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Stress of pH and acetate on product formation of fermenting polysaccharide-rich organic waste

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Abstract

Effects of stress of pH and/or acetate on the fermentation product formation of polysaccharide-rich organic waste were examined in this work, with externally dosed acetate (up to 333 mmol l^{-1}) and adjusted pH (5–9). Ethanol, acetate, and lactate were the main metabolites. Concentrations and dissociation states of acetate affected the productions of ethanol and lactate. With dosed acetate, ethanol was favorably produced at pH 6, but was completely inhibited at pH 5. On the other hand, lactate was less favorably produced with dosed acetate, but was also completely inhibited at pH 5. With no externally dosed acetate, the metabolism gradually shifted from acetate to lactate and ethanol-forming pathway with fermentation. With dosed acetate, except for at pH 5, the pathway to ethanol was strengthened with time. The acetate stress on product formation was stronger than from pH stress.

Keywords: Anaerobic processes; Fermentation; Acetic acid; Ethanol; Product inhibition; Anaerobic Digestion Model No. 1 (ADM1)

1. Introduction

Hydrolysis and acidogenesis of particulate organic waste was the key stage of anaerobic digestion for reducing the amount of solids phase and supplying favorable precursors for the subsequent biogas production, biological nutrient removal, polylactic-acid production, and others [1,2]. The diversity of product compositions was due to various metabolic pathways of microbial populations involved in fermentation, which were affected by several factors, such as substrate compositions, pH, temperature, retention times, organic loading rate, etc. [3–5].

Volatile fatty acids (VFA) were the major acidogenic products, and would impose product inhibition on acidogenesis, following an incompetitive pattern or non-competitive pattern [6,7]. Besides, VFA might also affect the metabolic pathways [8,9]. As pH dropped owing to acetate accumulation, microbes would limit internal acidification from metabolism by producing lactate or butyrate instead of acetate, or by reuptaking acetate to acetyl-coenzyme A [10,11]. Meanwhile, detailed knowledge on how high levels of VFA affected the fermentation pathways remained insufficient [12]. It is also unclear whether pH dropped or VFA themselves affected the metabolic pathways.

The pH was acknowledged to be a key parameter to regulate product distribution in fermentation [11,13,14]. The Anaerobic Digestion Model No. 1 (ADM1) proposed ethanol as an alternative metabolic pathway to acetic acid, propionic acid and butyric acid only to be considered at low pH (<5.0) [15]. However, it was also reported that higher ethanol was produced from glucose fermentation at pH 7.9 than at pH 4.5 [8]. For the fermentation of protein-type substrate (gelatin), the ethanol production was minor at less than 4.4% over pH 4–7 [5]. The inconsistent conclusions by these authors indicated that the regulation of pH on product distribution might not be independent and should be interfered by other factors.

The product inhibitory effects on acidogenesis were usually assumed to follow the same kinetics over the entire acidogenesis test period regardless of the metabolite species for the sake of model simplification [16]. Restated, a constant inhibition factor was accepted to the biomass concentration in the model, rather than to individual acidogenic products, within a broad range of pH value. That means acidogenic products are assumed to have fixed ratio, even when acidogenic biomass encounter inhibition. During the anaerobic treatment of solid waste, e.g. dry-type digestion or bioreactor landfill, low pH and acidogenic

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products accumulation were often the main problems (e.g. pH 3.9, 50 g l^{-1} VFA) [12,17]. It was unknown whether the acidogenic product formation/distribution would be altered at such kind of extremely inhibitory environments.

We demonstrated in this work that the production of different acidogenic products would be inhibited differently by the same inhibitor. The effects of pH and acetate on fermentative pathways of polysaccharide-rich organic waste were explored.

2. Materials and methods

2.1. Materials

Fresh potato was the testing substrate, cut into cubes of size 2–3 mm and stored at 4 °C for 12–24 h. The basic characteristics of the potato samples were listed in Table 1. The potato contained much carbohydrate and some proteins. Highly concentrated acetate (20 g l^{-1}) was externally added to some samples for investigating its inhibition effects on dissolution and acidogenesis of the given potato. The concentration ratio of total acetate and the substrate was estimated stoichiometrically using "$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$" [16]. The concentration of carbohydrate in tested potato was 60 g l^{-1} (based on Table 1), leading to a maximum yield of total acetate from tested potato of 40 g l^{-1}. Given that half carbohydrate had been fermented to acetate, the fermentative tests demonstrated the subsequent fermentation in a reactor accumulated with these acetates. The concentration of externally added acetate was 20 g l^{-1}.

. Fermentation test

Two batches of hydrolysis tests with different dosed acetate concentrations were conducted. The batch I test was conducted with no dosed acetate and at constant pHs (5–9), adjusted by adding NaOH and HCl solutions. The batch II test was conducted at constant pHs (5–9) and with 333 mmol l^{-1} of acetate (equal to 20 g l^{-1}). In each test, 20 g of wet potato was wrapped in gauze bags (80 mesh), and hung in sealed bottles with 50 ml above mentioned leaching liquors. The bottles were shaken at 100 rpm and the temperature was kept at 37 ± 0.2 °C. It was noted that fermentation could spontaneously start anaerobically or aerobically [2,18,19]. Therefore, no additional inoculums were added in the present tests. The microbial organisms dominant in the reactive system were indigenous and evolved selectively under certain circumstance, e.g. stress from pH or acetate. The leaching liquor was replaced by fresh one (fixed pH, dosed acetates, etc.) after 4, 8, 12, 16, 25, 36, 50, 74, and 98 h, respectively. With the liquor replacement, the changes in pH and the acetate level of suspension in the test were minimal. The yields reported were the concentrations of species noted in the collected samples subtracted by background. The oxidation and reduction potentials (ORP) were all at 54 ± 20 mV for all samples tested. Two replicates were set for each batch, with their averages reported.

Table 1
The physiochemical characteristics of experimental materials

Total solid (TS) (g g^{-1}-wet sample)	0.18
Volatile solid (VS) (g g^{-1}-TS)	0.96
Carbohydrate (g g^{-1}-VS)	0.87
Protein (g g^{-1}-VS)	0.12
Lipid (g g^{-1}-VS)	0.005
Element C (g g^{-1}-VS)	0.41
Element N (g g^{-1}-VS)	0.14

2.3. Analytic methods

The collected leaching liquors were filtered using 0.45 μm polyester film, and measured for pH, ORP, chemical oxygen demand (COD), and metabolites (formate, lactate, methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, acetate, propionate, *n*-butyrate, *i*-butyrate, *n*-valerate, and *i*-valerate). The pH and ORP of the suspension were measured by a pH/ORP meter (OAKTON Instruments, Illinois, USA). COD was measured with COD Reactor Model 45600 and DR/890 Datalogging Colorimeter (HACH Company, Colorado, USA). Formate and lactate were colorimetrically determined [20,21]. The colorimetric assays were verified to be reliable even in the presence of high concentrated acetate, when compared with the measurement by Liquid Chromatography Organic Analysis System (SHIMADZU Co. Ltd., Japan). Alcohols were measured with GC-102 gas chromatography (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China) equipped with flame ionization detector and a Ø5 mm × 3000 mm stainless-steel column packed with Porapack Q (60/80 mesh). The operational temperatures of the injection port, the column, and the flame ionization detector were 200, 145, and 230 °C, respectively. Helium gas was the carrier gas at a flow rate of 15 ml min^{-1}. The detectable levels were 20 mg l^{-1} for individual alcohols. The VFA were measured by GC-122 gas chromatograph (Shanghai Precision & Scientific Instrument Co. Ltd., Shanghai, China) equipped with flame ionization detector and a DM-FFAP capillary column (30 m × 0.32 mm i.d. × 0.25 μm). The operational temperatures for the injection port and the flame ionization detector were 200 and 230 °C. The temperature of the column was initially 80 °C for 4 min, followed by 130 °C for 2 min, and the temperature increment was 7.5 °C min^{-1}. H_2 was used as the carrier gas at a flow rate of 20 ml min^{-1}. The split ratio was 16:1. The detectable levels were 10 mg l^{-1} for individual VFA.

The gas pressure in the headspace of each sealed bottle was measured with syringes prior to liquid sampling. Minor gas production was noted over all testing periods.

3. Results and discussion

3.1. Experimental results

3.1.1. Hydrolysis

As shown in Fig. 1, the dissolved COD of batch I without external acetate addition reached a similar plateau of 31,000 ± 1200 mg l^{-1} in 25 h regardless of pH, except that at pH 5.0 (26,000 mg l^{-1}). When 333 mmol l^{-1} of acetate was

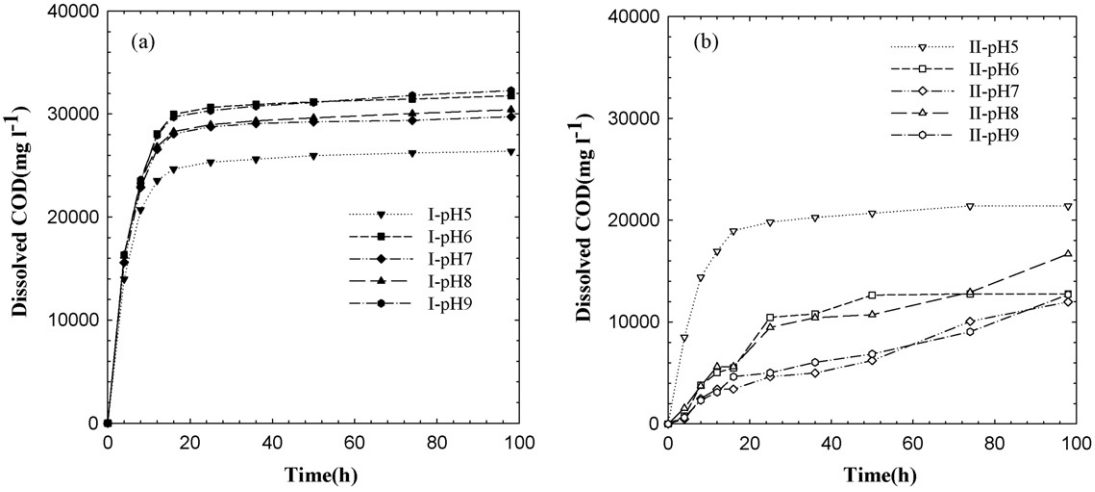

Fig. 1. Dissolved COD in liquor at different pH. (a) Without acetate; (b) with 333 mmol l^{-1} acetate.

added, the dissolved COD reached 21,000 mg l^{-1} at pH 5.0 in 25 h. The hydrolysis was much slower and reached a much lower plateau at pH >6. Since the dissociated acetate at pH 5.0 would be less than at elevated pHs, the dissociated acetate appeared a key inhibitor to potato hydrolysis. In literature, the undissociated organic acids were regarded inhibitive to microorganisms, because undissociated acid could pass through cell membranes more easily than dissociated form [15]. However, hydrolysis was catalyzed by extracellular enzymes [22], indicating that acid would inhibit hydrolysis without passing through cell membranes, then dissociated acetate could affect hydrolysis directly. Moreover, the starch could be acetylated by acetic acid or acetate anhydride at alkali conditions [23,24], while the acetylated starch has hydrophobic surface [25]. These occurrences might correspond to the lower hydrolysis rate noted in batch II tests.

Although the loss of carbon into gas was minimal, the dissolved CO_2 could be evaluated. When calculated on the metabolic reaction with the measured concentration of acidogenic products [15], the amount of by-produced CO_2 was about 7% of the dissolved COD. Hence, the gas phase loss was not significant to the present system.

3.1.2. Acidogenesis

Figs. 2–6 show the time evolutions of both batches on yields of methanol, ethanol, formate, acetate, and lactate, respectively. A constant yield over time indicates no further hydrolysis occurs in that period of time interval. The acetate concentrations of batch II tests were not reported herein since the initially dosed high level of acetate interfered reliable determination of the metabolized acetate. Other VFA and solvents were not detected in a large amount. In general, the production amounts of metabolites follow: acetate > lactate > ethanol > methanol > formate. The yields of metabolites after 98 h of fermentation were listed in Table 2.

As shown in Fig. 2, the externally dosed acetate had not only stimulated methanol yield from 0–1.6 mmol l^{-1} to 0.6–4.8 mmol l^{-1} with 333 mmol l^{-1} dosed acetate, but also shorten the initial induction period (8–12 h without and 0–4 h

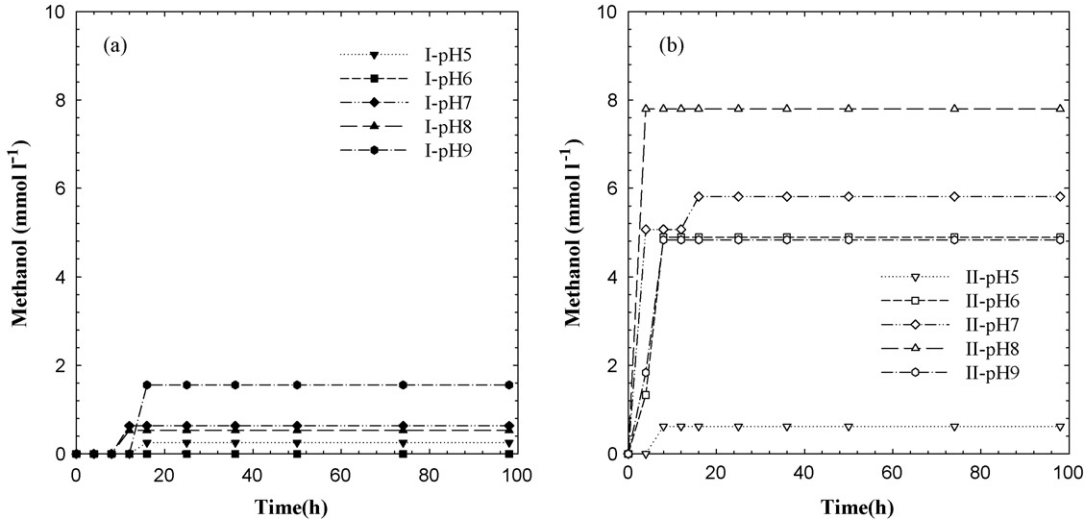

Fig. 2. Methanol yields at different pH. (a) Without acetate; (b) with 333 mmol l^{-1} acetate.

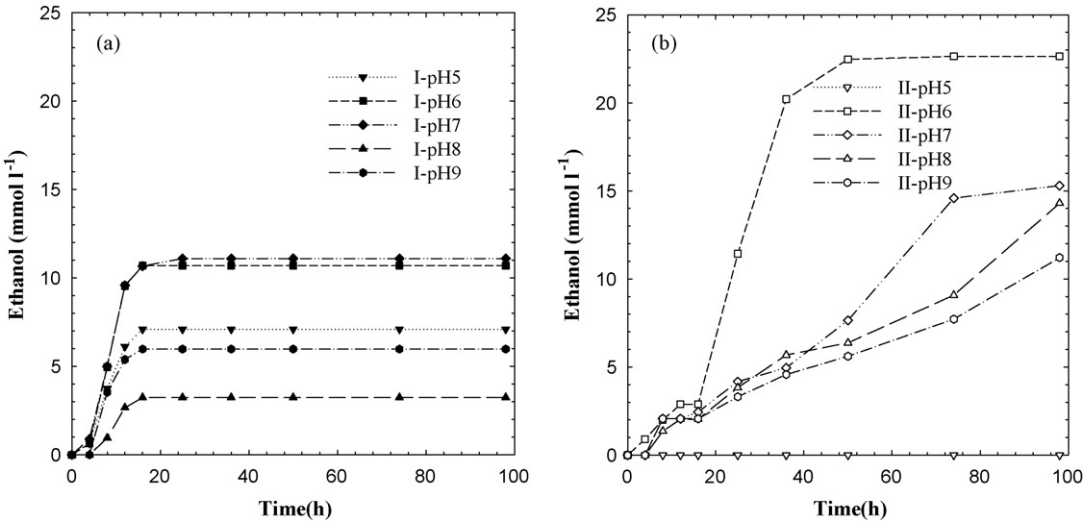

Fig. 3. Ethanol yields at different pH. (a) Without acetate; (b) with 333 mmol l^{-1} acetate.

with dosed acetate). Moreover, methanol was favorably produced under alkaline conditions.

Fig. 3 reveals that the ethanol was produced within 16 h without dosed acetate, following their plateaus at sequence: pH 6 ≈ pH 7 > pH 5 > pH 9 > pH 8. If 333 mmol l^{-1} of acetate was added, the ethanol would be yielded in a different way. Ethanol was essentially not present at pH 5. At pH 6, the ethanol was produced in a mass amount, particularly during the period of 16–44 h. Ethanol production decreased with increasing pH at alkaline condition.

Formate production was also rapidly formed within 12 h without dosed acetate, approaching plateaus following pH 7 > pH 8 ≈ pH 9 > pH 6 > pH 5 (Fig. 4). With acetate addition, again, formate was produced at a much higher level but took longer time to reach plateaus (>98 h). In general, neutral condition was favorable for formate production.

Without acetate addition, the acetate was produced within 8–12 h, with plateaus following: pH 6 ≈ pH 7 ≈ pH 8 > pH 9 > pH 5 (Fig. 5). The batch II tests with dosed acetate presented a high background for preventing correct estimate of acetate formation.

As shown in Fig. 6, the lactate productions reached plateaus within 16 h, following pH 6 > pH 9 > pH 7 ≈ pH 5 ≈ pH 8 with no acetate dosing. The presence of acetate suppressed lactate formation except for at pH 6, while particularly at pH 5 where no lactate was present.

Metabolic pathways change with the stress of acetate and/or pH. With no acetate addition, acetate was the dominant metabolite over pH 5–9, followed by lactate. But at pH 5–6, the production of lactate increased, resulting in lactate as important as acetate in the acidogenic phase. When high level of acetate presented, methanol and formate increased greatly; while the production of ethanol was impaired slightly at pH 7–9, enhanced at pH 6, and completely inhibited at pH 5. The production of lactate was impaired slightly at pH 6–9, and was completely inhibited at pH 5.

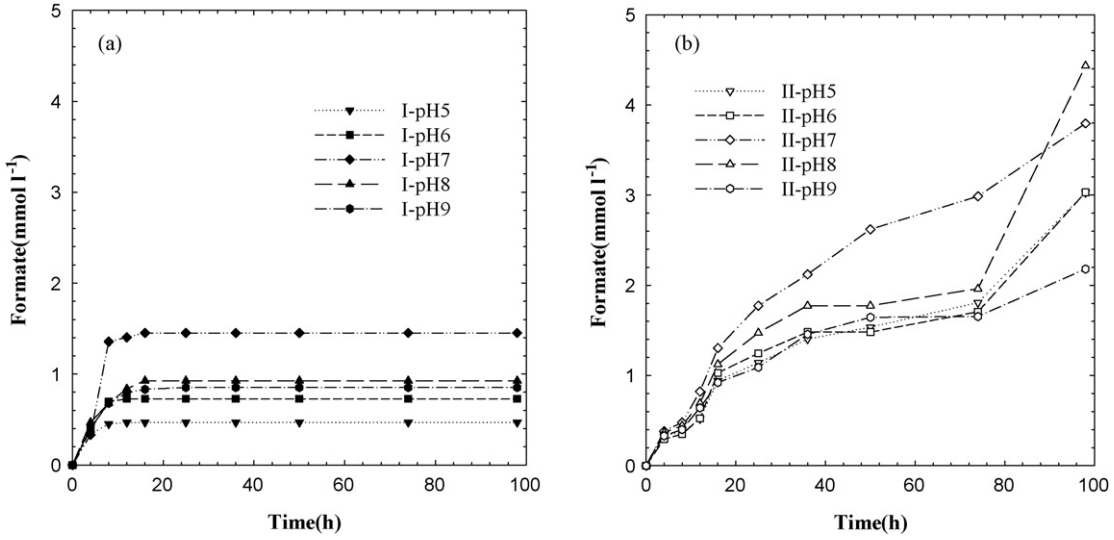

Fig. 4. Formate yields at different pH. (a) Without acetate; (b) with 333 mmol l^{-1} acetate.

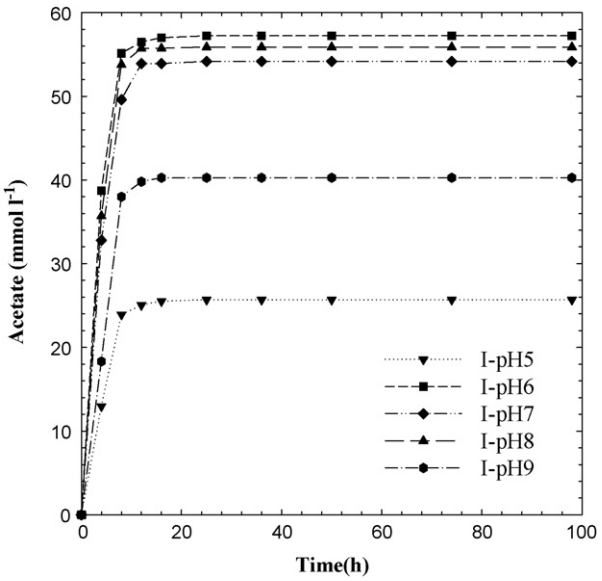

Fig. 5. Acetate yields without acetate addition at different pH.

3.2. Principal component analysis

Principal component analysis was applied to interpret the fermentation data, taking the yields of five metabolites as variables. The data were scaled and centered. The model was constructed based on 95% confidence intervals. Two principal components (PC) were extracted, explaining 50.4% and 29.4% of data derivation, respectively. Totally 79.8% of data derivation could be explained; therefore, the extracted model could present fairly the original data. The loading plot showed that production characteristics of five metabolites could be grouped into three characteristics: formate/methanol, acetate/lactate, and ethanol (Fig. 7a). The formate and methanol had positive loadings to PC1 and PC2. The others had positive loadings to PC1, but negative loadings to PC2. The ethanol had the highest loading to PC1. The score plot of the data at 25 h showed that the data could be classified into two groups: the data with acetate had positive scores in PC2, the data without acetate had negative scores in PC2 (Fig. 7b). Combined with the loading plot (Fig. 7a), the positive scores with acetate could be attributed to the increased production of methanol and formate. Likewise, the score plot of the data at 98 h presented similar patterns (Fig. 7c). Furthermore, the data with acetate were more scattered than that without acetate (Fig. 7b and c), suggesting more variable with acetate compared with corresponding pHs. In the case without acetate, the score points of pH 6 and 7 separated from other pHs in the direction of PC1. With acetate, the score points of pH 5 and 6 separated from other pHs mainly in the direction of PC1, suggesting that the deviation could be attributed to metabolites with high loadings to PC1, e.g. ethanol. Principal component analysis revealed that metabolic pathways changed with the stress of acetate and pH.

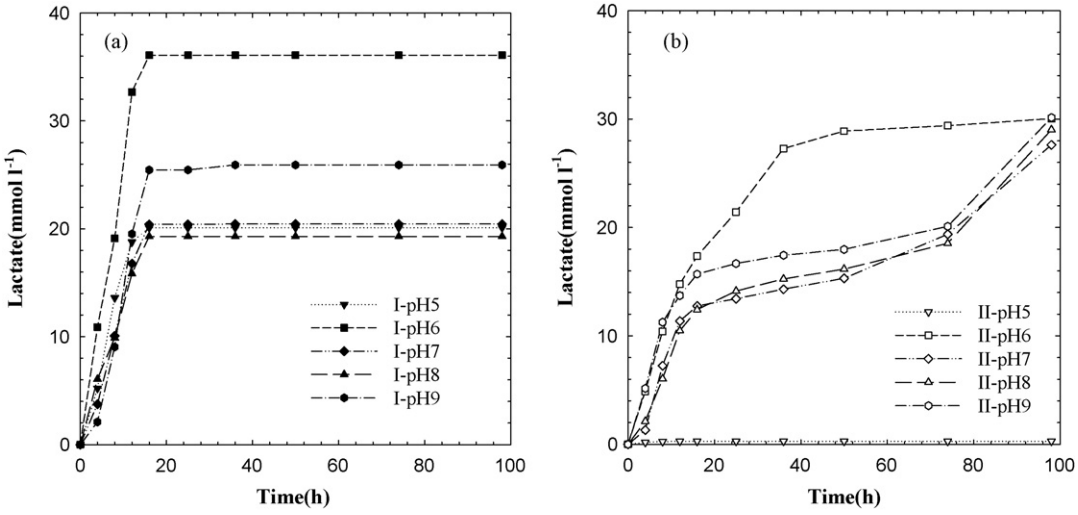

Fig. 6. Lactate yields at different pH. (a) Without acetate; (b) with 333 mmol l^{-1} acetate.

Table 2
The yields of metabolites after 98 h fermentation

Yields	Methanol (mmol l^{-1})		Ethanol (mmol l^{-1})		Formate (mmol l^{-1})		Acetate (mmol l^{-1})		Lactate (mmol l^{-1})	
With dosed acetate	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
pH 5	0.3	0.6	7.1	0.0	0.5	3.0	25.7	–	20.1	0.3
pH 6	0.0	4.9	10.7	22.6	0.7	3.0	57.2	–	36.1	30.0
pH 7	0.6	5.8	11.1	15.3	1.5	3.8	54.2	–	20.4	27.6
pH 8	0.5	7.8	3.2	14.3	0.9	4.4	55.9	–	19.3	29.0
pH 9	1.6	4.8	6.0	11.2	0.9	2.2	40.3	–	25.9	30.1

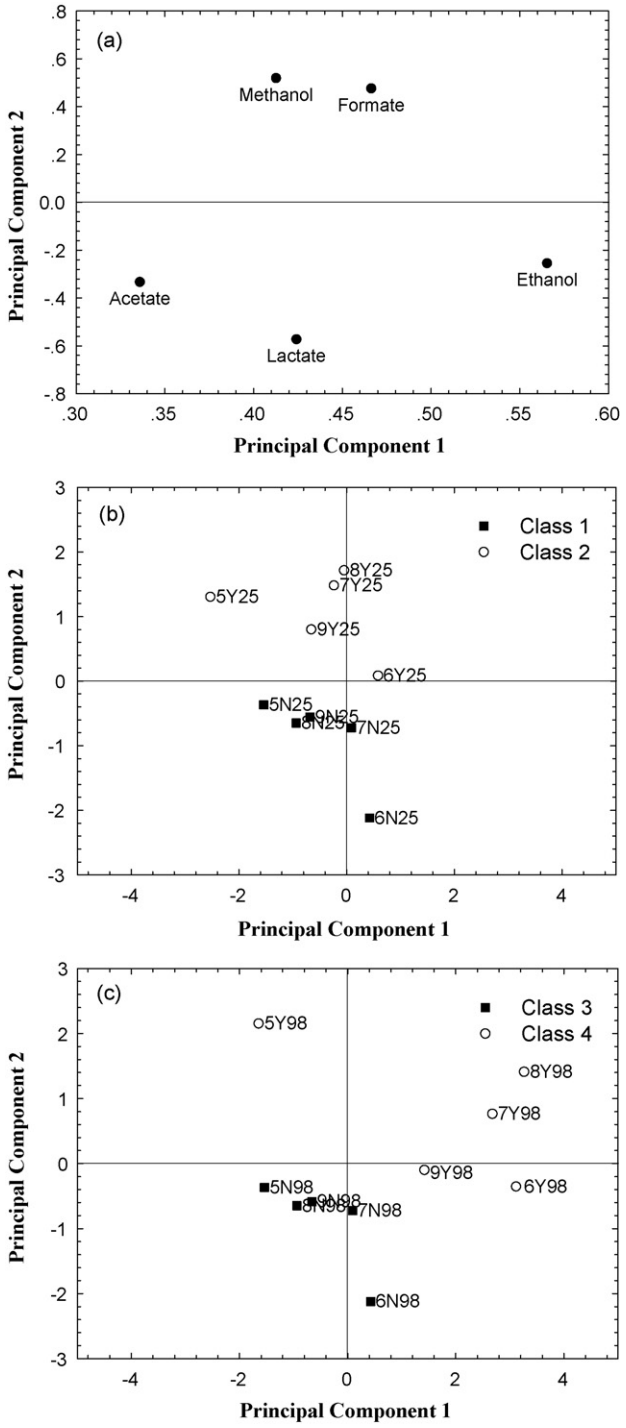

Fig. 7. Principal component analysis with the yields of metabolites. (a) Loading plot; (b) score plot of data at 25 h; (c) score plot of data at 98 h. Class 1 and Class 3: without acetate; Class 2 and Class 4: with 333 mmol l^{-1} acetate. Label of symbols: pH (5 or 7) – with (Y)/without (N) acetate – time (25 or 98 h).

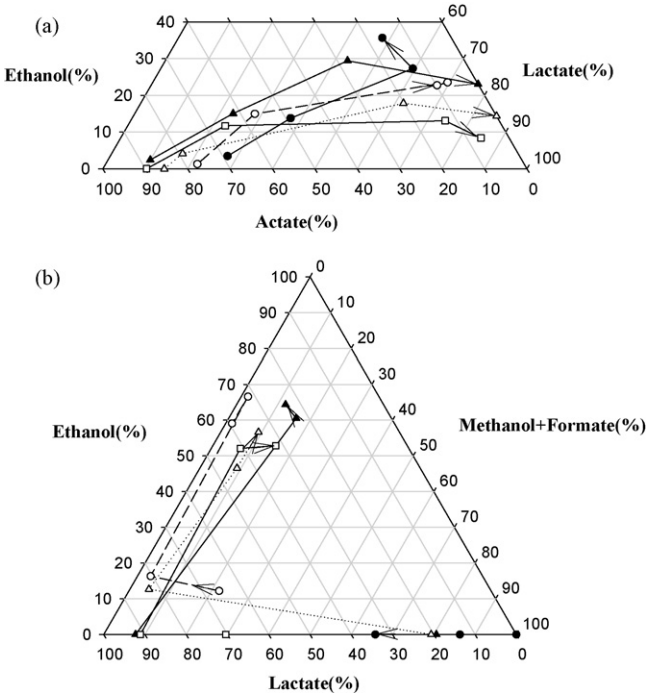

Fig. 8. "Phase" diagram of metabolic flux distribution at different pH. (a) Without acetate, the points along the direction of arrows mean the interval of 0–4, 4–8, 8–12, and 12–16 h; (b) with 333 mmol l^{-1} acetate, the points along the direction of arrows mean the interval of 0–4, 4–12, 12–25, and 25–36 h. (●) pH 5; (◯) pH 6; (▲) pH7; (△) pH 8; (□) pH 9.

3.3. Metabolites

Among the five detected metabolites, ethanol, acetate and lactate were the dominant products under stress of pH and acetate. Fig. 8a presents how the system without acetate evolved with time on a pseudo-three component "phase diagram". Generally, under very different experimental conditions, the "state" of the fermentative system evolved from left-corner (acetate-rich) to right-corner (lactate-rich), passing through an intermediate period at which ethanol was largely produced. Low pH (5–6) was preferred for the production of lactate, indicated by position of state locating right to most data. Most shifts happened in 8–12 h (point # 2–3 from the start). Hence, different metabolic patterns were noticeable in different fermentation stages.

Fig. 8b presents the "phase diagram" of the system with dosed acetate. Here, the flux was defined as the production rate of each metabolite versus the total sum of all four metabolites. The cases at pH 5 were distinguished from tests at other pHs with high methanol and formate production, no ethanol production, and low acetate production. The cases at other pHs were alike by shifting from lactate-rich to ethanol-rich product distributions, with crossing over points at 12 h (point 2# from the start). Restated, the metabolic pathways changed also with fermentation time in a batch mode. Han and Shin also utilized retention time to regulate the ratio of butyrate to acetate, and controlling the shift of predominant metabolic flow from hydrogen- and acid-forming pathway to solvent-forming pathway in a plug-flow like leaching-bed reactor [26].

3.4. Discussions

As revealed in this study, product inhibition by acetate (i.e. some kind of acid stress) affects not only the overall activity of microorganisms, as commonly assumed in literature works, but also the product distribution [27]. In the present work, high ratio of lactate to acetate was noted at pH 5–6, which was

attributable to the fact that lactic acid bacteria prefers acidic environment [28]. Under acetate stress, the production of methanol and formate was greatly enhanced at pH 5–9. Formate is normally produced under stress condition [29]. We also noted a mass production of methanol subjected to stress. Moreover, the form of acetate, undissociated or dissociated, correlated with ethanol production. Under the stress of 333 mmol l^{-1} acetate, the ethanol production was slightly impaired at pH 7–9, but greatly stimulated at pH 6, again completely inhibited at pH 5. At pH 7–9, the concentrations of undissociated acetate were below 1.8 mmol l^{-1}, but increased to 19 mmol l^{-1} at pH 6 and 127 mmol l^{-1} at pH 5. Then, a 10^0 mmol l^{-1} level of undissociated acetate slightly inhibited the microbial activities, 10^1 mmol l^{-1} level likely assisted the shift from acid-forming pathway to solvent-forming pathway, in order to escape from the stress [30], but 10^2 mmol l^{-1} level was completely lethal, confirmed by zero production of lactate and methanol at pH 5.

The transition of metabolic pathways in pure microbes had been studied by microcosmic assess of key enzymes, proteins and genes [9,10,31,32]. However, it is difficult to clarify whether the change in product distribution in a mixed-culture system was attributable to the change in microbes or in metabolic pathways [33]. Zoetemeyer et al. noted that the addition of 1100–1700 mg C l^{-1} butyrate could promote the production of lactate from glucose at pH 6, but reduce the production of butyric acid and propionate acid; while 2500 mg C l^{-1} butyrate would on the contrary reduce the lactate production [34]. The initial microbial inoculums could affect VFA degradation [35,36]. A steady-state metabolic model predicted the production of fermentative metabolites as functions of hydrogen partial pressure, pH, and substrate concentration [37], and ethanol was found to be preferably produced from high level of glucose at low pH. Recent studies revealed that pH regulated mix culture fermentation by affecting the bacterial community, and then leading to different fermentation products [38,39]. The problem was suggested to be aroused, especially when facing risk of acute accumulation of intermediate metabolites.

4. Conclusions

This work investigated how pH and externally dosed acetate could affect the fermentation reactions of potato, a polysaccharide-rich sample, particularly on the metabolites and their inhibitory effects. The tests were conducted at $37 \pm 0.2\,^{\circ}C$, with frequently replenished leaching liquors at fixed pH (5–9) and acetate levels (0 or 333 mmol l^{-1}). Methanol, ethanol, formate, acetate, and lactate were the five major metabolites, with production amount follow: acetate > lactate > ethanol > methanol > formate. Principal component analysis revealed that metabolic pathways changed with the stress of acetate and pH. The productions of ethanol or lactate were completely inhibited at pH 5 with 333 mmol l^{-1} of acetate dosed, while those of methanol and formate were mildly affected. With dosed acetate, ethanol was favorably produced at pH 6, but was completely inhibited at pH 5. On the other hand, lactate was less favorably produced with dosed acetate, but was also completely inhibited at pH 5. With no externally dosed acetate, the metabolism gradually shifted from acetate to lactate and ethanol-forming pathway with fermentation. With dosed acetate, except for at pH 5, the pathway to ethanol was strengthened with time. Hence, it was suggested that different inhibition factors should be considered for individual metabolites, besides the overall inhibitory kinetic factor to the biomass activity.

Acknowledgements

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