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Metabolic Flux Analysis of Hydrogen Production from Rice Starch by Anaerobic Sludge under Varying Organic Loading

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Metabolic flux analysis (MFA) was used to evaluate the impact of organic loading of rice starch fermentation by an anaerobic sludge on the H_2 production. H_2 production was conducted in 0.5 L batch reactor under varying organic loading in the range of 2.5 to 12.5 g/L, initial pH of 6 and 37°C. Maximum H_2 production, the percentage of starch hydrolysis and glucose utilization were observed at 5 g/L starch. Acetate was the main carbon fraction after 20 h fermentation for all cases. Acetate production, an objective function, was maximized with respect to H_2 production pathway. Results indicated that acetate production pathway was correlated not only to the H_2 production but it also the H_2 consumption through acetogenesis (R17). Intracellular butyrate oxidation (R26) was strongly correlated to the acetate production and H_2 production. The organic loading strongly impact the principal flux of H_2 production. Re-oxidizing ferredoxin pathway (R13) plays a significant role on the maximum H_2 flux only at low organic loading (≤ 5 g/L starch). However, H_2 flux obtained from butyrate oxidation reaction (R26) and re-oxidizing ferredoxin pathway (R13) was not significantly different at high organic loading (7.5 to 12.5 g/L). Butyrate oxidation could be used as a strategic reaction target to promote the performance of the involved microorganisms in the reactor to achieve the high H_2 production. MFA calculated from the *in silico* model was a crucial information for enhancing the H_2 production by the mixed culture.

1. Introduction

Dark hydrogen (H_2) fermentation has been attractive for bioenergy generation in recent decades. Many researchers have attempted to maximize the H_2 production to a theoretical yield of 12 mol H_2 per mol glucose (Eq.1), but this phenomenon has never been exploited in any known biological system up to now (Westermann et al., 2007; Das et al., 2009). Volatile fatty acids (VFAs) and/or alcohol are common end-products in the dark H_2 fermentation. The types of end-products are related to the metabolic pathways and H_2 yield. Maximum theoretical yield is 4 and 2 mol H_2 per mol glucose when acetate (Eq.2) and butyrate (Eq.3) are the sole end-products in the dark H_2 fermentation (Liu et al., 2008; Das et al., 2009). Optimization of environmental conditions in the fermentation process together with the selection of H_2 producers have been used to promote the H_2 fermentation. The low conversion yield might be involved with some crucial metabolic activity preferentially developed for cell growth instead of H_2 including some competitive reaction such as H_2 consumption or diversion to other products in which the currently proposed metabolic reactions and equations might be ignored (Hallenbeck and Benemann, 2002; Cai et al., 2010).

$$C_6H_{12}O_6 + 6H_2O \text{ ----> } 6CO_2+12H_2 \quad (1)$$
$$C_6H_{12}O_6 + 2H_2O \text{ ----> } 2CH_3COOH + 2CO_2 +4H_2 \quad (2)$$
$$C_6H_{12}O_6 \text{ ----> } CH_3CH_2CH_2COOH + 2CO_2 +2H_2 \quad (3)$$

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Available H_2 fermentation pathways have been mostly observed from *Clostridia spp*. fed glucose in several previous studies (Cai et al., 2010; Liu et al., 2010). Multiple metabolic pathways were constructed in the network reaction in accordance with the different environmental condition of fermentation and/or type of microorganisms (Levin et al., 2006; Wang et al., 2008). Metabolic flux analysis (MFA) is a powerful tool to calculate intracellular fluxes from extracellular fluxes and a useful tool to understand the flux direction in the H_2 fermentation (Cai et al., 2010). It has been used for analysing electron or carbon flux distribution patterns and maximizing the yield of valuable end-products such as organic acid, amino acids, polysaccharide and antibiotics (Stephanopoulos et al., 1998; Edwards et al., 2002). Based on the stoichiometric analysis and mass balances, MFA can be used to quantify intracellular fluxes to understand the overwhelming complexity of metabolic network of cellular responses (Stephanopoulos et al., 1998). It has been intensively used for analysing intracellular flux correlated with maximizing H_2 production and specific growth rate is selected for objective function. Most MFA studies have been focused on pure bacterial strains and simple form of substrate to analyse the capability of H_2 production and other valuable metabolites under different conditions by considering biomass formation as an objective function in an optimizing process. Biomass formation seemed to be a significant metabolic flux which directly corresponded to the overall process of H_2 synthesis. Little literature has been used the MFA for the H_2 production by a mixed culture with simple substrate and/or complex substrate.

Over the last decade, H_2 production from dark fermentation of food waste has been extensively reported (Kim et al., 2004; Pan et al., 2008) under various operational conditions, but its metabolic flux network has not been thoroughly elucidated. In this study, H_2 batch fermentation under varying initial rice starch (a main composition of the food waste) was setup. Biomass and major fermentative products in gaseous and aqueous samples were quantitatively measured. An *in silico* metabolic flux model, using a linear optimization program, for the anaerobic starch fermentation by an anaerobic sludge was generated to maximize the H_2 production.

2. Materials and Methods

2.1 Microbial inoculum and batch fermentation

Anaerobic sludge granule was obtained from a full-scale upflow anaerobic sludge blanket reactor treating cassava starch processing wastewater (Eiamburapa Industry Co. Ltd., Thailand). The sludge was sieved to the size <0.5 mm to remove coarse matters and then washed twice with tap water and re-cultivated in 0.5% (w/v) glucose solution for 24 h. Subsequently it was washed with the distilled water twice and heat-shocked by boiling at 100^{o}C for 1 h before using as a seed inoculum for the hydrogen fermentation. The heat shocked sludge contains total solid (TS) and total volatile solid (VS) of 66,227 and 38,000 mg L^{-1}. Batch fermentation system was set up in 0.5 L screw-cap bottles with a working volume of 0.5 L. The inoculum was fixed at 5.45 g VS per batch. The rice starch suspension was prepared in distilled water with final concentration in the range of 2.5-12.5 g/L. The initial pH was adjusted to 6.0 with 6 N NaOH or concentrated H_3PO_4. The system was flushed with nitrogen gas to generate anaerobic conditions. H_2 fermentation was conducted at 37°C with rotary shaking at 150 rpm. All experiments were setup in duplicate. During the fermentation experiment, total gas volume and composition were periodically monitored by gas counters and gas chromatography, respectively. The liquid samples were periodically analyzed for pH, VFAs, residual starch and glucose.

2.2 Analytical methods

The amount of generated biogas was monitored using liquid displacement gasometers. Biogas content (H_2, CH_4, and CO_2) was measured periodically every 5 h using a gas chromatograph. The analytical methods for the gas compositions and VFAs were previously described (Nathao et al., 2013). Glucose was analyzed by High Performance Liquid Chromatography (Agilent Technologies 1200 series, Germany) equipped with reflective index detector, UV detector and an Aminex HPX-87H column, 300 x 7.8 mm. (Biorad, USA). The mobile phase was 5mM H_2SO_4 at flow rate of 0.7 mL/min and the column temperature was 64^{o}C. For starch analysis, samples were acidified and boiled with concentrated acid (2.5 N HCl) for 3 h for complete hydrolysis of starch to sugar and the total sugar concentration was determined by using the phenol acid spectrometric method (Dubois et al., 1956). The biomass measurement was determined according to the standard method (Nathao et al., 2013).

2.3 MFA analysis and metabolic network constructed in silico model

The anaerobic starch metabolic network for the anaerobic sludge constructed in the *in silico* model was developed from the concept of universal bacterium under anaerobic degradation of organic matter. The network was constructed with 26 reactions, 14 intracellular metabolites and 12 extracellular compounds (Table 1). In order to avoid redundancy, the value of residual starch was ignored from the flux calculation. Biomass synthesis was not included into the model since negligible biomass increased was observed. Acetate production was selected as an objective function to be maximized to achieve the optimum H_2 production. MetaFluxNet (Version

1.8), a linear optimization program, was used to solve the system of linear equations. Hydrolysed starch, utilized glucose, and accumulated metabolites (e.g. acetate, butyrate, lactate, and ethanol) were defined in stoichiometric coefficients comparing to 1 mol of initial starch concentration were used as constraints in the *in silico* model. Carbon mass balance was evaluated to check the carbon recovery in the batch system.

Table 1. Reactions used in metabolic reaction network.

Reaction No.	Reaction
1	Starch ----> Glucose (external)
2	Starch ----> Starch (residual)
3	Glucose (external) ----> Glucose
4	Glucose ----> Biomass
5	Glucose ----> Glucose (residual)
6	Glucose + 2 NAD^+ ----> 2 Pyruvate +2 NADH
7	Pyruvate +NADH ----> Lactic acid +NAD^+
8	Lactic acid ----> Lactic acid (external)
9	Lactic acid +NADH ----> Propionic acid +NAD^+
10	Propionic acid ----> Propionic acid (external)
11	Pyruvate +CoA+2Fd^{2+} ----> Acetyl- CoA +CO_2+2Fd^+
12	NADH+2Fd^{2+} ----> NAD^++2Fd^+
13	2Fd^++2H^+ ----> 2Fd^{2+}+H_2
14	H_2 ----> H_2 (external)
15	Acetyl- CoA ----> Acetic acid +CoA
16	Acetic acid ----> Acetic acid (external)
17	4H_2+CO_2 ----> Acetic acid
18	Acetyl- CoA +2NADH ----> Ethanol+2NAD^++CoA
19	2 Acetyl- CoA ----> Acetoacetyl-CoA +CoA
20	Acetoacetyl-CoA +2NADH ----> Butyryl-CoA +2NAD^+
21	Butyryl-CoA ----> Butyric acid +CoA
22	Butyric acid ----> Butyric acid (external)
23	Acetic acid ----> CO_2+CH_4
24	CO_2+4H_2 ----> CH_4+2H_2O
25	CH_4 ----> CH_4 (external)
26	Butyric acid ----> 2 Acetic acid +2H_2

3. Results and Discussion

3.1 H_2 production profile and carbon mass balance

The correlation of the cumulative H_2 production, hydrolysis of starch and utilization of glycose versus fermentation time showed hyperbolic characteristics. These parameters rapidly increased within first 10 h fermentation and reached the saturation point after 20 h fermentation for all starch concentrations. The highest hydrolysis and utilization were observed at 5 g/L starch fermentation (Figure 1A). Acetate is the major end-product in the H_2 fermentation for all cases. The carbon mass balance and carbon recovery in the H_2 fermentation were evaluated to validate the data. Negligible biomass formation after 20 h fermentation (< 1%) was not included in carbon balance analysis and MFA model. The carbon balance is satisfactory as the estimated carbon recoveries against the initial carbon input are approximately 85.8-99.6%.

The flux of starch hydrolysis was decreased as the concentrations of starch was increased (Figure 1A). On the other hand, the flux of utilization of glucose appeared to increase at the higher concentrations while the flux of H_2 production reached to maximum at 5 g/L starch and continuously reduced at the higher concentrations (from 7.5 to12.5 g/L). The capability of hydrolysis and utilization process by microorganisms was the first step of the anaerobic degradation of organic substance to drive normal metabolism and finally to produce the combined volatile fatty acid and/or alcohol. In this study, H_2 production at 5 g/L starch gave the maximum production and the highest efficiency of hydrolysis process along with utilization process. The main carbon fraction of fermentation end-product in the starch fermentation reactor after 20 h fermentation was acetate for all cases (Figure 1B). Normally, fermentation end-products of anaerobic fermentation such as VFAs could be used as an indicator to understand microbial activity of hydrogen producing bacteria in a reactor (Nathao et al., 2013).

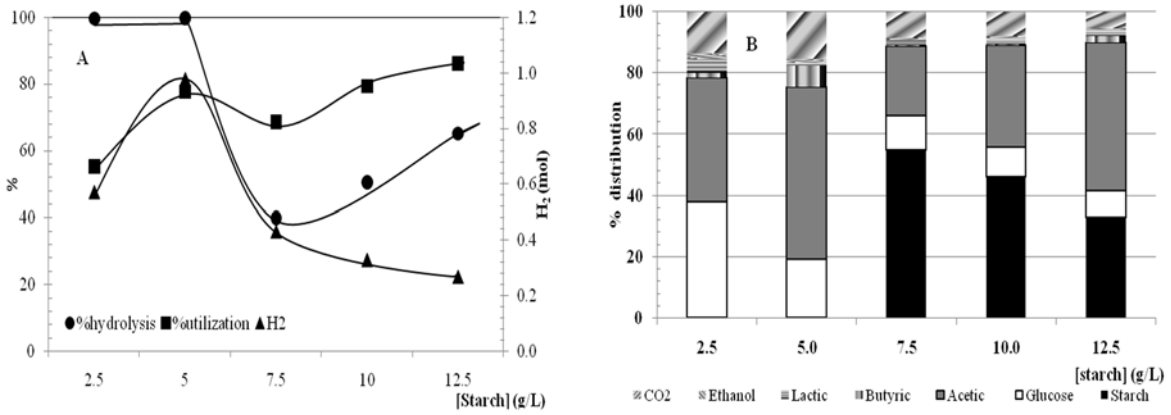

Figure 1: Hydrolysis, utilization and H_2 production (A), carbon balance analysis (B) after 20 h fermentation.

Acetate can be generated via either acidogensis, H_2 production pathway, or homoacetogenesis, H_2 consuming pathway (Eq. 4). The presence of homoacetogen (acitogenic hydrogen consumers) is the main factor resulting in the low yield of hydrogen production that it cannot be suppressed by thermal stress (104°C for 2 h) (Oh et al., 2003). Therefore, considering the pathway of acetate production together with hydrogen production could be examined to evaluate the feasibility of the net H_2 production and H_2 consumption through starch fermentation.

$$4H_2+2CO_2 \text{ ----> } CH_3COOH+2H_2O \quad (4)$$

3.2 Metabolic Flux Analysis

The metabolic reactions for H_2 production constructed in the *in silico* model and the optimum flux for different starch fermentation (2.5-12.5 g/L) were showed in Table 1. Fourteen intracellular and twelve extracellular compounds were constructed in configuring the network. Starch is hydrolyzed to glucose as the simple form by hydrolytic bacteria and then converts to pyruvate via glycolysis pathway and finally to hydrogen plus many end-products (e.g. VFAs and alcohol). No CH_4 was detected in this study. Acetate production pathway is defined as hydrogen producing pathway that can achieve the highest theoretical yield 4 mol H_2 per mol glucose if acetate is the sole end-product (Eq.2) (Das et al., 2009). However, acetate can be produced from acetogenesis pathway (Eq.1) and degradation of short chain fatty acid such as propionate (Eq.5), and butyrate (Eq.6) (Muller et al., 2003; Veldez-Vazquez et al., 2005) under the anaerobic condition. The anaerobic oxidation of propionate and butyrate to acetate, CO_2 and H_2 are highly endergonic for standard condition at 25°C ($\Delta G^0_{propionate}$ = +76.1 kJ/mol and $\Delta G^0_{butyrate}$ = +48.1 kJ/mol). However, these reactions can be achieved under the maintained condition of low H_2 partial pressure.

$$CH_3CH_2CH_2COOH \text{ ----> } 2\ CH_3COOH+2\ H_2 \quad (5)$$

$$CH_3CH_2COOH + 3H_2O \text{ ----> } CH_3COOH + 3H_2 + CO_2 \quad (6)$$

The results showed that the acetate is the main metabolite in the current study suggested that acetate formation in Eq. 6 is thermodynamically feasible under the experimental condition.

3.3 Maximizing H_2 production via acetate production pathway

The net production of acetate was obtained from acetyl CoA conversion (R15), homoacetogenesis (R17) and butyrate oxidation (R26*2). However, the results calculated from the *in silico* model and measured from the experiment had a very good agreement with respect to acetate production used as the objective function (Figure 2A). Although acetate was defined as the correlative pathway of hydrogen production in acidogenesis process,

the pathway of production could be intensively considered to understand the activity of the involved microorganism in fermentation reactor. The maximum flux of acetate production was observed from R26, R17 and R15 respectively (Figure 2B). Results indicated that butyrate oxidation reaction was the major pathway to generate acetate and H_2 as the final products. Acetogenesis was the H_2 consuming pathway in which 4 mol of hydrogen was consumed to produce 1 mol of acetate. Therefore, flux of acidogenesis (R17) affected directly in low yield of H_2 production whereas the increased H_2 yield resulted from flux of butyrate oxidation (R24). Typically, acetyl CoA (R15) would be converted to acetate and H_2 in normal condition (Kramer et al., 2006). The highest flux of R26 (1.2 mol) was observed at 5 g/L condition corresponding with the maximum net H_2 production (0.98 mol) from R14 (Figure 3A). However, re-oxidizing ferredoxin (R13) was the main flux for hydrogen production observed under low starch fermentation (2.5 and 5 g/L) and it was significantly higher than H_2 flux from butyrate oxidation (R26). On the other hand, trends in both R13 and R26 flux observed under high starch fermentation (7.5 - 12.5 g/L) were not significantly different (less than 10%). Results illustrated that the initial starch concentration highly impacted the major flux of H_2 production. The net H_2 production (R14) tended to

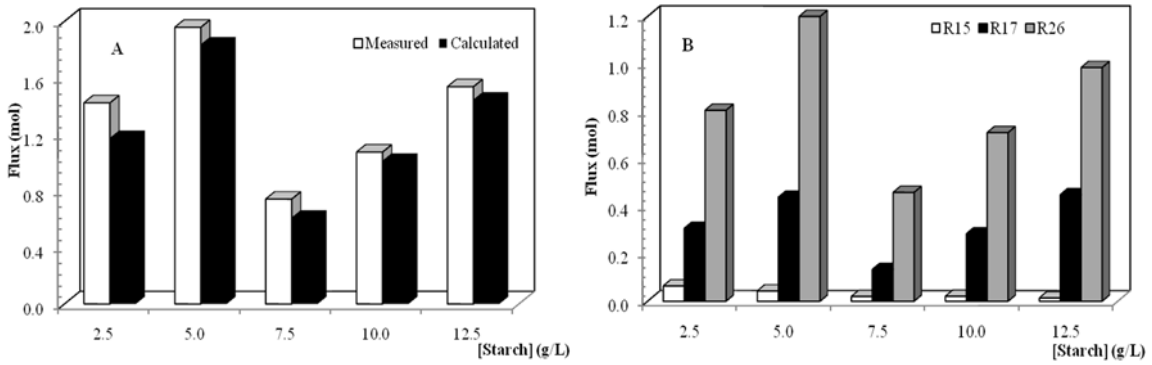

*Figure 2: Acetic acid production measured from experiment and calculated from flux distribution (A) and acetic acid production by ACCOA intermediate pathway (R15), acetogenesis (R 17) and butyric acid oxidation {(R26)*2} (B) during the starch fermentation.*

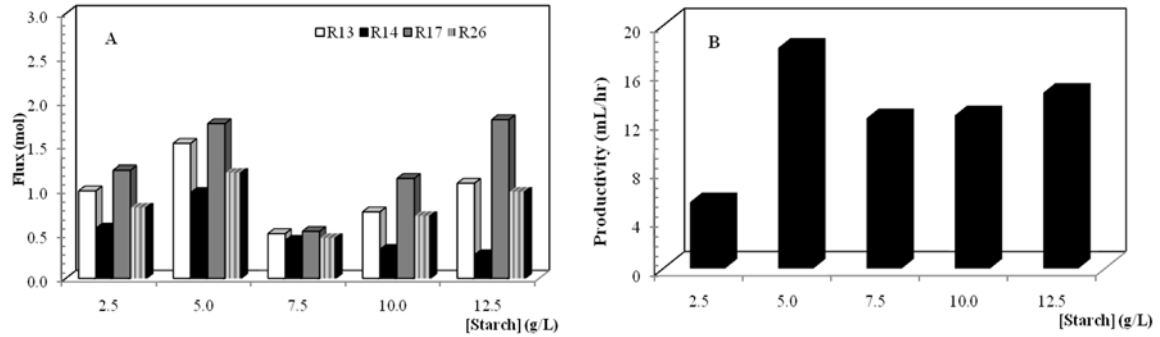

*Figure 3: H_2 production (R15) from ferredoxin pathway, butyric acid degradation (R26*2) and hydrogen consumption (R 17*4) fluxes (A) and H_2 productivity (B) during the starch fermentation.*

increase from 0.57 to 0.98 mol when starch concentration was increased (2.5 - 5 g/L) and dramatically reduced at high starch concentrations (7.5 - 12.5 g/L). Besides, homoacetogenesis is the major flux correlated with H_2 consumption in the anaerobic fermentation. Moreover, H_2 consuming acetogenic activity was not suppressed by thermal stress and pH adjustment (Chaganti et al., 2011). H_2 consuming flux (R17*4) observed in this study was higher than other hydrogen fluxes for all cases. This flux appeared to be a major reaction resulted in the low H_2 production that it was strongly affected in the high starch fermentation (10 and 12.5 g/L). H_2 productivity tended to increase with increased starch concentration from 2.5 to 5 g/L and decrease with the higher starch concentration (Figure 3B). The highest productivity was 18.15 mL H_2 /h at 5 g/L starch condition. Results illustrated that acetate production pathway was significantly correlated with H_2 synthesis. Therefore, not only H_2 production from normal flux: ferredoxin re-oxidation could be maintained in optimal condition of fermentation but also other intermediates correlated with H_2 flux such as acetogenesis (R17) and butyrate oxidation (R26) could be used as the major issues for enhancement the efficiency of hydrogen production.

4. Conclusion

Results from *in silico* metabolic flux model were agreed with those from the experiment with respected to acetate production using as the objective function. Maximizing acetate is not only correlated to the H_2 production but also the H_2 consumption through acetogenesis. Butyrate oxidation (R26) corresponded to the H_2 production under anaerobic fermentation. Acetate formation from R26 was the maximum flux for all cases of starch fermentation. The initial starch concentration resulted the major flux of H_2 production. R13 was the major H_2 formation flux observed in the low starch fermentation (2.5 and 5 g/L). The maximum H_2 productivity obtained at 5 g/L starch fermentation. To achieve high yield of hydrogen production from starch fermentation, besides normal acetate fermentation partway, butyrate oxidation could be a strategic target to promote the H_2 production.

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