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Effect of lignocellulose-derived weak acids on butanol production by *Clostridium acetobutylicum* under different pH adjustment conditions

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The effects of formic acid, acetic acid and levulinic acid on acetone–butanol–ethanol (ABE) fermentation under different pH adjustment conditions were investigated using *Clostridium acetobutylicum* as the fermentation strain. CaCO₃ supplementation can alleviate the inhibitory effect of formic acid on ABE production. The ABE titers from the medium containing 0.5 g L⁻¹ formic acid with pH adjusted by CaCO₃ and KOH were 11.08 g L⁻¹ and 1.04 g L⁻¹, which reached 64.8% and 6.3% of the control group, respectively. Compared with CaCO₃ pH adjustment, fermentation results with higher ABE titers and yields were obtained from the medium containing acetic acid or levulinic acid, when the pH was adjusted by KOH. When formic acid, acetic acid, and levulinic acid co-existed in the medium, better fermentation result was achieved by adjusting the pH by CaCO₃. Moreover, 12.50 g L⁻¹ ABE was obtained from the medium containing 2.0 g L⁻¹ acetic acid, 0.4 g L⁻¹ formic acid, and 1.0 g L⁻¹ levulinic acid as compared to 3.98 g L⁻¹ ABE obtained from the same medium when the pH was adjusted by KOH. CaCO₃ supplementation is a more favorable pH adjustment method for ABE medium preparation from lignocellulosic hydrolysate.

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1. Introduction

Recent concerns about the fossil fuel depletion and environmental deterioration have aroused significant interests in the field of renewable fuel production.¹ Butanol, a promising renewable fuel, is recognized as a potential substitute of gasoline due to its favorable fuel properties such as high energy density, low volatility and hygroscopicity, less corrosiveness as well as the high miscibility with gasoline at any ratio.² Butanol can be produced by acetone–butanol–ethanol (ABE) fermentation from lignocellulosic biomass by *Clostridium* strains (such as *C. acetobutylicum*).^{3–7} The pentoses and hexoses in the lignocellulosic hydrolysate can be utilized by *C. acetobutylicum* for butanol production.^{8,9} In addition, the cost of lignocellulosic biomass is about 3 to 5 times more economical than that of traditional starchy substrates such as corn.^{10,11} Therefore, lignocellulosic biomass is expected to be an ideal substrate for butanol production.¹²

Although lignocellulose is the most abundant feedstock for butanol production, it is not easy to obtain fermentable sugars

from the native lignocellulosic biomass.¹³ Diluted acid pretreatment has emerged as a favorable method for industrial scale polysaccharide degradation.¹⁴ The polysaccharides can be degraded to fermentable sugars (such as glucose, xylose, arabinose, etc.) by the dilute acid pretreatment. Butanol fermentation from dilute acid hydrolysate of lignocellulose was expected to be an ideal mode for its large-scale production. However, inhibitors in the hydrolysate impede the butanol refining efficiency. The inhibitors mainly include furan derivatives, phenolics, and weak acids. The furan derivatives are furan and 5-hydroxymethylfurfural (5-HMF), which are derived from pentoses and hexoses, respectively. Phenolic compounds (such as coumaric acid, ferulic acid, and syringaldehyde) originate from Klason lignin.¹⁵ Weak acids mainly include acetic acid, formic acid, and levulinic acid.¹⁶ Acetic acid is liberated from the acetyl groups of hemicellulose fraction.¹⁷ Formic acid is obtained from the degradation of furfural and 5-HMF, and levulinic acid is obtained from the degradation of 5-HMF.¹⁸ Thus, it is urgent to develop a simple and efficient process control method.

The inhibiting or promoting effects of acetic acid and/or formic acid on ABE fermentation have been reported.^{19–23} The undissociated weak acids can diffuse across the cell membrane, which will result in the drop of intracellular pH and the collapse of the transmembrane proton gradient.^{24,25} Most researchers have focused on the removal of weak acids from hydrolysate by chemical treatment or physical adsorption.^{26,27} There are few

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reports on the process control strategies for improving the ABE fermentation efficiency without weak acids removal. Our previous study has suggested that the inhibiting effect of weak acids (formic acid) on ABE fermentation can be alleviated by improving the buffering capacity of the medium.²⁸ Based on the weak electrolyte dissociation characteristics of weak acids, the medium buffering capacity was increased to improve the butanol fermentation efficiency from lignocellulose hydrolysate without the removal of the inhibitors. This study aimed at the investigation of the effects of weak acid inhibitors produced by the degradation of lignocellulose on the ABE fermentation under different pH adjustment conditions.

2. Materials and methods

2.1. Strains and seed culture

C. acetobutylicum CH02, obtained from *C. acetobutylicum* ATCC 824 by long-term adaptation, was used for butanol production in this research. The inoculum of *C. acetobutylicum* CH02 was prepared as described in our previous study.²⁹

2.2. Medium preparation and batch fermentation

The medium of the control group was composed of (per liter medium): glucose 25 g, xylose 25 g and wheat bran 11 g. To evaluate the influence of formic acid, acetic acid, and levulinic acid on ABE fermentation, each single acid at different concentrations (formic acid: 0.1, 0.2, 0.3, 0.4, and 0.5 g L⁻¹; acetic acid: 0.2, 0.5, 1.0, 2.0, and 5.0 g L⁻¹; levulinic acid: 0.2, 0.5, 1.0, 2.0, and 5.0 g L⁻¹) and combined acids (formic acid 0.4 g L⁻¹, acetic acid 2.0 g L⁻¹ and levulinic acid 1.0 g L⁻¹) were added to the media. The initial pH of the media after the addition of the acids was adjusted to 6.5 via the following two methods: (i) by adding 4 g L⁻¹ CaCO₃ and a small amount of CaO or (ii) by adding 6 M KOH. The initial pH of the media without the addition of the acids was adjusted to 6.5 with 20 mM sulfuric acid.

All batch fermentations were conducted in 250 mL serum bottles with the working volume of 200 mL in duplicates. The inoculation levels were controlled at 5% (v/v), and the bottles were incubated statically at 37 °C for 120 h.

2.3. Analytical methods

The solvents (acetone, butanol and ethanol) were analyzed using a gas chromatograph (GC, Agilent 7890A). The acids (formate, acetate, butyrate, and levulinate) and sugars (glucose and xylose) were measured by a high performance liquid chromatograph (HPLC, Waters 2695e) as in our previous report.²⁸ The pH of the medium was maintained by a pH meter. The ABE yield was calculated as given in the following eqn (1):

$$\text{ABE yield} = \frac{c_{\text{acetone}} + c_{\text{butanol}} + c_{\text{ethanol}}}{(c_{\text{glucose}}^i + c_{\text{xylose}}^i) - (c_{\text{glucose}}^r + c_{\text{xylose}}^r)} \quad (1)$$

where c_{acetone} , c_{butanol} and c_{ethanol} means the concentration of acetone, butanol, and ethanol in the fermentation broth, respectively. c_{glucose}^i and c_{xylose}^i means the initial concentration of glucose and xylose in the medium before fermentation,

respectively. c_{glucose}^r and c_{xylose}^r means the residual concentration of glucose and xylose, respectively.

3. Results and discussion

3.1. Effect of acetic acid on ABE fermentation under different pH adjustment conditions

To investigate the effect of acetic acid on butanol fermentation under different pH adjustment conditions, butanol fermentation was conducted in groups A1–A5 (containing 0.2, 0.5, 1.0, 2.0, and 5.0 g L⁻¹ acetic acid). In the control group B, butanol fermentation was conducted without the addition of acetic acid. Fig. 1 and 2 show the fermentation results with pH adjusted by CaCO₃ and KOH, respectively.

As can be seen from Fig. 1.1, with CaCO₃ supplementation, the solvent (acetone, ethanol, and butanol) titers were lower than those in the control group. However, the acid accumulation is relatively high. ABE fermentation by *C. acetobutylicum* is typically characterized by acidogenesis and solventogenesis.³⁰ The acetic acid concentrations after fermentation in group A1–A5 were higher than the native supplementation levels; this suggested that no net acetic acid was consumed. In the batch fermentation A5, 5.72 g L⁻¹ acetic acid and 5.42 g L⁻¹ butyric acid were accumulated after fermentation. On the one hand, the relatively high pH level, as shown in Fig. 1.3, caused by CaCO₃ supplementation can induce acid accumulation in acidogenesis.³¹ On the other hand, the dissociated acetic acid and butyric acid cannot be re-assimilated in solventogenesis.³² Therefore, the acid concentration after fermentation in A1–A5 was high. Interestingly, the accumulation trends of acetic acid and butyric acid in different batch fermentations were similar (as shown in Fig. 1.2). This was because ATP production from acetic acid pathway was inhibited by the high acetic acid supplementation level in the medium, and therefore, most of the ATP was produced through the butyric acid pathway.²⁸ The acetic acid and butyric acid accumulation consumed a large amount of the substrates, and the solvent yield was relatively lower than that in the control group, as shown in Fig. 3.3. The residual sugars after all batch fermentations were lower than 4 g L⁻¹ (Fig. 1.4); this suggested that the acetic acid supplementation lower than 5 g L⁻¹ did not cause significant inhibition of sugar consumption.

When the pH was adjusted by KOH, the fermentation results were obviously different. As illustrated in Fig. 2.1, the ABE titers increased slightly when the acetic acid supplementation was increased from 0.2 to 1.0 g L⁻¹, and the ABE titers reached 17.54 g L⁻¹ when 1.0 g L⁻¹ acetic acid was added to the medium. However, the ABE titers decreased when acetic acid supplementation further increased to 5.0 g L⁻¹. Compared with Fig. 1.2, the re-assimilation of acetic acid was obvious. As shown in Fig. 2.2, 17.88%, 31.20% and 69.97% supplemented acetic acid was consumed in the batch fermentations A3–A5. More undissociated acetic acid and butyric acid were re-assimilated by *C. acetobutylicum* when the medium pH was at a relatively low level, as shown in Fig. 2.3. Acetic acid, glucose, and xylose can serve as the co-substrates for solvent production. Solvent yield calculated from the sugar in Fig. 2.3 was higher than that



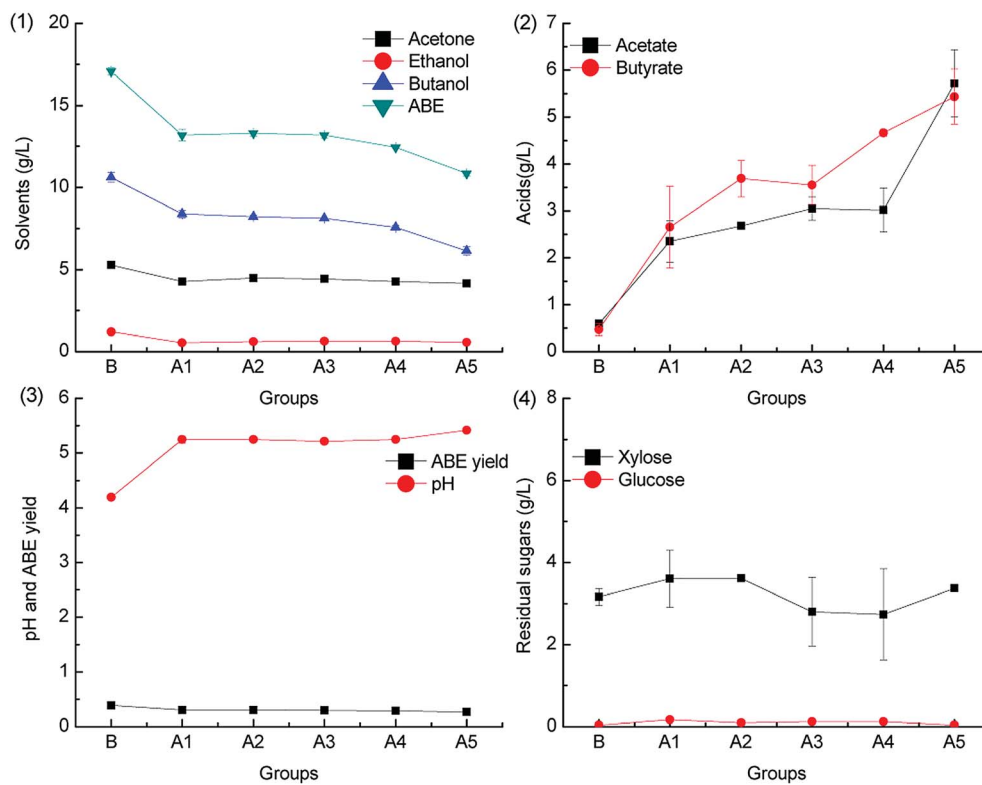


Fig. 1 Effect of acetic acid on ABE fermentation with pH adjusted by CaCO_3 .

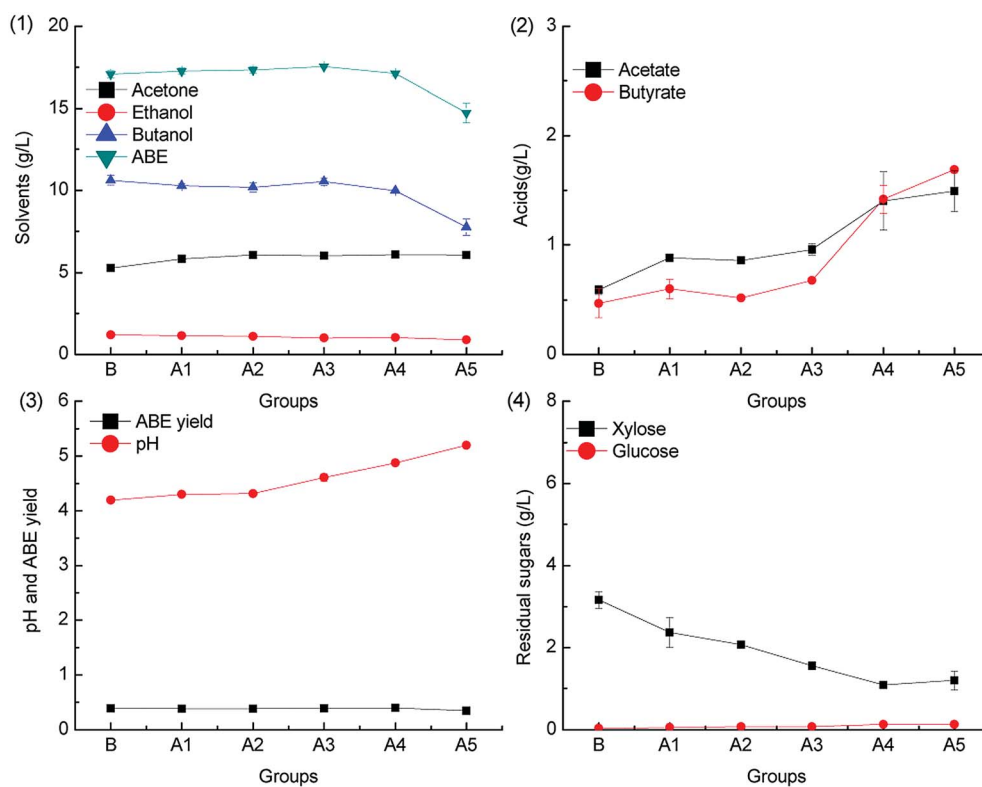


Fig. 2 Effect of acetic acid on ABE fermentation with pH adjusted by KOH .



in Fig. 1.3. As illustrated in Fig. 2.4, glucose was almost completely consumed after fermentation, and the residual xylose was lower than 4 g L^{-1} . It can be seen from the comparison results that when the medium is adjusted by KOH, acetic acid can be re-assimilated at a relatively high level, which will result in a high solvent yield.

3.2. Effect of formic acid on ABE fermentation under different pH adjustment conditions

Formic acid is deleterious to ABE fermentation by *C. acetobutylicum* because it can trigger acid crash,²⁰ but the inhibitory effect can be alleviated by CaCO_3 supplementation.²⁸ The inhibitory effect of formic acid on ABE fermentation with different concentration was evaluated herein, and the results are shown in Fig. 3 and 4.

To investigate the effect of formic acid on butanol fermentation under different pH adjustment conditions, batch fermentations were conducted in the groups F1–F5 (containing 0.1, 0.2, 0.3, 0.4, and 0.5 g L^{-1} formic acid). The ABE titers obtained from the medium F1–F5 were 84.5%, 80.5%, 77.2%, 70.0% and 64.8% of the control group B when the pH was adjusted by CaCO_3 . However, the ABE titers in the batch fermentation of F1–F5 reached only 63.6%, 44.7%, 26.7%, 16.4% and 6.1% of the control group when the pH was adjusted by KOH. The results are in agreement with our previous report that CaCO_3 can alleviate the inhibitory effect of formic acid on butanol fermentation.²⁸ The acids production with different pH adjustment methods is obviously different. As shown in Fig. 3.2,

acetic acid concentration accumulated in the medium was higher than that of butyric acid in batch fermentation of groups F1–F5, whereas the acetic acid and butyric acid accumulation in the control group was almost the same. In contrast, the differences in acids accumulation in groups F1–F5 in Fig. 4.2 were not obvious. Acids production was accompanied by ATP generation, and ATP generation efficiency from acetic acid production was higher than that from butyric acid production.³³ The uncoupling effect caused by the undissociated acids can be alleviated by the over-production of acetate. The ABE yield was between 0.3 g g^{-1} and 0.4 g g^{-1} , as shown in Fig. 3.3, which was higher than that shown in Fig. 4.3 from the same medium.

3.3. Effect of levulinic acid on ABE fermentation under different pH adjustment conditions

Levulinic acid is produced from hexoses through the intermediate 5-HMF under acid pretreatment condition.³⁴ Prior to butanol production from the hemicellulose hydrolysate, levulinic acid is often removed as the fermentation inhibitor.^{35,36} In this study, we have found that levulinic acid can be metabolized by *C. acetobutylicum*. To the best of our knowledge, there is no report on the catabolism pathway of levulinic acid in *C. acetobutylicum*. The metabolism pathway of levulinic acid in other microorganisms such as *Cupriavidus necator* has been reported, and it is found that levulinic acid is metabolized *via* conversion to acetyl-CoA and propionyl-CoA by an inducible levulinic acid acyl-CoA synthetase.³⁷ The ABE fermentation results with different levulinic acid supplementation levels in groups L1–L5

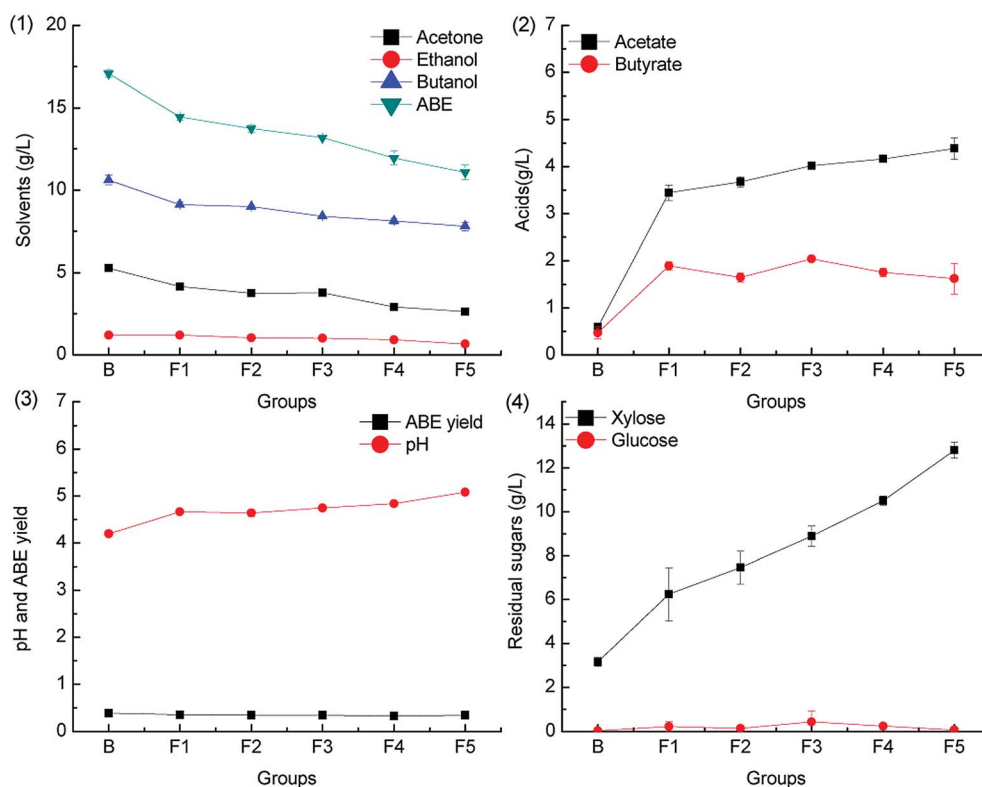


Fig. 3 Effect of formic acid on ABE fermentation with pH adjusted by CaCO_3 .



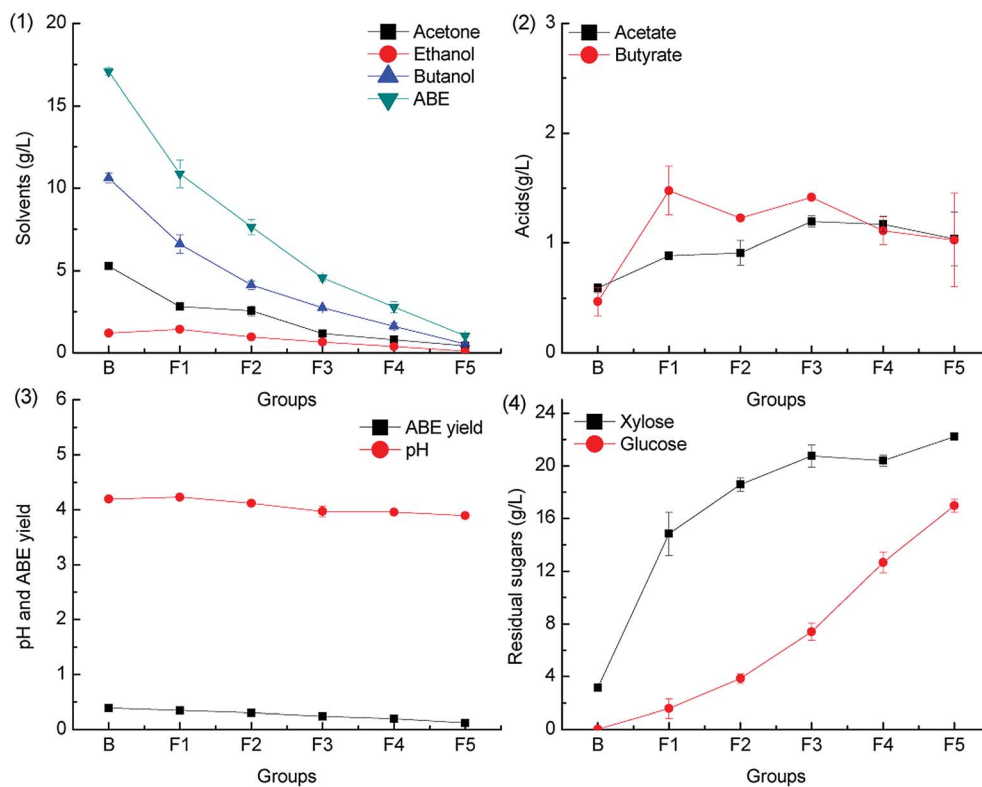


Fig. 4 Effect of formic acid on ABE fermentation with pH adjusted by KOH.

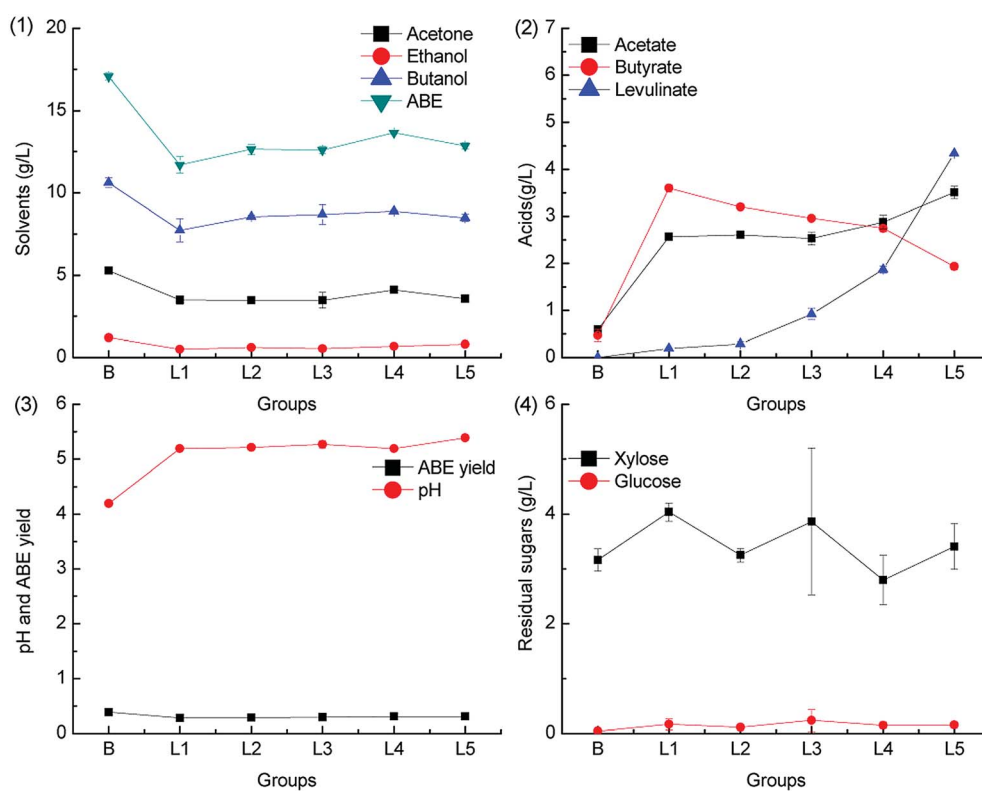


Fig. 5 Effect of levulinic acid on ABE fermentation with pH adjusted by CaCO_3 .



(0.2 g L⁻¹, 0.5 g L⁻¹, 1.0 g L⁻¹, 2.0 g L⁻¹ and 5.0 g L⁻¹) are illustrated in Fig. 5 and 6.

As illustrated in Fig. 5, the ABE titers were lower than those in the control group when the pH was adjusted by CaCO₃. On the other hand, more acetic acid and butyric acid were accumulated, and only a small quantity of levulinic acid was consumed at the end of the fermentation. When CaCO₃ was supplemented in the medium, the pH was maintained at a relatively high level due to the pH buffering capacity of CaCO₃, as shown in Fig. 5.3. Therefore, more acetic acid and butyric acid were produced and accumulated in the dissociated state, which could not be re-assimilated. This also resulted in low ABE yield. No obvious inhibitory effects on the sugar consumption were observed when 5.0 g L⁻¹ levulinic acid was added to the fermentation medium.

When the fermentation pH was adjusted by KOH, the ABE titers increased slightly as compared to the case of the control group, with the levulinic acid supplementation level ranging from 0.2 g L⁻¹ to 2.0 g L⁻¹; the results are shown in Fig. 6. The maximum concentration of 18.17 g L⁻¹ ABE was produced when 1.0 g L⁻¹ levulinic acid was added to the medium, which was 6.5% higher as compared to that achieved from the control group. When the levulinic acid supplementation level was higher than 2.0 g L⁻¹, the ABE titers decreased significantly, and the phenomenon could be explained from the acid production. The acetic acid and butyric acid accumulation increased with the levulinic acid supplementation level; this was due to the pH buffering capacity of potassium levulinate. As illustrated in

Fig. 6.3, the final pH level increased with the levulinic acid supplementation level. The accumulated acetic acid and butyric acid were in the dissociated state and could not be re-assimilated for ABE production; therefore, the ABE titers and yield decreased as compared to that in the control group.

3.4. Effect of the co-presence of acetic acid, formic acid, and levulinic acid on ABE fermentation under different pH adjustment conditions

To investigate the co-effect of acetic acid, formic acid, and levulinic acid on ABE fermentation under different pH adjustment conditions, the acids concentrations were selected according to the wheat straw hydrolysate composition of our previous study with some changes.³⁸ Batch fermentations with the co-presence of 2.0 g L⁻¹ acetic acid, 0.4 g L⁻¹ formic acid, and 1.0 g L⁻¹ levulinic acid were performed.

Herein, 0.09 g L⁻¹ acetic acid, 0.03 g L⁻¹ formic acid, and 0.08 g L⁻¹ levulinic acid were detected in the control medium because of the auto-hydrolysis of cellulose and hemicellulose component in wheat bran during the autoclave process. The fermentation results with the co-presence of three kinds of acids are illustrated in Fig. 7.

As shown in Fig. 7.1 and 7.2, formic acid cannot be metabolized under different pH adjustment conditions due to the lack of formate dehydrogenase (FDH) in *C. acetobutylicum*¹⁹. The levulinic acid consumption ratios in batch fermentations with pH adjusted by CaCO₃ and KOH were 8.9% and 42.7%, respectively. More levulinic acid was consumed when the

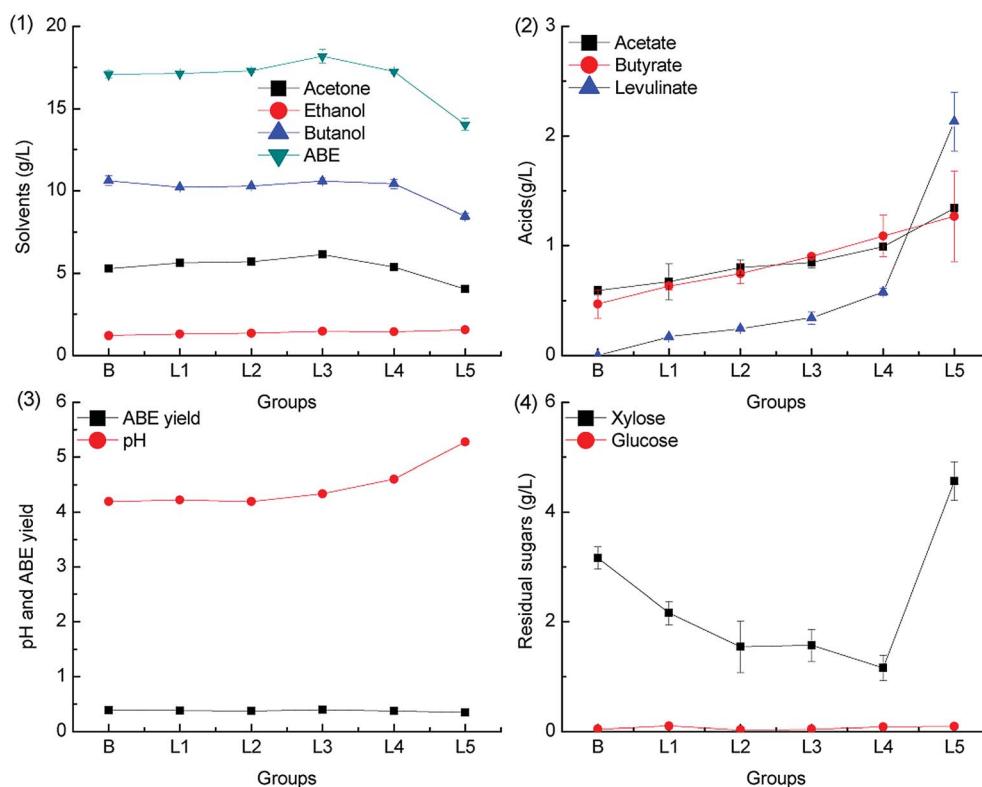


Fig. 6 Effect of formic acid on ABE fermentation with pH adjusted by KOH.



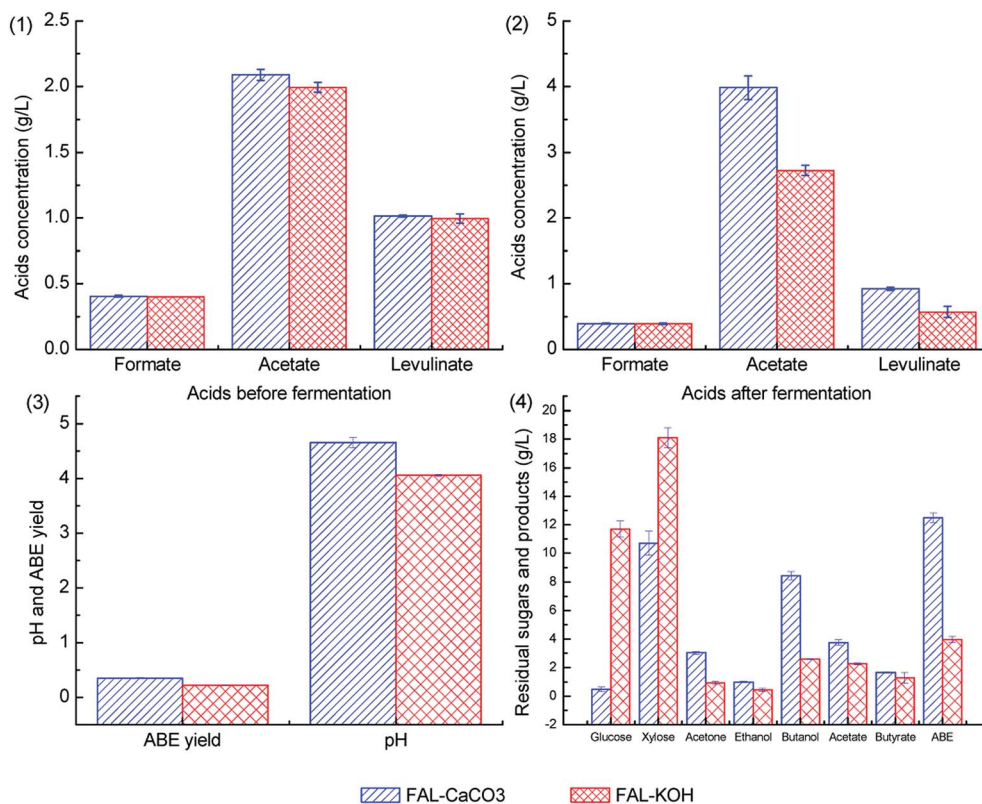


Fig. 7 Effect of co-presence acetic acid, formic acid and levulinic acid on ABE fermentation under different pH adjustment conditions. FAL-KOH, co-presence of formic acid, acetic acid and levulinic acid with pH adjusted by KOH. FAL-CaCO₃, co-presence of formic acid, acetic acid and levulinic acid with pH adjusted by CaCO₃.

fermentation pH was adjusted by KOH, this was because more levulinic acid was present in the dissociated state when CaCO₃ was supplemented to improve the pH buffering capacity. For the ABE production shown in Fig. 7.3, ABE titers in batch fermentations with pH adjusted by CaCO₃ and KOH were 12.50 g L⁻¹ and 3.98 g L⁻¹, which reached 73.2% and 23.3% of the control group, respectively. As shown in Fig. 7.4, the ABE yield in batch fermentations with pH adjusted by CaCO₃ and KOH was 0.35 g g⁻¹ and 0.22 g g⁻¹, respectively. Among the three kinds of weak acids, formic acid exhibits fatal inhibitory effect on the ABE production. Compared with the batch fermentation results of group F4 in Fig. 4.1, the inhibitory effect of formic acid can be alleviated by acetic acid and levulinic acid supplementation. On the one hand, acetate and levulinate can increase the medium's pH buffering capacity. On the other hand, acetic acid can play a role in preventing degeneration and enhancing the CoA transferase activity.^{21,39} Acetic acid and butyric acid accumulation was also different because of the different pH buffering capacity of KOH and CaCO₃, and more acetic acid and butyric acid were accumulated when CaCO₃ was supplemented. Cho *et al.* reported that the inhibition of formic acid on *C. acetobutylicum* can be alleviated by acetic acid supplementation, and the results in this study are in agreement with these conclusions.¹⁹ Weak acids such as formic acid, acetic acid, and levulinic acid often co-exist in the biomass hydrolysate.⁴⁰ Previous studies have often been focused on the removal of fermentation inhibitors (weak acids included). Poor

fermentation results with low ABE titers and yields were achieved when the medium pH was adjusted by KOH or other alkali without pH buffering capacity. Qureshi *et al.* reported that only 1.48 g L⁻¹ of ABE was obtained from the switchgrass hydrolysate when the pH was adjusted by NaOH.⁴¹

4. Conclusions

Among the three kinds of weak acids, formic acid exhibits fatal inhibitory effect on the ABE production. The inhibitory effect of formic acid can be alleviated by CaCO₃ supplementation. However, pH adjustment with KOH was more suitable when a single acid such as acetic acid or levulinic acid existed in the medium. Satisfactory fermentation results were achieved with pH adjusted by CaCO₃ in the medium containing the three kind of weak acids. Formic acid, acetic acid, and levulinic acid often co-exist in the lignocellulosic hydrolysate. pH controlling strategy by CaCO₃ is compatible with industrial applications because of its economic and technical feasibility. On the one hand, CaCO₃ is more affordable than KOH or other common alkaline materials. On the other hand, pH adjustment by CaCO₃ is technically feasible because their supplementation level controls is easy, and even an excessive dose of CaCO₃ supplementation will not result in a pH increase. Therefore, CaCO₃ supplementation is the more favorable pH adjustment method for ABE medium preparation from lignocellulosic hydrolysate.



Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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