Biodiesel and Biohydrogen Co-Production with Treatment of High Solid Food Waste

Final Report

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This report is available on the Department of Ecology's website at www.ecy.wa.gov/beyondwaste/organics. The reader may be interested in the other project reports supported by Organic Waste to Resources and Waste to Fuel Technology funding sponsored by Ecology. These are also available on the "organics" link. The Washington State University Extension Energy Program will make this report accessible in its broader library of bioenergy information at www.pacificbiomass.org.

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1. Executive summary

Great public and development interest exists in potential use of biofuel for transportation. The goal of this project was to evaluate possibilities converting food waste to biohydrogen and yeast as biodiesel feedstock through biological processing. The project results contribute to technology development and technical data for policy making in developing Washington's bioeconomy using the available low-cost organic resources.

A two-step process was developed in this project as a potential technology to produce hydrogen and biodiesel feedstock using the food waste. The first step of this process is dark fermentative hydrogen production, in which the fermentative bacteria use glucose derived from waste carbon to produce hydrogen and volatile fatty acids (VFA; e.g. acetate or butyrate). One third of the carbon is converted to carbon dioxide in the first-step while two thirds of the carbon is converted to VFA. In the second step, carbon in the form of VFA is used to feed yeast for simultaneous carbon sequestration and production of biodiesel feedstock from the oil-enriched microbial biomass.

The work conducted in this project proved the concept with laboratory scale experiments. Further study needs to be conducted to scale up this process and assess its economical viability. The following conclusions were drawn from the results of this project:

1) pH adjustment proved to be an effective way to increase the concentration of VFA in the hydrogen production broth (HPB). The increased VFA concentration could then produce higher concentrations of yeast biomass and ultimately improve the efficiency of the yeast culture process with HPB.
2) pH and substrate concentration were key factors for the yeast *C. curvatus* culture when VFA was used as carbon source. The optimal pH for yeast growth was 8.0 and VFA concentration needed to be kept at a low concentration to support optimal growth of the yeast.
3) There was no inhibitor to the culture of *C. curvatus* present in the HPB. This yeast showed very good growth with HPB culture medium, and produced 15%-30% of lipids in the dry biomass.
4) It was proved in this study that both the hydrogen production process and yeast culture process can be conducted in a continuous manner, thus improving efficiency and automation. Most COD in the HPB broth was consumed in the yeast culture process, and there were no odors in the discharged water.
5) With the developed process, it was estimated that 18 million gallons of biodiesel and 147 millions of cubic meters of hydrogen could be produced from the 2 million dry tons of organic waste produced annually in Washington State.

Background

Dark fermentative hydrogen production from sugar requires no light energy (Das and Veziroglu, 2000; Hallenbeck and Benemann, 2002), and has no oxygen limitation problems because the process does not require oxygen (Nandi and Sengupta, 1998). A variety of carbon sources can be used as substrates in the process, including negatively valued raw materials such as sugar-containing wastewater (Claassen et al., 2005). Such hydrogen production has been reported, using waste sources such as bean curd manufacturing waste, rice winery wastewater, starch wastewater, livestock waste, olive oil waste, food processing, and domestic wastewater. Mixed culture has also been widely utilized, requiring no sterilization in the process.

In this dark fermentative process, the fermentative bacteria use glucose derived from the organic waste carbon to produce hydrogen and volatile fatty acids (VFA; e.g. acetate or butyrate) through their anaerobic metabolic pathway (see following equations) (Levin et al., 2003).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$$
$$C_6H_{12}O_6 \rightarrow 2H_2 + CH_3CH_2CH_2COOH + 2CO_2$$

The major hydrogen producing microorganisms using anaerobic metabolism are *Clostridia* (Hawkes et al., 2002; Hawkes et al., 2007), a series of gram-positive, spore-forming, rod-shaped bacteria. *Clostridia* are very resistant to heat or harmful chemicals, so acid treatment, heat treatment, and alkaline treatment can kill methanogens that co-reside with the *Clostridia* in the mixed anaerobic culture and thus 'turn off' the last step in the anaerobic conversion process where either H_2 or VFA are converted to methane. By turning this last step off, both the H_2 and the VFA remain for utilization during the proposed process (Hu and Chen, 2008). Only one third of the available carbon is converted to carbon dioxide in the above process while two thirds of the carbon is converted to volatile fatty acids, which is still a threat to the environment if it is released without further treatment. In this process, though, the remaining carbon in the form of VFA is used as a carbon feed to microalgae and/or yeast for simultaneous vector reduction and production of biodiesel. A particular yeast as described below has been chosen for both its ability to readily take up VFA as its carbon source and its ability to metabolize the carbon into accumulated high content lipids inside the cells, thereby allowing for the development of a high rate single cell oil production process.

Although it is experiencing considerable growth, the biodiesel industry recognizes that its traditional sources of oil, such as vegetable oil, plant seeds, and waste animal grease will not be able to meet future demand. Single cell oil from microorganisms such as microalgae and yeast has garnered increasing attention because of its advantages in high efficiency use of land and its use of organic waste as raw materials. Some species of yeast, such as *Cryptococcus curvatus* and *Rhodosporidium toruloides* have been successfully used to produce cocoa butter substitutes with fermentative processes since the 1980s (Davies, 1988). *C. curvatus* can be cultured on many different carbon sources such as sugars (Evans and Ratledge, 1983), whey permeate (Ykema et al., 1988), oil (Davies, 1988), and glycerol (Meesters et al., 1996). The oil content in *C. curvatus'* dry biomass is in the range of 25-37% (Meesters et al., 1996). This high content of C18:1 (a fatty acid with a chain of 18 carbons and 1 unsaturated double bond) is preferred by the biodiesel industry since it decreases the cloud point of the produced biodiesel.

If the VFA produced in the hydrogen production process is used as a feedstock for oleaginous yeast culture (Figure 2.1), it not only reduces the chemical oxygen demand (COD) in the wastewater, but also produces oil-enriched yeast biomass that can be used as feedstock for biodiesel production. Such a strategy will improve the economical viability compared with the traditional glucose based yeast fermentation process because the feedstock used according to this strategy has a negative value. *C. curvatus* takes a variety of carbon sources such as 6-carbon compound glucose, 5-carbon compound xylose and lactose, 3-carbon glycerol, as well as 2-carbon compound ethanol. When ethanol is used as carbon source, it is converted into acetate via acetaldehyde. Then, acetyle-CoA will be activated from acetate, and feed into glyoxylate and tricarboxylic acid cycles to produce oxaloacetate (Palmieri et al., 1997). Thus, the microorganism has the capability to utilize ethanol can take acetate as carbon source, and can be used for testing this biohydrogen-biodiesel production concept.

This project was funded by the Washington State Department of Ecology to investigate an alternative technology for biodiesel production from organic waste under the program of Organic Waste to Resources – Research for the period from January 2008 through June 2009.

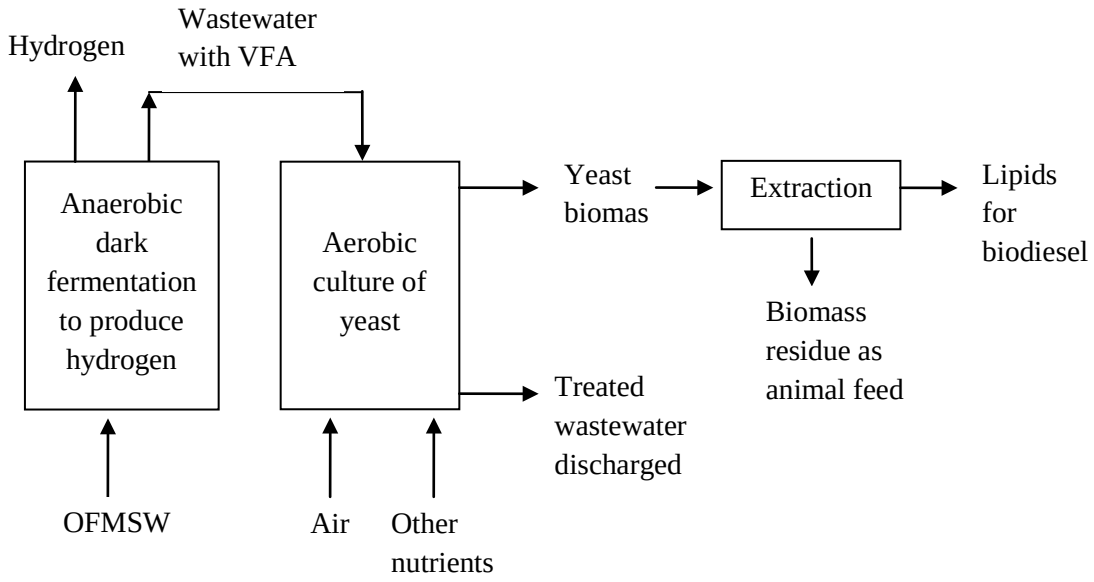

Figure 2.1 Schematic plot of bio-hydrogen and biodiesel co-production system

2. Methods

In this project, a variety of methods were developed to convert food waste and other municipal organic waste into hydrogen and VFA enriched broth, and later be used to culture yeast in order to produce oil. Oil can be extracted from the produced yeast biomass and processed into biodiesel, and the leftover yeast biomass may be used as animal feed.

3.1 H_2 producing sludge

In order to obtain hydrogen producing bacteria, anaerobic sewage sludge was taken from the wastewater treatment plant in Pullman, WA, and pretreated using heat pretreatment to kill the methanogenic species (Oh et al., 2003). In this process, 100 g solid sewage sludge was heated at 100°C in an oven for 2 hours.

3.2 Dark fermentation bio-hydrogen production

200 ml of the heat pretreated sludge was used as seed to inoculate the dark fermentation process for hydrogen production. An 8 L sealed stirred tank with 6 L culture medium was used for this culture (Table 3.2.). The agitation was accomplished using a magnetic stir bar in the tank. The process was carried out at room temperature (25-30°C).

Table 3.1. Culture medium used in dark fermentation for hydrogen production

Component	Quantity	Unit
Sucrose	20	g/L
NH_4Cl	500	mg/L
Yeast extract	50	mg/L
K_2HPO_4	250	mg/L
KH_2PO_4	250	mg/L
$NaHCO_3$	1000	mg/L
$MgSO_4.7H_2O$	320	mg/L
$CaCl_2$	50	mg/L
$NiSO_4$	32	mg/L
$FeCl_2$	20	mg/L
$CoCl_2 \cdot 6H_2O$	21	mg/L
$CuCl_2 \cdot H_2O$	10	mg/L
$ZnCl_2$	23	mg/L
$MnCl_2 \cdot 4H_2O$	30	mg/L
$Na_2BO_4 \cdot H_2O$	7	mg/L
$Na_2MoO_4 \cdot 2H_2O$	14	mg/L
pH = 7.5		

3.3 Biogas analysis methods

The composition of biogas was measured using a gas chromatograph (GC; CP-3800, Varian Inc., Walnut Creek, CA). The fatty acid methyl esters (FAME), cell density, and dry weight analyses were the same as previously reported (Chi et al., 2007). The VFA was determined using a GC (Shimadzu Corporation, Japan). Details of the analytical method are described in Appendix Π.

3.4 Continuous culture for H_2 production

An upflow reactor was maintained in batch mode for three days, and then switched to continuous mode with a hydraulic retention time (HRT) of 13 hours. The HRT was adjusted then quickly to 5.3 hours and kept operational for three days until it ran constantly (details in Appendix Π).

3.5 High solid food waste anaerobic digestion

Food waste was collected from a student-run restaurant at Washington State University in Pullman, WA. It contained fruits, vegetables, meat, cream, rice, and noodles. The food waste was ground with a food processor and used in the culture medium for hydrogen production. The characteristics are shown in Table 3.2.

High solid food waste anaerobic digestion was conducted in order to produce a high concentration VFA for yeast culture. The pH had a great impact on the hydrolysis process and VFA concentration in the effluent (Chen et al., 2007). Thus, pH values from 4.0 to 11.0 were investigated in the batch culture (details in Appendix II).

Table 3.2. Characteristics of substrate and inoculum

Parameter	TS	VS	VS/TS
Food waste	31.67 (%, w/w)	29.98 (%, w/w)	55.70 (%)
Digester sludge	17.12 (g/L)	11.87 (g/L)	69.29 (%)

TS indicate total solids and VS indicate volatile solids

3.6 HPB pretreatment

After anaerobic digestion of the food waste, the HPB was autoclaved at 121 ℃ for 20 minutes to kill all the bacteria and endospores and then centrifuged to remove most of the solid content. The supernatant was kept as feedstock for the yeast culture.

3.7 Yeast cell strain and culture

Cryptococcus curvatus (ATCC #20509) was cultured to produce biomass with high oil content. The seed cells were pre-cultured in a medium composed of 20 g/L malt extract and 5 g/L peptone. The cells were grown in 250 ml Erlenmeyer flasks, each containing 50 ml of medium and incubated at 20^{o}C for 4 days in an orbital shaker set to170 rpm. Subcultured cells were used as inoculum for future studies. The inoculum volume was 10% of the total liquid volume in each flask.

3.8 Statistical experiment for optimization of oleaginous yeast culture condition

A Plackett–Burman design was used to screen the factors that have significant effects on the biomass production from acetate (Wen and Chen, 2001). The variables to be evaluated are listed in Table 3.3 (Appendix I). Each independent variable was investigated at a high and a low level. The design matrix is shown in Table 3.4 (Appendix I).

Once the significant factors were identified in the Plackett–Burman design, a central composite design was used to optimize their levels. The coded and real values of each parameter are shown in Table 3.5 and the design matrix is shown in Table 3.6 (both in Appendix I). Based on results

obtained in Table 3.6, the responses of dry cell weight and final pH were correlated as functions of variables by a second-order polynomial equation (Equation 1).

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_i x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (1)$$

In Equation 1, *Y* is the predicted response, β are the coefficients of the equation, and *xi* and *xj* are the coded levels of variables *i* and *j*, respectively. The software Design-Expert (Stat-Ease Inc. Minneapolis, MN) was used for this correlation through non-liner regression. The *F*-test was used to evaluate the significance of the models.

3.9 Fed batch culture of oleaginous yeast using a fermentor

For the experiments conducted in fermentors, Bioflo 110 glass tank fermentors (New Brunswick Scientific, Edison, NJ) with 7.5 L or 1.3 L capacities were used, with working volumes of 2.0 L and 0.5 L, respectively. The broth from the hydrogen production was centrifuged to separate the bacterial biomass, and the supernatant was filtered with a 0.22 µm filter for sterilization. When the cells began to grow and pH increased to 8.1, acetic acid was fed to the culture continuously. Sodium hydroxide (5 M) was used to adjust the pH if the pH decreased to 7.95, and was controlled by the Bioflo 110 contoller.

4. Results and Discussion

4.1 Laboratory scale continuous bio-hydrogen production process

In this study, we investigated the effect of the long-term continuous operation factors upon the performance of the upflow reactor. As HRT decreased, hydrogen productivity gradually increased. The yield of hydrogen fermentation, however, decreased when the HRT was shorter than three hours, owing to the overloading of the upflow reactor (Figure 4.1).

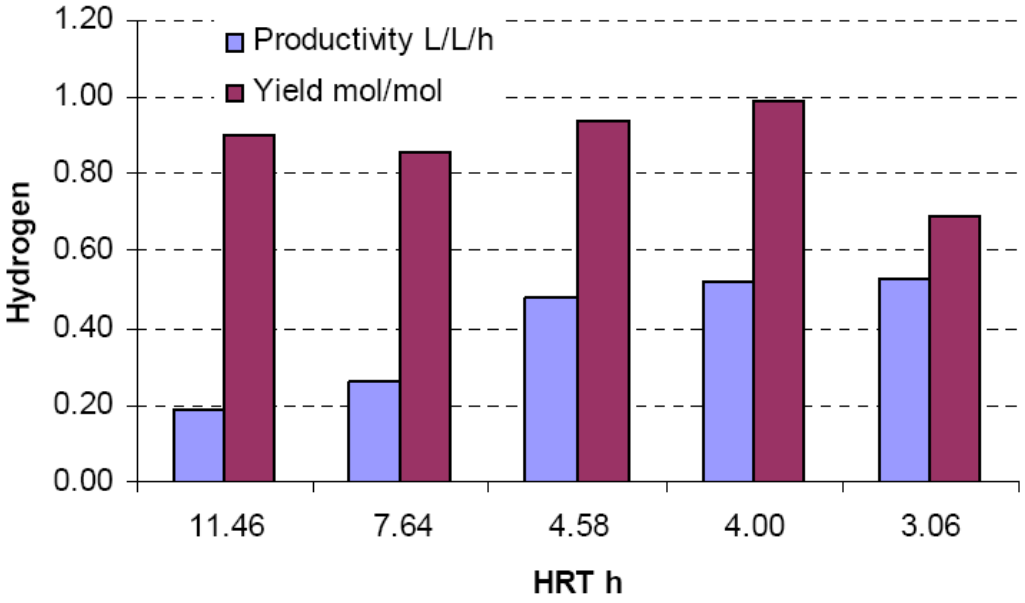

Figure 4.1. Influence of HRT on the performance of the upflow hydrogen fermentation. Glucose culture medium contained 20 g COD/L at initial pH 8.0. This figure was taken from [8]

Figure 4.2 demonstrated that substrate concentration influenced the continuous granulized fermentation. With an increase in glucose concentration and the COD loading of the upflow reactor, hydrogen productivity reached maximum levels and then decreased. There was no significant difference in hydrogen productivity between glucose concentration at 20 g COD/L and at 30 g COD/L. When the glucose concentration was over 20 g COD/L, however, the overall yield decreased gradually. Fermentation with high substrate concentrations are preferred because many raw materials (such as whey or manure) have high COD content, and high concentrations of substrate are easy to heat and handle. Inhibition, however, was found at higher feedstock concentrations (Van Ginkel et al., 2005). In this study, cultures with a substrate concentration below 20 g COD/L had similar yields; however, at higher substrate concentrations, overloading occurs, causing a decrease in hydrogen productivity and overall yield.

4.2 High concentration VFA production process from food waste

VFA and gas production at different pH levels are shown in Table 4.1. After 10 days fermentation, the VFA concentrations at various pH levels were as follows: 28.8 g/L (pH 8) > 24.5 g/L (pH 7) > 20.1 g/L (pH 9) > 17.5 g/L (pH 6) > 8.2 g/L (pH 5) > 5.5 g/L (pH 4) > 4.1 g/L (pH 11) > 3.9 g/L (pH 10). These results show that the production of VFA was highest when the pH of the fermentation was 8.0. The VFA production increased linearly between pH 4.0 and 9.0,

while further increasing the pH at strong alkaline condition resulted in a decrease of VFA production. The concentration of VFAs at pH 8.0 was approximately five times higher than in acidic and strong alkaline conditions. The reason for less VFA produced at pH 10 and 11 may be attributed to the toxic effects of stronger alkalinity to acidogenic (acid producing) bacteria. Further investigation revealed that gas production was closely related to VFA production, which is in accordance with the metabolic pathway of acidogenic bacteria.

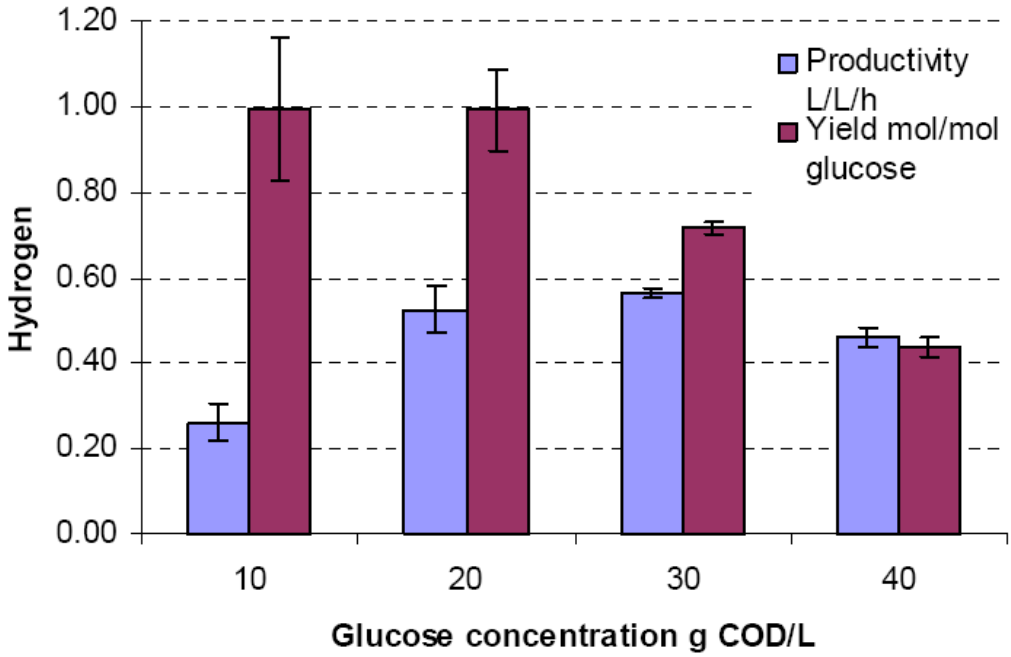

Figure 4.2**.** Influence of glucose concentration on the performance of the upflow hydrogen fermentation reactor. Glucose culture medium conditions: initial pH 8.0 and HRT 4 h. This figure was taken from [8]

Table 4.1. VFA concentrations of food waste anaerobic digestion at different pH values.

pH	**4.0**	**5.0**	**6.0**	**7.0**	**8.0**	**9.0**	**10.0**	**11.0**
VFAs (g/L)	5.5	8.2	17.5	24.5	28.8	20.1	3.9	4.1
Hydrogen (ml)	4166	4481	5594	7046	5837	4868	3316	580

4.3 Optimized culture conditions for the lipid-producing yeast Cryptococcus curvatus using VFA as the carbon source

4.3.1 Screening the significant factors

Experiments were initiated for screening the significant factors according to the Plackett-Burman design. Yeast cells were grown under 20 different conditions, with cell dry weight being taken as the responses for each run (Table 3.4, Appendix I). With the experimental data, the effects of the variables on the responses, and their associated *t*-values were calculated (Table 4.2). Based on the effects of dummy variables (see Appendix I), the standard error of the Plackett-Burman design was determined as 0.04 g/L for cell dry weight. The effects with $p < 0.10$ were accepted as significant, which has a *t* value of 2.132. It was found that among the 12 factors investigated,

potassium acetate (CH_3COOK), initial pH, EDTA, $MgSO_4 \cdot 7H_2O$, and NH_4Cl had significant effects on cell weight (Table 4.2).

Acetate is the most significant factor influencing the yeast dry biomass. Carbon source is a very important factor for yeast growth because it provides energy for its metabolism. Another very significant factor was initial pH. The reason may be that pH will affect the ionization equilibrium for acetate and different formation of the carbon source may have different impacts on the cell growth.

Table 4.2 Result of Placket-Burman design

	KH_2PO_4 (g/L)	NH_4Cl (g/L)	Na_2HPO_4 (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	Peptone (g/L)	Yeast extract (g/L)	EDTA (g/L)	glucose (g/L)	Solution A (ml/L)	Acetate (g/L)	pH	Temp (°C)
DCW	A	B	C	D	E	F	G	H	I	J	K	L
low	1.14	1.08	1.10	1.18	1.18	1.10	1.06	1.10	1.16	0.80	0.98	1.08
high	1.12	1.18	1.16	1.08	1.08	1.16	1.20	1.16	1.10	1.46	1.28	1.18
E	-0.02	0.10	0.06	-0.10	-0.10	0.06	0.14	0.06	-0.06	0.66	0.30	0.10
t = E/SE	-0.475	2.376	1.426	-2.376	-2.376	1.426	3.326	1.426	-1.426	15.681	7.128	2.376

E indicates error, and SE indicates standard error

4.3.2 Optimization of the significant factors

As shown in Table 3.6 (Appendix I), the central point in the central composite design was repeated six times (runs 27 – 32), and the standard deviation of the six replicates was used to determine the experimental errors for cell dry weight as 0.02 g/L. The experimental data of dry cell weight in Table 4.3 were correlated as functions of the four variables by a second-order polynomial equation (Equation 1) using Design-Expert. To obtain a more accurate model, the coefficient whose p-level was higher than 0.1000 was excluded in the final model. The coefficients included in the final model and their corresponding F-value and p-level are shown in Table 4.3.

Table 4.3. Values of the coefficients in Equation1 and their *F*-values and *P*-values

		Dry cell weight			Final pH		
coefficient	variable	Estimate	F-value	p-level	Estimate	F-value	p-level
β_0	constant	0.30			6.65		
β_1	A: NH_4Cl	0.27	6.750	0.017			
β_2	B: $MgSO_4 \cdot 7H_2O$	-0.03	0.057	0.8135			
β_3	C: EDTA	-0.24	5.342	0.0316	-0.24	5.145	0.0319
β_4	D: CH_3COOK	-0.68	42.084	< 0.0001	-0.44	17.457	0.0003
β_5	E: Initial PH	0.54	26.178	< 0.0001	1.15	121.494	< 0.0001
β_{33}	C^2	0.31	11.140	0.0033	0.26	7.768	0.0098
β_{44}	D^2	0.34	13.379	0.0016	0.27	8.528	0.0071
β_{14}	AD	-0.42	10.884	0.0036			
β_{15}	AE	0.42	10.628	0.0039			
β_{23}	BC	-0.42	10.884	0.0036			
β_{45}	DE	-0.61	22.503	0.0001			
model			14.391	< 0.0001		31.875	< 0.0001

The coefficients in this table correspond to Equation 1. The variables in this table are assigned the letters A – E and are defined above. C^2 indicates twice the amount of EDTA in the reaction, and AD indicates NH_4Cl and CH_3COOK in the reaction. Only the reactions that yielded the most significant results (as indicated by p-level) are shown.

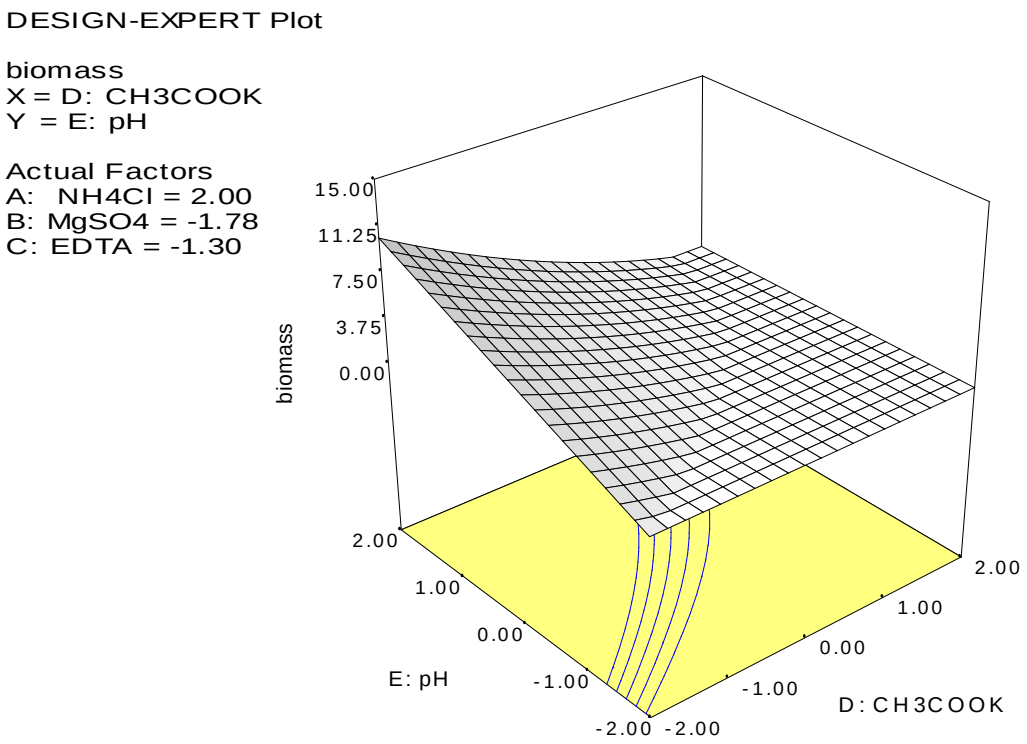

Figure 4.3. Three dimensional surface response plot of cell dry weight as a function of potassium acetate concentration and initial pH. The coded levels of pH and potassium acetate concentration correspond to the coded levels in Table 3.6 (Appendix I)

4.3 g/L of yeast biomass was obtained in the best culture condition in this experiment. The pH in the culture changed significantly, however, reaching a value as high as 9.0, and inhibited further growth. The potassium acetate was used in this culture as a carbon source. With the consumption of acetate, some of protons were consumed. The remaining hydroxide ions increased the pH.

Table 4.4. Predicted values and experimental results for the set parameters

	Predicted values							Experiment values	
	NH_4Cl	$MgSO_4 \cdot 7H_2O$	EDTA	CH_3COOK	Initial PH	Dry cell weight	Final pH	Dry cell weight	Final pH
	(g/L)	(g/L)	(g/L)	(g/L)		(g/L)		(g/L)	
1	1.6	0.5	0.04	20	6.9	8.59	10.0	3.93±0.23	9.4±0.1
2	1.6	0.3	0.12	20	7.1	8.39	10.0	3.67±0.12	9.5±0.1
3	1.6	0.4	0.12	25	7.3	8.13	10.0	4.27±0.12	9.5±0.1

The optimal values of the four parameters were derived by Design-Expert as show in the first row in Table 4.4. The other two conditions were selected at random. A three dimensional surface

response plot illustrates the relationships between the responses and the variables in Figure 4.3. pH and potassium acetate concentration have the most significant effects on biomass production. A significant interaction between these two factors was also observed. Higher initial pH and lower concentrations of potassium acetate produce higher yields of yeast biomass.

4.3.3 Verification of optimized culture conditions

The verification experiment for the predicted optimal condition and an additional two sets of conditions was conducted. The results were far from the predicted values. This may be caused by pH inhibition. Although a final pH as high as 10.0 was predicted, the actual final pH was only 9.4, which was already very high for most yeast species.

4.4 Culture of *C. curvatus* with VFA from the hydrogen production process as the carbon source

Acetate, butyrate, and residual sugar were analyzed in this experiment. 2.05 g/L acetic acid and 4.06 g/L butyric acid were produced in the biohydrogen production process, and the residual sugar concentration was 0.16 ± 0.03 g/L.

10 g/L glucose was used in the initial medium, and all other nutrients were at the same level as the predicted optimal values as shown in Table 4.4. The temperature was controlled at 30°C, and the dissolved oxygen was controlled at a minimum of 10%. The pH was controlled at 5.5, since it was the optimal pH value for the *C. curvatus* being cultured with glucose. After 24 hours of culturing, half of the broth was discharged and supplemented with same volume of HPB. The pH was then adjusted to 8.0. The final biomass production was 12.4 g/L (Figure 4.4a). We speculate that to keep feeding this batch would produce higher concentrations of biomass. This data shows that the HPB feeding certainly had no inhibitory effect to the growth of *C. curvatus*.

A second experiment was conducted with HPB as the initial medium, supplemented with the same nutrients as in the first batch, except the carbon source. Thus, the only carbon source available to the yeast was from HPB. The pH was controlled at 8.0. When the cells started to grow and the pH began to increase correspondingly, pure acetic acid was fed to increase the biomass production, as well as to prevent the possible limitation of carbon available in the initial medium. 16.9 g/L yeast biomass was produced in this batch (Figure 4.4b). This further proved that the HPB can be a good carbon source for *C. curvatus*.

Continuous fermentation was conducted using a 1.3 L fermentor. The initial batch culture was started with a 500 ml working volume of medium containing 10 g/L glucose. The temperature was controlled at 30°C, the dissolved oxygen was controlled at 50%, and the pH was controlled at 5.5. After 29 hours, the pretreated HPB (containing 18.8 g/L VFA) was added without any other nutrients and the pH was controlled at 8.0. The data after 82 hours is shown in Figure 4.5.

The dilution rate (D) for this continuous culture was 0.86 d^{-1}. The steady-state values for biomass, lipid, and VFA are shown in Table 4.5. The HPB rich in VFA, nitrogen, and phosphorous is a potential environmental concern if it is released without further treatment. After being treated by the continuous yeast culture system, the VFA was completely removed, the COD was reduced approximately 8 times, and the nitrogen decreased from 2.6 g/L to 1.25 g/L (Table 4.6).

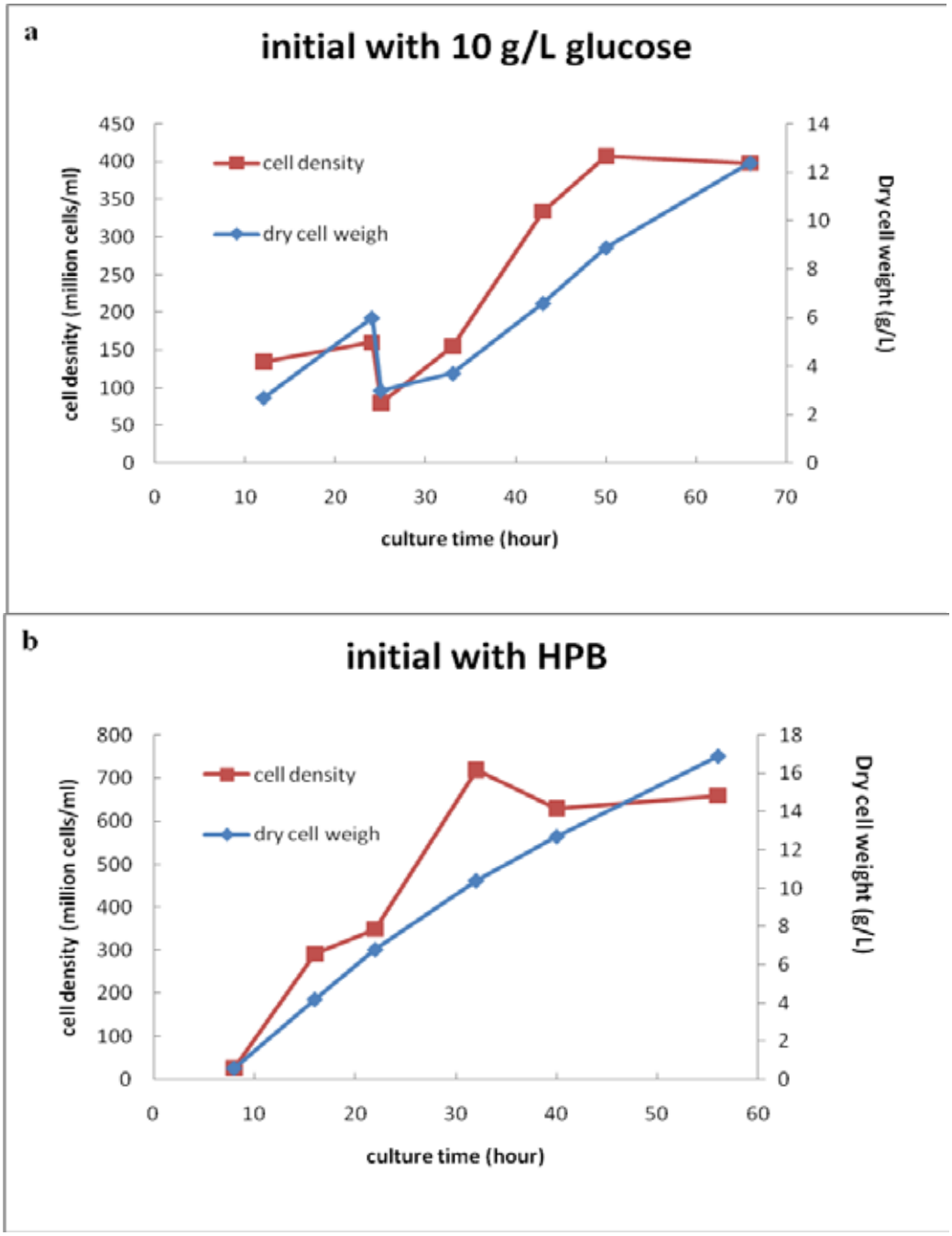

Figure 4.4. *C. curvatus* culture supplemented with HPB (a) and HPB as the initial medium (b). Both plots support HPB as a good carbon source for *C. curvatus* growth.

4.5 Continuous culture of *Cryptococcus curvatus*

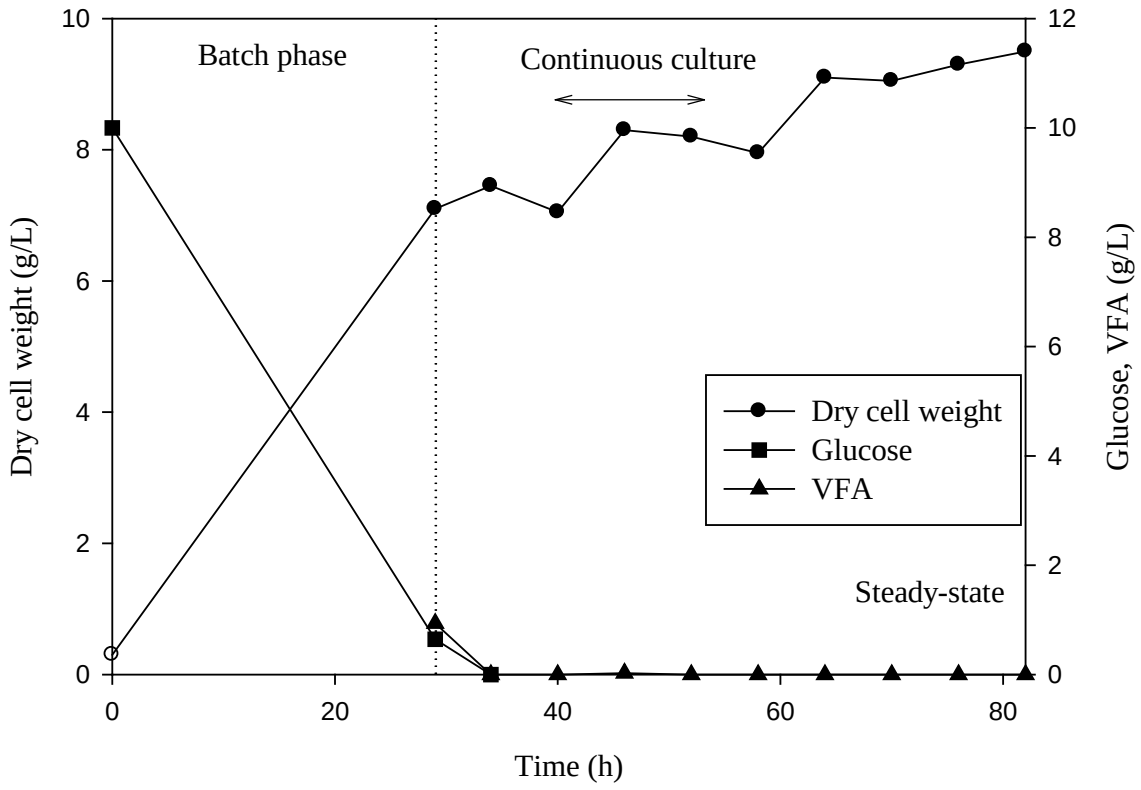

Figure 4.5. Continuous culture of *C. curvatus* by supplementing with hydrogen production broth (HPB)

Table 4.5. Steady-state values for continuous yeast culture

D (d^{-1})	Biomass yield (g_{DW}/g_{VFA})	Biomass yield (g_{DW}/g_{COD})	Biomass productivity (g/L/d)	Lipid content (mg/g DW)	Lipid yield (mg/g_{VFA})	Lipid productivity (mg/L/d)
0.86	0.49	0.24	8.0	150	72	1200

D indicates the dilution rate per day in the continuous culture

Table 4.6. Continuous yeast culture treatment of HPB

	HPB			Yeast effluent		
D (d^{-1})	VFA (g/L)	COD (g/L)	Nitrogen (g/L)	VFA (g/L)	COD (g/L)	Nitrogen (g/L)
0.86	18.8	43.0	2.6	0	5.2	1.25

D indicates the dilution rate per day in the continuous culture

4.6 Potential biomass yields from Washington State feedstocks

Based on the data obtained, calculations of potential biodiesel and hydrogen production from feedstocks available in Washington State can be found in Table 4.7. The amounts of wastes available were listed in the Washington State Biomass Inventory (Frear et al., 2005).

Table 4.7. Potential biomass production from waste listed in the Washington State Biomass Inventory (Frear et al., 2005)

Waste type	Total volume in Washington State (dry tons/year)	Biodiesel production (gallons/year)	Hydrogen production (m^3/year)
Food waste	246,000	1,863,000	15,206,000
Cheese whey	44,000	335,000	2,735,000
Cull potatoes	91,000	692,000	5,650,000
Cull apples	41,000	310,000	2,536,000
Grape pomace	19,000	145,000	1,190,000
Dairy manure	457,000	3,462,000	28,249,000
Cattle manure	242,000	1,836,000	14,983,000
Horse manure	407,000	3,084,000	25,166,000
Poultry manure	784,000	5,943,000	48,495,000
Potato solids	19,000	145,000	1,185,000
Apple pomace	27,000	210,000	1,717,000
Total:	2,380,000	18,031,000	147,116,000

Calculations for biodiesel and hydrogen production are based on the following assumptions: There is 50% degradable carbon in the waste sources; a 60% conversion rate of the carbon source to VFA; a 28% conversion rate of VFA to yeast biomass; 30% oil content in the yeast biomass; and a hydrogen production of 1 mol H_2/mol glucose.

5. Implications and impacts

The data from this project shows that at the lab scale, the originally proposed process of co-production of biodiesel and biohydrogen from organic municipal waste is technically feasible. The hydrogen production broth, which contained high concentrations of VFA, could be used as the feedstock for yeast culture, and the VFA could be converted into single cell oil.

Both steps in this process were optimized and the efficiency of each step was improved upon in this study. For the hydrogen production process with food waste, a digestion process with high substrate concentration was developed to produce hydrogen and VFA in an efficient manner using the pH adjustment and continuous culture process developed in this study. For the yeast culture process, the culture conditions were optimized using statistical methods to provide culture conditions that yielded a higher density of yeast cells. The continuous nature of the method using hydrogen production broth as the feedstock will improve efficiency and reduce labor costs.

Although it is experiencing considerable growth, the biodiesel industry recognizes that its traditional sources of oil, such as vegetable oil, plant seeds, and waste animal grease, will not be able to meet future demands. Single cell oil from microorganisms such as microalgae and yeast has garnered increasing attention because of its advantages in high efficiency land use and its use of organic waste as raw materials. Using the organic waste as the feedstock offers an alternative since their value is negative. The process developed in this project showed that the concept of using organic waste is valid approach for single cell oil production.

This process can be scaled up in future studies, with more efforts on testing the various feedstocks, as well as a production cost analysis and economic assessment. If successful, the application of this technology will have a variety of benefits on the environment, renewable enenergy supply, and economics development of the state.

5.1 Waste materials utilization

The process developed in this project converts food waste and wastewater into hydrogen that can be used as clean biofuel, lipids that can be used as biodiesel production feedstock, and yeast biomass that can be used as animal feed. These are all valuable products. The process can be applied to other organic fraction waste stream.

5.2 Environment benefits

If high solids wastes are not treated well and appropriately discharged to the environment, methane and carbon dioxide will be produced. Un-reacted VFA, which leads to air quality concerns, may also be produced. Methane is a relatively potent greenhouse gas with a high global warming potential, which is measured as a warming effect compared to carbon dioxide. When averaged over 100 years, each kg of CH_4 warms the Earth 23 times as much as the same mass of CO_2. The process developed in this project recovers the organic carbon from the waste and converts it to biomass with high oil content, as was shown when VFA was completely removed by yeast. In this process, nitrogen, phosphorous, and other metal elements, which would otherwise be potential threats to the environemnt, are absorbed by the biomass and utilized. Hydrogen and biodiesel are produced in this process, which provide renewable energy and biofuel.

5.3 Local economy

The proposed project has the potential to benefit the local economy by converting wastes to valuable products. Producing and distributing these products will provide job opportunities and stimulate total economic growth. As the international oil market fluctuates due to concerns over the limited reserve and increasing demand, producing bio-hydrogen and biodiesel from waste offers energy security and an increased number of jobs in Washington State.

6. Conclusions

This project developed a process to produce hydrogen and biodiesel using food waste as feedstock. The work in this phase proved the concept with laboratory scale experiments, and further study needs to be conducted to scale up this process and assess its economical viability. With this work, the following conclusions were made:

6) pH adjustment proved to be an effective way to increase the concentration of VFA in the hydrogen production broth (HPB). The increased VFA concentration could then produce higher concentrations of yeast biomass and ultimately improve the efficiency of the yeast culture process with HPB.
7) pH and substrate concentration were key factors for the yeast *C. curvatus* culture when VFA was used as carbon source. The optimal pH for yeast growth was 8.0 and VFA concentration needed to be kept at a low concentration to support optimal growth of the yeast.
8) There was no inhibitor to the culture of *C. curvatus* present in the HPB. This yeast showed very good growth with HPB culture medium, and produced 15%-30% of lipids in the dry biomass.
9) It was proved in this study that both the hydrogen production process and yeast culture process can be conducted in a continuous way, thus improving efficiency and automation. Most COD in the HPB broth was consumed in the yeast culture process, and there were no odors in the discharged water.
10) With the developed process, it was estimated that 18 million gallons of biodiesel and 147 millions of cubic meters of hydrogen could be produced from 2 million dry tons of organic waste annually in Washington State.

Appendix I – Tables from Section 3.8 on statistical experimental design

Table 3.3. Variables to be screened using the Plackett–Burman design

Designation	Variables	Unit	Low( )	High(+)
A	KH_2PO4	g/L	2	8
B	NH_4Cl	g/L	0.2	0.8
C	Na_2HPO_4	g/L	1	2
D	$MgSO_4.7H_2O$	g/L	0.3	1.5
E	Peptone	g/L	0.5	1.5
F	Yeast extract	g/L	0	0.5
G	EDTA	g/L	0	0.1
H	Glucose	g/L	0	0.5
I	PI metal solution	ml/L	30	60
J	CH_3COOK	g/L	20	100
K	Initial pH	–	4.5	5.5
L	Temperature	℃	25	30

Low and High values are those concentrations investigated in the optimization of the oleaginous yeast culture conditions. Designations A through L are referred to in Table 3.4

Table 3.4. The Plackett–Burman design matrix with cell dry weight (DW) as the response

Run	A	B	C	D	E	F	G	H	I	J	K	L	D1	D2	D3	D4	DW g/L
1	+	+	−	−	+	+	+	+	−	+	−	+	−	+	+	−	1.6
2	−	+	+	−	−	+	+	+	+	−	+	−	−	−	+	+	1.2
3	+	−	+	+	−	−	+	+	+	+	−	+	−	−	−	+	1.4
4	+	+	−	+	+	−	−	+	+	+	+	−	−	−	−	−	1.4
5	−	+	+	−	+	+	−	−	+	+	+	+	+	−	−	−	1.6
6	−	−	+	+	−	+	+	−	−	+	+	+	−	+	−	−	1.8
7	−	−	−	+	+	−	+	+	−	−	+	+	+	−	+	−	0.8
8	−	−	−	−	+	+	−	+	+	−	−	+	−	+	−	+	0.6
9	+	−	−	−	−	+	+	−	+	+	−	−	+	−	+	−	1.2
10	−	+	−	−	−	−	+	+	−	+	+	−	+	+	−	+	1.8
11	+	−	+	−	−	−	−	+	+	−	+	+	+	+	+	−	1.0
12	−	+	−	+	−	−	−	−	+	+	−	+	+	+	+	+	1.2
13	+	−	+	−	+	−	−	−	−	+	+	−	−	+	+	+	1.4
14	+	+	−	+	−	+	−	−	−	−	+	+	−	−	+	+	1.0
15	+	+	+	−	+	−	+	−	−	−	−	+	+	−	−	+	0.8
16	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	−	0.6
17	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	0.6
18	−	−	+	+	+	+	−	+	−	+	−	−	+	−	+	+	1.2
19	+	−	−	+	+	+	+	−	+	−	+	−	+	+	−	+	0.8
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0.6

A through L correspond to the designations assigned in Table 3.3. D1 through D4 are dummy variables assigned in order to aid in the statistical calculations. No experimental changes were made regarding D1 through D4. The effect of each variable on response was determined by subtracting the average response of the low level from the high level.

Table 3.5. The coded levels and real values of the independent variables in the central composite design

	A	B	C	D	E
level	NH_4Cl	$MgSO_4 \cdot 7H_2O$	EDTA	CH_3COOK	Initial PH
	g/L	g/L	g/L	g/L	
2	1.6	0.5	0.20	100	7.5
1	1.2	0.4	0.15	80	7.0
0	0.8	0.3	0.10	60	6.5
-1	0.4	0.2	0.05	40	6.0
-2	0	0.1	0.00	20	5.5

Table 3.6. The central composite design of the variables (in coded levels) with cell dry weight as the response

Run	NH_4Cl	$MgSO_4 \cdot 7H_2O$	EDTA	CH_3COOK	Initial PH	biomass	Final pH
1	-1	-1	-1	-1	1	1.24	8.9
2	-1	-1	-1	1	-1	0.24	5.9
3	-1	-1	1	-1	-1	0.24	5.9
4	-1	-1	1	1	1	0.36	8.2
5	-1	1	-1	-1	-1	0.28	6.0
6	-1	1	-1	1	1	0.32	8.0
7	-1	1	1	-1	1	1.00	9.0
8	-1	1	1	1	-1	0.20	5.9
9	1	-1	-1	-1	-1	0.24	5.9
10	1	-1	-1	1	1	0.32	7.7
11	1	-1	1	-1	1	4.60	9.1
12	1	-1	1	1	-1	0.20	5.9
13	1	1	-1	-1	1	4.32	9.0
14	1	1	-1	1	-1	0.20	6.0
15	1	1	1	-1	-1	0.24	5.9
16	1	1	1	1	1	0.28	7.2
17	-2	0	0	0	0	0.24	6.6
18	2	0	0	0	0	0.24	6.5
19	0	-2	0	0	0	0.24	6.6
20	0	2	0	0	0	0.24	6.5
21	0	0	-2	0	0	3.12	9.1
22	0	0	2	0	0	0.24	6.4
23	0	0	0	-2	0	3.36	9.2
24	0	0	0	2	0	0.24	6.4
25	0	0	0	0	-2	0.24	5.4
26	0	0	0	0	2	1.36	9.4
27-32	0	0	0	0	0	0.26	6.5

Appendix II – Detailed methods

Biogas analysis methods

Biogas volume and compositional analysis

Biogas was released from the fermentor into a U-tube with water. The biogas volume produced during the fermentation was determined by measuring the water pressured out of the U-tube. The composition of biogas (H_2, CO_2, CH_4, and H_2S) in the headspace of the reactor was measured using a gas chromatograph (GC; CP-3800, Varian, Walnut Creek, CA) equipped with detectors, which included a thermal conductivity detector for H_2 and CO_2; a flame ionization detector for CH_4; a Valco Instrument pulsed discharge detector run in helium ionization mode D2 for H_2S; an 18' × 1/8" HayeSep Q 80/100 Mesh Silcosteel column for CO_2, H_2, and CH_4 with nitrogen as the carrier gas; and a 50 m × 0.53 mm × 4 μm SilicaPLOT column for H_2S with helium as the carrier gas. The time to analyze one sample was approximately 8 minutes.

Cell density

Cell density was determined by counting the cells on a light microscope with a hemacytometer. To determine cell dry weight, 5 ml cell suspension sample was transferred to a centrifuge tube and centrifuged at 2500 rpm for 5 min. The cell pellet was then washed twice with distilled water, and then dried in a pre-weighed aluminum dish at 105°C for 3 hours. Glucose was measured using a D-glucose Assay Kit (Megazyme International, Ireland).

FAME analysis

Fatty acid methyl ester (FAME) preparation and GC analysis were same as previously reported (Chi et al., 2007). Yeast cells were harvested and freeze-dried for fatty acid analysis. The method involves a 4 ml mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0, v/v/v) being added into a tube containing 20 mg of dried cell biomass and 1 mg heptadecanoic acid (C17:0) as an internal standard. The tubes were heated in a water bath at 90°C for 40 min, and then cooled to room temperature, at which point 1 ml of distilled water was added. The liquid in the tubes was thoroughly mixed by vortexing for 1 min, and then settled for separation of the two phases. The lower phase containing the FAME was transferred to a clean vial and dried with anhydrous Na_2SO_4. 0.5 ml dried solutions were transferred into a vial and analyzed using gas chromatography.

A Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used for FAME analysis. The GC was equipped with a flame ionization detector and a Sol Gel-Wax^{TM} capillary column (30 m × 0.25 mm × 0.25 mm; SGE Analytical Science, Australia). The injector was kept at 250°C, with an injection volume of 1 ml by split injection mode (ratio: 10:1). The profile of the column temperature was as follows: 80°C for 0.5 min, raised to 175°C at 30°C/min, raised to 260°C at 5°C/min, maintained for 6 min; raised to 280°C at 30°C/min, and maintained for 1 min. Helium was used as the carrier gas. The detector temperature was kept at 300°C. The fatty acids of the yeast sample were identified by comparing the retention times with those of standard fatty acids (Sigma-Aldrich Corp., St. Louis, MO). To quantify the fatty acids, the response factor of each fatty acid was first determined by GC FAME analysis of the fatty acid and the internal standard at equal amounts, then comparing the peak area of the fatty acid to that of the internal standard (C17:0). Here, we set response factor for the

internal standard (17:0) as 1, the response factors of the representative fatty acids were determined as 0.99 for C14:0, 0.99 for C16:0, 0.97 for C18:0, 0.87 for C22:5, and 0.87 for C22:6. Based on these response factors, the fatty acids of the yeast sample were quantified by comparing their peak area with that of the internal standard (C17:0), and correcting by their response factors.

VFA analysis

Concentrations of VFA in the mixture were analyzed via headspace chromatographic (HS-GC) analysis (Cruwys et al., 2002). A GC (Shimadzu Corporation, Japan) equipped with a flame ionization detector and a HP-INNOWax Polyethylene glycol (PEG) capillary column (Agilent Technologies, Santa Clara, CA) was used in this analysis. The VFA analyzed included acetate, propionate, butyrate, and valerate. An AOC-5000 auto injector (Shimadzu GC-2014, Japan) was used for vial incubation and automatic sampling, and 500 µL samples were injected. The PEG capillary column was first heated from 140°C to 200ºC at 6ºC/min and then held at 200ºC for 1 min. The temperatures of the injector and detector were 200°C and 250ºC, respectively. Nitrogen was used as the carrier gas at a flow rate of 25 ml/min.

Continuous culture for H_2 production

An upflow reactor with a height of 18 cm was set up with a volume of 450 ml. 50 ml methanogenic granules were treated with 0.1% chloroform following the method mentioned previously. Chloroform treated granules were packed in the upflow reactor as an expanded bed. Glucose culture medium at 20 g COD/L was fed into the reactor at the bottom with a pump. Biogas and fermentation broth flowed out of the reactor at the top and were separated in a separation bottle. Water replacement bottles were connected to the separation bottle in order to collect the biogas and measure the biogas volume. The reactor was maintained in batch mode for three days, and then switched to continuous mode with a hydraulic retention time (HRT) of 13 hours. The HRT was adjusted quickly to 5.3 hours and kept operational for three days until it ran constantly (as indicated when the pH of the effluent, the biogas and hydrogen production, the glucose conversion, and VFA production were constant for more than 12 hours).

High solid food waste anaerobic digestion

A series of batch experiments were conducted in 1000 ml serum bottles to investigate bio-hydrogen and VFA production from food waste at different pH levels. Nine bottles were filled with 500 g food waste and 100 ml digester sludge. Total solids, food waste concentration, and sludge concentration in each bottle was 17.55% (w/w), 149.90 g/L, and 1.19 g/L, respectively. The pH value in bottles 1 – 8 was controlled at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0, respectively, by adding 2 M sodium hydroxide or 2 M hydrochloric acid. Bottle 10 was only filled with 500 g food waste and bottle 11 was only filled with 100 ml sludge. The pH value in bottle 9 – 11 was not adjusted and then used as controls.

Appendix III – Abbreviations

COD: Chemical oxygen demand
DW: Dry weight
FAME: Fatty acid methyl esters
GC: Gas chromatography
HPB: Hydrogen production broth
HRT: Hydraulic retention time
OFMSW: Organic fraction municipal solid waste
PEG: Polyethylene glycol
VFA: Volatile fatty acids

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