Enhanced Biobutanol Production from Fern Root using *Clostridium Acetobutylicum* CGMCC1.0134 with Yeast Extract Addition

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Fern root (FR) was used in the biofuel production for the first time. However, fermentation of the enzymatic hydrolysate of FR starch (EHFS) directly to butanol by *Clostridium acetobutylicum* CGMCC1.0134 resulted in a low butanol production and yield with high content of starch residual. After adding yeast extract (YE) solution (with a 3 g/L final concentration in broth) into EHFS, the butanol production, productivity, and yield were raised by 174%, 250%, and 183%, respectively. Monitoring changes of free amino acid concentration in the fermentation broth indicated that aspartic acid families and serine families were stimulated to accumulate by YE addition. Gene expression analysis further revealed that ctfB coding CoA-transferase was induced by adding YE into EHFS. It was concluded that EHFS could be a promising substrate for butanol fermentation.

Keywords: Clostridium acetobutylicum CGMCC1.0134; Butanol fermentation; Fern root starch; Yeast extract; Real-time fluorescence quantitative PCR

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INTRODUCTION

Butanol ($C_4H_{10}O$) is a four-carbon, straight-chained molecule, and it is an important chemical precursor for paints, polymers, and plastics (Jiang *et al.* 2015). Compared with ethanol, butanol is easier to blend with gasoline or other hydrocarbon products; it contains a higher proportion of hydrogen and carbon, which is approximately the same amount as a 25% increment in harvest energy (Nigam and Singh 2011; Ibrahim *et al.* 2018). In 2012, butanol consumption in China accounts for approximately 34.8% of the global amount, and mainly relies on imports (around 60%) (Jiang *et al*. 2015).

Recently, increasing worldwide attention on the energy crisis and global climate change has led to further development of alternative energy as a substitute for fossil fuel (Himmel *et al*. 2007). Biofuels are environmentally friendly energy sources to fulfill the global energy demand (Nigam and Singh 2011). Specifically, biobutanol has attracted attention due to the rising price of butanol caused by the steep increase in international oil prices since 2006 (Chiao and Sun 2007; Lee *et al*. 2008; Ni and Sun 2009).

Biobutanol is fermented during an anaerobic pathway by *Clostridium spp*., accompanying the production of acetone and ethanol as byproducts (Jones and Woods 1986). Its fermentation process can be divided into two phases: the acidogenic phase, when organic acids accumulate and the value of pH drops rapidly, and the solventogenic phase, when the organic acids re-assimilate to acetone and butanol (Jones and Woods 1986).

In butanol fermentation the cost of feedstocks can account for up to 75% of the total cost (Jiang *et al*. 2015). Due to its decisive role in the economics, finding an inexpensive

substrate is a persistent pursuit in the fermentation industry. Corn and molasses, as preferred substrates often have been used in traditional butanol fermentation, while corn mainly has been used in China (Jiang *et al*. 2015). In recent years, the price of corn increased rapidly due to the expanding biological refining industry in China (Qiu *et al*. 2010). Other substrates, such as sago starch (Al-Shorgani *et al.* 2011), cassava (Tran *et al*. 2010; Lu *et al*. 2012), sweet sorghum (Cai *et al*. 2013), wheat B-starch (Luo *et al*. 2018), and Jerusalem artichoke (Sarchami and Rehmann 2015), are also applicable as substrates in butanol fermentation. Currently, using starch feedstock for biofuel is still widely favored. Alternative feedstocks include cellulose, but the saccharification and detoxification processes of cellulose are very complicated, which directly leads to a greatly increased cost and severely limited production (Green 2011; Ibrahim *et al.* 2018). Moreover, some feedstocks with high starch content, such as cassava, are also used for industrial bioethanol and other biofuels production in China (Qiu *et al.* 2010). However, the use of competitive substrates with ethanol fermentation inevitably results in the increase of the feedstock cost.

Fern root (FR) is derived from the rhizome of *Pteridium aquilinum* (L.) Kuhn var. *latiusculum* (Desv.) Underw, whose growth is not compatible with arable land. Ferns are abundantly distributed in South China with more than 2,600 species, accounting for one-fifth of the world's reserves, with most of them growing in the mountains (Wu and Raven 2013). At present, ferns are mainly derived from wild resources. In recent years, artificial cultivation techniques become more popular in production of ferns. According to reports, under the current cultivation conditions, fern roots can yield a harvest of 37,500 kg/hm^2 every 2 to 3 years (Huang *et al.* 2016). The reported extremely high starch content (35% to 45%) in a wild-growing FR enables the possibilities of using FR for biofuel production. Furthermore, to avoid the difficulty of pretreatment and the production of toxic substances, currently researchers use FR starch of which the cellulose content has already been removed from FR. The resulting FR starch is made of 0.09% protein, 0.12% crude fat, 88.84% starch, and trace elements such as magnesium, zinc, and strontium (Du *et al*. 2016).

Unfortunately, not much attention has been paid to the use of FR starch as substrate for butanol fermentation, although FR starch has a high starch content and is suitable for biofuel fermentation. Domestic research on FR starch is mostly limited to its basic physical and chemical properties (Du *et al*. 2016). Similarly, it only has been reported in the industrial field that oxidized FR starch binder is a natural binder with strong adhesion, no toxicity, and low price (Liu *et al*. 1999; Rohan *et al*. 2018). The FR starch has also been processed into food. However, some studies have shown that FR has toxicity. For instance, a variety of tumors can be induced in different test animals such as mice (Jarrett *et al*. 1978), guinea pigs (Bringuier *et al*. 1995), baboons (El-Mofty *et al*. 1987), and rats (Pamukcu *et al.* 1980) by feeding on fern. Therefore, using FR starch in food production is not popular in China.

Taking high starch content without cellulose in FR starch into account, a high-quality fermentation substrate solution is available by simple enzymatic operation. In this study, FR starch was used as a substrate in butanol fermentation by *Clostridium acetobutylicum* CGMCC1.0134 for the first time. This study was to solve the non-negligible problem of poor performance in butanol fermentation by FR starch so that it can be utilized as a substitute for corn. By monitoring the butanol fermentation process, adding an appropriate amount of yeast extract (YE) was found to be able to enhance butanol production, shorten fermentation time, and increase starch utilization. Furthermore, to investigate the underlying mechanism of enhanced performance in butanol fermentation

after the addition of YE, changes of amino acid concentration in the fermentation broth were monitored to explore whether the addition of YE promoted the secretion of certain amino acids by bacteria after excluding possible effects caused by the components of YE. Expression levels of genes coding key enzymes were also measured simultaneously to determine whether the addition of YE could promote the expression of certain key enzyme genes in cells.

EXPERIMENTAL

Microorganism and Culture Medium

The bacterial strain used in this study was *Clostridium acetobutylicum* CGMCC1.0134, which was obtained from the China General Microbiological Culture Collection Center (Beijing, China). Seed was maintained as spore suspension in a 5% corn meal medium at 4 °C (Li *et al*. 2014).

Preparation of Enzymatic Hydrolysate of FR Starch (EHFS)

The preparation steps of enzymatic hydrolysate of FR starch (EHFS) are shown in Fig. 1, and briefly described as follows. The FR starch was pretreated by adding a small amount of α-amylase (8 U/g-corn, heated in boiling water bath for 45 min) after being sieved up to a mesh size of 40. The medium was autoclaved with neutral pH at 121 °C for 15 min. The FR starch used for study was purchased from a local market. Alpha-amylase (20,000 U/mL) was purchased from Aladdin Industrial Corporation (Shanghai, China) (Li *et al*. 2012).

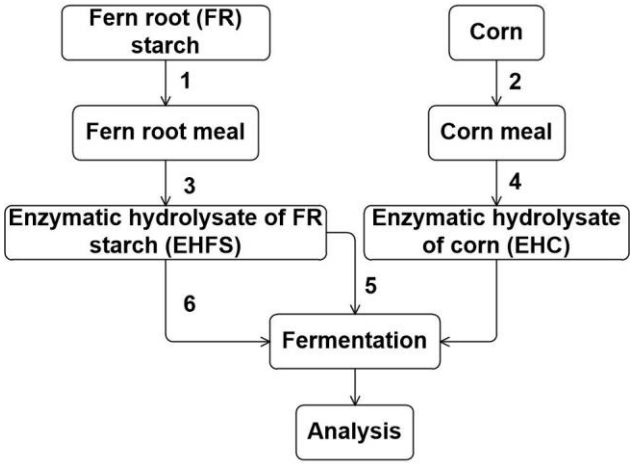

Fig. 1. The experimental design. 1 and 2: Sieved up to a mesh size 40, then boiling water bath for 30 min; 3 and 4: Pretreated by adding a minuscule amount of alpha-amylase (8 U/g-corn, heated in boiling water bath for 45 min); 5: Adding urea solution (final concentration in medium was 3 g/L-broth); 6: Adding yeast extract (final concentration in medium was 3 g/L-broth)

Butanol Fermentation

Seed culture was conducted in a 250 mL fermentation bottle with a 150 mL working volume incubated in an anaerobic incubator (YQX-II, Shanghai Heng Yue Medical Devices Corporation, Shanghai, China) at 37 °C for 20 h using corn meal (5%, W/V) as

the substrate. Butanol fermentation was conducted by adding 15 mL of seed liquid into a 250 mL fermentation bottle with 150 mL of fermentation medium (7%, W/V) in anaerobic incubator (YQX-II, Shanghai Heng Yue Medical Devices Corporation, Shanghai, China). The cell growth, pH value, acetic acid concentration (C_{Ac}), butyric acid concentration (C_{Bu}), solvents concentration, glucose concentration (C_{Glu}), and residual starch concentration (C_S) were measured during the fermentation process. Triplicate fermentation treatments were carried out under the same conditions for each substrate.

Analytical Methods

Cell growth was determined as cell DNA. The DNA content was analyzed colorimetrically in deproteinized trichloroacetic acid (TCA, $C_2HCl_3O_2$) extracts (Martin and McDaniel 1975). A 30 mL fermentation broth was placed in ice water bath for 10 min, and then centrifuged at 0 °C, 9000 rpm for 10 min. The precipitate was added with 30 mL of TCA (5%, W/V), and shaken thoroughly. The precipitate was centrifuged at 0 °C, 9000 rpm for 10 min, and washed again according to the previous step. Next, the precipitate was added to 30 mL of TCA (5%, W/V) and shaken thoroughly. It was then extracted in a water bath at 80 °C for 25 min, cooled in an ice water bath, and centrifuged at 0 °C, 9000 rpm for 10 min. The supernatant was diluted using the appropriate multiple with TCA (5%, W/V). For UV spectrophotometry, 3 mL of TCA (10%, W/V) was used as a blank control, and diluted samples were read with appropriate multiples for each test. The absorbance was kept between 0.1 and 0.8 by adjusting the sample concentration. Absorbance was measured at 260 nm. The supernatant obtained after centrifugation (5000 rpm for 5 min) of fermentation broth was diluted by an appropriate multiple, then injected into a biosensor (SBA-40D, Institute of Biology, Shandong Academy of Sciences, Jinan, China) to detect glucose concentration (C_{Glu}).

For the C_S measurement steps, starch was hydrolyzed by 2 M hydrochloric acid solution (heated in boiling water bath for 45 min). After the starch was completely hydrolyzed to glucose solution, the pH was adjusted to neutral using a 6 M NAOH solution. Then C_{Glu} in the solution was detected by biosensor. Next, C_S was calculated from the final glucose concentration ($C_S = C_{Glu} \times 0.9$) (Luo *et al*. 2016). Acetic acid, butyric acid, acetone, butanol, and ethanol were determined by gas chromatography (GC-2014C, Shimadzu Corporation, Kyoto, Japan) with a C_{18} column (ZKAT-FFAP). The above analysis was carried out under the following conditions: 1) oven temperature: the initial temperature was 40 °C. After retaining for 1 min, it was warmed to 70 °C with a heating gradient of 3 °C/min. Then the temperature was retained for another 1 min, followed by heating up to 140 °C at 5 °C/min. The temperature was retained again for 1 min, then heated up to 200 °C with a gradient of 15 °C/min, and kept for 15 min; 2) injector temperature: 160 °C; 3) detector temperature: 220 °C; 4) carrier gas (nitrogen) flow rate: 2 mL/min; 5) hydrogen flow rate: 40 mL/min; 6) air flow rate: 400 mL/min. Free amino acid concentrations in the broth were determined by an automatic amino acid analyzer L-8900 (HITACHI Construction Machinery Corporation, Tokyo, Japan). Sample pretreatment was the same as described in a previous study (Li *et al*. 2012).

RNA Isolation and Real-Time Fluorescence Quantitative PCR

For RNA isolation, 50 mL of the cultures growing on fermentation medium at different times (*i.e*., 12 h, 24 h, 48 h, 72 h, and 84 h, as shown in Fig. 6) were harvested with centrifugation at 5000 rpm, for 5 min at 4 °C. Total RNA was extracted using the RNAprep pure Cell/Bacteria Kit (DP430, TIANGEN Biotech (Beijing) Corporation,

Beijing, China). The RNA quantity and concentration were determined by electrophoresis (Mini Pro 300 V Power Supply, Major Science, Saratoga, USA) and ND2000 (Nano Drop 2000 Fluorospectrometer, Thermo Fisher Scientific, Waltham, USA), respectively. For cDNA synthesis, 2 μg total RNA samples were treated with 1 μL of Oligo (dT) (50 uM) and 1 μL of dNTP Mix (10 mmol/L), then RNase free dH_2O was added until a total volume of 10 μL was reached. After being evenly mixed, it was placed in a 65 °C water bath for 5 min, 0 °C for 1 min, and then centrifuged. The authors further discarded the supernatant, added the reverse transcription reaction solution, and mixed well. It was reacted at 42 °C for 1 h, and then transferred to a 95 °C warm bath for 5 min until the reaction was over. The cDNA samples were stored at -20 °C for use. Subsequently, real-time PCR reactions were carried out on a real-time PCR system (TIB8600, TIB Biosciences Corporation, Beijing, China) under the following reaction system: 2×SYBR real-time PCR premixture 10 μL, upstream and downstream primers (both 10 μmol/L) 0.4 μL each, cDNA template 1 μL, and RNase free dH_2O added up to a 20 μL total volume. The primers were adhE-S (5′-agaggaatttgtaaaacgaggat-3′), adhE-A (5′-ttcaacagattgtacttcgccta-3′), bdhA-S (5′-ctgatgattacgaggctagagct-3′), bdhA-A (5′-ctattccccaaacatttattcca-3′), ctfB-S (5′-agaaaacggaatagttggaatgg-3′), ctfB-A (5′-tgaccaccacggattagtgaaa-3′), 16S-S (5′-ttgagccaaaggatttattcg-3′), and 16S-A (5′-gaccgtgtctcagttccaatg-3′), respectively. The conditions were one cycle of 95 °C for 5 min, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. Triplicate PCR reactions were carried out. The amplification plot and melt curve are shown in Fig. S1 and Fig. S2, respectively.

RESULTS AND DISCUSSION

Butanol Fermentation on Enzymatic Hydrolysates of FR Starch and Corn (EHFS and EHC)

In this study, EHFS were subjected to butanol fermentation by *Clostridium acetobutylicum* CGMCC 1.0134 directly in an anaerobic workstation at 37 °C to compare the fermentation performance with EHC that operated under the same conditions. The process of feedstock treatment is shown in Fig. 1. The cell growth, pH, acid concentration, butanol concentration (C_{Bt}), starch concentration (C_S), and glucose concentration (C_{Glu}) in EHC and EHFS are shown in Fig. 2. The bacteria entered the logarithmic phase at 20 h, and reached the highest DNA content (0.36 mg/mL) at 42 h when cultivated in EHC. In comparison, it reached the highest DNA content (0.17 mg/mL) in EHFS slowly (Fig. 2A). Obviously, the cell growth rate in EHC was far faster than EHFS from 24 h to 54 h.

Figure 2A also shows that the pH in EHC declined to a lowest point (4.33) at 24 h, and went up again rapidly, but the pH in EHFS remained almost constant after dropping to a relatively lower point (4.05) at 72 h. It has been reported that the initiation of solvent production occurs only after the pH of the medium decreases to values at around 4.5 to 5.0 (Jones and Woods 1986). Thus, solvent generation is possible in EHC or EHFS.

Figure 2B illustrates the evolution of acid concentration. The curve trend of acetic acid in EHC was increased, decreased, and increased again, which was consistent with butyric acid. In comparison, acetic acid concentration (C_{Ac}) in EHFS continued to rise and reached a very high level (3.8 g/L) at 72 h, while butyric acid concentration (C_{Bu}) slowly increased and was always below 2 g/L. Luo *et al*. (2015; 2016) published that acetic acid and butyric acid were not only byproducts but also carbon sources for solventogenesis. A noticeable decrease of acid concentration was observed in EHC, but this phenomenon did

not occur in EHFS. It was inferred that a large amount of organic acid was not reabsorbed to form solvents, but continuously accumulated. In EHC, the relatively higher total organic acid concentration (C_{Tacid}, C_{Tacid}= C_{Bu} + C_{Ac}) was only around 2.5 g/L at 12 h, among which C_{Bu} was 2.3 g/L. In EHFS, C_{Tacid} was comparable with that in EHC at 12 h. However, the C_{Bu} was only 0.8 g/L at 12 h. Subsequently, acetic acid and butyric acid had been accumulated continuously, with a total C_{Tacid} over 4 g/L at 72 h. Hüsemann and Papoutsakis (1988) have reported that the initial solventogenesis depended on the undissociated C_{Bu} but did not require minimum undissociated C_{Ac}. At pH 6.0, only 6% of the total amount of butyric acid was in the undissociated form, while 66% occurred in the undissociated form at pH 4.5 (Jones and Woods 1986). Monot *et al*. (1984) found that, when the concentration of undissociated butyric acid reached a level of 0.5 g/L to 0.8 g/L, cell growth was inhibited and the induction of solvents occurred when the concentration of undissociated butyric acid reached a level of 1.5 g/L to 1.9 g/L. Based on the published experimental data from the above literature, it was evaluated that undissociated C_{Bu} in EHC was already up to 1.5 g/L at 12 h, which favored the initiation of solventogenesis. But in EHFS, undissociated C_{Bu} was below 1.5 g/L throughout the whole fermentation process. It was inferred that the poor fermentation performance in EHFS resulted from inhibition by solvents.

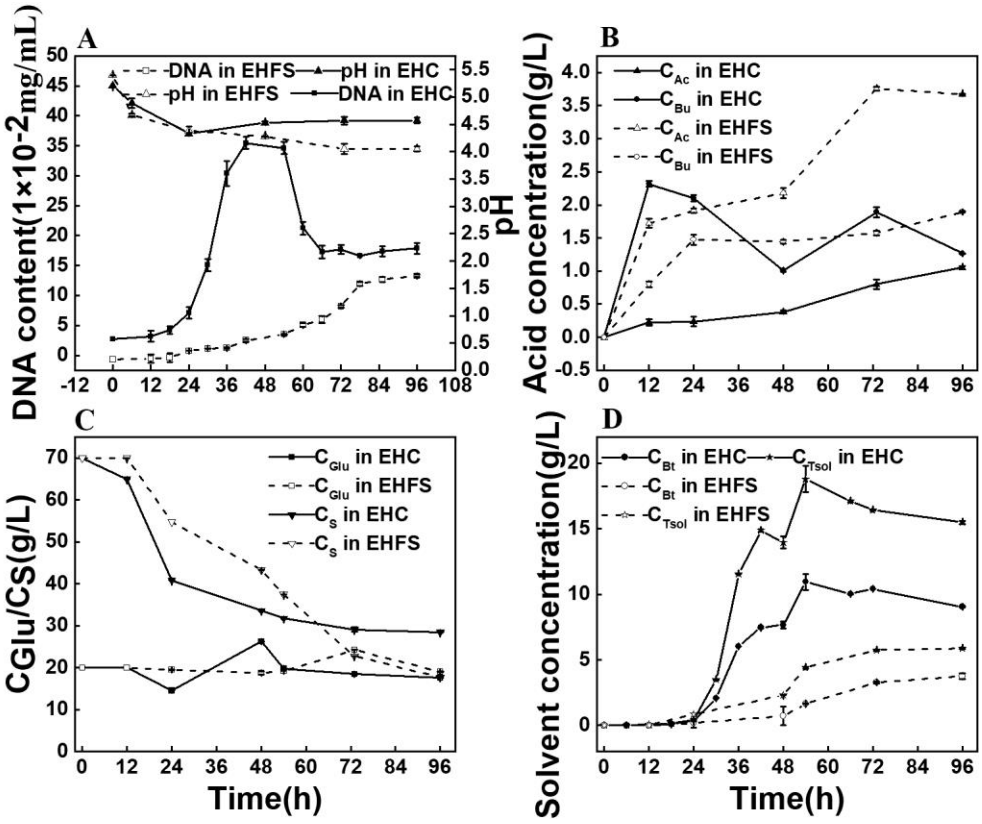

Fig. 2. The profiles of flask fermentation for butanol production using EHC and EHFS. C_{Ac}, C_{Bu}, C_{Glu}, C_S, C_{Bt}, and C_{Tsol}: the concentrations of acetic acid, butyric acid, glucose, starch, butanol, and total solvent in the fermentation broth

The C_S, C_{Glu}, and C_{Bt} in EHC and EHFS were plotted in Fig. 2C and Fig. 2D. The starch consumption rate between EHC and EHFS was comparable (4.72 g/L/h and 3.63 g/L/h, respectively). The fermentation ended with higher residual starch concentrations in

EHC than in EHFS, but SCR and glucose consumption rate were faster than EHFS. Butanol production and productivity in EHC were 10.95 g/L and 0.20 g/L/h, respectively. In comparison, butanol production in EHFS (3.74 g/L) was only 34% of that in EHC, and the productivity was even lower when compared to the control. Although the mechanism from acidogenesis to solventogenesis is not understood now (Li *et al*. 2012), it was speculated that excess organic acid accumulation without any re-assimilation contributed to the poor fermentation performance, which was mainly reflected in lower pH point, lower butanol production, and higher residual starch.

Based on the above description, it was feasible that EHFS could be used as a fermentation substrate for butanol fermentation. However, EHFS could not be used widely in the industry production, unless the defects of longer fermentation time, lower butanol production, and lower productivity were overcome. The FR starch has a higher carbohydrate content, but lower proportion of other ingredients (Du *et al*. 2016) compared to corn. During the process of microorganism growth, the type and concentration of nitrogen sources also play crucial roles (Gouveia and Oliveira 2009). In this study, free amino acids contents were measured to consider issues related to nitrogen sources. Free amino acids contents in FR starch and corn after complete hydrolysis are shown in Table 1. Free amino acids contents in EHFS were less than 50% of those in EHC. Moreover, amino acids concentrations in FR starch (430.7 mg/L) were remarkably lower than those in corn (7713.6 mg/L) medium after enzymatic pre-treatment (Table 1).

Table 1. Amino Acids Concentrations in Corn/FR Starch after Complete Proteolysis and in EHFS/EHC

Amino Acid	Corn (mg/100 g-corn starch [a]	FR Starch (mg/100 g-FR starch) [a]	EHFS (mg/L)[b]	EHFS+ Yeast Extract (mg/L)[b]	EHC (mg/L)[b]
Asp	616.5	14.3	1.5	1.6	36.4
Thr	293.7	75.3	4.3	47.4	3.1
Ser	396.5	46.4	10.3	29.1	5.6
Glu	1529.9	23.7	2.1	92.8	44.1
Gly	294.7	25.2	3.8	36.0	2.7
Ala	586.7	30.3	25.2	69.6	2.5
Cys	117.3	21.7	0.0	10.3	2.8
Val	365.7	25.5	0.6	86.4	13.0
Met	107.4	51.4	10.0	27.9	18.4
Ile	269.3	15.9	12.7	73.8	1.9
Leu	873.8	13.7	19.0	124.4	16.1
Tyr	287.7	6.5	1.5	7.2	4.3
Phe	371.6	15.6	2.2	89.6	0.4
Lys	284.0	11.9	5.8	75.2	1.2
His	201.9	3.2	5.3	8.3	5.9
Arg	423.8	21.4	2.9	62.0	5.2
Pro	693.1	28.7	4.7	12.5	66.4
Totals	7713.6	430.7	111.9	854.2	230.0

Asp: aspartic acid; Thr: threonine; Ser: serine; Glu: glutamic acid; Gly: glycine; Ala: alanine; Cys: tryptophan; Val: valine; Met: methionine; Ile: isoleucine; Leu: leucine; Tyr: tyrosine; Phe: phenylalanine; Lys: lysine; His: histidine; Arg: arginine; Pro: proline.

[a]: Detection of amino acids contents after complete proteins hydrolysis of corn or FR starch meal (g/100 g-corn or FR starch meal).

[b]: The test for free amino acids concentrations in corn or FR starch medium after enzymatic pre-treatment (g/L) (C_0).

It could be inferred that FR starch was severely deficient in nitrogen source compared to corn. Nair *et al*. (1999) has reported that due to the observation spore formation in *Clostridia* is not triggered when glucose and/or ammonia limitation exists. Accordingly, the shift to the solventogenic phase will not occur either if there is not enough nutrient. The authors proposed that the lack of nitrogen source was likely to be the main reason for the poor fermentation performance of butanol fermentation in FR starch.

EHFS Fermentation with Exogenous YE Solution Addition

Both organic and inorganic nitrogen sources promote the growth of *Clostridium acetobutylicum* and solvent production (Madihah *et al*. 2001). To circumvent poor fermentation performance, YE (organic nitrogen source) and urea (inorganic nitrogen source) were added as the supplementary nutrients. The experiment fermented on EHFS (without any exogenous addition) was used as a control to compare the fermentation performance with that of adding YE ($EHFS_{YE}$) and urea (EHFSurea). Figure 3 depicts the fermentation performance of EHFS with urea/YE solution (final concentration in medium was 3 g/L) addition. The pH curve trend in EHFSurea was similar, but the value was higher throughout the fermentation except the initial pH when compared to the control (Fig. 3A). The C_{Ac} and C_{Bu} were both lower than the control (Fig. 3B). Very little butanol was found in the fermentation broth (Fig. 3C). In contrast, the pH in $EHFS_{YE}$ rebounded quickly after it declined to the bottom level, and the changes of acid concentration were raised up, dropped down, and raised up again (Fig. 3A). The final C_{Bt} reached 6.94 g/L, which indicated a remarkable increase compared to the control, which was comparable to the field in the EHC (Fig. 3C). Observations of the above phenomena indicated that the butanol fermentation was smoothly triggered by adding the appropriate YE solution into EHFS.

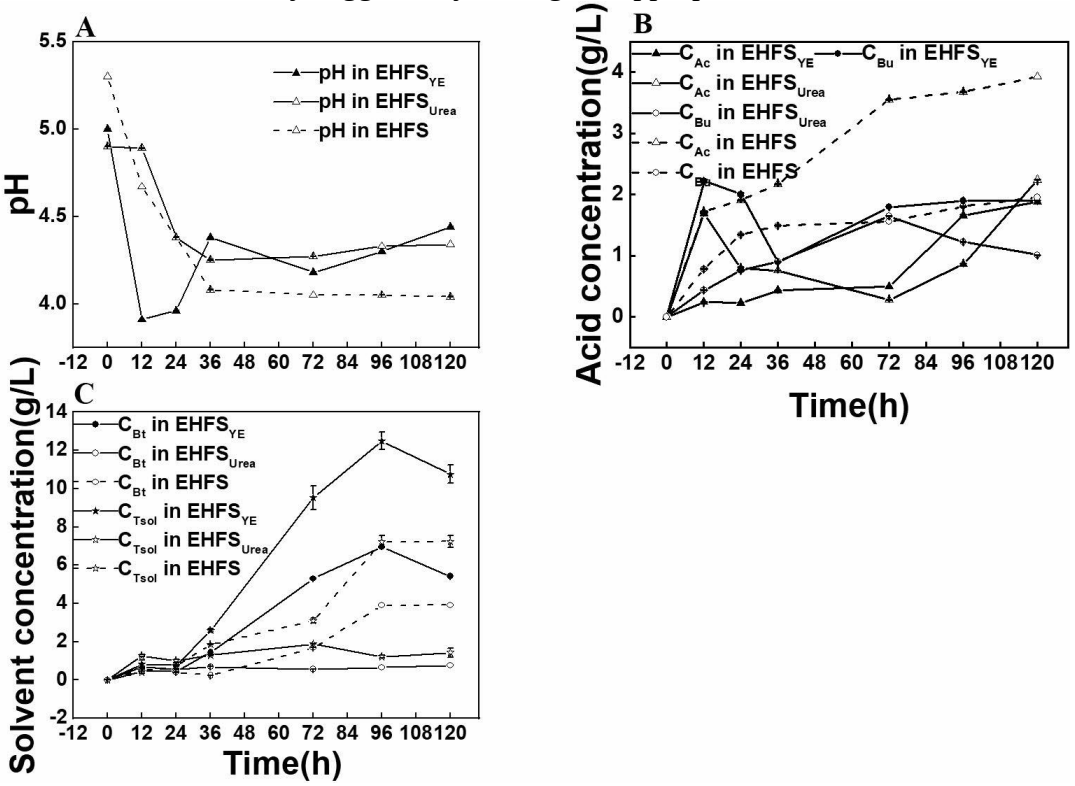

Fig. 3. The profiles of flask fermentation for butanol production using EHFS with or without yeast extract addition or with urea addition

Effects of Amino Acids on Butanol Synthesis

After YE was added into the medium, fermentation on $EHFS_{YE}$ was triggered from acidogenesis to solventogenesis smoothly. Butanol and total solvents concentration (productivity) in $EHFS_{YE}$ reached 11.8 g/L and 20.4 g/L (0.16 g/L/h and 0.28 g/L/h), respectively, whose values were 4.3 g/L and 7.8 g/L (0.04 g/L/h, 0.08 g/L/h) in EHFS (Fig. 4). Meanwhile, the final total solvents yield in $EHFS_{YE}$ (EHFS) reached 0.29 g/g-starch (0.11 g/g-starch), which was almost equivalent to those in EHC. These observations revealed that the fermentation performance was greatly improved as fermentation time shortened remarkably and starch utilization rate improved due to the addition of YE.

Amino acids, as the important nutrients and metabolic intermediates, were vital for bacterial growth and development (Xiao *et al*. 2017). It has also been verified that butanol synthesis and cell growth can benefit from certain self-generated or exogenously added amino acids (Mason *et al*. 1987). According to Heluane *et al*. (2011), methionine has a remarkable effect on improving solvents production. Similar results have been obtained where over-secreted aspartic acid families enhanced butanol synthesis noticeably (Li *et al*. 2012). To test whether amino acids helped to enhance butanol production in $EHFS_{YE}$, major amino acids were measured in butanol fermentation broth. As shown in Fig. 5, concentrations of threonine, methionine, isoleucine, aspartic acid, serine, and glycine had grown remarkably (C_{Thr}, C_{Met}, C_{Ile}, C_{Asp}, C_{Ser}, and C_{Gly}). Maximum net increment ΔC was defined as the maximum amino acid concentration after the start of fermentation (C_{max}) minus the amino acid concentration of fermentation initial medium (C_0, values shown in Table 1) (*i.e.*, $\Delta C = C_{max} - C_0$).

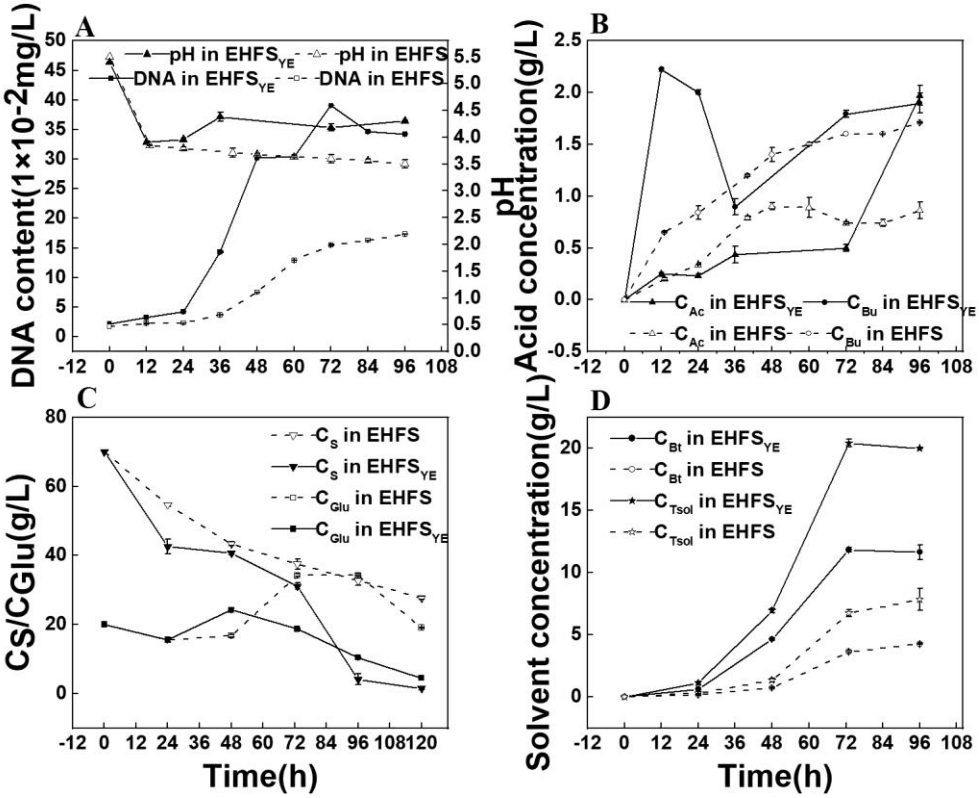

Fig. 4. The profiles of flask fermentation for butanol production using EHFS with/without yeast extract addition

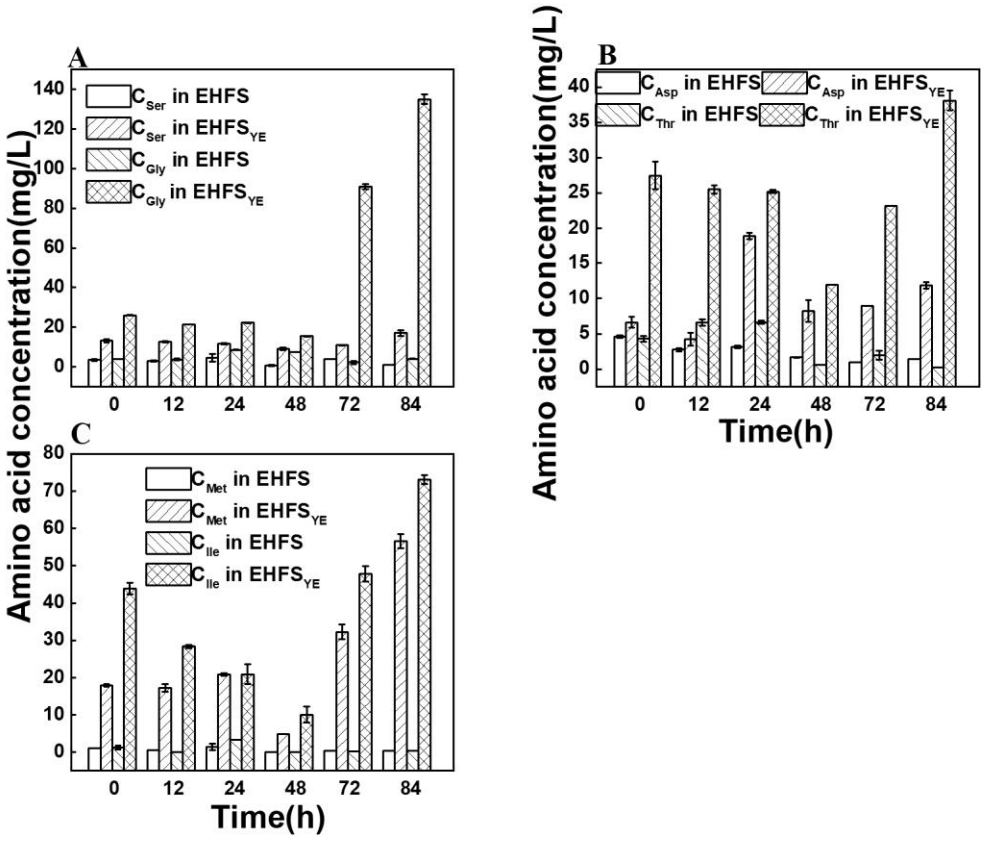

Fig. 5. Changes of different amino acids in fermentation broth fermented on EHFS/EHFS$_{YE}$. C_{Ser}, C_{Gly}, C_{Asp}, C_{Thr}, C_{Met}, and C_{Ile}: the concentrations of serine, glycine, aspartate acid, threonine, methionine, and isoleucine in the fermentation broth, respectively, fermented on EHFS/ EHFS$_{YE}$

Corresponding values of concentrations of threonine, methionine, isoleucine, aspartic acid, serine, and glycine (ΔC_{Thr}, ΔC_{Met}, ΔC_{Ile}, ΔC_{Asp}, ΔC_{Ser}, and ΔC_{Gly}) in the EHFS$_{YE}$ during the fermentation were 5 times, 115 times, 15 times, 3 times, 4 times, and 14 times greater than that in EHFS during the fermentation time, respectively. Through the above calculations, the effects of amino acids were excluded directly from exogenous YE addition in the experiment. Hence, it was surmised that these additional increased amino acids concentrations were derived from the secretion of bacteria rather than the components of YE. Among aforementioned amino acids, threonine, methionine, isoleucine, and aspartic acid belonged to aspartic acid families, while serine and glycine were from serine families. It was speculated that the addition of YE into EHFS stimulated the secretion of aspartic acid families and serine families. It was concluded that aspartic acid families and serine families were stimulated to accumulate with YE added into the EHFS, which played an important role in butanol fermentation.

Differentially Expressed Genes of *Clostridium Acetobutylicum* CGMCC 1.0134 in EHFS and EHFS$_{YE}$ Detected by Real-Time Fluorescence Quantitative PCR

To explore the molecular basis of improved fermentation performance in EHFS$_{YE}$, gene expression analysis was performed using real-time fluorescence quantitative PCR on the cells of *Clostridium acetobutylicum* CGMCC 1.0134 cultured in both EHFS and

$EHFS_{YE}$ at different fermentation times. According to the fermentation results, adding YE greatly increased starch consumption and butanol production. Nölling *et al*. (2001) proposed a metabolic map indicating the mechanism of butanol biosynthesis by *C. acetobutylicum* ATCC824. Here, three genes involved in metabolism, including ctfB, adhE, and bdhA, were selected for the experiment. The transcriptional levels of these genes are shown in Fig. 6. The ctfB gene encodes CoA-transferase, which is the most important enzyme during the phase shift from acidogenic to solventogenic phase. CoA-transferase re-assimilates the formed organic acids and converts acetic acid and butyric acid into acetyl-CoA and butyryl-CoA, which are the precursors of ethanol and butanol, respectively. It also converts acetoaceyl-CoA to acetoacetic acid, which is the precursor of acetone.

In this study, the expression level of ctfB in $EHFS_{YE}$ was 13 times higher than that in EHFS at 72 h. A significance test result showed that the impact on the expression level of ctfB by YE addition was significant ($P = 0.01 < 0.05$). Thus, the expression of the ctfB gene might be induced by the addition of YE. The adhE gene and bdhA gene encoded the butyraldehyde dehydrogenase and butanol dehydrogenase, which are two key enzymes directly associated with butanol biosynthesis (Gheshlaghi *et al*. 2009). However, the impact of YE addition on the expression of adhE and bdhA was not significant. The expression level of adhE and bdhA in $EHFS_{YE}$ were only 1.5 times and 1 times higher than that in EHFS at 72 h, which confirmed that the expression of adhE and bdhA genes of *Clostridium acetobutylicum* could not be induced by YE addition.

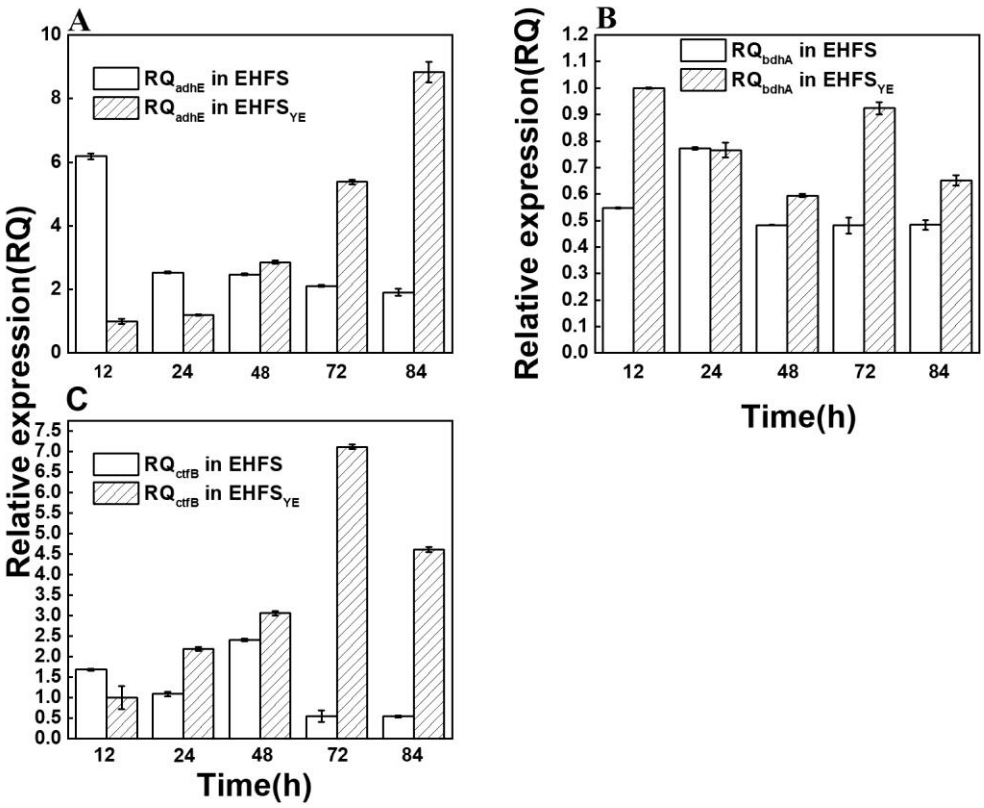

Fig. 6. Changes in relative expression (RQ) of adhE, bdhA, and ctfB fermented on EHFS/ $EHFS_{YE}$. RQ_{adhE}, RQ_{bdhA}, and RQ_{ctfB}: relative expression of adhE, bdhA, and ctfB in the fermentation broth respectively fermented on EHFS/$EHFS_{YE}$

CONCLUSIONS

1. Butanol fermentation by *Clostridium acetobutylicum* CGMCC1.0134 on enzymatic hydrolysate of fern root starch (EHFS) directly showed a low solvent production and productivity, high starch residual, and long fermentation time. With yeast extract (YE) addition into EHFS, butanol fermentation went smoothly with remarkably improved solvent production and productivity, mostly exhausted starch, and greatly shortened fermentation time.
2. Numerous accumulations of aspartic acid families and serine families promoted butanol fermentation transition from the acidogenic phase to the solventogenic phase.
3. At the molecular level, gene expression level of the gene ctfB was elevated after YE was added into EHFS, confirming that YE addition induced the expression of ctfB.
4. After calculation, the butanol production was approximately 62.05kg per ton fern root. These findings demonstrated that EHFS was a promising substrate for butanol fermentation.

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REFERENCES CITED

Al-Shorgani, N. K. N., Kalil, M. S., and Yusoff, W. M. W. (2011). "Fermentation of sago starch to biobutanol in a batch culture using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564)," *Ann. Microbiol*. 62(3), 1059-1070. DOI: 10.1007/s13213-011-0347-x

Bringuier, P. P., Piaton, P., Berger, N., Debruyne, F., Perrin, P., Schalken, J., and Devonec, M. (1995). "Bracken fern-induced bladder tumors in guinea pigs – A model for human neoplasia," *Am. J. Patbol.* 147(3), 858-868.

Cai, D., Zhang, T., Zheng, J., Chang, Z., Wang, Z., Qin, P.-Y., and Tan, T.-W. (2013). "Biobutanol from sweet sorghum bagasse hydrolysate by a hybrid pervaporation process," *Bioresource Technol*. 145(10), 97-102. DOI: 10.1016/j.biortech.2013.02.094

Chiao, J. S., and Sun, Z. H. (2007). "History of the acetone-butanol-ethanol fermentation industry in China: Development of continuous production technology," *J. Mol. Microbiol. Biotechnol*. 13(1-3), 12-14. DOI: 10.1159/000103592

Du, C. L., Wang, L., Pan, M. M., and Guo, Z. Y. (2016). "Study on granule shape and pasting properties of fern root starch," *Zhongguo Liangyou Xuebao* [*Journal of the Chinese Cereals and Oils Association*] 31(7), 46-50.

El-Mofty, M., Sadek, I., Soliman, A., Mohamed, A., and Sakre, S. (1987). "α-Ecdysone, a new bracken fern factor responsible for neoplasm induction in the Egyptian toad (*Bufo regularis*)," *Nutr. Cancer* 9(2-3), 103-107. DOI: 10.1080/01635588709513917

Gheshlaghi, R., Scharer, J., Moo-Young, M., and Chou, C. P. (2009). "Metabolic pathways of *Clostridia* for producing butanol," *Biotechnol. Adv*. 27(6), 764-781. DOI: 10.1016/j.biotechadv.2009.06.002

Gouveia, L., and Oliveira, A. C. (2009). "Microalgae as a raw material for biofuels production," *J. Ind. Microbiol. Biotechnol*. 36(2), 269-274. DOI: 10.1007/s10295-008-0495-6

Green, E. M. (2011). "Fermentative production of butanol—The industrial perspective," *Curr. Opin. Biotechnol.* 22(3), 337-343. DOI: 10.1016/j.copbio.2011.02.004

Heluane, H., Evans, M. R., Dagher, S. F., Bruno-Bárcena, J. M. (2011). "Meta-analysis and functional validation of nutritional requirements of solventogenic *Clostridia* growing under butanol stress conditions and coutilization of D-glucose and D-xylose," *Appl. Environ. Microbiol*. 77(13), 4473-4485. DOI: 10.1128/AEM.00116-11

Himmel, M. E., Ding, S.-Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007). "Biomass recalcitrance: Engineering Plants and enzymes for biofuels production," *Science* 315(5813), 804-807. DOI: 10.1126/science.1137016

Huang, S. W., Dai, Z. H., and Ni, S. (2016). "Study on the growth and cultivation techniques of wild *Pteridium aquilinum* under *Myrica rubra* Forests, *Chinese Wild Plant Resources*, 35(2), 74-77. (in Chinese)

Hüsemann, M. H., and Papoutsakis, E. T. (1988). "Solventogenesis in *Clostridium acetrobutylicum* fermentations related to carboxylic acid and proton concentrations," *Biotechnol. Bioeng*. 32(7), 843-852. DOI: 10.1002/bit.260320702

Ibrahim, M. F., Kim, S. W., and Abd-Aziz, S. (2018). "Advanced bioprocessing strategies for biobutanol production from biomass," *Renew. Sust. Energ. Rev*. 91(8), 1192-1204. DOI: 10.1016/j.rser.2018.04.060

Jarrett, W. F. H., McNeil, P. E., Grimshaw, W. T. R., Selman, I. E., and McIntyre, W. I. M. (1978). "High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papilloma virus," *Nature* 274(5668), 215-217. DOI: 10.1038/274215a0

Jiang, Y., Liu, J., Jiang, W., Yang, Y., and Yang, S. (2015). "Current status and prospects of industrial bio-production of n-butanol in China," *Biotechnol. Adv*. 33(7), 1493-1501. DOI: 10.1016/j.biotechadv.2014.10.007

Jones, D. T., and Woods, D. R. (1986). "Acetone-butanol fermentation revisited," *Microbiol. Rev*. 50(4), 484-524.

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., and Jung, K. S. (2008). "Fermentative butanol production by *Clostridia*," *Biotechnol. Bioeng*. 101(2), 209-228. DOI: 10.1002/bit.22003

Li, X., Li, Z., Zheng, J., Shi, Z., and Li, L. (2012). "Yeast extract promotes phase shift of bio-butanol fermentation by *Clostridium acetobutylicum* ATCC824 using cassava as substrate," *Bioresource Technol*. 125(338), 43-51. DOI: 10.1016/j.biortech.2012.08.056

Li, X., Shi, Z., and Li, Z. (2014). "Increasing butanol/acetone ratio and solvent productivity in ABE fermentation by consecutively feeding butyrate to weaken metabolic strength of butyrate loop," *Bioprocess Biosyst. Eng*. 37(8), 1609-1616. DOI: 10.1007/s00449-014-1133-5

Liu, X. J., Li, Q. S., Fu, Y. Z., and Zhang, L. (1999). "The development and use of the

brake starch," *Chemical Industry Times*, 13(9), 20-22. (in Chinese)

Lu, C., Zhao, J., Yang, S.-T., and Wei, D. (2012). "Fed-batch fermentation for *n*-butanol production from cassava bagasse hydrolysate in a fibrous bed bioreactor with continuous gas stripping," *Bioresource Technol*. 104(1), 380-387. DOI: 10.1016/j.biortech.2011.10.089

Luo, H., Ge, L., Zhang, J., Ding, J., Chen, R., and Shi, Z. (2016). "Enhancing acetone biosynthesis and acetone-butanol-ethanol fermentation performance by co-culturing *Clostridium acetobutylicum*/*Saccharomyces cerevisiae* integrated with exogenous acetate addition," *Bioresource Technol*. 200(1), 111-120. DOI: 10.1016/j.biortech.2015.09.116

Luo, H., Ge, L., Zhang, J., Zhao, Y., Ding, J., Li, Z., He, Z., Chen, R., and Shi, Z. (2015). "Enhancing butanol production under the stress environments of co-culturing *Clostridium acetobutylicum*/*Saccharomyces cerevisiae* integrated with exogenous butyrate addition," *PLoS One* 10(10), 1-25. DOI: 10.1371/journal.pone.0141160

Luo, W., Zhao, Z., Pan, H., Zhao, L., Xu, C., and Yu, X. (2018). "Feasibility of butanol production from wheat starch wastewater by *Clostridium acetobutylicum*," *Energy* 154(7), 240-248. DOI: 10.1016/j.energy.2018.04.125

Madihah, M. S., Ariff, A. B., Sahaid, K. M., Suraini, A. A., and Karim, M. I. A. (2001). "Direct fermentation of gelatinized sago starch to acetone-butanol-ethanol by *Clostridium acetobutylicum*," *World J. Microbiol. Biotechnol*. 17(6), 567-576. DOI: 10.1023/a: 1012351112351

Martin, J. F., and McDaniel, L. E. (1975). "Kinetics of biosynthesis of polyene macrolide antibiotics in batch cultures: Cell maturation time," *Biotechnol. Bioeng*. 17(6), 925-938. DOI: 10.1002/bit.260170611

Masion, E., Amine, J., and Marczak, R. (1987). "Influence of amino acid supplements on the metabolism of *Clostridium acetobutylicum*," *FEMS Microbiol. Lett*. 43(3), 269-274. DOI: 10.1111/j.1574-6968.1987.tb02156.x

Monot, F., Engasser, J.-M., and Petitdemange, H. (1984). "Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*," *Appl. Microbiol. Biotechnol*. 19(6), 422-426. DOI: 10.1007/BF00454381

Nair, R. V., Green, E. M., Watson, D. E., Bennett, G. N., and Papoutsakis, E. T. (1999). "Regulation of the *sol* Locus genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 by a putative transcriptional repressor," *J. Bacteriol*. 181(1), 319-330.

Ni, Y., and Sun, Z. (2009). "Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China," *Appl. Microbiol. Biotechnol*. 83(3), 415-423. DOI: 10.1007/s00253-009-2003-y

Nigam, P. S., and Singh, A. (2011). "Production of liquid biofuels from renewable resources," *Prog. Energy Combust. Sci.* 37(1), 52-68. DOI: 10.1016/j.pecs.2010.01.003

Nölling, J., Breton, G., Omelchenko, M. V., Makarova, K. S., Zeng, Q., Gibson, R., Lee, H. M., Dubois, J., Qiu, D., Hitti, J., *et al*. (2001). "Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*," *J. Bacteriol.* 183(16), 4823-4838. DOI: 10.1128/JB.183.16.4823-4838.2001

Pamukcu, A. M., Yalçiner, S., Hatcher, J. F., and Bryan, G. T. (1980). "Quercetin, a rat intestinal and bladder carcinogen present in bracken fern (*Pteridium aquilinum*)," *Cancer Res.* 40(10), 3468-3472.

Qiu, H., Huang, J., Yang, J., Rozelle, S., Zhang, Y., Zhang, Y., and Zhang, Y. (2010). "Bioethanol development in China and the potential impacts on its agricultural economy," *Appl. Energy* 87(1), 76-83. DOI: 10.1016/j.apenergy.2009.07.015

Rohan, R., Kuo, T.-C., Chiou, C.-Y., Chang, Y.-L., Li, C.-C., and Lee, J.-T. (2018). "Low-cost and sustainable corn starch as a high-performance aqueous binder in silicon anodes *via in situ* cross-linking," *J. Power Sources* 396(16), 459-466. DOI: 10.1016/j.jpowsour.2018.06.045

Sarchami, T., and Rehmann, L. (2015). "Optimizing acid hydrolysis of Jerusalem artichoke-derived inulin for fermentative butanol production," *BioEnergy Res*. 8(3), 1148-1157. DOI: 10.1007/s12155-014-9568-8

Tran, H. T. M., Cheirsilp, B., Hodgson, B., and Umsakul, K. (2010). "Potential use of *Bacillus subtilis* in a co-culture with *Clostridium butylicum* for acetone–butanol–ethanol production from cassava starch," *Biochem. Eng. J.* 48(2), 260-267. DOI: 10.1016/j.bej.2009.11.001

Xiao, C., Fan, W., Du, S., Liu, L., Wang, C., Guo, M., Zhang, L., Zhang, M., and Yu, L. (2017). "A novel glycosylated solution from *Dioscorea zingiberensis* C.H. Wright significantly improves the solvent productivity of *Clostridium beijerinckii*," *Bioresource Technol.* 241(20), 317-324. DOI: 10.1016/j.biortech.2017.03.176

Wu, Z. Y., and Raven, P. (2013). *Flora of China*, Missouri Botanical Garden Press, St. Louis, MO, USA.

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