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Consolidated processing of biobutanol production from food wastes by solventogenic *Clostridium* sp. strain HN4

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ABSTRACT

In this study, biobutanol production from glucose, starch and food waste by newly identified *Clostridium* sp. strain HN4 was comprehensively investigated, which is capable of secreting amylase indigenously for the following acetone-butanol-ethanol fermentation. With pH adjustment, strain HN4 could produce 5.23 g/L of butanol from 60 g/L of starch with secretion of 1.95 U/mL amylase through consolidated bioprocessing. Further supplementation of 3 g/L of $CaCO_3$ and 0.5% non-ionic surfactant of Tween 80 could stimulate both amylase activities and the final butanol titer, leading to 17.64 g/L of butanol with yield of 0.15 g/g. Fed batch fermentation integrated with *in situ* removal could further improve the butanol titer to 35.63 g/L with yield of , representing the highest butanol production and yield from food waste. These unique features of *Clostridium* sp. strain HN4 could open the door to the possibility of cost-effective biobutanol production from food waste on a large scale.

1. Introduction

The growing concerns regarding the climate change and greenhouse effect, together with the limited availability and fluctuating price of fossil fuels urge researchers to develop more efficient and greener systems to produce advanced biofuels from sustainable, safe and low expense sources. Currently, bioethanol is the most widely used biofuel; however, biobutanol shows better characteristics over bioethanol, such as higher energy content, lower volatility, less hydroscopic, less corrosive and better compatibility with current car engines (Jones and Woods, 1986; Xin et al., 2018). In addition, butanol has been regarded as an important chemical of substantial industrial interest, as it can be used as the solvent for the production of a variety of compounds, such as hormones, drugs, antibiotics, cosmetics, and vitamins (Lee et al., 2008). Currently, one of main bottlenecks hindering the scaling up of biobutanol production through acetone-butanol-ethanol (ABE) fermentation is the high cost of traditional substrates, which are mainly starchy based materials, such as corn and cassava (Xin et al., 2017). Therefore, the adoption of low expense substrates for the cost-competitive production of butanol is still a matter of priority research.

During the past decades, numerous investigations regarding ABE production using low cost substrates mainly focused on lignocellulosic biomass, such as wheat straw, sugar cane bagasse, corn stover et al. Meanwhile, there also has been isolated reports regarding butanol production from non-lignocellulosic wastes, such as waste glycerol, whey, apple pomace, and Jerusalem artichoke et al (Jiang et al., 2017; Xue et al., 2017; Lee et al., 2016; Sun et al., 2018). Food wastage is currently a global problem occurring at different stages in food production systems, starting from the harvesting of food to storage, packaging and end of life (Gioannis et al., 2013; Pleissner et al., 2013). In Europe, it is estimated that approximately 88 million tons of food are wasted and of these, 57 million tons are from households and food service companies (Banks et al., 2011; De Gioannis et al., 2013). In the United States, the Environmental Protection Agency (EPA) estimated that approximately 37 million tons of food ended up in municipal solid waste systems in 2013, which was approximately 14% of all waste in the United States (Han et al., 2015; Huang et al., 2015). If alternative methods of converting food wastes into value-added products are developed, such as biobutanol, the energy stored in these wastes can be essentially transformed (Han and Shin, 2004; Zhang et al., 2012).

Generally, polysaccharides stored in food wastes are in the form of macromolecules, e.g., starch, cellulose/hemicellulose and lipid, which need to be hydrolyzed to their components e.g., glucose and fatty acid before microbial fermentation (Zhang et al., 2012; Banks et al., 2011).

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Various pretreatment methods towards food wastes have been investigated for enhancing the hydrolysis of food wastes, including ultrasonication, microwave, thermochemical and enzymatic hydrolysis (Banks et al., 2011; Han et al., 2015). Especially, commercial enzymes mainly carbohydrases, such as amylase, arabinase, and β-glucanase have been widely used to improve the hydrolysis of starch in food waste (Zhang et al., 2012; Burhan et al., 2003). However, it should be noticed that the high cost of commercial enzymes (e.g., about USD120 for treating 1 ton of FW with amylase at 10 U/g food waste) hinders the economic value of this process. Different from traditional separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), in which hydrolytic enzymes need to be supplemented, consolidated bioprocessing (CBP) offers such a more promising way, in which amylase production, enzymatic hydrolysis and microbial fermentation are completed in a single step by one microorganism (Jiang et al., 2018). This would significantly reduce the overall cost of the process, with elimination of complex and costly amylase separation and purification steps.

Most wild-type solventogenic *Clostridium* strains including *C. acetobutylicum* or *C. beijerinckii* contain some polysaccharide degrading enzymes, such as amylase; however, few *Clostridium* strains could efficiently synthesize butanol from starch through CBP (Lee et al, 2008). Therefore, this study aims to (i) isolate an amylase producer with capability of butanol production, (ii) investigate single step butanol production from food wastes without supplementation of commercial amylases; (ii) further improve butanol titer and yield via enhancement of amylase expression.

2. Materials and methods

2.1. Isolation and culture conditions

During the experiments, microorganisms originating from the food waste collected form the canteen of Hunan University of Science and Engineering were cultivated in the reduced mineral salts medium. The procedure to prepare the medium is briefly as follows: the serum bottles were spiked with mineral salts medium containing 1 g/L of NaCl, 0.5 g/L of $MgCl_2{\cdot}6H_2O$, 0.2 g/L of KH_2PO_4, 0.3 g/L of NH_4Cl, 0.3 g/L of KCl, 0.015 g/L of $CaCl_2{\cdot}H_2O$, 0.2 g/L of $MgSO_7{\cdot}H_2O$, and amended with 30 g/L of food waste. In addition, 1 mL of trace element solution, 1 mL of Na_2SeO_3-Na_2WO_4 solution, and 10 mg of resazurin were added per liter of medium (Xin et al., 2017). After the medium was boiled and cooled down to room temperature (20–25 °C) under N_2, the reductants Na_2S, L-cysteine, and DL-dithiothretol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively. Subsequently, $NaHCO_3$ was added to the medium and the pH was adjusted to 7.0. Then, 50-mL liquid medium was dispensed into 160-mL bottles, which were sealed with butyl stoppers, autoclaved for 20 min, and cooled down to room temperature (20–25 °C). After five transfers in starch -amended medium, the enrichment culture (0.1 mL; successively diluted to 10^{-5} times) was repeatedly streaked on agar plates poured with mineral salts medium containing starch as the sole carbon source. After incubation for six days, iodine staining was used to indicate amylase activities of the colonies (Chung et al., 1992). The amylase activity of each colony was determined by measuring the zone of clearance on the agar plates. Through such processes, a pure bacterial culture, designated as strain HN4 was obtained. All the enrichment, isolation and cultivation were performed in an anaerobic chamber filled with mixed gas (90% N_2, 5% CO_2 and 5% H_2) and operated at 25 °C.

2.2. Molecular characterization of strain HN4

Genomic DNA of the cultures was extracted and purified with DNeasy tissue kit (Qiagen, Germany) according to manufacturer's instructions. The genomic DNA was used as a template for PCR amplification of the 16S rRNA gene with a pair of universal bacterial primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1392R (5′-ACGGGCGGTG TGT-3′) (Xin et al, 2017). The obtained PCR products were purified with a PCR purification kit (Qiagen, Germany) and sequenced using an ABI DNA sequencer (Applied Biosystems, USA). The 16S rRNA gene sequence was aligned using the BLAST algorithm and deposited in the GenBank database with an accession number of MH260007.

2.3. Fermentation conditions

Food wastes were collected from the school canteen of Hunan University of Science and Engineering, China. After picking up bones, vegetables, tissues and plastics, food wastes were blended and dried for overnight at 50 °C in the oven. Fermentation studies were conducted in a 3.0-L bioreactor (Bioflo110, USA) equipped with a pH probe containing the modified P2 medium with 3 g/L of $CaCO_3$. Prior to autoclaving the medium, the pH was adjusted to 6.2 using 5 M NaOH. The medium containing carbon sources (glucose, starch or food waste) was sterilized at 121 °C for 15 min. On cooling to 35 °C under oxygen-free nitrogen atmosphere (in an anaerobic chamber), filter-sterilized P2 stock solutions [(buffer: KH_2PO_4, 50 g/L; K_2HPO_4, 50 g/L; ammonium acetate, 220 g/L), and (mineral: $MgSO_4{\cdot}7H_2O$, 20 g/L; $MnSO_4{\cdot}H_2O$, 1 g/L; $FeSO_4{\cdot}7H_2O$, 1 g/L; NaCl, 1 g/L)] were added (1 mL each), followed by the inoculation with highly active cells of *Clostridium* sp. HN4 (5 mL cell suspension in 100 mL medium).

To further improve the final butanol titer from food wastes, a fed-batch fermentation strategy using biodiesel as *in situ* extractant with a volume ratio of 1:1 (fermentation medium:biodiesel = 1:1; added at the beginning) was carried out in a 3.0-L bioreactor (Bioflo110, USA) at 35 °C with an agitation rate of 150 rpm. 750 mL (O_2 free) biodiesel as the extractant of biobutanol was added into 750 mL fermentation medium. After 144 h of fermentation, 80 g/L of food waste was added to the fermentation broth.

2.4. Amylase assay from Clostridium sp. strain HN4

The assay for amylase activity involved measurement of the reducing sugar from the enzymatic hydrolysis of soluble starch (Chung et al., 1992). In brief, the reaction mixture consisted of 1.0 mL of 2% soluble starch, 0.5 mL of 0.5 M citrate buffer (pH 5.5), and 0.5 mL of supernatant sample. To detect the activation of calcium ions for amylase activity, 10 mM of EDTA was added. After incubation at 35 °C for 10 min, the reaction was stopped by boiling the mixture at 100 °C for 10 min. The control was carried out in the same manner using a sample previously inactivated by boiling for 10 min. The liberated reducing sugars were estimated by the dinitrosalicylic acid (DNS) method with glucose as the standard (Miller, 1959). One unit (U) of amylase activity was defined as the amount of enzyme that released 1 μmol of glucose equivalent per min under the defined conditions.

2.5. Analytical methods

Samples were extracted from batch cultures for the quantification of fermentation products including solvents (acetone, butanol, ethanol) and VFAs (acetic and butyric acids) and glucose. In details, culture broths were centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatants were stored at −20 °C until further analysis. Glucose was analyzed by a 1200 Series HPLC system (Agilent Technologies Inc.) equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) and a Refractive Index Detector (RID). The samples were run at 75 °C with 0.6 mL/min eluent of 5 mM sulfuric acid. Solvents and VFAs were measured by a gas chromatography (GC, model 7890A; Agilent Technologies, U.S.A.) equipped with a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm; J&W, U.S.A.) and a flame ionization detector (FID). The oven temperature was initially held at 60 °C for 2 min, increased at 15 °C/min to 230 °C, and held for 1.7 min. Helium was used as the carrier gas, with a column flow rate of 1.5 mL/min. Standard