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Production of butyric acid by a cellulolytic actinobacterium *Thermobifida fusca* on cellulose

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ABSTRACT

Thermobifida fusca not only produces cellulases, hemicellulases and xylanases, but also excretes butyric acid. In order to achieve a high yield of butyric acid, the effect of different carbon sources: mannose, xylose, lactose, cellobiose, glucose, sucrose and acetates, on butyric acid production was studied. The highest yield of butyric acid was 0.67 g/g C (g-butyric acid/g-carbon input) on cellobiose. The best stir speed and aeration rate for butyric acid production were found to be 400 rpm and 2 vvm in a 5-L fermentor. The maximum titer of 2.1 g/L butyric acid was achieved on 9.66 g/L cellulose. In order to test the production of butyric acid on lignocellulosic biomass, corn stover was used as the substrate, on which there was 2.37 g/L butyric acid produced under the optimized conditions. In addition, butyric acid synthesis pathway was identified involving five genes that catalyzed reactions from acetyl-CoA to butanoyl-CoA in *T. fusca*.

1. Introduction

Butyric acid, a 4-carbon short chain fatty acid, is widely used in chemical, food, and pharmaceutical industries [1]. Current industrial production of butyric acid is based on petroleum chemical synthesis. However, with the rising oil price, concerns of environmental pollution caused by petrochemicals, and consumer's preference for bio-based natural ingredients for foods, cosmetics, and pharmaceuticals, the production of butyric acid from renewable biomass has become an attractive alternative [2]. Historically, bioprocess production of butyric acid has mainly utilized anaerobic bacteria such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* in acetone–butanol–ethanol fermentation (ABE fermentation), which was once the second largest industrial fermentation in the world [1]. However, anaerobic bacteria are not a good platform for industrial manufacturing because they need more complicated and expensive equipment and they have low growth rate and energy level compared to aerobes.

Cellulose is the most common organic compound on the earth which can be potentially used as a source material for chemical production but its recalcitrant nature hinders the efficiency of degrading it to fermentable sugars [3]. Cellulolytic microorganisms that produce different cellulases are able to degrade cellulose to sugars. While generally poorly-characterized, cellulolytic microbes possess great physiological and biochemical diversity making them interesting organisms to study from both a basic and applied perspective [4]. The reported anaerobes producing butyric acid were not able to directly ferment cellulose, instead, they could only use pretreated biomass to produce butyric acid but there were a variety of inhibitors preventing clostridia from fast growth and high production yield [5]. There are few reports on producing butyric acid from aerobic bacteria, which have great advantages compared to anaerobes, such as an easy fermentation operation, low device cost, low operation cost, high basal energetics, and ease of laboratory manipulation.

Thermobifida fusca is an aerobic, moderately thermophilic, filamentous soil bacterium [6]. It is a major degrader of plant cell walls in heated organic materials [7]. The extracellular enzymes produced by *T. fusca*, including cellulases, have been studied extensively because of their thermostability, utility through a broad pH range (4–10) and high activity [8]. Recently, the genomic sequence of *T. fusca* has been published and the regulation and characteristics of it have been studied [3,9–11]. Moreover, *T. fusca* has been found to directly ferment lignocellulosic biomass to chemicals and was engineered to produce 1-proponal directly from biomass without pretreatment [11].

Although most of the recent research on *T. fusca* is concentrated on regulation of cellulase-related genes and synergies in cellulase activity, few studies about the effect of general culture conditions

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on by-product productions have been published. In previous studies, *T. fusca* was found to naturally produce significant amounts of butyric acid [10,11]. In this study a variety of culture conditions were explored to increase butyric acid productivity in *T. fusca* muS. In order to produce the highest yield of butyric acid, a series of carbon sources were studied in the shaken flasks; the fermentation process was then optimized in a 5-L fermentor and the pathways to produce butyric acid were identified by various methods. To our knowledge, this is the first example of an aerobic cellulolytic bacterium producing butyric acid.

2. Materials and methods

2.1. Culture conditions

T. fusca muS was grown in Hagerdahl medium [12]. For experiments conducted in Erlenmeyer flasks, 50 mL pre-cultures of *T. fusca* were grown at 55 °C and 250 rpm for 12–24 h in a 500 mL Erlenmeyer flask. Growth cultures for testing were inoculated using 5% of the pre-culture and grown at 55 °C and 250 rpm for 42–48 h. For fermentor experiments, 200 mL pre-cultures were grown for 12–24 h and used to inoculate the bioreactor with a 5 L working volume. The fermentor had a jacketed glass vessel with stainless steel-top plate and baffle insert. The fermentor experiments were conducted with four baffles and two six-bladed-disk impellers for mixing. Cells were cultured in the bioreactor at 55 °C for 42–68 h. Stir speeds and aeration rates varied depending upon the specific experiment [3]. To determine the oxygen supply in the bioreactor, the volumetric oxygen transfer coefficient (K_La) was calculated at different agitation and aeration rates [13].

2.2. Cell density and by-product measurement

Due to the growth physiology of *T. fusca* (filamentous cells that aggregate), the culture density of *T. fusca* was determined by measuring cytoplasmic protein content. 1 mL culture was centrifuged at 10,000 × *g* for 5 min. The pellets were re-suspended in fresh media and centrifuged at 10,000 × *g* for 5 min again. Sediments were dissolved in 200 μL 50 mM Tris–HCl buffer (pH6.8) containing 2% SDS, 0.1 M DTT and 50% glycerol. Samples were then pulsar sonicated at 70% strength for 10 min in an ice bath. After centrifuging at 10,000 × *g* for 5 min, the proteins in the supernatant were measured by the Bradford protein assay [14]. The dry cell weight (DCW) is proportionally related to the overall protein content.

Byproducts were detected using an HPLC system equipped with Bio-Rad HPX-87H ion exclusion column. The mobile phase was 0.005 mol/L H_2SO_4 at the rate of 0.6 mL/min and IR and UV detectors were used.

The definition of the yield of butyric acid was defined: gram butyric acid produced per gram carbon entering the cell.

2.3. Adding precursors of butyric acid into washed cell system

T. fusca was grown in shaken flasks to mid-log phase, when cells were obtained and washed by fresh media without carbon source three times and then cells were transferred to other shaken flasks with fresh media without carbon source to grow. After adding cells to the media, 0.1% of butanoyl-CoA and butanoyl-P were added into the flasks, respectively, and then *T. fusca* cells were cultured for another 12 h. After cultivation, the supernatants were used for detection of butyric acid.

2.4. Enzyme activity assays

2.4.1. Cellulase activity

Cellulase activity was measured according to the published protocols [3].

2.4.2. Butyryl-CoA transferase activity

The assay mechanism involves the condensation of the formed acetyl-CoA with oxaloacetate, and the subsequent liberation of CoA-SH under the influence of the citrate synthase. CoA-SH reacted with 5,5′-dithio-bis-(2-nitrobenzoate) to form the yellow thiophenolate anion, 2-nitro-5-mercaptobenzoate. Enzyme activity was determined by following the formation of this colored product at 410 nm at 25 °C and pH 7 [15].

The enzyme assay was adapted for microtiter plate measurements. A 50 mM solution of Ellman's reagent was freshly prepared and kept on ice until use. 20 μL of the enzyme solution at the appropriate dilution was placed in a well of a 96-well plate. The reaction was started by mixing thoroughly 4 μL of Ellman's reagent to the reaction mix and adding immediately to the enzyme solution in the well. A master-mix was prepared for several reactions and a volume of Ellman's reagent was added accordingly before dispensing 180 μL of the mix into each well-containing enzyme of the microtiter plate. The increase in absorbance was followed at 410 nm every 30 s over 5 min with a thermo-regulated microtiter plate spectrophotometer set at 25 °C and data were recorded. The plate was briefly shaken before each reading. The negative control contained all reagents but butyryl-CoA.

2.5. RNA preparation and real-time PCR

To study molecular-level differences in the cultures of *T. fusca*, gene expression was studied using real-time PCR using cells harvested at the mid-log growth phase and early stationary phase corresponding to experimental points. Cells at the selected points were centrifuged at 10,000 × *g* for 5 min. The cell pellets were resuspended in RNA protect bacteria reagent (QIAGEN, Valencia, CA) as proscribed by the manufacturer. After incubation at room temperature for 5 min, the cells were pulsar sonicated at 10% strength for 2 min. RNeasy Midi kits (QIAGEN, Valencia, CA) were then used to isolate RNA using the protocol suggested by the manufacturer. The real-time PCR measurements were performed in the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan® One Step PCR Master Mix Reagents Kit (P/N: 4309169). The cycling conditions were: 48 °C/30 min; 95 °C/10 min; and 40 cycles of 95 °C/15 s and 60 °C/1 min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). The genes were measured along one housekeeping gene (Tfu_02001404) that was used as a control and all reported transcript levels were normalized to this housekeeping gene. The probes were labeled at the 5′ end with FAM (6-carboxyfluoresceine) and at the 3′ end with TAMRA (6-carboxytetramethylrhodamine) [3]. All the data points were measured in triplicate.

3. Results and discussion

3.1. Effect of carbon sources on butyric acid fermentation

T. fusca produced a variety of cellulases, hemicellulases and xylanases, which degraded most of lignocellulosic biomass [7]. *T. fusca* was not only able to produce cellulolytic enzymes but also produce value added chemicals such as butyric acid. Although *T. fusca* is a cellulolytic bacterium, it has the ability to uptake a variety of carbon sources to support growth [10]. Prior to this study, the effect of different carbon courses on chemical productivity