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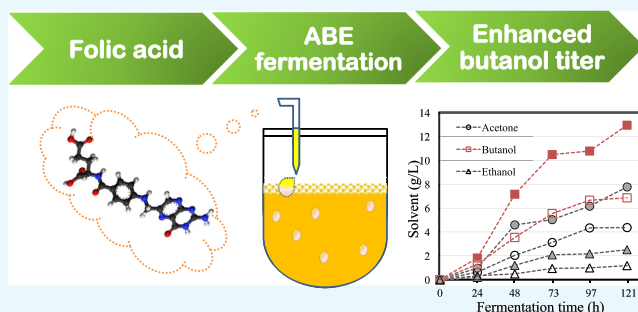
Enhanced Biobutanol Production in Folic Acid-Induced Medium by Using *Clostridium acetobutylicum* NRRL B-527

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ABSTRACT: The conventional acetone–butanol–ethanol fermentation process suffers from several key hurdles viz. low solvent titer, insufficient yield and productivity, and solvent intolerance which largely affect butanol commercialization. To counteract these issues, the effect of stimulator, namely, folic acid was investigated in the present study to improve butanol titer. Folic acid is involved in biosynthesis of a diverse range of cellular components, which subsequently alter the amino acid balance. Therefore, different concentrations of folic acid were screened, and 10 mg/L supplementation resulted in a maximum butanol production of 10.78 ± 0.09 g/L with total solvents of 18.91 ± 0.21 g/L. Folic acid addition at different time intervals was also optimized to get additional improvements in final butanol concentration. Overall, folic acid supplementation resulted in two-fold increase in butanol concentration and thus could be considered as a promising strategy to enhance solvent titers.



1. INTRODUCTION

Butanol has been extensively used as an industrial intermediate from past few decades and now attracted public attention for being an advanced biofuel.¹ Based on fuel properties, butanol shows better compatibility with gasoline as compared to different liquid fuels.² However, the traditional acetone–butanol–ethanol (ABE) fermentation process has numerous pitfalls such as complex bioprocess, low butanol concentration, and end product inhibition.² Hence, more research efforts in field of biobutanol production are needed to explore butanol as next-generation biofuel. Incidentally, various efforts including mutagenesis, metabolic engineering approaches, and integrated fermentation with competent product recovery were reported recently to enhance product titer.³ Moreover, the metabolic regulation to elevate butanol production has been carried out by incorporating organic acids and electron carrier together.⁴

Several researchers have implemented certain precursors/cofactors/electron carriers and indeed, considerable successes have been achieved in raising butanol titer.^{5,6} Co-culturing of *Clostridium acetobutylicum*/*Saccharomyces cerevisiae* along with butyrate addition has maximized butanol concentration upto 16.3 g/L which is 46.8% higher than control.⁷ On the other hand, addition of calcium carbonate together with yeast extract promotes butanol synthesis to increase amino acid concentrations, and thereby stimulate cell growth.⁸ Similarly, butanol concentration can be upregulated by incorporation of substrate supplements such as butyrate, acetate, and allopurinol.⁹ Besides, the pH-controlled approach could be one of the promising techniques to increase sugar consumption rate and

final solvent titer.¹⁰ Nonetheless, butanol toxicity during fermentation is again a key hurdle that significantly affects process yield and productivity. Therefore, augmenting strain robustness is of prime importance. Interestingly, upregulation of heat shock proteins could be helpful in adapting clostridial strains to high butanol concentration.¹¹ Additionally, few amino acids that are either self-generated or exogenously incorporated, enhance the cell's survival rate and butanol synthesis.¹²

Attempts were made in the current study to improve biobutanol production along with strain tolerance during batch fermentation by supplementing folic acid in production medium. Folic acid is mainly involved in the amino acid biosynthesis in microorganisms.¹³ The micro-organisms metabolically convert folic acid into tetrahydrofolic acid. This intermediate could then be diverted to amino acid or purine/thymidine synthesis. Hence, it was thought desirable to supplement folic acid in the fermentation medium, in order to induce amino acid generation during ABE biosynthesis. These self-generated amino acids in growing clostridia eventually trigger the biobutanol production. Furthermore, folic acid addition at different fermentation times was also investigated to improve the overall performance with respect to butanol/acetone (B/A) ratio.

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2. RESULTS AND DISCUSSION

2.1. Effect of Folic Acid on Biobutanol Production.

Folic acid is a stable synthetic form of folate, which is required by prokaryotic/eukaryotic cells for the production of cellular materials.¹⁴ Thus in present study, folic acid was incorporated in the clostridial ABE fermentation medium to investigate its stimulatory effect. It has been observed that precursors/cofactors/stimulators are very effective when present at low concentration in the fermentation medium.^{15,16} Also, our previous study demonstrated significant improvement in butanol titer when trace elements were supplemented in the concentration range of 1–100 mg/L.¹⁷ Therefore, folic acid was selectively incorporated in different concentrations as 1, 10, 50, and 100 mg/L. Figure 1 depicts ABE production under varied folic acid concentrations.

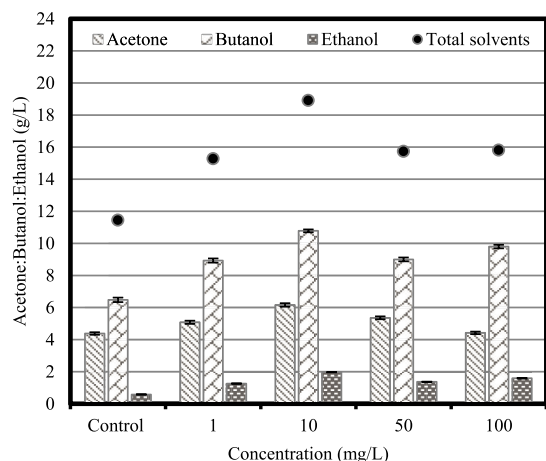


Figure 1. Effect of folic acid during ABE fermentation by using *C. acetobutylicum* NRRL B-527 after 120 h.

Interestingly, folic acid supplementation has positively triggered ABE fermentation, thus resulting considerable increment in total solvent production. Control (without folic acid) experiment produced around 6.48 ± 0.14 g/L of butanol with total ABE to be 11.44 ± 0.23 g/L. Conversely, 10 mg/L folic acid supplementation resulted in maximum butanol production up to 10.78 ± 0.09 g/L with ABE of 18.91 ± 0.21 g/L. Addition of folic acid beyond 10 mg/L led to drop in solvent titer, especially because of detrimental effects at higher concentration. Virk et al.¹⁸ also showed similar detrimental impact on *Clostridium elegans* ageing because of increased folate synthesis, and bacterial-dependent chronic toxicity.

The upsurge in solvent concentrations is may be due to stimulation of clostridial cells by folic acid for amino acid self-generation.¹³ It has been reported that effective amino acid assimilation yields higher butanol level along with a greater B/A ratio.¹² The positive influence of folic acid was further evaluated by their addition at several fermentation time intervals.

2.2. Effect of Time-Wise Folic Acid Addition.

Optimized concentration of folic acid (10 mg/L) was incorporated at fermentation time intervals of 0, 4, 8, 24, and 31 h. The conventional clostridial growth profile shows that the exponential phase begins at 4–6 h which lasts until 32–36 h just before the stationary phase. *C. acetobutylicum* are known to enter in the solventogenic phase after 36 h to initiate ABE production. Therefore, the folic acid addition was not

extended beyond 31 h. As can be seen in Figure 2, the highest butanol production (12.94 ± 0.09 g/L) was reached when folic

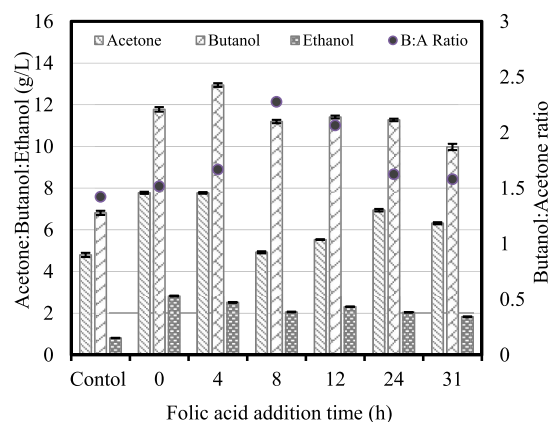


Figure 2. Time-course of folic acid addition in production media of *C. acetobutylicum* NRRL B-527 with a total fermentation time of 120 h.

acid was incorporated after 4 h of fermentation. However, further delay in addition time resulted into relatively lower solvent production. This is because when microorganisms just start to enter in the exponential growth phase, their cellular activities are at peak and additional folic acid can immediately be utilized in butyric acid production under a favorable acidogenic phase. Interestingly, notable changes were observed in acetone production, while ethanol production was slightly altered with different folic acid addition times. Acetone production was lowered when folic acid was added at 8 and 12 h of fermentation (Figure 2). The lowered acetone production was attributed to diversion of flux toward butanol.¹⁹

Lowered acetone production obviously resulted in fluctuation in the B/A ratio in the presence of folic acid. From Figure 2, it was found that the maximum B/A ratio (2.27) was reached when folic acid was added after 8 h of fermentation. Although it was not evaluated in the current study, it was believed that the lowered acetone flux was eventually transferred to the butanol production pathway that ultimately improved the butanol titer. This is evident from the altered butanol/ethanol ratio, especially because of higher butanol production and steady ethanol accumulation. Overall, these findings clearly suggest that folic acid incorporation at the specific clostridial growth phase (beginning of exponential phase) is essential to elevate butanol titer. Besides, several researchers also pointed out the effectiveness of “precursor addition time” for increased biobutanol production.^{6,17,20} Furthermore, fermentation kinetics of control and folic acid-supplemented medium were also studied in order to assess the fermentation performance.

2.3. ABE Fermentation Profiles in the Presence of Folic Acid.

Folic acid (10 mg/L) was added after 4 h of clostridial fermentation and ABE profile was studied with respect to growth, pH, and sugar consumption. The growth and production behavior of clostridia was significantly affected by addition folic acid with improved butanol yield and productivity of approximately two-fold. From Figure 3A, it was observed that clostridial growth in the presence of folic acid is consistently higher than control, throughout the fermentation. A lag period of ~4 h was observed, in which cells acclimatize to fermentation conditions. Thereafter, cells enter into exponen-

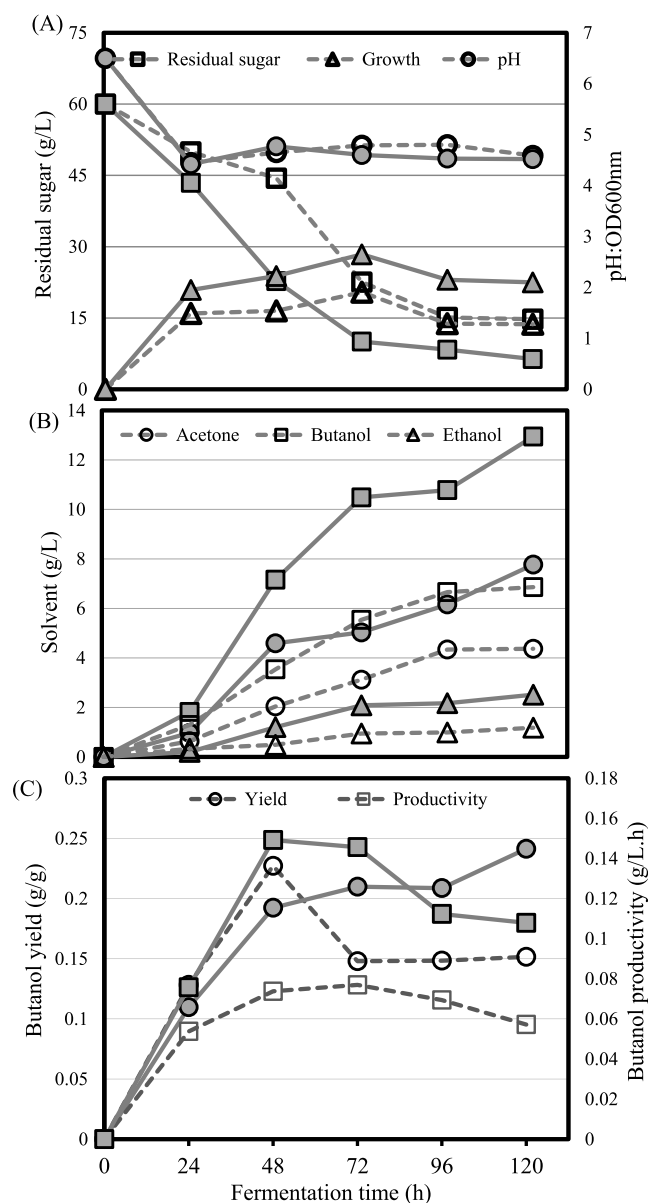


Figure 3. ABE fermentation performance in the presence of folic acid by using *C. acetobutylicum* NRRL B-527: (A) residual sugar, pH, and clostridial growth profile; (B) solvent profiles; (C) butanol yield and productivity: empty symbols with dotted lines represent control readings, whereas filled symbols with solid lines represent folic acid added results. The data represent the average of triplicate determinations with SD < $\pm 0.5\%$.

tial growth phase showing steep increase in cell density. After 48 h, the stationary phase begun wherein cell growth slowed and maximum solvent production took place. In control experiment, a similar growth pattern was observed while cell density was relatively lowered as compared to folic acid-supplemented medium. Interestingly, the pH profile during ABE fermentation which is a measure to analyze the shift from the acidogenic phase to solventogenic phase of clostridia was unaffected (Figure 3A). This signifies that, although folic acid was believed to alter cellular activities of clostridia, it did not significantly change the microbial phase behavior.

Folic acid supplementation resulted into rapid sugar consumption of around 19% higher than control (Figure 3A). The sugar utilization rate was quite fast within 24–72 h of

fermentation, which can relate to rapid acid assimilation to solvents. These results are in agreement with our previous report that demonstrated the addition of nickel chloride aided almost complete sugar utilization with increased butanol titer.¹⁷ Also, incorporation of nicotinic acid has promoted the glucose consumption rate by ~9% than the corresponding control value.²¹ This increase in consumption rate is attributed to large amounts of reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated and subsequently being utilized during fermentation.

Figure 3B shows solvent production profiles by *C. acetobutylicum* NRRL B-527. The highest butanol and ABE as 12.94 ± 0.09 and 23.23 ± 0.17 g/L, respectively, were achieved by folic acid addition. This improvement is because of variety of amino acid induction that is solely responsible for increased strain tolerance, thereby better solvent production.²² These outcomes are in-line with other studies which showed that overproduction of NADH and aspartic acid is responsible for the enhanced butanol level in yeast extract-supplemented cassava medium.²³ Luo et al.¹² also demonstrated that secretion of lysine is favorable to *C. acetobutylicum*'s survival under high butanol concentration. Furthermore, it was observed that the solvent productions were drastically improved after 24 h of fermentation (Figure 3B) as a result of the shift from acidogenesis to solventogenesis. Moreover, folic acid incorporation also aided increment in acetone and ethanol concentrations. Ding et al.²⁰ reported that supplementation of electron receptors ($\text{Na}_2\text{SO}_4/\text{CaSO}_4$) altered the distribution of electron/proton in the intracellular electron transport shuttle system to accumulate amino acids favorable for clostridial survival/butanol synthesis.

Figure 3C emphasizes variations in specific butanol yield and productivity during the entire fermentation period. The maximum butanol yield of 0.24 g/g and productivity of 0.14 g/L.h were achieved in batch fermentation with folic acid addition. The statistical analysis by using single-factor ANOVA was performed to evaluate significant difference between studied parameters. The p value < 0.05 demonstrated that the folic acid supplementation significantly affected clostridial growth, fermentation pH, and ABE production profiles positively. ABE fermentation was also carried out in a bioreactor supplemented with folic acid (after 4 h fermentation). As a result, butanol concentration of 12.31 ± 0.03 g/L with total solvents of 21.50 ± 0.20 g/L was obtained. Interestingly, folic acid traces were not detected after fermentation was over (120 h). This clearly implies that folic acid added during fermentation was utilized as a stimulator to alter essential cellular components. Previously, folic acid supplementation altered the *C. elegans* development and longevity via bacterial uptake of breakdown products.²⁴ Besides, vitamins (A, B1, B2, B3, and C) are found to support bacterial growth which eventually improved butanol titer (21.56 g/L).²⁵ Another study also revealed that two basic vitamins, namely, biotin and *p*-aminobenzoic acid are required to maintain active cell growth.²⁶ Increasing these vitamin concentrations by 8-fold has triggered growth as well as the solvent formation rate. Similarly, other medium precursors have also been reported to elevate the butanol production (Table 1). This study will be continued further with several bioprocess modifications such as fed-batch and continuous process monitoring in order to see its large scale feasibility.

Table 1. Comparative Evaluation of the Present Study with Other Literature Reports

precursors supplemented in ABE fermentation	butanol (g/L)	total ABE (g/L)	butanol yield (g/g)	references
folic acid	12.94	23.23	0.24	current study
trace elements (nickel)	10.08	18.17	0.17	Nimbalkar et al. ¹⁷
benzyl viologen and butyric acid	18.05	20.17	0.50	Nasser Al-Shorgani et al. ⁶
yeast extract, NH ₄ NO ₃	21.70	31.73	0.39	Abd-Alla and Elsadek El-Enany ²⁵
cysteine, FeSO ₄ , yeast extract	9.15	11.24	0.30	Diez-Antolínez et al. ²⁷
methyl viologen, CaCO ₃	11.54		0.29	Kumar and Banerjee ²⁸

3. CONCLUSIONS

The biobutanol process, although very old, is still struggling to be commercialized for its biofuel application, especially because of its lower production titers and end production inhibition. Hence, modification in metabolic stimulators like folic acid is especially important in order to divert the flux toward butanol production. Current work shows the importance of metabolic stimulators that affect the biobutanol production profile in clostridia with improved tolerance toward solvent toxicity. Butanol production stimulators such as folic acid increased the overall titer and fermentation performance. The folic acid supplementation yielded nearly two-fold rise in butanol concentration, thus reaching specific butanol yield of 0.24 g/g. This study also detailed an impact of folic acid addition time on butanol production and B/A ratio. Furthermore, fermentation profiles demonstrated that butanol production was greatly triggered in the stationary phase of clostridia. Maximum butanol concentration of 12.94 ± 0.09 g/L was achieved in presence of folic acid. Incidentally, the current study supported the butanol production pathway with addition of folic acid which is a precursor to many amino acid biosynthesis in microorganisms. The specific upsurge in butanol concentration with the help of folic acid would be an alternative and feasible approach which can be implemented with further modifications to strengthen ABE fermentation economics.

4. MATERIALS AND METHODS

4.1. Clostridial Strain and Its Maintenance. The bacterium, *C. acetobutylicum* NRRL B-527 was obtained from ARS (Agricultural Research Services) Culture Collection, USA. Freeze-dried cells were stimulated in sterile revival media (RM) containing (g/L): glucose (5.0), beef extract (10), yeast extract (3.0), peptone (10), sodium chloride (5.0), sodium acetate (3.0), soluble starch (1.0), L-cysteine hydrochloride (0.5), and resazurin (0.001), at pH 6.8. The RM culture bottles were incubated at 37 ± 2 °C for 48 h. Furthermore, the clostridial spore suspension was prepared according to Nimbalkar et al.²⁹ and used whenever required.

4.2. Inoculum and Production Media. The seed culture was prepared by inoculating 2% (v/v) spores in sterile 80 mL-reinforced clostridial medium (RCM) through heat shock treatment. Further, the RCM culture bottles were kept in an incubator for 18–20 h at 37 °C. The fermentation medium (P2) used in the current study contained the following (g/L):

glucose (60), ammonium acetate (2.2), dipotassium hydrogen phosphate (0.5), potassium dihydrogen phosphate (0.5), magnesium sulfate (0.2), *p*-aminobenzoic acid (0.1), thiamin (0.1), manganese sulfate (0.01), iron sulfate (0.01), sodium chloride (0.01), and biotin (0.01) at pH 6.5. The nitrogen was purged through fermentation media and autoclaved subsequently at 121 °C for 20 min. Analytical grade chemicals were used in the present study.

4.3. Fermentation Experiments. The batch experiments were carried out in 100 mL glass bottles with 80 mL production medium. Folic acid (at varied concentration range of 1–100 mg/L) was added in the production medium by filter sterilization before inoculation. Fermentation was initiated by inoculating 5% (v/v) actively growing clostridial cells and maintained at 37 °C for 120 h. The standard P2 medium was used as a control. Batch fermentation was also performed in a 3 L bioreactor (Dhruv Fabrotech, India) fitted with pH probe, temperature sensor, and working volume of 1.5 L. All other fermentation conditions for the bioreactor were kept similar as explained earlier unless otherwise stated. The effect of folic acid was determined by analyzing samples after each 24 h interval. The data points were plotted to compare the fermentation performance. All the experiments were performed at least in triplicate and results presented are with average value ± standard deviation (SD).

4.4. Analytical Techniques. Total solvents (acetone, butanol, and ethanol) and acids (acetic acid and butyric acid) were quantified by analyzing samples in gas chromatography (Agilent Technologies 7890B) equipped with AB-INNOWax capillary column (30 m × 0.32 mm × 1 mm) and flame ionization detector. The oven program was set as 90 (5 min hold) to 200 °C at 40 °C/min rise (5 min hold). The injector (0.5 μL injection volume) and detector were maintained at 200 and 250 °C, respectively. Unutilized glucose was determined by the dinitrosalicylic acid method.³⁰ A UV-visible spectrophotometer (3000+, Labindia) was used to monitor clostridial growth by measuring optical density at 600 nm. Besides, a fermentation medium pH was also recorded with the help of a laboratory pH meter (Global, India). Folic acid concentration after fermentation was estimated according to Matias et al.³¹ Statistical analysis (ANOVA) was also performed to evaluate the significant difference between the parameters studied.

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Wang, Z.; Qi, W.; Zhu, S.; Feng, P.; Qin, L. Microbial Butanol Production. In *Microbial Energy Conversion*; Yuan, Z., Ed.; De Gruyter: Berlin, 2018; pp 210–269.
- (2) Bankar, S. B.; Survase, S. A.; Ojamo, H.; Granström, T. Biobutanol: the outlook of an academic and industrialist. *RSC Adv.* **2013**, *3*, 24734–24757.
- (3) Jiménez-Bonilla, P.; Wang, Y. In situ biobutanol recovery from clostridial fermentations: a critical review. *Crit. Rev. Biotechnol.* **2018**, *38*, 469–482.
- (4) Jiang, C.; Cao, G.; Wang, Z.; Li, Y.; Song, J.; Cong, H.; Zhang, J.; Yang, Q. Enhanced butanol production through adding organic acids and neutral red by newly isolated butanol-tolerant bacteria. *Appl. Biochem. Biotechnol.* **2016**, *180*, 1416–1427.
- (5) Li, X.; Shi, Z.; Li, Z. Increasing butanol/acetone ratio and solvent productivity in abe fermentation by consecutively feeding butyrate to weaken metabolic strength of butyrate loop. *Bioprocess Biosyst. Eng.* **2014**, *37*, 1609–1616.
- (6) Nasser Al-Shorgani, N. K.; Kalil, M. S.; Wan Yusoff, W. M.; Shukor, H.; Hamid, A. A. Improvement of the butanol production selectivity and butanol to acetone ratio (B:A) by addition of electron carriers in the batch culture of a new local isolate of *Clostridium acetobutylicum* YM1. *Anaerobe* **2015**, *36*, 65–72.
- (7) Luo, H.; Zeng, Q.; Han, S.; Wang, Z.; Dong, Q.; Bi, Y.; Zhao, Y. High-efficient n-butanol production by co-culturing *Clostridium acetobutylicum* and *Saccharomyces cerevisiae* integrated with butyrate fermentative supernatant addition. *World J. Microbiol. Biotechnol.* **2017**, *33*, 76.
- (8) Magalhães, B. L.; Grassi, M. C. B.; Pereira, G. A. G.; Brocchi, M. Improved n-butanol production from lignocellulosic hydrolysate by clostridium strain screening and culture-medium optimization. *Biomass Bioenergy* **2018**, *108*, 157–166.
- (9) Maiti, S.; Gallastegui, G.; Sarma, S. J.; Brar, S. K.; Le Bihan, Y.; Drogui, P.; Buelna, G.; Verma, M. A re-look at the biochemical strategies to enhance butanol production. *Biomass Bioenergy* **2016**, *94*, 187–200.
- (10) Gao, M.; Tashiro, Y.; Wang, Q.; Sakai, K.; Sonomoto, K. High acetone-butanol-ethanol production in pH-stat co-feeding of acetate and glucose. *J. Biosci. Bioeng.* **2016**, *122*, 176–182.
- (11) Xue, C.; Zhao, J.; Chen, L.; Yang, S.-T.; Bai, F. Recent advances and state-of-the-art strategies in strain and process engineering for biobutanol production by *Clostridium acetobutylicum*. *Biotechnol. Adv.* **2017**, *35*, 310–322.
- (12) Luo, H.; Ge, L.; Zhang, J.; Zhao, Y.; Ding, J.; Li, Z.; He, Z.; Chen, R.; Shi, Z. Enhancing butanol production under the stress environments of co-culturing *Clostridium acetobutylicum*/*Saccharomyces cerevisiae* integrated with exogenous butyrate addition. *PLoS One* **2015**, *10*, No. e0141160.
- (13) LeBlanc, J. G.; Laiño, J. E.; del Valle, M. J.; Vannini, V.; van Sinderen, D.; Taranto, M. P.; de Valdez, G. F.; de Giori, G. S.; Sesma, F. B-group vitamin production by lactic acid bacteria – current knowledge and potential applications. *J. Appl. Microbiol.* **2011**, *111*, 1297–1309.
- (14) Bermingham, A.; Derrick, J. P. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. *Bioessays* **2002**, *24*, 637–648.
- (15) Kousha, M.; Yeganeh, S.; Keramat Amirkolaie, A. Effect of sodium selenite on the bacteria growth, selenium accumulation, and selenium biotransformation in *Pediococcus acidilactici*. *Food Sci. Biotechnol.* **2017**, *26*, 1013–1018.
- (16) He, Y.; Chen, Z.; Liu, X.; Wang, C.; Lu, W. Influence of trace elements mixture on bacterial diversity and fermentation characteristics of liquid diet fermented with probiotics under air-tight condition. *PLoS One* **2014**, *9*, No. e114218.
- (17) Nimbalkar, P. R.; Khedkar, M. A.; Parulekar, R. S.; Chandgude, V. K.; Sonawane, K. D.; Chavan, P. V.; Bankar, S. B. Role of trace elements as cofactor: an efficient strategy toward enhanced biobutanol production. *ACS Sustainable Chem. Eng.* **2018**, *6*, 9304–9313.
- (18) Virk, B.; Jia, J.; Maynard, C. A.; Raimundo, A.; Lefebvre, J.; Richards, S. A.; Chetina, N.; Liang, Y.; Helliwell, N.; Cipinska, M.; Weinkove, D. Folate acts in *E. coli* to accelerate *C. elegans* aging independently of bacterial biosynthesis. *Cell Rep.* **2016**, *14*, 1611–1620.
- (19) Liu, D.; Chen, Y.; Ding, F.-Y.; Zhao, T.; Wu, J.-L.; Guo, T.; Ren, H.-F.; Li, B.-B.; Niu, H.-Q.; Cao, Z.; Lin, X.-Q.; Xie, J.-J.; He, X.-J.; Ying, H.-J. Biobutanol production in a *Clostridium acetobutylicum* biofilm reactor integrated with simultaneous product recovery by adsorption. *Biotechnol. Biofuels* **2014**, *7*, 5.
- (20) Ding, J.; Luo, H.; Xie, F.; Wang, H.; Xu, M.; Shi, Z. Electron receptor addition enhances butanol synthesis in ABE fermentation by *Clostridium acetobutylicum*. *Bioresour. Technol.* **2018**, *247*, 1201–1205.
- (21) Li, T.; Yan, Y.; He, J. Reducing cofactors contribute to the increase of butanol production by a wild-type clostridium sp. strain BOH3. *Bioresour. Technol.* **2014**, *155*, 220–228.
- (22) Wu, J.; Dong, L.; Zhou, C.; Liu, B.; Feng, L.; Wu, C.; Qi, Z.; Cao, G. Developing a coculture for enhanced butanol production by *Clostridium beijerinckii* and *Saccharomyces cerevisiae*. *Bioresour. Technol. Rep.* **2019**, *6*, 223–228.
- (23) Li, X.; Li, Z.; Zheng, J.; Shi, Z.; Li, L. Yeast extract promotes phase shift of bio-butanol fermentation by *Clostridium acetobutylicum* ATCC824 using cassava as substrate. *Bioresour. Technol.* **2012**, *125*, 43–51.
- (24) Maynard, C.; Cummins, I.; Green, J.; Weinkove, D. A bacterial route for folic acid supplementation. *BMC Biol.* **2018**, *16*, 67.
- (25) Abd-Alla, M. H.; Elsadek El-Enany, A.-W. Production of acetone-butanol-ethanol from spoilage date palm (*Phoenix dactylifera* L.) fruits by mixed culture of *Clostridium acetobutylicum* and *Bacillus subtilis*. *Biomass Bioenergy* **2012**, *42*, 172–178.
- (26) Soni, B. K.; Soucaille, P.; Goma, G. Continuous acetone-butanol fermentation: influence of vitamins on the metabolic activity of *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.* **1987**, *25*, 317–321.
- (27) Díez-Antolínez, R.; Hijosa-Valsero, M.; Paniagua, A. I.; Gómez, X. Effect of nutrient supplementation on biobutanol production from cheese whey by ABE (Acetone-Butanol-Ethanol) fermentation. *Chem. Eng. Trans.* **2016**, *49*, 217–222.
- (28) Kumar, S.; Banerjee, R. Enzymatic delignification and saccharification of *Bambusa bambos* for biobutanol production. *Ind. Crops Prod.* **2018**, *125*, 386–394.
- (29) Nimbalkar, P. R.; Khedkar, M. A.; Gaikwad, S. G.; Chavan, P. V.; Bankar, S. B. New Insight into Sugarcane Industry Waste Utilization (Press Mud) for Cleaner Biobutanol Production by Using *C. acetobutylicum* NRRL B-527. *Appl. Biochem. Biotechnol.* **2017**, *183*, 1008–1025.
- (30) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428.
- (31) Matias, R.; Ribeiro, P. R. S.; Sarragaça, M. C.; Lopes, J. A. A UV spectrophotometric method for the determination of folic acid in pharmaceutical tablets and dissolution tests. *Anal. Methods* **2014**, *6*, 3065–3071.