Continuous biohydrogen production from waste bread by anaerobic sludge

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Abstract: In this study, continuous biohydrogen production from waste bread by anaerobic sludge was performed. The waste bread was first hydrolyzed by the crude enzymes which were generated by *Aspergillus awamori* and *Aspergillus oryzae* via solid-state fermentation. It was observed that 49.78 g/L glucose and 284.12 mg/L free amino nitrogen could be produced with waste bread mass ratio of 15% (w/v). The waste bread hydrolysate was then used for biohydrogen production by anaerobic sludge in a continuous stirred tank reactor (CSTR). The optimal hydrogen production rate of 7.4 L/(Ld) was achieved at chemical oxygen demand (COD) of 6000 mg/L. According to the results obtained from this study, 1 g waste bread could generate 0.332 g glucose which could be further utilized to produce 109.5 mL hydrogen. This is the first study which reports continuous biohydrogen production from waste bread by anaerobic

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1 sludge.

2 **Keywords:** waste bread; biohydrogen production; continuous stirred tank reactor;
3 chemical oxygen demand

5 **1. Introduction**

6 Hydrogen is regarded as an ideal energy source in the future since it is clean and
7 renewable (Show et al., 2012). Furthermore, the energy density of hydrogen is 122 kJ/g
8 which is 2.75 times higher than that of fossil fuel (Urbaniec and Grabarczyk, 2014).
9 Currently, more than 70% of hydrogen is generated by steam reforming of natural gas
10 which is not a clean and sustainable way because fossil fuels are used as feedstock
11 (Acar and Dincer, 2015). Dark fermentation, which could produce hydrogen with mild
12 operation, is considered to be an alternative way (Logan et al., 2002). At present, dark
13 fermentative hydrogen production is still under investigation and could not apply into
14 industrial application due to the low hydrogen yield and high cost (Guerrero-Lemus et al.,
15 2010). Utilization of raw organic waste (such as food waste) as feedstock for
16 biohydrogen production seems to be a promising solution (Kim et al., 2011; Redondas
17 et al., 2012).

18 Food waste, which is one of the largest parts of the municipal solid waste, is
19 considered to be a serious global issue (De-Gioannis et al., 2013; Van-Ginkel et al.,

2005). It is reported that around 1.3 billion of food waste could be produced per year in the world (Alibardi and Cossu, 2015). So, it is important to take actions to alleviate the social, economic and environmental problems caused by the food waste (Elbeshbishy et al., 2011). Waste bread, which contains a great amount of nutrients for biofuels production, is one of the most common food wastes in Asian and Europe countries (Lee et al., 2010). So, utilization of no-value waste bread for valuable products, such as hydrogen, has attracted great attentions (Han et al., 2016a). However, compared to the soluble waste, it is difficult to directly use waste bread for hydrogen production since the solid waste bread should be solubilized to enhance the nutrient conversion efficiency (Alibardi and Cossu, 2016; Castillo-Hernandez et al., 2015). Furthermore, the nutrients stored in the waste bread are in the form of the macromolecules (such as starch and protein) which have to be broken into monomers (glucose and free amino nitrogen) before being used by microorganisms for hydrogen production (Han et al., 2015b). Hydrolysis of macromolecules is considered to be the rate-limiting step in the overall biohydrogen production process (Kim et al., 2010). In light of the above technical analysis, there is little information about biohydrogen production from waste bread.

In this study, a novel bioprocess of hydrogen production from waste bread was developed. The waste bread was hydrolyzed by the crude enzymes which were generated by *Aspergillus awamori* and *Aspergillus oryzae* via solid state fermentation.

The waste bread hydrolysate was then used as substrate for biohydrogen production by heat pretreated sludge in a continuous stirred tank reactor (CSTR). The effect of the chemical oxygen demand (COD) on the performance of the CSTR was also examined.

2. Materials and methods

2.1 Raw material and microorganism

The waste bread used in this study was provided by the local Wumei Supermarket and immediately brought to laboratory for processing. In order to get the smaller physical size of the substrate, the waste bread was grounded triplicate by the food blender. The pretreated waste bread was then stored at -4 °C. The characteristics of waste bread (Table 1) were measured according to Standard Method (APHA et al., 1998).

Microorganism strain of *Aspergillus awamori* and *Aspergillus oryzae* used in this study were purchased from Shanghai Beinuo Biotechnology Co., Ltd. and utilized in solid-state fermentation to produce crude enzymes (glucoamylase and protease). The hydrogen-producing sludge used in this study was collected from a local municipal wastewater treatment plant (Hangzhou, Zhi Jiang Wastewater Treatment Plant). The sludge was heat pretreated in a water bath at 100 °C for 6 h before inoculation to inhibit the activity of methane-producing bacteria. The volatile suspended solids (VSS) concentration of the seed inoculum was 3.6 g/L.

1

2 2.2 Solid-state fermentation and waste bread hydrolysate

3 Solid-state fermentation was carried out in two Petri dishes containing 5 g ground waste
4 bread with 1ml of cryopreserved spores of *A. awamori* (4×10^6 spores/mL) or *A. oryzae*
5 (1×10^6 spores/mL) spreading evenly on waste bread. The mixtures were cultured in an
6 incubator without stirring at 30 °C for 96 h to obtain the fermented solid mash which
7 were rich in glucoamylase and protease, respectively.

8 Fermented mash obtained from solid-state fermentation was transferred into a 3 L
9 bioreactor which was equipped with automatic temperature controller and stirrer for
10 enzymatic hydrolysis. The agitation speed was 500 rpm. Waste bread (15%, w/v) was
11 added into bioreactor when the temperature reached 55 °C. The conditions for
12 solid-state fermentation and enzymatic hydrolysis have been approved to be optimal by
13 our previous publications (Han et al., 2015a; Han et al., 2016b). Samples were taken
14 every hour to analyze the glucose and free amino nitrogen (FAN) production. The
15 resultant broth was centrifuged at 10000 rpm for 30 min and filtered by Whatman No. 1
16 filter paper to obtain the waste bread hydrolysate which was used as substrate for
17 subsequent biohydrogen production. In order to investigate the effect of COD
18 concentration on biohydrogen production, the waste bread hydrolysate was diluted by
19 distilled water to certain COD concentration (2000-8000 mg/L).

1

2 2.3 Continuous stirred tank reactor

3 Continuous biohydrogen production from waste bread hydrolysate was performed in a
4 3.5 L continuous stirred tank reactor (CSTR) with a working volume of 2.8 L. The
5 CSTR was constructed by transparent plexiglas with a gas-liquid-solid separating
6 device. The influent flow rate was controlled by a feed pump to regulate the HRT at 6 h
7 in the CSTR. The generated gas was collected with a waterlock and measured by a wet
8 gas meter. Fermentation pH was kept above 4 by using 5 M NaOH solution. The CSTR
9 was operated in batch mode until gas was produced. Reactor was then switched to
10 continuous mode (HRT = 6 h) with COD concentration of 2000 mg/L until steady state
11 conditions were obtained. Steady state conditions were based on the constant products
12 with a variation of less than 10%. The CSTR was sampled at a fixed COD over at least
13 three days. The COD was then increased to the next level and the CSTR was operated
14 until steady state conditions were achieved as noted above. All the samples obtained
15 from this study were analyzed at least in triplicate.

16

17 2.4 Analytical methods

18 The glucose concentration produced in the waste bread hydrolysate was quantified
19 using the high performance liquid chromatography (HPLC) system which was equipped

1 with a BIO-RAD column (HPX-87H), a refractive index (RI) detector and a photodiode
2 array (PDA) analyzer. The ninydrin reaction method was used to analyze the free
3 amino nitrogen (FAN) production in the waste bread hydrolysate. The detailed
4 procedure of glucose and FAN measurements were described by our earlier publications
5 (Han et al., 2015a; Han et al., 2016b).

6 COD was monitored and measured daily according to Standard Methods (APHA et
7 al., 1998). The produced gas was analyzed using a gas chromatography (GC) equipped
8 with a thermal conductivity detector (TCD) and a stainless steel column (2m × 5mm)
9 filled with Porapak Q (50-80 meshes). Nitrogen was used as the carrier gas at a flow
10 rate of 30 ml/min. A dose of injected sample was 0.5 ml each time. Gas samples were
11 compared to four standard levels of pure gas injections. Based on the percentage of
12 hydrogen in the produced gas, the hydrogen yield could be calculated. The soluble
13 microbial products (SMPs) in the fermentation solution were analyzed by another GC
14 using flame ionization detector (FID). A 2-m stainless steel column was packed with the
15 supporter GDX-103 (60-80 meshes). The temperatures of the injection port, oven and
16 detector were 220 °C, 190 °C and 220 °C, respectively. The carrier gas was nitrogen at a
17 flow rate of 30 ml/min. Liquid samples were refrigerated before analysis and compared
18 to five standard levels of pure chemical injections.

3. Results and discussion

3.1 Enzymatic hydrolysis of waste bread

Fig. 1 showed the production of glucose and FAN in the enzymatic hydrolysis of waste bread. It was observed that both the glucose and FAN increased in the waste bread hydrolysate with time. The maximum glucose of 49.78 g/L and FAN of 284.12 mg/L production were obtained at 24 h, respectively. Linear regression results showed that glucose (y_1) and FAN (y_2) production in the waste bread hydrolysate with time (x_1 , x_2) can be expressed as $y_1 = 14.21\text{Ln}(x_1) + 1.9863$ ($R^2 = 0.986$) and $y_2 = 80.623\text{Ln}(x_2) + 8.5786$ ($R^2 = 0.982$), respectively. The duration of the hydrolysis reactions were similar to the hydrolysis of bakery pastry as reported in our previous study (Zhang et al., 2012). However, as shown in Table 2, the glucose yield obtained from this study was 0.332 g glucose/g waste bread which was higher than previous study reported (0.18 g glucose/g pastry) because the starch content in the waste bread was higher than the pastry. On the other hand, the waste bread used in this study consisted of around 72.9 g starch. The theoretical glucose yield of 81 g glucose could be calculated based on the molar basis of starch hydrolysis. The conversion ratio from starch to glucose reached 61.4% which was higher than that study reported (around 36.5%). This is probably because the physical size of the substrate (grounded triplicate) was much smaller than the substrate used in previous study. The waste bread hydrolysate obtained from this study is rich in glucose

and FAN which could be directly used as substrate for subsequent biohydrogen production.

3.2 Continuous biohydrogen production from waste bread hydrolysate

Various parameters, such as hydraulic retention time, pH and cyclic duration, have been investigated for continuous biohydrogen production. COD is one of the most important parameters affecting the performance of biohydrogen production. Efficient biohydrogen production could be achieved with appropriate COD concentration which could reduce the hydrogen production cost for industrial scale. Therefore, the effect of different COD concentration on the performance of continuous biohydrogen production from waste bread hydrolysate was investigated.

Fig. 2 showed the variation of COD (a) and biogas and hydrogen production rate (b). Both the biogas and hydrogen production rate increased when COD concentration increased from 2000 mg/L to 6000 mg/L due to the limited carbon source. The best biogas and hydrogen production rate of 23.5 L/(Ld) and 7.4 L/(Ld) were obtained at COD concentration of 6000 mg/L. However, the remarkable decrease in biogas and hydrogen production rate was observed when the COD concentration further increased to 8000 mg/L because of the inhibition caused by high substrate concentration and washout of hydrogen-producing microorganism (Ren et al., 2010). The least biomass

concentration observed at COD concentration of 8000 mg/L also confirmed this result (Table 3). Moreover, it was interesting to find that the hydrogen-producing system needed less time to adapt to the new conditions at the lower COD concentration (2000-6000 mg/L). However, it is difficult to recover for the hydrogen-producing system when the COD concentration further increased to 8000 mg/L (Fig. 2). Higher COD concentration could be beneficial to enhance the productivity of hydrogen, but too high COD concentration (8000 mg/L) could be detrimental to the hydrogen-producing system due to the washout of hydrogen-producing bacteria and substrate inhibition. Therefore, the COD concentration of 6000 mg/L was considered to be the optimal value for continuous biohydrogen production from waste bread hydrolysate in the CSTR.

An economically feasible hydrogen production process should be not only dependent on the high hydrogen production efficiency, but also the efficiency of substrate utilization (Han et al., 2016a). Consequently, the effect of COD concentration on substrate utilization was also investigated. The substrate utilization efficiency and soluble microbial products under steady-state with different COD concentrations were summarized in Table 3. The glucose utilization reached 84.5%-92.3% when the COD concentration increased from 2000 mg/L to 6000 mg/L. However, further increasing COD concentration to 8000 mg/L inhibited the activity of the hydrogen-producing microorganism, and thus, the glucose utilization decreased sharply to 43.4%. Consistent

with the glucose utilization, biomass concentrations increased from 4.25 g/L to 6.7 g/L with COD concentration increasing from 2000 mg/L to 6000 mg/L. Further increasing COD concentration to 8000 mg/L led to biomass concentration decreased to 5.2 g/L due to the substrate inhibition (Logan et al., 2002).

Biohydrogen production is always accompanied with soluble microbial products (SMPs). The distribution of SMPs has been successfully used as indicators for monitoring hydrogen-producing system. Table 3 summarized the yield and distribution of SMPs obtained under steady-state conditions at each COD concentration. When the COD concentration increased from 2000 mg/L to 6000 mg/L, ethanol and acetate were the main SMPs along with little amount of butyrate and propionate. Therefore, the bacterial metabolism in the CSTR belonged to ethanol-acetate type fermentation (Eq. (1)) which is regarded as the best fermentation type for biohydrogen production.

However, when the COD concentration increased to 8000 mg/L, the hydrogen-producing system had undergone a transition of fermentation types. The mixed-acid-type fermentation was formed, and the hydrogen-producing microorganism was inactive. During this period, ethanol production decreased from 25.44 mmol/L to 7.28 mmol/L, while propionate production increased from 0.54 mmol/L to 3.83 mmol/L (Table 3). It is known that propionate pathway (Eq. (2)) could consume molecular hydrogen which resulted in the reduced of hydrogen yield (Han et al., 2015b; Ren et al.,

2007).

Fig. 3 showed the carbon (a) and material (b) balance of biohydrogen production from waste bread in the CSTR with the optimal COD concentration (6000 mg/L). The carbon content in the waste bread included 38.6% for undigested waste bread and 61.4% used by microorganism for biogas and SMP production. Among the consumed carbon, the ethanol (19.79%) and carbon dioxide (13.5%) accounted for the largest parts because they were the main soluble and gaseous products for biohydrogen production from waste bread in the CSTR. According to the results shown in Fig. 1 and Fig. 2 (b), 1 g waste bread could produce 0.332 g glucose which could further convert to 109.5 mL hydrogen. To our knowledge, this is the first study which reports hydrogen production from waste bread by anaerobic sludge.

4. Conclusions

The waste bread (15%, w/v) could be hydrolyzed into glucose (49.78 g/L) and free amino nitrogen (284.12 mg/L) by the crude enzymes which were produced by *Aspergillus awamori* and *Aspergillus oryzae* via solid state fermentation. Then, the produced waste bread hydrolysate was used as substrate for biohydrogen production in

a continuous stirred tank reactor. The best hydrogen production rate and hydrogen yield of 7.4 L/(Ld) and 109.5 ml hydrogen/g waste bread were obtained at COD concentration of 6000 mg/L. The proposed process could be successfully applied into continuous biohydrogen production by anaerobic sludge.

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Figure legends

Table 1 Composition of waste bread used in this study (per 100 g).

Table 2 Comparison of theoretical glucose production and conversion rate from waste bread and pastry hydrolysates.

Table 3 Performance of soluble microbial products and carbon recovery under steady state with different COD concentration.

Fig. 1 Glucose and free amino nitrogen productions in the enzymatic hydrolysis of waste bread.

Fig. 2 The variation of chemical oxygen demand (a) and biogas and hydrogen production rate (b).

Fig. 3 Carbon (a) and material (b) balance of biohydrogen production from waste bread in the CSTR with COD concentration of 6000 g/L.

Table 1 Composition of waste bread used in this study (per 100 g).

Component	Value (g)	Component	Value (g)
Moisture	24.3 ± 0.8 g	Starch	48.6 ± 1.5 g
Protein ($N \times 5.7$)	9 ± 0.1 g	Phosphorous	0.08 ± 0.01 g
Total organic nitrogen	1.5 ± 0.1 g	Ash	2.3 ± 0.2 g

1 **Table 2** Comparison of theoretical glucose production and conversion rate from waste
2 bread and pastry hydrolysates.

Substrate	Glucose	Glucose	Starch	Starch	Theoretical	Conversion	References
(g)	production	yield (g/g	content	(g)	glucose	ratio (%)	
	(g)	substrate)	(%)		production		
					(g)		
150	49.78	0.332	48.6	72.9	81	61.4	This study
(waste							
bread)							
300	54.2	0.18	44.6	133.8	148.67	36.5	Zhang et
(pastry)							al., 2012

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Table 3 Performance of soluble microbial products and carbon recovery under steady state with different COD concentration.

Parameters	COD concentration (mg/L)			
	2000	4000	6000	8000
Glucose utilization (%)	92.3	89.7	84.5	43.4
Consumed glucose, g/L (mmol/L)	1.74	3.38	4.73	3.27
	(9.67)	(18.7)	(26.3)	(18.2)
Consumed carbon (mmol/L)	58.02	112.2	157.8	109.2
Ethanol (mmol/L)	8.44	15.7	25.44	7.28
Acetate (mmol/L)	4.8	8.74	10.48	6.7
Butyrate (mmol/L)	1.1	3.12	3.7	3.96
Propionate (mmol/L)	0.8	0.68	0.54	3.83
Carbon dioxide (mmol/L)	13.2	26.8	34.7	24.9
Carbon recovered in ethanol, acetate, butyrate, propionate and CO ₂ (mmol/L)	48.08	91.56	124.04	87.85
Volatile suspended solid (g/L)	4.25	5.37	6.7	5.2
Carbon recovery (%)	82.8	81.6	78.6	80.4

Fig. 1 Glucose and free amino nitrogen productions in the enzymatic hydrolysis of waste bread.

Fig. 2 The variation of chemical oxygen demand (a) and biogas and hydrogen production rate (b).

Fig. 3 Carbon (a) and material (b) balance of biohydrogen production from waste bread in the CSTR with COD concentration of 6000 mg/L.