Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2017

www.elsevier.com/locate/jbiosc

Amylolysis is predominated by cell-surface-bound hydrolase during anaerobic fermentation under mesophilic conditions

Shiue-Lin Li,[1,2,*] Yu-Hsuan Wang,[1] Yu-Chieh Chao,[1] Ming-Der Bai,[1,3] and Sheng-Shung Cheng[1]

Department of Environmental Engineering, National Cheng Kung University, No. 1, University Road, Tainan 701, Taiwan, ROC,[1] Department of Bio-industrial Mechatronics Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan, ROC,[2] and New Energy Technology Division, Green Energy and Environmental Research Laboratories, Industry Technology Research Institute, Chutung, Hsinchu 31040, Taiwan, ROC[3]

Received 5 July 2017; accepted 21 October 2017
Available online xxx

While knowing the amylolysis mechanism is important to effectively decompose corn starch fed into an anaerobic digestor, the objective of this study was to detect the activities and locations of α-amylase in a continuous reactor and batch cultures. In the continuous reactor operated at 35 °C, the greatest cell-bound α-amylase activity was found to be 4.7 CU mL^{-1} at hydraulic retention time (HRT) = 9 h, while the greatest volumetric hydrogen production rate (r_{H2}) was observed at HRT = 3 h as 61 mmol L^{-1} day^{-1}. In the batch tests, the cell-bound α-amylase activities increased when the carbohydrate concentration decreased, and no significant reducing sugar accumulation was found in the serum bottles. By examining the specific hydrogen production rate (q_{H2}) against different corn starch concentrations, the half-saturation constant (K_{Sta}) and the maximum q_{H2} were regressed to be 0.47 g L^{-1} and 6 mmol g-VSS^{-1} d^{-1}, respectively. The electronic microscopic images showed that the microbes could colonize on the starch granules without the disturbance of any floc-like materials. Conclusively, by excluding the methanogens and floc matrix, the secreted α-amylases are predominately bound on the cell surfaces and enabled the microbial cells favorably attach on large substrates for hydrolysis under the mesophilic condition.

[**key words:** α-Amylase; Anaerobic digestion; Cell-bound activities; Corn starch; Extracellular hydrolase]

Anaerobic digestion (AD) refers to the process that uses complex microbial communities that are capable of degrading organics to produce methane (1–3). This microbial process usually contains into three biochemical stages, namely hydrolysis, acidogenesis, and methanogenesis. It is generally believed that hydrolysis is the rate-limiting step (4–7), which requires extracellular hydrolase to cleavage large molecules into ones smaller than 1000 Da molecular to readily cross the cell membrane (8). With regard to the large particulates, for example, it is significant that the cellulose contributes almost half of the organic materials entering AD (9), and this fact attracts many efforts to study cellulolytic bacteria over the last few decades (10–12). In addition to cellulosic materials, starch is also an important and abundant carbohydrate in nature, which is primarily composed of linear amylose and highly branched amylopectin (13) and laid down in all higher plants (14). α-Amylase (1,4-α-D-glucan-glucanhydrolase, EC. 3.2.1.1) is the most important enzyme in breaking down the α-1,4 glycosidic bonds when digesting starch, and it then work to progressively form oligomers and monomers (15). This enzyme is universally distributed throughout all the biological kingdoms, and work effectively within a wide range of pH (16).

Extracellular hydrolases could be categorized into two different forms: freely suspended in the water, and cell-surface-/sludge-matrix-bound (4). To achieve better hydrolysis performance, it is suggested that the strategy of process operation should be determined according to the enzyme location. For example, when the cell-bound form is predominating, slow mixing and biofilm based operations should be used, to favor an effective cell attachment on the polymers and activated enzymes staying in the reactor (17), respectively. If the suspended form is predominating, a rapid mixing is suggested to enhance the mass transfer with a high efficiency.

Hydrogen fermentation, also known as dark fermentation (18), indicates a bioprocess converting biodegradable materials into organic acids and hydrogen, in which the methanogens are thoroughly removed by pretreating the seeding sludge by boiling (19), adding acid/base (20), and adding methanogen inhibitor (21). According to investigations of the related microbial communities, some bacteria like *Clostridium* sp., *Lactobacillus* sp., and *Megasphaera* sp. (22–26), are commonly present in both the hydrogen-fermentation and AD processes. Therefore, by excluding methanogens, the step of hydrolysis plus acidogenesis could perform individually, and so the hydrolysis mechanisms could be further clarified.

Our past studies have shown the variation of amylase activity in a continuous thermophilic fermentor operated under different conditions (27). Here in the present study, using a commercial assay kit, we provide direct evidence showing that under mesophilic

* Corresponding author at: Department of Bio-industrial Mechatronics Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan, ROC. Tel.: +886 929392250.
E-mail address: reedli0227@ntu.edu.tw (S.-L. Li).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2017.10.013

conditions the α-amylase is localized differently to those detected under thermophilic conditions, whether in the continuous fermentor at different hydraulic retention times (HRTs), or in a batch culture. Moreover, the microscopic images obtained in this study also illustrate the interaction between microbes and starch granules during fermentation.

MATERIALS AND METHODS

Operation of continuous stirred tank reactor A continuous stirred tank reactor (CSTR), as shown in Fig. 1, was used in this study to enrich hydrogen producing bacteria at different HRTs. The total volume of the CSTR is 25 L, with working volumes of 15, 10, 5 L, as varied with different HRTs. A complete-mix condition was achieved using a mechanic propeller at an agitation speed of 300 rpm. The amount of gases produced in the CSTR due to fermentation was measured with a wet-gas flow-meter (W-NK-0.5B, Shinagawa Corporation, Tokyo, Japan). The CSTR and the off-gas flow meter were kept in an air-bath incubator and the temperature was maintained at 35 °C. The CSTR was fed with 12 g L^{-1} corn starch and 8 g L^{-1} peptone for all experimental runs. The corn starch was pretreated by boiling to make it porridge-like. Each liter of the influent feed was supplemented with 528 mg $MgCl_2 \cdot 6H_2O$, 380 mg KCl, 73.4 mg $CaCl_2 \cdot 6H_2O$, 5.8 mg $MnCl_2 \cdot 4H_2O$, 8.8 mg $CoCl_2 \cdot 6H_2O$, 1.6 mg H_3BO_3, 0.8 mg $CuCl_2 \cdot 2H_2O$, 24.8 mg $FeCl_2 \cdot 4H_2O$, 0.74 mg Na_2MoO_4, and 0.62 mg $ZnCl_2$. The influent medium solution was stored at 4 °C in a refrigerator at all time and fed continuously into the CSTR using a peristaltic pump (MasterFlex Peristaltic pump drive 7553-80 equipped with a controller, Cole Parmer, Vernon Hills, IL, USA). In order to avoid undesired bioreactions occurring in the feed tank and to neutralize the mixed liquid in the CSTR, 120 mL of 70% liquid sodium hydroxide was added to every 20 L of influent media. No additional pH control was used for the CSTR, and the pH values ranging from 5.3 to 5.5 were observed in the CSTR in all experimental runs. The seeding microorganisms for the CSTR in each experimental run were obtained from the cattle manure and the undigested grass residue from sheep rumina which were collected from the livestock farm located in Changhua County and a slaughterhouse located in Kaohsiung City, Taiwan, respectively. The microorganisms in these samples were quite active, that produced a large amount of biogas in situ. To obtain hydrogen producing bacteria, within 24 h after sampling, the samples were thoroughly boiled (19) to kill undesired microorganisms, and subsequently were enriched in the media containing 3 g L^{-1} corn starch and 2 g L^{-1} peptone to obtain the seeding sources for continuous reactor operation.

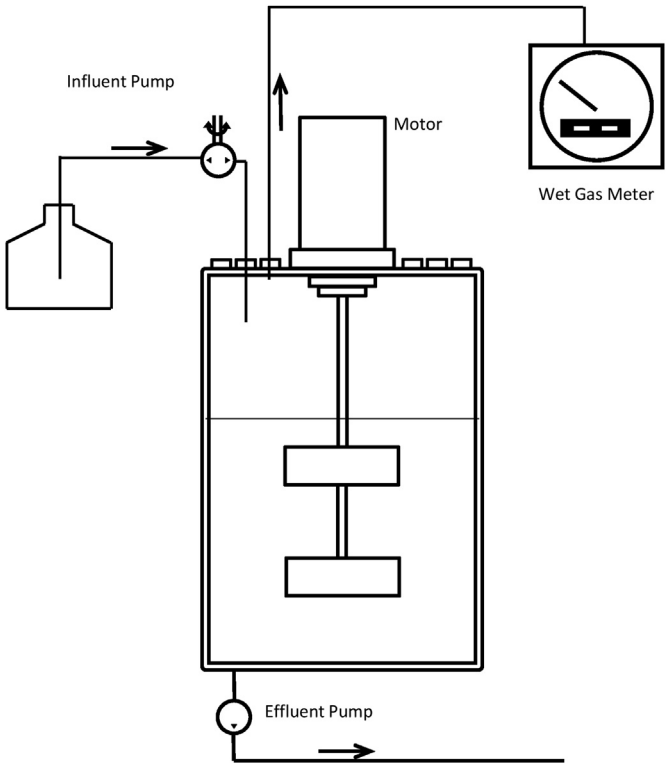

FIG. 1. Schematic illustration of the biohydrogen reactor.

Batch test The batch tests were carried out in a series of 120 mL serum bottles. To each bottle with a liquid working volume of 80 mL, 3 g L^{-1} corn starch and 2 g L^{-1} peptone were added as the fermentation substrates. In addition to the supplement chemicals, 217 mg L^{-1} of sodium thioglycolate and 175 μg L^{-1} of resazurin were added in each bottle to adjust the redox potentials and indicate the redox-status, respectively. The medium pH was adjusted to 7.2 using NaOH and HCl. The bottles were flushed with oxygen-free nitrogen gas, capped tightly with rubber septum stoppers using crimp tops, and then sterilized in an autoclave. The mixed microorganisms (adding approximately 28 mg VSS in the serum bottles at the initial conditions) collected from the bioreactor were then inoculated into the bottles and incubated on an orbital shaker with a rotational rate of 120 rpm and a temperature of 35 ± 1 °C. To analyze the kinetics of hydrogen producing bacteria degrading corn starch, a serial batch tests with different concentrations were carried out. The corn starch concentrations at initial conditions were designed to be 0, 0.2, 0.4, 0.8, 1.2, 1.5, and 2 g L^{-1}, respectively, which were supplemented by peptone with the same ratio as mentioned in the former paragraph (i.e., corn starch:peptone = 3:2). The kinetic model was described as a modified Monod equation:

$$q_{H2} = q_{max-H2} \times C_{Sta}/(K_{Sta} + C_{Sta}) \quad (1)$$

where q_{H2} is the specific hydrogen production rate (mmol g-VSS^{-1} d^{-1}), $q_{max\text{-}H2}$ is the maximum specific hydrogen production rate, C_{Sta} is the corn starch concentration (g L^{-1}), and K_{Sta} is the half saturated constant (g L^{-1}).

Analytical methods The composition of biogas in the headspace was analyzed using a gas chromatograph (GC 8900, China Chromatography, Taipei, Taiwan) equipped with a thermal conductivity detector. A 2 m stainless column packed with Hayesep Q (60/80 mesh) was installed in a 60 °C oven. The operational temperatures of the injection port, the oven, and the detector were all set at 60 °C. Nitrogen was used as the carrier gas at a flow rate of 15 mL min^{-1}. The carbohydrate was analyzed using the phenol-sulfuric acid method (28), and the reducing sugar was analyzed using the dinitrosalicylic acid reagent method (29). The pH, and volatile suspended solids (VSS), were measured according to Standard Methods (30).

α-Amylase assay α-Amylase activity was measured using the α-Amylase Assay Procedure kit (Megazyme, Bray, Ireland). Each vial of the kit contains blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) and thermostable α-glucosidase as a substrate and auxiliary enzyme, respectively. After a series of reactions occur between the target enzyme (i.e., α-amylase), thermostable α-glucosidase, and BPNPG7, free p-nitrophenol molecules will be produced from the cleaved BPNPG7, which can be detected under the wavelength of 400 nm (31). The α-amylase activity (expressed as ceralpha units, CU) is determined by the p-nitrophenol production rate during a certain testing period (i.e., 10 min in the present study). Some portions of the mixed-liquid samples taken from the bioreactor were filtered through a cellulose filter (DISMIC-25SS020RS, 0.2 μm pore size, Advantec, Tokyo, Japan), and the rest were preserved to have bacterial cells left in the samples. The filtrated liquid was used to determine the free α-amylase (cell-free) activities, and the cell-containing liquid was used to determine the gross α-amylase activities. Finally, the activities of the α-amylase attached to the cells (cell-bound) were determined by subtracting the cell-free activities from the gross ones.

Scanned electronic microscope The mixed liquids were taken from the reactor, dropped onto a nylon membrane (0.2 μm pore size, 47 mm diameter, Whatman, Buckinghamshire, United Kingdom), and then left for a few mins to dry. The resulting specimens were soaked in a 4% glutaraldehyde solution containing 100 mM phosphate buffer (pH 7.2) for 8 h at 4 °C for fixing. The specimens were then soaked in the phosphate buffer three times (for 10 min each time) to remove the excess glutaraldehyde. Dehydration was carried out using a series of gradually increasing ethanol concentrations: 50%, 75%, 85%, 95%, and 100%. Finally, the dehydrated biopellets were dried using the critical point drier (HCP-2, Hitachi, Tokyo, Japan) and then coated with gold by ion sputter (JFC-1100, JEOL, Akishima, Tokyo, Japan). Microscopic observations were carried out and photographed using Jxa-840 scanned electronic microscope (SEM) (JEOL).

RESULTS

Reactor performance of the anaerobic hydrogen fermentor at different HRTs The experimental results of substrate utilization, biomass concentrations, and biogas production in starch fed bioreactors operated with different dilution rates are presented in Table 1. This process included three operational stages: 24 d for stage 1, 31 d for stage 2, and 17 d for stage 3. A complete washout was observed at the HRT = 2 h (data not listed), which agrees well with our previous study (32). The low volumetric hydrogen production rates (r_{H2}) of 0.3 and 1.9 mmol L^{-1} d^{-1} were

Please cite this article in press as: Li, S.-L., et al., Amylolysis is predominated by cell-surface-bound hydrolase during anaerobic fermentation under mesophilic conditions, J. Biosci. Bioeng., (2017), https://doi.org/10.1016/j.jbiosc.2017.10.013