From raw cassava pulp to L-lactic acid via fermentation by *Rhizopus oryzae*

Nuttha Thongchul[1], Surapong Navankasattusas[1] and Shang-Tian Yang[2]*
[1]Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand
[2]Department of Chemical and Biomolecular Engineering, The Ohio State University,OH, U.S.A.

Abstract

Currently the natural crude oil depletion has been brought into awareness. This affects many industries including petrochemical manufacturing. As a result, there are many attempts to develop novel processes to produce substitutes to such petrochemical products including plastics. Polylactic acid, made from polymerization of optically pure isomer of lactic acid, is one of such examples. In addition to the application in food and pharmaceutical industries, lactic acid can be used as a chemical building block for plastics and solvents. Nowadays lactic acid is produced via fermentation by lactic acid bacteria, which require complex media and produce a racemic mixture of lactic acid. In contrast, *Rhizopus oryzae* is capable of producing an optically pure L-lactic acid from simple and inexpensive media consisting of starchy materials and pentose sugars. Raw cassava pulp, containing approximately 50% starch (dry basis), is one of the starchy materials which are abundant in Thailand. Potentially, it can be used as a carbon substrate in L-lactic acid fermentation by *R. oryzae*. Therefore, in this study we investigated L-lactic acid fermentation from raw cassava pulp. Raw cassava pulp was treated by acid hydrolysis before fermentation. The effects of hydrolysis conditions, including acid type and concentration, and time, on starch recovery and glucose production were observed. Among the conditions studied, the maximum starch recovery (88%) and the highest glucose production (98 g/L) were obtained when hydrolyzing cassava pulp at 121°C for 15 min with 1.0 M HCl (1 g dry pulp to 9 mL HCl solution). The kinetics of L-lactic acid fermentation using acid treated cassava pulp hydrolysates (ACPH) from different conditions as the carbon source was also studied. It was found that free ions present in the hydrolysates affected L-lactic acid fermentation. *R. oryzae* was capable of growing in the fermentation medium containing HCl treated cassava pulp hydrolysate (HACPH). However, instead of L-lactic acid, ethanol was produced as a major product in the fermentation. While no growth and product formation were observed in the fermentation of H_2SO_4 treated cassava pulp hydrolysate (SACPH) and H_3PO_4 treated cassava pulp hydrolysate (PACPH).

Introduction

In addition to the application in food and pharmaceutical industries, lactic acid can be used as a chemical building block for plastics and solvents (Vink et al., 2004; Zhan et al., 2003). Nowadays lactic acid is produced via fermentation by lactic acid bacteria, which require complex media and produce a racemic mixture of lactic acid. This somewhat makes bacterial fermentation uneconomically attractive. Many works reported that *Rhizopus oryzae* was capable of utilizing various agricultural residues including solid agro-industrial wastes to produce a variety of products including L-lactic acid (Cata et al., 1999; Christen et al, 2000; Pandey et al., 2000; Yu and Hang, 1989). For examples, rice brans, wheat brans, sugarcane bagasse, apple pomaces, corncobs, sorghum, soyabean, potato wastes, paper pulp sulfite liquor, steam-exploded wood hydrolysate, and cassava pulp have been used as the carbon sources of *R. oryzae* in many studies (Aikat and Bhattacharyya, 2001; Huang et al., 2003; Ruengruglikit and Hang, 2003; Sun and Xu, 2008; Taherzadeh et al., 2003; Tani, et al., 1988; Woiciechowski et al., 1999). It is noted that the processing of agro-industrial raw material like cassava produces a large amount of wastes whose accumulation leads to the environmental pollution problem. At present, cassava pulp

discharged from the cassava milling plant is used as an additive for making cassava pellets for animal feed although it can be used as a carbon substrate in bioprocessing (Nishise et al., 1988; Soccol and Vandenberghe, 2003). Cassava pulp is a starchy-rich lignocellulosic residue which contains approximately 50% starch (by weight, dry basis). Since it provides a rich organic nature and low ash content compared with other crop residues such as rice straw and wheat straw, it can be used as a substrate for bioconversion processes for the production of value-added products (Pandey et al., 2000; Sriroth et al., 2000). Therefore, application of cassava pulp in bioprocessing not only provides an alternated substrate, but also solves the pollution problem.

Thailand is one of the world's largest cassava exporters of cassava products. As a result, there has been a plenty of cassava pulp discharged daily from the cassava milling industry. Bioprocessing was used to convert starch remained in cassava pulp to value-added product, lactic acid. Due to the ability of *R. oryzae* to produce and secrete amylases, lactic acid fermentation of cassava pulp hydrolysates was investigated in this study. Before use in fermentation, raw cassava pulp was treated by acid. The effects of hydrolysis conditions, including acid type and concentration, and time, on starch recovery and glucose production were observed. In addition, the effect of the fermentation medium containing different acid treated cassava pulp hydrolysates (ACPH) on lactic acid fermentation by *R. oryzae* was investigated.

Materials and Methods

Starch recovery from raw cassava pulp

Oven-dried cassava pulp was hydrolyzed by 3 different mineral acids (HCl, H_2SO_4, and H_3PO_4) at 121°C and 15 psig. The effects of acid concentration, ratio of dry pulp to acid solution (g/mL), and hydrolysis time on reducing sugar concentration and yield were determined. After hydrolysis, the samples were centrifuged and the supernatants were collected for analysis. The reducing sugar present in the hydrolysate was analyzed by Dinitrosalicylic acid assay (Miller, 1959) and the yield of reducing sugar liberated from the pulp was determined.

Lactic acid fermentation from the hydrolysates of raw cassava pulp

Lactic acid fermentation from different ACPH by *R. oryzae* was carried out in free cell culture in the shaken flask culture at 30°C, 200 rpm. The fermentation kinetics as well as the cell growth and product formation were observed.

Microorganism

Rhizopus oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid obtained from the Northern Regional Research Center, Peroria, IL, was used in this study. The sporangiospores were collected from the 7-day culture on a potato dextrose agar (PDA) plate by shaving and extracting the spores with sterile water. The spore concentration was determined by spore counting using a haemacytometer. The spore suspension was then adjusted to desired concentration by dilution with sterile water.

Fermentation medium

Lactic acid production by *R. oryzae* consists of 2 phases, i.e. growth and production. During the growth phase, the medium consisted of 50 g/L carbon source and 5 g/L yeast extract was used for spore

germination and initial cell growth where the control of pH was not necessary during this phase. After the growth phase, the growth medium was replaced by the production medium for enhancing lactic acid production. The production medium contained 70 g/L carbon source, 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.088 g/L $ZnSO_4 \cdot 7H_2O$, and 0.3 g/L urea. 5 M NaOH was used for pH control (Thongchul and Yang, 2003). 2 groups of the carbon source were used in this study. Glucose was used as the typical carbon source in the fermentation. Another is the ACPH. The hydrolysates obtained from hydrolyzing the pulp with HCl, H_2SO_4, and H_3PO_4 were referred later as HACPH, SACPH, and PACPH, respectively.

Analytical methods

Moisture content and ash content of raw cassava pulp

The moisture content of raw cassava pulp was determined. Exact weight of raw pulp was recorded. The raw pulp was then dried at 105°C until constant weight. The moisture content (wet basis) was determined. Exact weight of moisture-free cassava pulp was recorded. Then the moisture-free pulp was put into the muffle at 600°C for 4h. After that the burnt pulp (ash) was weighed. The ash content (dry basis) was determined by dividing the weight of the ash by the exact weight of moisture-free cassava pulp.

Cell biomass

Cell biomass was harvested from the culture and washed with water to remove the residues. Washed biomass was dried at 105°C until constant weight was obtained.

Substrate and product concentrations

Glucose and L-lactic acid were analyzed by YSI 2700 glucose analyzer (Yellow Spring Instrument Co., Inc.). Before measurement, the sample was centrifuged and diluted with distilled water. High performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, xylose, arabinose, lactic acid, fumaric acid, and ethanol) present in the samples. Samples were centrifuged, filtered through cellulose acetate membrane, and diluted with double distilled water. 20 µL diluted particle-free samples were injected into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm×7.8mm) maintained at 45°C in a column oven. 0.005M H_2SO_4 was used as an eluant at 0.6 mL/min flow rate. A refractive index detector was used to detect the organic compounds. A standard containing 2 g/L of each component was injected as a reference to determine the sample concentration. The peak area was used for the comparison basis. It is noted that HPLC can detect both L(+) and (D)-lactic acids.

Dextrose equivalent (%DE)

Reducing sugar present in the hydrolysate was measured using dinitrosalicylic acid assay as described in Miller (1959). The reducing sugar was determined by comparing the absorbency at 540 nm of the reaction mixture to a standard curve of glucose solution. Dextrose equivalent was determined by the following eq.1

$$\%DE = \frac{[RS]}{[TS]} \times 100 \qquad \text{eq.1}$$

Where [*RS*] represents reducing sugar concentration liberated from raw cassava pulp (g/L) and [*TS*] is the total solid content in the hydrolysate (g/L). Total solid content was determined from drying hydrolysis solution at 105°C until constant weight. The weight amount of solid remained after drying is referred as total solid content.

Results and discussion

Raw cassava pulp

Cassava pulp obtained from cassava mill contains approximately 56.0% starch, 35.9% fiber, 5.3% protein, 2.7% ash, and 0.1% fat (dry basis) (Sriroth et al., 2000). The moisture content of raw pulp used in this study was 74.66% and the ash content was 1.21%.

Acid hydrolysis

Reducing sugar yield obtained from acid hydrolysis of oven-dried pulp at 121°C, 15 psig was observed. It was reported that steaming released uronic acids and acetyl groups in the form of acetic acid. Under these acid steaming conditions, the hemicelluloses in cassava pulp were hydrolyzed with concomitant removal of the hemicelluloses-lignin matrix (Agu et al., 1997). In addition, H^+ ion in acid molecule attacks not only starchy materials but also the lignocellulosic structure in the pulp, it should be noted that the reducing sugar as well as glucose liberated in the hydrolysate come from starch and lignocellulosic structure in cassava pulp (Urbaneja et al., 1996; Zhu et al., 2002).

Effects of reaction time and acid concentration

The reducing sugar in the hydrolysate obtained from acid hydrolysis of cassava pulp was determined. Cassava pulp was treated with 3 types of acid at different concentration under 121°C, 15 psig. Tables 1-3 show the effects of acid concentration and reaction time on reducing sugar yield. It was found that increasing acid concentration increased reducing sugar yield due to more H^+ ions dissociated; thus more chance of starch molecules in cassava pulp to be attacked (Chamy et al., 1994; Stryer, 1995). When excess H^+ ions were present at high acid concentration, reducing sugar concentration and yield were decreased indicating that reducing sugar was further hydrolyzed resulting in unwanted compounds which may inhibit microbial growth in fermentation (Malester et al., 1988; Woiciechowski et al., 1999). Potential fermentation inhibitor compounds include furan aldehydes formed by degradation of sugars, organic acid released from hemicellulose side-groups, and aldehydes and phenolics released from lignin (Nichols et al., 2008). The results also indicate that using less acid concentration required longer reaction time to reach high reducing sugar yield. It was found that the ratio of dry pulp to acid solution (g/mL) did not influence reducing sugar yield; however, the lower ratio gave lower reducing sugar concentration of which required concentration step before use in fermentation.

Type of acid and its dissociation constant

Among 3 acids, HCl provided the highest reducing sugar yield while H_3PO_4 gave the lowest yield (Tables 1-3). Comparing the dissociation constant (K_a) of these 3 acids, it is found that K_a of HCl is higher than H_2SO_4 and H_3PO_4, respectively. Therefore, more H^+ ions in HCl were readily dissociated leading to the higher chance of the starch molecules as well as the lignocellulosic material in cassava pulp to be attacked and broken down (Chamy et al., 1994; Urbaneja et al., 1996). This resulted in the

higher reducing sugar concentration obtained and consequently the higher yield with less acid concentration used at the same reaction time.

Table 1 Reducing sugar present in the hydrolysate of oven-dried cassava pulp after hydrolysis by HCl under different conditions

Hydrolysis time (min)	HCl conc. (M)	Ratio of oven-dried cassava pulp to acid solution (g/mL)			
		1 g/9 mL		1 g/18 mL	
		Reducing sugar in hydrolysate (g/L)	Reducing sugar yield (% wt; g RS/g DP)	Reducing sugar in hydrolysate (g/L)	Reducing sugar yield (% wt; g RS/g DP)
15	0.25	86.28	77.66	46.35	83.44
	0.50	109.28	98.35	53.90	97.01
	0.75	108.32	97.49	**53.97**	97.15
	1.00	**111.50**	100.35	51.83	93.29
	1.25	101.66	91.50	50.87	91.56
	1.50	100.26	90.23	52.20	93.95
	1.75	103.59	93.23	42.81	77.05
	2.00	98.19	88.37	43.54	78.38
30	0.25	102.85	92.56	37.85	68.13
	0.50	**103.88**	93.49	47.09	84.77
	0.75	102.55	92.30	45.47	81.84
	1.00	98.93	89.04	**49.39**	88.90
	1.25	93.01	83.71	45.39	81.71
	1.50	90.50	81.45	44.00	79.18
	1.75	98.41	88.57	43.03	77.45
	2.00	90.05	81.05	39.11	70.39
45	0.25	83.40	75.06	42.07	75.72
	0.50	**102.55**	92.30	44.80	80.64
	0.75	91.09	81.98	45.76	82.37
	1.00	99.15	89.23	**47.69**	85.83
	1.25	88.21	79.39	46.43	83.57
	1.50	88.43	79.59	46.21	83.17
	1.75	91.83	82.65	38.00	68.40
	2.00	92.57	83.31	37.26	67.07
60	0.25	**110.46**	99.42	41.70	75.05
	0.50	108.91	98.02	41.62	74.92
	0.75	104.40	93.96	**47.09**	84.77
	1.00	98.85	88.97	46.58	83.84
	1.25	97.52	87.77	45.98	82.77
	1.50	93.68	84.31	44.51	80.11
	1.75	90.50	81.45	43.62	78.51
	2.00	94.05	84.64	37.33	67.20

Remark:

- 3 replicates of hydrolysis were conducted at each hydrolysis condition

Table 2 Reducing sugar present in the hydrolysate of oven-dried cassava pulp after hydrolysis by H_2SO_4 under different conditions

Hydrolysis time (min)	H_2SO_4 conc. (M)	Ratio of oven-dried cassava pulp to acid solution (g/mL)			
		1 g/9 mL		1 g/18 mL	
		Reducing sugar in hydrolysate (g/L)	Reducing sugar yield (% wt; g RS/g DP)	Reducing sugar in hydrolysate (g/L)	Reducing sugar yield (% wt; g RS/g DP)
15	0.25	83.10	74.79	33.85	60.93
	0.50	83.98	75.59	40.73	73.31
	0.75	83.98	75.59	41.84	75.31
	1.00	91.08	81.97	**43.61**	78.50
	1.25	90.64	81.57	39.17	70.51
	1.50	**93.08**	83.77	37.84	68.12
	1.75	87.09	78.38	37.84	68.12
	2.00	88.20	79.38	38.44	69.18
30	0.25	73.19	65.87	23.06	41.50
	0.50	78.22	70.39	35.63	64.13
	0.75	77.11	69.40	35.77	64.39
	1.00	74.08	66.67	38.07	68.52
	1.25	82.51	74.25	**47.01**	84.62
	1.50	**93.01**	83.70	45.16	81.30
	1.75	71.86	64.67	39.40	70.91
	2.00	70.38	63.34	38.44	69.18
45	0.25	70.67	63.61	26.31	47.36
	0.50	69.34	62.41	35.03	63.06
	0.75	75.11	67.60	36.96	66.52
	1.00	**75.78**	68.20	**39.91**	71.85
	1.25	73.56	66.20	20.39	36.71
	1.50	70.90	63.81	18.18	32.72
	1.75	69.64	62.68	35.33	63.59
	2.00	67.42	60.68	34.07	61.33
60	0.25	55.59	50.03	35.33	63.39
	0.50	66.46	59.81	37.54	67.59
	0.75	66.24	59.61	36.96	66.52
	1.00	66.83	60.15	36.66	65.99
	1.25	74.37	66.93	41.10	73.98
	1.50	76.44	68.80	33.70	60.67
	1.75	77.55	69.80	**41.39**	74.51
	2.00	**87.68**	78.91	34.89	62.80

Remark:

- 3 replicates of hydrolysis were conducted at each hydrolysis condition

Table 3 Reducing sugar present in the hydrolysate of oven-dried cassava pulp after hydrolysis by H_3PO_4 under different conditions

Hydrolysis time (min)	H_3PO_4 conc. (M)	Ratio of oven-dried cassava pulp to acid solution (g/mL)			
		1 g/9 mL		1 g/18 mL	
		Reducing sugar in hydrolysate (g/L)	Reducing sugar yield (% wt; g RS/g DP)	Reducing sugar in hydrolysate (g/L)	Reducing sugar yield (% wt; g RS/g DP)
15	0.25	46.58	41.92	26.39	47.50
	0.50	63.95	57.56	30.53	54.96
	0.75	86.65	77.99	38.74	69.73
	1.00	88.80	79.92	39.18	70.53
	1.25	85.17	76.66	42.07	75.72
	1.50	95.38	85.84	44.21	79.58
	1.75	**97.74**	87.97	**47.83**	86.10
	2.00	95.67	86.11	40.81	73.46
30	0.25	24.10	21.69	11.01	19.82
	0.50	37.26	33.53	17.89	32.20
	0.75	48.20	43.38	23.43	42.18
	1.00	54.71	49.24	27.13	48.83
	1.25	70.68	63.61	28.02	50.43
	1.50	68.46	61.62	**50.87**	91.56
	1.75	73.42	66.08	43.84	78.91
	2.00	**74.82**	67.34	49.98	89.96
45	0.25	15.89	14.30	20.03	36.06
	0.50	36.81	33.13	25.72	46.30
	0.75	46.28	41.65	29.94	53.89
	1.00	52.86	47.58	31.34	56.42
	1.25	58.48	52.63	33.49	60.28
	1.50	62.70	56.43	32.01	57.62
	1.75	**73.64**	66.28	33.34	60.01
	2.00	71.20	64.08	**36.89**	66.40
60	0.25	36.22	32.60	9.75	17.56
	0.50	37.26	33.53	26.98	48.57
	0.75	51.23	46.11	30.75	55.35
	1.00	59.22	53.30	33.12	59.61
	1.25	62.92	56.63	39.40	70.93
	1.50	**75.27**	**67.74**	**41.25**	74.25
	1.75	68.17	61.35	40.07	72.13
	2.00	73.05	65.74	36.22	65.20

Remark:

- 3 replicates of hydrolysis were conducted at each hydrolysis condition

Glucose production by acid hydrolysis

Reducing sugar yield obtained from acid and enzymatic hydrolysis of cassava pulp was compared. The highest reducing sugar concentration of 111.50 g/L with up to 100% reducing sugar yield was obtained when hydrolyzing cassava pulp at 121°C, 15 psig for 15 min using 1.0 M HCl solution at the ratio of 1 g dry pulp to 9 mL acid solution comparing with other conditions studied in acid hydrolysis (Tables 1-3). Nevertheless, these products from acid hydrolysis also contained plenty of acid salts which may harm the microorganisms and consequently inhibit desired product formation when directly be used as the carbon source in fermentation (Woiciechowski et al., 1999).

L-lactic acid fermentation

Different ACPH obtained from digesting the pulp with 1.0 M HCl, 1.5 M H_2SO_4, and 1.75 H_3PO_4 at 121°C, 15 psig, 15 min were used as the sole carbon source in the fermentation medium. The fermentation kinetics of *R. oryzae* cultivation in the media containing different ACPH was compared with the cultivation in the simple glucose medium.

L-lactic acid fermentation from glucose

Lactic acid fermentation consisted of 2 phases. In growth phase, yeast extract was used as a nitrogen source for promoting spore germination and initial cell growth. While during the production phase, urea was used as the nitrogen source with the help of $ZnSO_4$ for limiting growth and maintaining fungal activity to produce lactic acid. Fermentation kinetics with glucose as the carbon source is shown in Figure 1. It was found that ethanol was observed as a major product (0.2991 g ethanol/g glucose) in the growth phase instead of cell growth. While during the production phase, lactic acid was a major product (0.3097 g lactate/g glucose). Cell biomass and ethanol production was still found in the production phase resulting in less lactic acid yield and productivity than expected (Table 4).

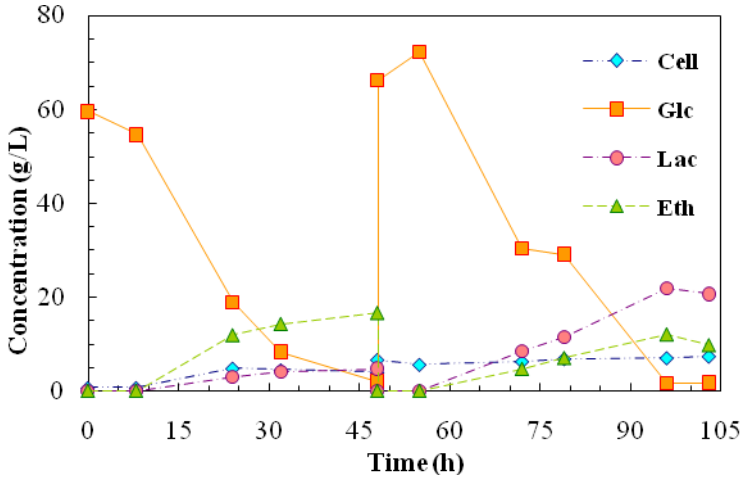

Figure 1 Fermentation kinetics of lactic acid production by *R. oryzae* at 30°C, 200 rpm using glucose as a carbon source

Table 4 Cell biomass and product formation in lactic acid fermentation using glucose as the carbon source

Phase	Max. lactate (g/L)	Yield (g/g)			Productivity (g/L·h)		
		Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
Growth	4.77	0.1068	0.0835	0.2991	0.0873	0.1135	0.3998
Production	21.82	0.0139	0.3097	0.1606	0.0183	0.4322	0.2207

L-lactic acid fermentation from the acid hydrolysates

HACPH, SACPH, and PACPH were used in lactic acid fermentation. Glucose and reducing sugar concentrations in the hydrolysates were determined. The pH of the hydrolysates was approximately 0.3. Before fermentation, pH of the hydrolysate was adjusted to 6.0 (an optimal pH of *R. oryzae*) using concentrated NaOH solution. It was observed that the largest amount of NaOH was required in adjusting pH of PACPH whereas the smallest amount of NaOH was used in adjusting pH of HACPH. This was because the difference in HCl (1.0 M) and H_3PO_4 (1.75 M) concentrations were used in cassava pulp hydrolysis resulting in the difference in H^+ ions present in the hydrolysates. Therefore, the different amount of OH^- ions from NaOH solution was required in adjusting the pH of the hydrolysates. After adjusting the pH and diluting to the desired concentration, acid hydrolysates were used as the carbon source in the fermentation medium. It was found that growth and product formation were observed in fermentation of HACPH while no growth was found in fermentation of SACPH and PACPH. It is believed that a large amount of the remaining ions (Na^+, SO_4^{2-}, and PO_4^{3-}) in the hydrolysates caused growth inhibition resulting in no growth and product formation in the fermentation of SACPH and PACPH. By detoxification of these hydrolysates, it would help improve microbial growth and product formation in the fermentation process. The examples of the detoxification of the hydrolysates include adsorption on resins or activated charcoal, precipitation and filtration, and extraction with an organic solvent system such as Alamine 336 and Aliquat 336 dissolved in octanol (Gamez et al., 2006; Grzenia et al., 2008; Nichols et al., 2008; Qureshi et al., 2008).

Fermentation kinetics of *R. oryzae* grown in the medium containing HACPH is shown in Figure 2. Instead of lactic acid, ethanol was found as the major product in both growth and production phases. In this work, we were able to produce high cell density in the growth phase; however, in the production phase a large amount of cell biomass converted glucose to ethanol (Table 5). This was probably due to an operating condition in the shaken flask culture of *R. oryzae* grown in the HACPH medium favored ethanol production (Taherzadeh et al., 2003). In addition, oxygen limitation inside the mycelial clump in free cells caused metabolic shift to anaerobic route to yield ethanol as the major product. Previous study on lactic acid fermentation by pelletized *R. oryzae* indicated that lactic acid production was improved when culturing in a bioreactor due to better oxygen transfer and mixing compared to the culture in the shaken flask (Liu et al., 2006). Therefore, it is believed that with better oxygen transfer in the fermentation system, it is possible to achieve high cell production in the growth phase. Consequently

with high cell biomass with good morphological control and sufficient oxygen supply, lactic acid production will be promoted in the production phase. Therefore, controlling oxygen supply is one of the major keys in lactic acid fermentation.

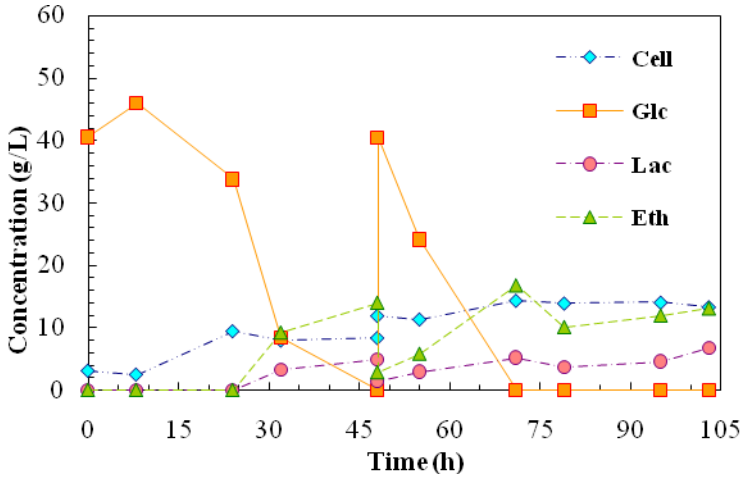

Figure 2 Fermentation kinetics of lactic acid production by *R. oryzae* at 30°C, 200 rpm using cassava pulp hydrolysate obtained from hydrolysis with 1.0 M HCl at 121°C, 15 psig, 15 min as a carbon source

Table 5 Cell biomass and product formation in lactic acid fermentation using HCl treated cassava pulp hydrolysate as the carbon source

Phase	Max. lactate (g/L)	Yield (g/g)			Productivity (g/L·h)		
		Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
Growth	4.87	0.1090	0.1100	0.3150	0.1367	0.1068	0.3065
Production	6.67	0.0648	0.0926	0.3537	0.1193	0.1588	0.6201

Comparison of the sole carbon source used in lactic acid fermentation

It was observed that the lower cell biomass production was obtained in fermentation of glucose (the highest concentration of 7.38 g/L) as compared with the production from HACPH (14.23 g/L). High ethanol production was observed as a consequence of high cell biomass production in fermentation of HACPH (16.80 g/L during the growth phase). Taherzadeh et al. (2003) reported that poor medium compositions and cultivation conditions resulted in high lactic acid yield whereas ethanol and cell biomass yields were high in a rich medium. They found that high ethanol and cell biomass production was achieved when growing *R. oryzae* in paper pulp sulfite liquor containing 45% dry weight materials including 60% lignosulfonate, 12 g/L galactose, 21 g/L glucose, 75 g/L mannose, 25 g/L xylose, and 6

g/L acetic acid. They indicated that the rich medium resulted in faster sugar uptake and a higher ethanol yield whereas lactic acid yield was lower. In contrast, a poor medium (a synthetic medium containing glucose as the sole carbon source) resulted in slower sugar uptake, higher lactic acid yield, and lower ethanol yield. Thongchul (2005) reported that high cell biomass was produced with low lactic acid yield and productivity in the fermentation of corn fiber hydrolysate by *R. oryzae* NRRL395 in a Rotating Fibrous Bed bioreactor. Raw cassava pulp used in this study contained approximately 56% starch, 35.9% fiber, 5.3% protein, 2.7% ash, and 0.1% fat (dry basis) (Sriroth et al., 2000). Hydrolysis product of cassava pulp contained such materials in the soluble forms which probably promoted cell biomass and ethanol production; therefore, resulting in higher cell biomass and ethanol production but lower lactic acid production as compared with those obtained from culture in the glucose medium.

Conclusion

This study showed that the hydrolysis products of cassava pulp could be used as a sole carbon source by *R. oryzae* to produce cell biomass, lactic acid, and ethanol. Acid hydrolysis at high temperature and pressure yielded high concentration of reducing sugar liberated from cassava pulp within a short time. However, the presence of the remaining ions (Na^+, SO_4^{2-}, and PO_4^{3-}) in the hydrolysates caused growth inhibition resulting in no growth and product formation in the fermentation of SACPH and PACPH. By detoxification, it is believed that *R. oryzae* can grow in detoxified SACPH and PACPH. Instead of lactic acid, cell biomass and ethanol were the major products found in the fermentation of HACPH while *R. oryzae* produced lactic acid as the major product from the simple glucose medium. This was perhaps due to some proteins and fibers in the pulp remained soluble in the hydrolysates enriched the fermentation medium. Thus, the fermentation medium containing the hydrolysates favored cell biomass production and consequently led to ethanol formation due to excess growth and oxygen limitation. Oxygen limitation could be diminished by high agitation and aeration in the bioreactor and lactic acid production could be improved as compared to that in the shaken flask culture.

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References

1. Agu, R.C., Amadife, A.E., Ude, C.M., Onyia, A., Ogu, E.O., Okafor, M., 1997. Combined heat treatment and acid hydrolysis of cassava grate waste (CGW) biomass for ethanol production. Waste Management. 17, 91-96.
2. Aikat, K., Bhattacharyya, B.C., 2001. Protease production in solid state fermentation with liquid medium recycling in a stacked plate reactor and in a packed bed reactor by a local strain of *Rhizopus oryzae*. Process Biochemistry. 36, 1059-1068.
3. Cata, F.S., Soccol, C.R., Ramos, L.P., Fontana, J.D., 1999. Production of fumaric acid by fermentation of enzymatic hydrolysates derived from cassava bagasse. Bioresource Technology. 68, 23-28.
4. Chamy, R., Illanes, A., Aroca, G., Nunez, L., 1994. Acid hydrolysis of sugar beet pulp as pretreatment for fermentation. Bioresource Technology. 50, 149-152.

5. Christen, P., Bramorski, A., Revah, S., Soccol, C.R., 2000. Characterization of volatile compounds produced by *Rhizopus* strains grown on agro-industrial solid wastes. Bioresource Technology. 71, 211-215.
6. Gamez, S., Gonzalez-Cabriales, J.J., Ramirez, J.A., Garrote, G., Vazquez, M., 2006. Study of the hydrolysis of sugar cane bagasse using phosphoric acid. Journal of Food Engineering. 74,78-88.
7. Grzenia, D.L., Schell, D.J., Wickramasinghe, S.R., 2008. Membrane extraction for removal of acetic acid from biomass hydrolysates. Journal of Membrane Science. 322, 189-195.
8. Huang, L.P., Jin, B., Lant, P. and Zhou, J., 2003. Biotechnological production of lactic acid integrated with potato wastewater treatment by *Rhizopus arrhizus*. Journal of Chemical Technololgy and Biotechnology. 78, 899-906.
9. Liu, Y., Liao, W., Liu, C., Chen, S., 2006. Optimization of L-(+)-lactic acid production using pelletized filamentous *Rhizopus oryzae* NRRL395. Applied Biochemistry and Biotechnology. 129-132, 844-853.
10. Malester, A.I., Green, M., Kimchie, S., Shelef, G., 1988. The effect of the neutralizing capacity of cellulosic materials on the kinetics of cellulose dilute acid hydrolysis. Biological Waste. 26, 115-124.
11. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31, 426-428.
12. Nichols, N.N., Sharma, L.N., Mowery, R.A., Chambliss, C.K., 2008. Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate. Enzyme and Microbial Technology. 42, 624-630.
13. Nishise, H., Fuji, A., Ueno, M., Vongsuvanlert, V., Tani, Y., 1988. Production of raw cassava starch-digestive glucoamylase by *Rhizopus* sp. in liquid culture. Journal of Fermentation Technology. 66, 397-402.
14. Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T., Vandenberghe, L.P.S., Mohan, R., 2000. Biotechnological potential of agro-industrial residues. II: cassava bagasse. Bioresource Technology. 74, 81-87.
15. Qureshi, N., Ezeji, T.C., Ebener, J., Dien, B.S., Cotta, M.A., Blaschek, H.P., 2008. Butanol production by *Clostridium beijerinckii*. Part I: use of acid and enzyme hydrolyzed corn fiber. Bioresource Technology. 99, 5915-5922.
16. Ruengruglikit, C. and Hang, Y.D., 2003. L(+)-lactic acid production from corncobs by *Rhizopus oryzae* NRRL-395. LWT Food Science and Technology. 36, 573-575.
17. Soccol, C.R., Vandenberghe, L.P.S., 2003. Overview of applied solid-state fermentation in Brazil. Biochemical Engineering Journal. 13, 205-218.
18. Sriroth, K., Chollakup, R., Chotineeranat, S., Piyachomkwan, K. and Oates, C.G., 2000. Processing of cassava waste for improved biomass utilization. Bioresource Technology. 71, 63-69.
19. Stryer, B., 1995. Biochemistry, fourth ed. W.H. Freeman and Company, New York.
20. Sun, S.Y., Xu, Y., 2008. Solid-state fermentation for 'whole-cell synthetic lipase' production from *Rhizopus chinensis* and identification of the functional enzyme. Process Biochemistry. 43, 219-224.
21. Taherzadeh, M.J., Fox, M., Hjorth, H., Edebo, L., 2003. Production of mycelium biomass and ethanol from paper pulp sulfite liquor by *Rhizopus oryzae*. Bioresource Technology. 88, 167-177.
22. Tani, Y., Fuji, A., Nishise, H., 1988. Production of raw cassava starch-digestive glucoamylase by a 2-deoxyglucose-resistant mutant of *Rhizopus* sp. Journal of Fermentation Technology. 66, 545-551.
23. Thongchul, N. Lactic acid production by immobilized *Rhizopus oryzae* in a rotating fibrous bed bioreactor. PhD thesis. The Ohio State University. Columbus, Ohio. 2005.

24. Thongchul, N., Yang, S.-T., 2003. Controlling filamentous fungal morphology by immobilization on a rotating fibrous matrix to enhance oxygen transfer and L(+)-lactic acid production by *Rhizopus oryzae*, in: Saha, B.C. (Ed.), Fermentation Biotechnology. Oxford University Press, MA, 2003, pp. 36-51.
25. Urbaneja, G., Ferrer, J., Paez, G., Arenas, L., Colina, G., 1996. Acid hydrolysis and carbohydrates characterization of coffee pulp. Renewable Energy. 9, 1041-1044.
26. Vink, E.T.H., Rabago, K.R., Glassner, D.A., Springs, R., O'Corner, R.P. and Gruber, P.R., 2004. The sustainability of NatureWorks™ polylactide polymers and Ingeo™ polylactide fibers[a]: an update of the future. Macromolecular Bioscience. 4, 551-564.
27. Woiciechowski, A.L., Soccol, C.R., Ramos, L.P. and Pandey, A., 1999. Experimental design to enhance the production of L-(+)-lactic acid from steam-exploded wood hydrolysate using *Rhizopus oryzae* in a mixed-acid fermentation. Process Biochemistry. 34, 949-955.
28. Yu, R.-C., Hang, Y.D., 1989. Kinetics of direct fermentation of agricultural commodities to L(+)-lactic acid by *Rhizopus oryzae*. Biotechnology Letters. 11, 597-600.
29. Zhan, X., Wang, D., Tuinstra, M.R., Bean, S., Seib, P.A., Sun, X.S., 2003. Ethanol and lactic acid production as affected by sorghum genotype and location. Industrial Crop and Product. 18, 245-255.
30. Zhu, Y., Wu, Z. and Yang, S.-T., 2002. Butyric acid production from acid hydrolysate of corn fiber by *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. Process Biochemistry. 38, 657-666.