gas phase. The ratio of membrane surface area to culture volume was 0.5:1, 1:1, 1.5:1, 2.0:1 and 2.5:1 (cm^2:cm^3), respectively. After immobilization, the membrane was taken out, rinsed with pure water, and dried under vacuum at ambient temperature. Seed loading was defined as the amount of secondary seed (mg) per gram immobilized on the membrane, and was determined by mass difference of the polypropylene microfiber membrane before and after immobilization. Each value was the mean of three parallel experiments at least, and the standard deviation was within ca. ±5%.

2.5. Succinic acid production in anaerobic bottles

The medium for anaerobic bottle fermentation was composed of glucose (concentration varied from 20 to 80 g/L), corn steep liquor (2.5 wt.%), $CaCl_2$ (0.02 wt.%), $K_2HPO_4{\cdot}3H_2O$ (0.25 wt.%), $NaH_2PO_4{\cdot}2H_2O$ (0.25 wt.%), $MgCl_2{\cdot}6H_2O$ (0.02 wt.%) and Na_2S (0.01 wt.%). The medium was sterilized at 121 °C for 20 min and $MgCO_3$ (sterilized at 170 °C for 2 h) was used to tailor the medium pH to 6.5. The cell-immobilized polypropylene microfiber membrane was put into 50 mL of fermentation medium. The fermentation process was carried out under CO_2 phase at 38 °C until glucose was depleted.

2.6. Reusability test of the cell-immobilized polypropylene microfiber membrane

In the measurement of reusability, cell-immobilized membrane was first recovered and then thrown into a fresh fermentation broth. Similar fermentation processes were conducted for 6 times. Each time the final succinic acid production and optical density (OD) of the fermentation broth were measured.

2.7. Construction of microfiber membrane bioreactor for succinic acid production

The bioreactor was constructed based on a 3 L stirred fermenter (Biotech-3BG, Shanghai, China) equipped with thermometer and pH meter. The cell-immobilized polypropylene microfiber membrane was tethered on a stainless steel screen (10 cm in pore diameter) and then mounted to the agitation shaft, as were shown in Fig. 1. The fermenter was autoclaved twice at 121 °C for 30 min. The initial fermentation broth volume was 1.5 L with the composition similar to that described in Section 2.5 except for a 50 g/L of initial glucose concentration. $MgCO_3$ (sterilized at 170 °C for 2 h) was used to buffer the pH and also served as indirect CO_2 resource. There was a pH meter detecting the pH value of the fermentation system, and when the pH value dropped below 6.0, $MgCO_3$ was added into the fermentation system until the pH value increased to 6.0–6.5. When the glucose concentration was lower than 10 g/L, a concentrated 300 g/L glucose solution was fed into the medium through a peristaltic pump (Longer BT100-2J, Hubei, China) at a flow rate of 30 mL/min. The concentrated glucose solution was sterilized at 115 °C for 15 min in advance.