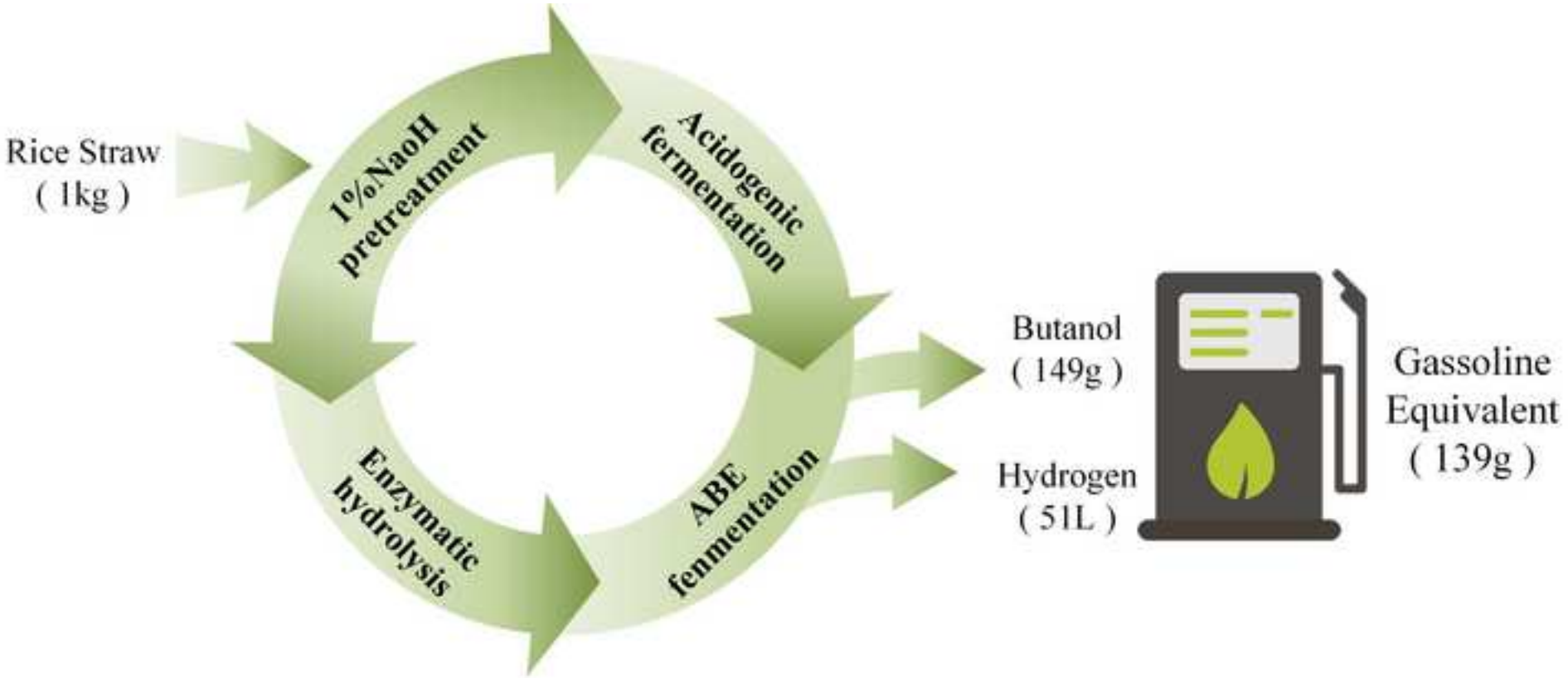
Highlights

- Staged acidogenic and solventogenic fermentation boost up biofuel production;
- Butanol titer increased from 6.2 g/L to 15.9 g/L from pretreated rice straw;
- Genes expression confirmed the enhanced activities after staged fermentation;
- Energy yield increased by 51% with enzyme consumption reduced by 3 fold.

Graphical Abstract (for review)



Features of a Staged Acidogenic/Solventogenic Fermentation Process to Improve Butanol Production from Rice Straw

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Abstract

This study introduced an innovative two-stage fermentation process to maximize sugar utilization and biobutanol production from alkaline pretreated rice straw. The new bioconversion process was composed of an acidogenic fermentation process followed by an acetone-butanol-ethanol (ABE) fermentation process. A sugar-rich hydrolysate (90.4 g/L) and a high acid content fermentation broth (33.9 g/L butyric acid), both produced from rice straw, was mixed together to increase the yield of the biofuels in the ABE fermentation process. Butyric and acetic acids generated from the acidogenic fermentation process play a critical role in the ABE fermentation process, which were confirmed by gene expression analysis of five messenger RNAs. In comparison with the conventional process the final butanol concentration of the new process increased from 6.2 to 15.9 g/L after 72 h of fermentation. The new process resulted in an enhanced production of 149 g butanol and 36 L hydrogen gas from 1 kg rice straw, which is equivalent to approximately 124 and 15 g gasoline, respectively. The total consumption of cellulase enzyme was reduced by 3 fold in the new process. The novel two-stage fermentation process was an effective and economic new approach for energy generation from lignocellulosic biomass.

Keywords: Rice straw; Butanol, Butyric acid, Acidogenic fermentation; ABE fermentation

1. Introduction

Fossil fuels are the driving force of human civilization, but increasing reliance of this non-renewable resource has risked the sustainability of global environment [1]. Combustion of fossil fuels increases greenhouse gases (GHGs) emissions and consequently aggregate the climate changes [2]. Many green techniques have been developed to mitigate this problem through withdrawing energy from renewable sources, such as wind, solar, and biomass. In line with all the renewable energy biofuels are carbon-neutral resources derived from agricultural or waste recycling industries. They have become an attractive substitution of fossil fuels for decarbonizing aviation, ocean shipping, and long-haul trucking [3]. While developing rapidly in the last decade, the biorefinery techniques for biofuel production have faced significant economic obstacles toward large-scale application. The replacing rate of fossil fuels accounted only approximately 0.8% of the total energy consumption in 2016 (82,306 Mt) [4], and most of the contributions are from controversial food-based feedstocks. Further development and optimization of the related techniques has become a task of the new generation for energy and fuel industries.

Bio-butanol production through the acetone-ethanol-butanol (ABE) fermentation processes has attracted growing attention for biomass conversion. Butanol is a proper liquid fuel with many outstanding characteristics such as high energy value, low hygroscopicity, and low carbon emissions. It can be used in most of the exiting internal combustion engines and can serve as precursor in many chemical processes [5, 6]. The projected global demand of butanol in 2018 is approximately 5 million tonnes [7], of which majority generated from petrochemical processing. The existing sugar-based biorefinery process is economically and socially less competitive than the conventional

process. The sugary or starchy feedstock is costly and can lead into the well-known "food vs. fuels" conflicts [8, 9]. Lignocellulosic butanol is preferable as it can be derived from recovered wastes like rice straw. Rice straw is a major agricultural by-products in many countries [10]. The annual rice straw production in the world is approximately 950 million tonnes, and approximately 33.1-42.6% contributed from the East Asia [11, 12]. In China, large amounts of the rice straw are burned in open fields for nutrient recovery, but this practice has resulted in severe air pollution problem. Successful development of a feasible technique to convert rice straw into liquid fuel shall create major impacts to environmental management [13].

Bioconversion of rice straw includes four major processes, *i.e*., pretreatment, hydrolysis, fermentation, and distillation [14-16]. The pretreatment process aims to broken down the complex plant cell wall structure of lignocellulosic biomass and is mandatory to improve the accessibility of cellulose to cellulase [17]. Hydrolysis is then carried out to produce monosaccharides in fermentation. The costs of commercial enzymes involved in conventional hydrolysis processes hinders the benefit of the cheap feedstocks [18]. Developing alternative fermentation strategies to reduce or eliminate enzyme consumption can provide a new insight to rice straw biobutanol production.

ABE fermentation is a biphasic process [19], *i.e*., acetic and butyric acids are first produced with rapid growth of fermentation cell and then converted into the solvents (*i.e*., acetone, butanol and ethanol) [20]. The butyric acid produced in the first phase reaction plays a vital role in many reactions, *i.e*., triggering the transcription of solvent formation genes, decreasing acetone/butanol ratio, enhancing substrates utilization and preventing strain degeneration [21-23]. Butyric acid has been mixed externally into the fermentation broth to stimulate butanol production in the ABE process [21-25].

This study introduced an innovative two-stage fermentation (TSF) strategy to enhance the productivity of biobutanol from rice straw (**Fig 1**). The TSF process included an acidogenic fermentation process and an ABE fermentation process which carried out by three different strains. *Clostridium thermocellum* and *Clostridium thermobutyricum* were applied together in the acidogenic fermentation process. In the co-culture *C. thermocellum* can hydrolyze the cellulose and hemicelluloses and *C. thermobutyricum* can produce butyric acid from the hydrolysate. The fermentation broth with high butyric acid was then used as a triggering substance in ABE fermentation. It was expected that reduced enzyme consumption and high product yield can be obtained from the TSF process. A conventional separate hydrolysis and fermentation (SHF) process was conducted to compare with the performances of the new TSF process. The relative genes expressions were detected by reverse transcription polymerase chain reaction (RT-PCR) to reveal the metabolic switches of the two fermentation approaches. The overall energy conversion and mass balance efficiency were also calculated to demonstrate the improvement between the two processes.

2. Materials and methods

2.1 Raw materials

Rice straw was collected at a local farm nearby Harbin, China (45°56'39.4"N, 126°26'40.8"E). The biomass contains 39.7% cellulose, 24.8% hemicellulose and 15.3% lignin. It was air dried and cut into 10-15 cm in length then stored in plastic bags at room temperature for further use. Commercial cellulase (Heshibi Biological Technology Co., LTD, China) was used for hydrolysis of pretreated rice straw. Filter paper activity of the cellulase was measured before the SHF and TSF, following the method reported

by Ghose [26]. The activity of the enzyme was approximately 58.0 filter paper unit (FPU/g).

2.2 Pretreatment

The rice straw was pretreated with 1% (*w/v*) NaOH-water solution with a solid: liquid ratio of 1:15 (*w/v*) (based on dry weight) [27]. The pretreatment was conducted in a tank-reactor with 20 L working volume and incubated at 50° C for 72 h. After solid-liquid separation, the residual solid was rinsed repeated by tap water until pH reach 7.0. The delignified rice straw was stored at 4° C, and air-dried in an oven (Bo Xun Industrial Co., Ltd, Shanghai, China) at 105° C for 24 h before further experiments.

2.3 Fed-batch enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated rice straw was carried out in a 1 L tank-reactor loaded with 80 g/L substrate and 500 mL 0.05 mol/L sodium acetate buffer (pH 4.8). The cellulase loading was 30 FPU per gram oven-dried substrate. Hydrolysis experiments were conducted at 50° C and 60 runs per minutes (rpm) for 72 h. At 12^{th} and 24^{th} h of the hydrolysis process, 20 g substrate and 10 FPU/g cellulase was added in the reactor to get a final substrate concentration of 120 g/L. Samples were withdrawn every 12 h for sugar analysis. After hydrolysis the reactor was autoclaved at 115° C for 20 min. The supernatant of the rice straw hydrolysate (SRH) was separated from the hydrolysate by solid-liquid separation before ABE fermentation.

2.4 Microorganism and inoculum preparation

2.4.1 Microbial consortium for lignocellulosic butyric acid production

The microbial consortium for acidogenic fermentation was a thermophilic co-culture of *C. thermocellum* ATCC 27405 and *C. thermobutyricum* ATCC 49875. Both strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). More details of the co-culture refers to Chi et al., [28]. For long-term maintenance, the strains were maintained in the form of spores in an 1191 medium at 4°C. To obtain the inoculum, a preculture was prepared for the strain cultivation. The stored spore suspensions of the two strains were transferred into fresh 1191 medium containing 6 g/L cellobiose or 10.0 g/L glucose, respectively. After incubation at 55°C and 140 rpm for 22-24 h, the liquid culture was used as the inoculum for fermentation. The Medium 1191 contains the following amounts of compounds: 3.0 g yeast extract, 1 g $NaHCO_3$, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g NH_4Cl，0.18 g $MgCl_2 \cdot 6H_2O$，0.00125 g $FeSO_4 \cdot 7H_2O$，0.5 g D-cysteine, 1 mL trace-element solution [29] and 1 L distilled water.

2.4.2 Strain for ABE fermentation

The strain for ABE fermentation *C. beijerinckii* NCIMB 8052 was obtained from the China General Microbiological Culture Collection Center (CGMCC). The strain was maintained in the form of spores in fresh peptone-yeast glucose (PYG) medium at 4°C [30]. The stored spore suspension was transferred into fresh P2 medium (3.0 g yeast extract, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.001 g p-aminobenzoic acid, 0.001 g thiamine, 0.0001 g biotin, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g NaCl and 1L distilled water) with 10 g/L glucose and incubated at 37°C with a mixing speed of 140 rpm for 18-22 h. The incubated cells were harvested by centrifugation at

3000 rpm for 5 min. The harvested cells were suspended in P2 medium as inoculum for fermentation.

2.5 Fermentation

2.5.1 Fed-batch acidogenic fermentation

The acidogenic fermentation was performed in a 3 L stirred-tank reactor with 1 L 1191 medium containing 20 g substrate. The broth was autoclaved at 115°C for 20 min and flushed with N_2 for 20 min before inoculation. The reactor was inoculated with 10% (v/v) *C. thermocellum* ATCC 27405 and 1% (v/v) *C. thermobutyricum* ATCC 49875. The fermentation was conducted at 55°C and 60 rpm for 22 days. Since the 3rd day of fermentation, 20 g substrate and 1 g yeast extract were fed into the reactor once per every two or three days until the 20th day of operation. Biogas, cell density and aqueous products were analyzed daily. Weight loss of the fed substrate were measured at the end of fermentation. After the acidogenic fermentation, the supernatant of fermentation culture (SFC) was collected after solid-liquid separation and used for butanol production in the ABE fermentation process.

2.5.2 ABE fermentation

ABE fermentations were performed with *C. beijerinckii* NCIMB 8052 in a 3 L stirred-tank reactor. The fermentation broth was composed of 150 mL SFC, 350 mL SRH, 500 mL distilled water, and the P2 medium. The initial pH of the fermentation broth was adjusted to 5.0 by 4 M HCl solution. For the TSF process SFC and SRH were pumped into the reactor in sequence and then flushed with N_2 for 20 min. For the SHF process, a P2 medium containing 35% (*v/v*) SRH with nature pH of 6.68 was prepared

in a similar fashion as control fermentation. The bacterial suspension of *C. beijerinckii* NCIMB 8052 was inoculated in the reactor down at room temperature. The inoculation amount of the strain was determined by the optical density at 600 nm (OD_{600}) of 1.0 in the broth. The culture was incubated at 37°C and 140 rpm for 6 days. The biogas, cell density, sugars, volatile fatty acids (VFAs) and solvents were read or analyzed daily.

2.6 Analytical procedures

The biogas produced in the fermentation processes was measured by a wet gas meter (LML-1, Changchun Automotive Filter Co., LTD., Changchun, China). The composition of the biogas was analyzed with a gas chromatograph (SP-6800A, Shandong Lunan Instrument Factory, China) equipped with a thermal conductivity detector and a 2-m stainless column packed with Porapak Q (60/80 mesh, Lanzhou ZhongKeKaiDi Chemical Newtech Co., Ltd, China). The carrier gas was N_2. The temperature of the column oven was set at 50°C, and the temperature for both the injection port and the detector were 80°C. The concentrations of VFAs and solvents in the culture supernatant were determined by another gas chromatograph (SP-6800, Shandong Lunan Instrument Factory, China) equipped with a flame ionization detector and a 30 m FFAP capillary column (i.d.0.32 mm, SHIMADZU, Japan), also with N_2 as carrier gas. The oven temperature was 160°C and both temperatures of the injector and the detector were 210°C. The concentrations of xylose, glucose and cellobiose were detected by a high-performance liquid chromatography (HPLC) (LC-10AVP, SHIMADZU, Japan) equipped with a refraction index detector (RID-10A, Waters, USA) and an HPX-87H column (Bio-rad, Hercules, CA). The operating temperature was 70°C and the mobile phase was 0.005 M H_2SO_4. The flow rate was 0.6 mL/min, and the

injection sample volume was 50 μL.

The weight loss of rice straw during the acidogenic fermentation was calculated as described previously [27].The component of rice straw was measured following the method reported by Van Soest [31]. The concentrations of the reducing sugars were determined following the DNS-method [26]. Cell density in the two fermentation systems were estimated by the optical density at 600 nm (OD_{600}) using a spectrophotometer (UV-2450; SHIMADZU, Japan). The correlation between OD_{600} and dry cell weight (DCW) was measured and defined before fermentation. One unit of OD_{600} was approximately equivalent to 0.60 and 0.59 g DCW per liter for the acidogenic co-culture and *C. beijerinckii* NCIMB 8052, respectively.

2.7 Reverse transcription polymerase chain reaction

Expressions of selected messenger RNA (mRNA) genes were measured to clarify the influences of SFC on ABE fermentation. The genes include butyrate kinase (*buk*), acetate kinase (*ack*), coenzyme A (CoA) transferase (*ctf*A), acetoacetate decarboxylase (*adc*) and alcohol dehydrogenase (*adh*E). A 10 mL sample was collected from the ABE fermentation broth every 12 h. The bacterial cells were harvested by centrifugation at 4°C and 3000 rpm for 5 min then immediately snap-frozen in liquid nitrogen. The frozen cells were stored at -80°C and sent to Sangon Biotech (Shanghai) Co., Ltd (China) for RNA purification, cDNA synthesis and real-time fluorescence quantitative PCR (qPCR) analysis. The total RNA was used as the template to synthesize cDNA. The cDNA products were amplified by qPCR with primers as shown in **Table 1**. The reported Cycle threshold (Ct) values were taken from the average of three technical replicates. The levels of respective mRNA were normalized to the peptidase T [32] and

relative quantification was performed by comparative cycle threshold ($2^{-\Delta\Delta CT}$) method and presented as representative graphs of triplicate experiments [33].

3. Results and discussion

3.1 Pretreatment

The efficiency of enzymatic hydrolysis of lignocellulosic biomass can vary significantly among the type and conditions of pretreatment [5, 17]. The selected alkaline pretreatment process is among the most widely applied processes which can improve the digestibility of lignocellulosic biomass without damaging the carbohydrates [34, 35]. In this study, approximately 35.3% solid was removed after the alkaline pretreatment, in which 68.6% lignin was removed with 7.1% and 5.4% losses of hemicellulose and cellulose, respectively. The chemical composition of the pretreated substrate was composed of 51.4% cellulose, 38.3% hemicellulose, 8.0% lignin and 1.5% ash. The pretreatment process minimized the losses of hemicellulose in the substrate and increased the solid recovery in comparison with the acid based pretreatment processes [36, 37].

3.2 Enzymatic hydrolysis and acidogenic fermentation

Fed-batch hydrolysis and/or fermentation were widely applied strategies to increase the concentration of carbon source in the fermentation broth. It can increase the total solid content of the hydrolysate while avoiding the mass/heat transfer problems resulted from high-density fibrous suspension [38]. Fed-batch processes were applied in this study for both the enzymatic hydrolysis and acidogenic fermentation processes, which both aimed to decompose the cell wall into fermentable monomers. In the

enzymatic hydrolysis process the pretreated rice straw was hydrolyzed at 50°C for 72 h. The total substrate concentration reached 120 g/L in the enzymatic hydrolysis process, resulting in a high reducing sugar production of 90.4 g/L containing 6.7, 63.1 and 20.6 g/L cellobiose, glucose and xylose, respectively (**Fig. S1**). The yield of the enzymatic hydrolysis was approximately 77.7%.

Acidogenic fermentation is a feasible approach for direct conversion of lignocellulose for butyric acid [39, 40]. During the fermentation period a total of 180 g/L substrate was fed into the reactor and the profile of fermentation for butyric acid production was presented in **Fig. 2**. Butyric acid was the dominant product throughout the fermentation process while both ethanol and acetic acid were the by-products. The accumulated conversion of the substrate was approximately 42% and the specific butyric acid yield from the consumed substrate was 0.45 g/g. This process showed high selectivity for butyric acid conversion (78%), and the final titer of the product was 33.9 g/L. The concentrations of the fermentation by-products were 5.4 g/L for ethanol and 2.7 g/L for acetic acid, respectively. The microbial co-culture showed high efficiency converting the pretreated rice straw substrate into butyric acid. The lignocellulosic substrate was decomposed by *C. thermocellum* ATCC 27405 and formed into easily degradable sugars, *i.e.*, cellobiose, glucose and xylose. The sugars were immediately utilized by the bacteria and therefore cannot be detected in the fermentation broth. With rapid cell growth the carbohydrates were converted into acetic acid, ethanol (C2), and butyric acid (C4) during the primary phase of fermentation (first 7 days). The acetic acid and ethanol was further converted into butyric acid by *C. thermobutyricum* ATCC 49875. The detailed metabolism pathway of the bioconversion process through the carboxylate platform have been well-documented in the previous studies [41, 42].

3.3 ABE fermentation

The hydrolysate of the enzymatic hydrolysis and acidogenic fermentation processes was used for ABE fermentations with *C. beijerinckii* NCIMB 8052. The SHF process was carried out using the SRH liquor with the related medium, and the TSF processes used both SRH and SFC in addition to the fermentation medium. The cell growth, substrates consumption and products concentrations of the two processes were presented in **Fig. 3(A)** to **Fig. 3(D)**. Supplementing the SFC in the ABE fermentation process resulted in significantly improvement in the butanol and hydrogen production. The butanol concentration (red solid lines with circle symbols) in the TSF process (**Fig. 3(C))** was approximately 156% higher than one measured in the SHF process **(Fig. 3(C))**. The ratio of acetone per butanol, meanwhile, reduced considerably from 0.56 to 0.19 in the TSF process. This reduction represented a 13% reduction of unwanted fermentation by-products in the ABE process. With the butanol production the hydrogen production also increased significantly when including SFC in the fermentation broth.

Cell density (blue lines with star signs) increased rapidly at the initial phase (0-24 h) of the SHF process (**Fig. 3(B))**, while a lagging phase was discovered for cell growth in the THF process (**Fig. 3(D))**. It took approximately 12 more hours to allow the cell density reaching the stabilization phase in the fermentation broth. The delayed cell growth may be due to inhibition effects caused by the organic acids in the SFC [43]. The cell density reached a maximum of 4.17 g/L at the 36^{th} h of fermentation, and slightly decreased to 3.60 g/L at the 72^{nd} h (**Fig. 3(D)**). Existence of the organic acids did not affect the final yield of total cell or butanol. The final cell densities of the two fermentation processes both ranged between 3.8 g/L and 4.0 g/L without significant difference between each other.

The SHF fermentation process experienced both acidogenesis and solventogenesis phases in sequence in the first 12 h of operation. The pH dropped rapidly to 5.5 (results not shown) during this period and was remained at constant by a pH control system. NaOH solution was dosed into the reactor to prevent continuous dropping of pH. Small amount of acetic and butyric acids started to be produced and accumulated in the reactor. The accumulation of acids and decrease of pH triggered the solventogenesis [23]. Acetone and butanol were formed rapidly between the 12^{th} and 36^{th} h, and reached the maximum concentrations at 3.00 and 6.23 g/L, respectively. The sugar consumption rate and butanol productivity during this growing period were 1.03 and 0.24 g/L/h, respectively. The fermentation activities slowed down significantly after 36^{th} h of fermentation, leaving more than 5 g/L reducing sugars in the system.

Compared with the control fermentation, the TSF process produced higher amount of butanol throughout the whole fermentation period. The SFC effectively triggered the formation of butanol at the beginning phase of fermentation, resulting in approximately 2.2 g/L after 12 h of operation. The butanol production increased exponentially and reached 14.04 g/L at the 36^{th} h. Butyric acid from the SFC and sugars from the SRH were consumed simultaneously during this period with corresponding rates/yeild of butanol production. The strain *C. beijerinckii* is capable to consume both glucose and xylose, although the consumption rates of xylose and butyric acid were slightly lower than glucose especially in the beginning phase of fermentation. The maximum producing rate of butanol was 0.51 g/L/h, and the consumption rates of butyric acid and sugar were 0.18 and 0.86 g/L/h, respectively. The butyric acid containing in the SFC could convert to butanol without carbon loss and therefore more butanol can be produced with less sugar consumption, when compared with the SHF process. By the

end of the ABE fermentations, approximately 6.20 and 15.86 g/L butanol were produced with 25.46 and 33.44 g/L consumed sugars in the SHF and TSF processes, respectively. The yield of fermentation in the TSF process (0.47 g/g consumed sugar) was significantly higher than that of the SHF process (0.24 g/g consumed sugar). Butanol formation had been remarkably prompted by the SFC [23, 44].

3.4 Genes expression

The mRNA genes expressions of *ack*, *buk*, *ctf*, *adc*, and *adh*E of *C. beijerinckii* NCIMB 8052 were determined by qRT-PCR during the two fermentation processes. The liquid samples were taken at six time points throughout the fermentation processes and compared with the control inoculum (**Fig.5**). In general all the gene expressions were higher in the TSF than in the SHF process. In the TSF process the genes involved in the acid formation pathways, including *ack* for acetic acid formation and *buk* for butyric acid formation were up-regulated slightly in the first 12 h of fermentation. The expressions of those genes increased rapidly to 6.7 and 17.2-fold at 36 h for *ack* and *buk*, respectively. In the SHF process, *ack* and *buk* were up-regulated slightly first from 0-36 h and 0-24 h, respectively. The expressions of the two genes then declined gradually throughout the whole process.

Based upon the experiment results of gene expression the related impacts to the two processes were demonstrated in **Fig. 5**. During the ABE fermentation, acetic acid production is more favorable to synthesize adenosine 5′-triphosphate (ATP) [20]. Addition of acetic acid containing in the SFC had feedback inhibited the enzymes involving in the acetic acid formation. This inhibition resulted in the remarkable up-regulation of *ack* expression to promote acetic acid production for synthesis of ATP.

In the theoretical ABE metabolic pathway, the butyric acid was re-assimilated via *adc*/*ctf*AB-dependent pathway, but previous researches have been proved that the butyric acid uptake in *C. acetobutylicum* and *C. beijerinckii* were not correlated with acetone production, implying that butyric acid synthesis pathway is reversible, *i.e.* the enzymes involved in butyric acid formation might be also responsible for butyric acid re-assimilation [23, 45, 46]. In the present research, although the accumulation of butyric acid was not observed during the TSF process, the expressions of *buk* gene were up-regulated. These results supported the view of previous studies that the conversion of butyric acid to butanol in *C. beijerinckii* NCIMB 8052 may be responsible by the butyric acid formation genes.

In the TSF process, gene expression related to solventogenesis was up-regulated to higher level when comparing to that in the SHF process, reflecting the higher solvent production as shown in **Fig. 3(C)**. A *sol* operon organized in the order of *ald* (Cbei_3832, encoding aldehyde dehydrogenase)-*ctf*A (Cbei_3833)-*ctf*B (Cbei_3834)-*adc* (Cbei_3835) was revealed in *C. beijerinckii* 8052 [23], and therefore highly coordinated expression patterns were observed for the *sol* operon genes in both fermentation processes. In the TSF process *ctf*A and *adc* expression were induced from the very beginning of fermentation, and further up-regulated to the highest level of 2.8-9.0 fold at 48 h then downregulated to 2.0-2.5 fold at the end of fermentation. Without the supplemented SFC, much lower expression levels of *ctf*A and *adc* were obtained throughout the control process. Expression of *adh*E was induced to high levels from the very beginning of the fermentation with SFC, which was associated with the higher butanol formation than the SHF process. Expression of *adh*E was up-regulated to 468.6 fold at the 36^{th} h and then down-regulated by 3.9 fold at the 48^{th} h. In the SHF

process *adh*E expression was up-regulated to 33.6 fold at 36th h and kept at highest level afterwards with a slight decrease at the 72nd h.

3.5 Energy conversion efficiency and mass balance

To further evaluate the performance of the TSF process, the overall mass balances of the two butanol producing processes are compared in **Fig. 6**. The TSF process can produce a total of 149 g butanol (124 g gasoline equivalent based on its heating value) and 51 L hydrogen (15 g gasoline equivalent) from each kg of rice straw. These values are higher than the SHF process, of which approximately 96 g butanol (80 g gasoline equivalent) and 39 L hydrogen (11 g gasoline equivalent) were obtained from each kg of rice straw. The rice straw has been previously evaluated for ethanol production, of which approximately 99-176 g of ethanol (62-110 g gasoline equivalent) can be harvested from each kg rice straw [47-49]. The energy recovered from rice straw in the form of butanol by the TSF process is remarkably higher than that obtainable in the form of bioethanol. Enzymatic hydrolysate of rice straw has been used as feedstock for butanol production by ABE fermentation. A total amount of 45.2-80.3 g butanol could be obtained from each kg rice straw, of which each g butanol product consumed 221.8-224.5 FPU cellulase [14, 50]. The cost of enzymes involved in the hydrolysis process almost counteracts the benefit of the cheap feedstocks [18]. If the cellulase enzyme is purchased from Chinese enzyme market at 1.9 USD/kg, with the FPA of 145.0 FPU/g [51], the cost of enzyme for each kg butanol production would counted to be approximately 2.9 USD [14, 50]. In the TSF process producing 1 g butanol only requires 79.3 FPU cellulase; hence the enzyme cost may be reduced remarkably by approximately 3 fold to1.0 USD for 1 kg butanol.

The total energy conversion efficiencies of the SHF and the TSF process were shown in **Fig .7**. The energy conversion efficiency was defined as the ratio of the total heating value in hydrogen (kJ) and aqueous product (kJ) to the total heating value of the rice straw fed (kJ). The energy equivalences of the processes were calculated based on the heating values of different components, *i.e*., rice straw (16.4 kJ/kg), butanol (2,673 kJ/mol), hydrogen (286 kJ/mol), ethanol (1,368 kJ/mol), acetic acid (874 kJ/mol), butyric acid (2,181 kJ/mol). The total energy conversion efficiencies of the TSF and SHF process accounted approximately to the fed rice straw by 44% and 35%, respectively. Most of the energy was stored in the forms of biofuels, *i.e*. butanol, hydrogen and ethanol. For the SHF process, approximately 24% of the energy containing in rice straw was converted and stored in the biofuels, with 21%, 3% and 0% in butanol, hydrogen and ethanol, respectively. The biofuels energy conversion efficiency was notably improved to 39% in the TSF process, with 33%, 4% and 2% in butanol, hydrogen and ethanol, respectively. The results indicate that the TSF process is an efficient energy conversion process for advanced biofuels. The energy in rice straw was mainly converted and stored in biofuels, and only few of the energy was used to generate VFAs.

Conclusion

An innovative TSF process was developed to convert alkaline pretreated rice straw into biobutanol, hydrogen, and lignin. The proposed staged fermentation process was composed of a joint process of acidogenic fermentation plus an enzymatic hydrolysis processes, followed by an ABE fermentation process. The beneficial effects of butyric and acetic acids in the ABE fermentation process were confirmed through the superior

performances of product yield and gene expression techniques. The new process resulted in higher butanol/hydrogen production, lower residual sugars, and lower enzyme doses over the conventional SHF process. The new process eventually resulted in a total yield of 149 g butanol, 36 L hydrogen, and 101 g lignin per kg rice straw. This finding provides a new direction to rice straw biorefinery toward a more efficient and economical operation.

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Figure Captions

Fig 1. Flow diagram of the new fermentation process to convert rice straw into bio-butanol;

Fig 2. Performance of acidogenic fermentation with fed-batch mode;

Fig 3. Performances of two ABE fermentation processes. Subfigures A and B, SHF process; C and D, TSF process. The TSF produced higher titer of butanol than the SHF process;

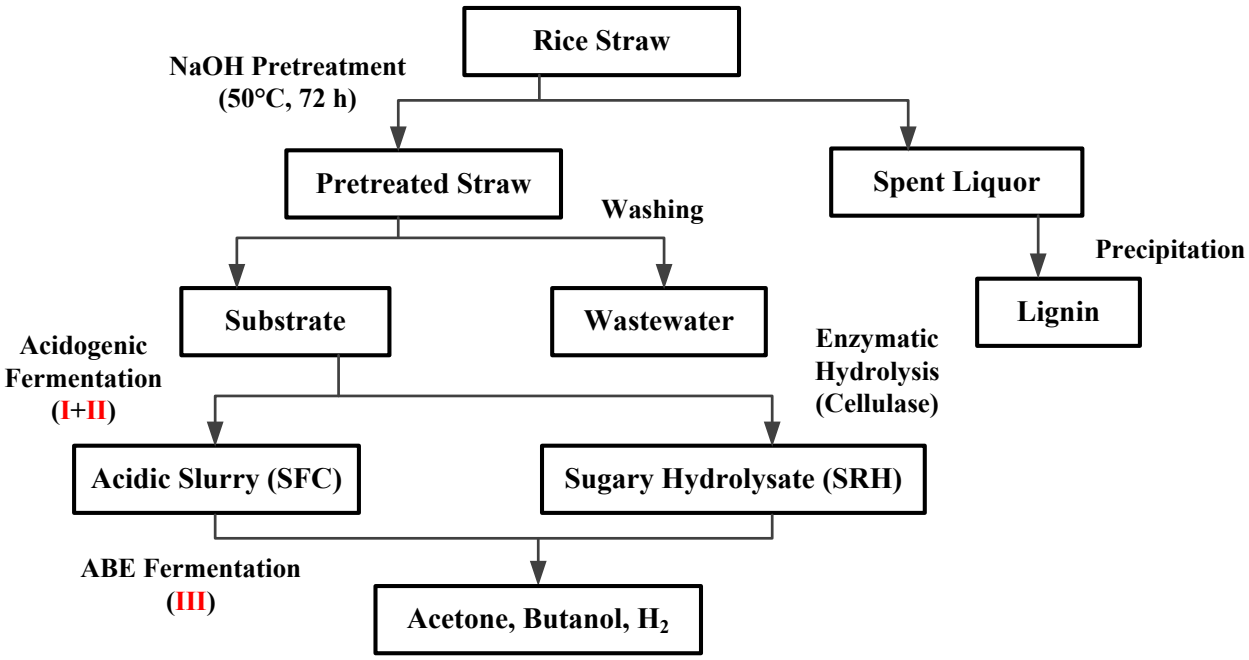
Fig 4. Gene expression of *C. beijerinckii* NCIMB 8052 during ABE fermentation;

Fig 5. Simplified pathway diagram of the fermentation processes. Reaction 1: cellulose hydrolysis; 2: hemicellulose hydrolysis; 3: xylose/arabinose uptake and subsequent breakdown via the transketolase-transaldolase sequence producing fructose 6-phosphate and glyceraldehydes 3-phosphate with subsequent metabolism by the Embden-Meyerhof-Parnas (EMP) pathway; 4: glucose uptake by the phosphotransferase system (PTS) and conversion to pyruvate by the EMP pathway; 5: pyruvate-ferrodoxin oxidoreductase; 6: thiolase; 7: 3- hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase; 8: phosphate acetyltransferase; 9: acetate kinase; 10: acetaldehyde dehydrogenase; 11: ethanol dehydrogenase; 12: acetoacetyl-CoA:acetate/butyrate : CoA transferase; 13: acetoacetate decarboxylase; 14: phosphate butyltransferase; 15: butyrate kinase; 16: butyraldehyde dehydrogenase; 17: butanol dehydrogenase; and 18: consortium TDCB;

Fig 6. Mass balances of the two tested fermentation processes: (A) SHF; and (B) TSF;

Fig 7. Comparison of energy conversion efficiencies of two butanol producing processes.

Figure



Strain I = *C. thermocellum*; **Strain II** = *C. thermobutyricum*; **Strain III** = *C. beijerinckii*

Fig 1.

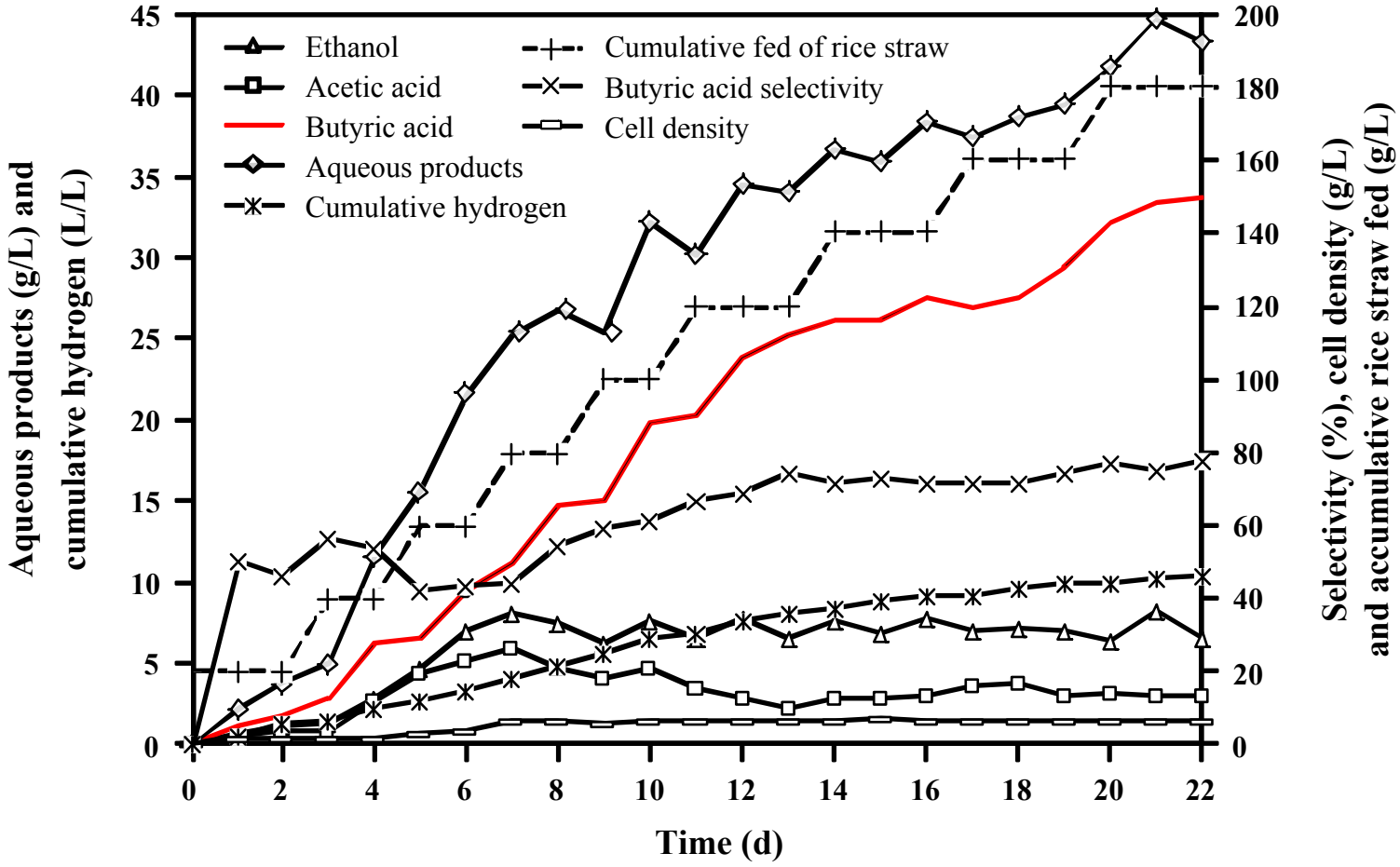

Ethanol
Acetic acid
Butyric acid
Aqueous products
Cumulative hydrogen
Cumulative fed of rice straw
Butyric acid selectivity
Cell density
Aqueous products (g/L) and cumulative hydrogen (L/L)
Selectivity (%), cell density (g/L) and accumulative rice straw fed (g/L)
Time (d)
0
5
10
15
20
25
30
35
40
45
0
20
40
60
80
100
120
140
160
180
200
0
2
4
6
8
10
12
14
16
18
20
22

Fig 2.

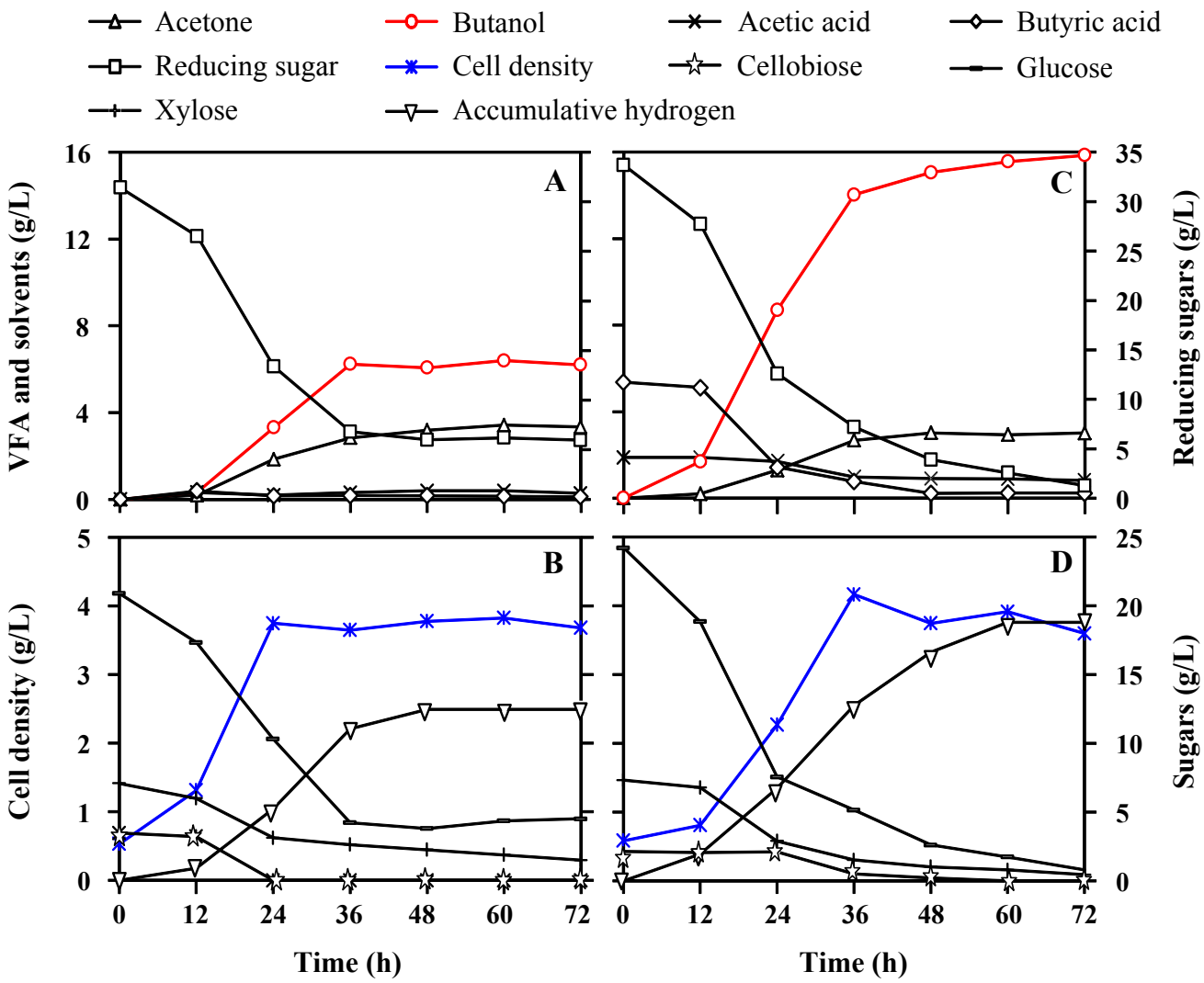

Acetone
Butanol
Acetic acid
Butyric acid
Reducing sugar
Cell density
Cellobiose
Glucose
Xylose
Accumulative hydrogen
A
B
C
D
VFA and solvents (g/L)
Cell density (g/L)
Reducing sugars (g/L)
Sugars (g/L)
Time (h)
Time (h)

Fig 3.

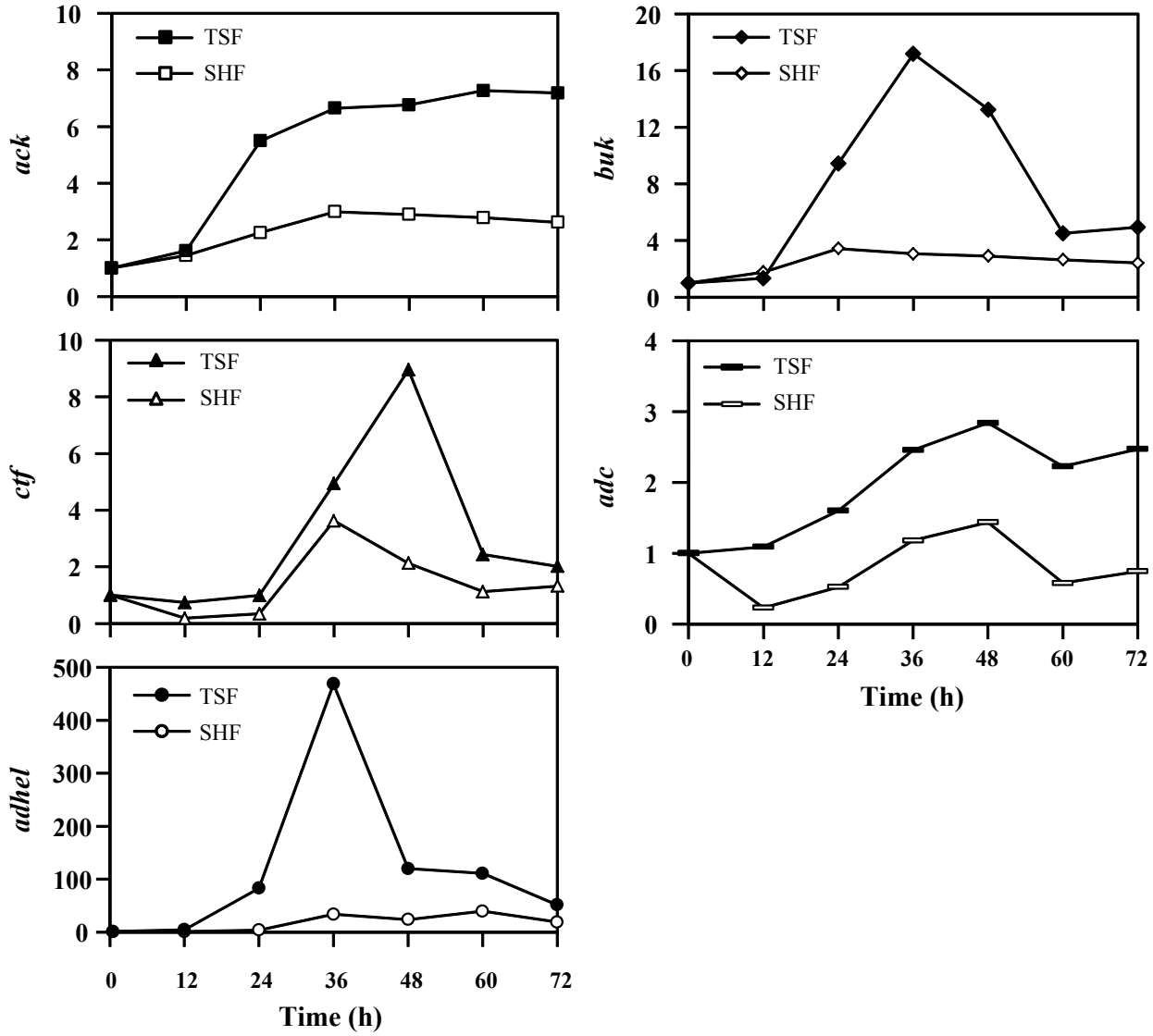

ack
TSF
SHF
buk
TSF
SHF
ctf
TSF
SHF
adc
TSF
SHF
adhel
TSF
SHF
Time (h)
Time (h)

Fig 4.

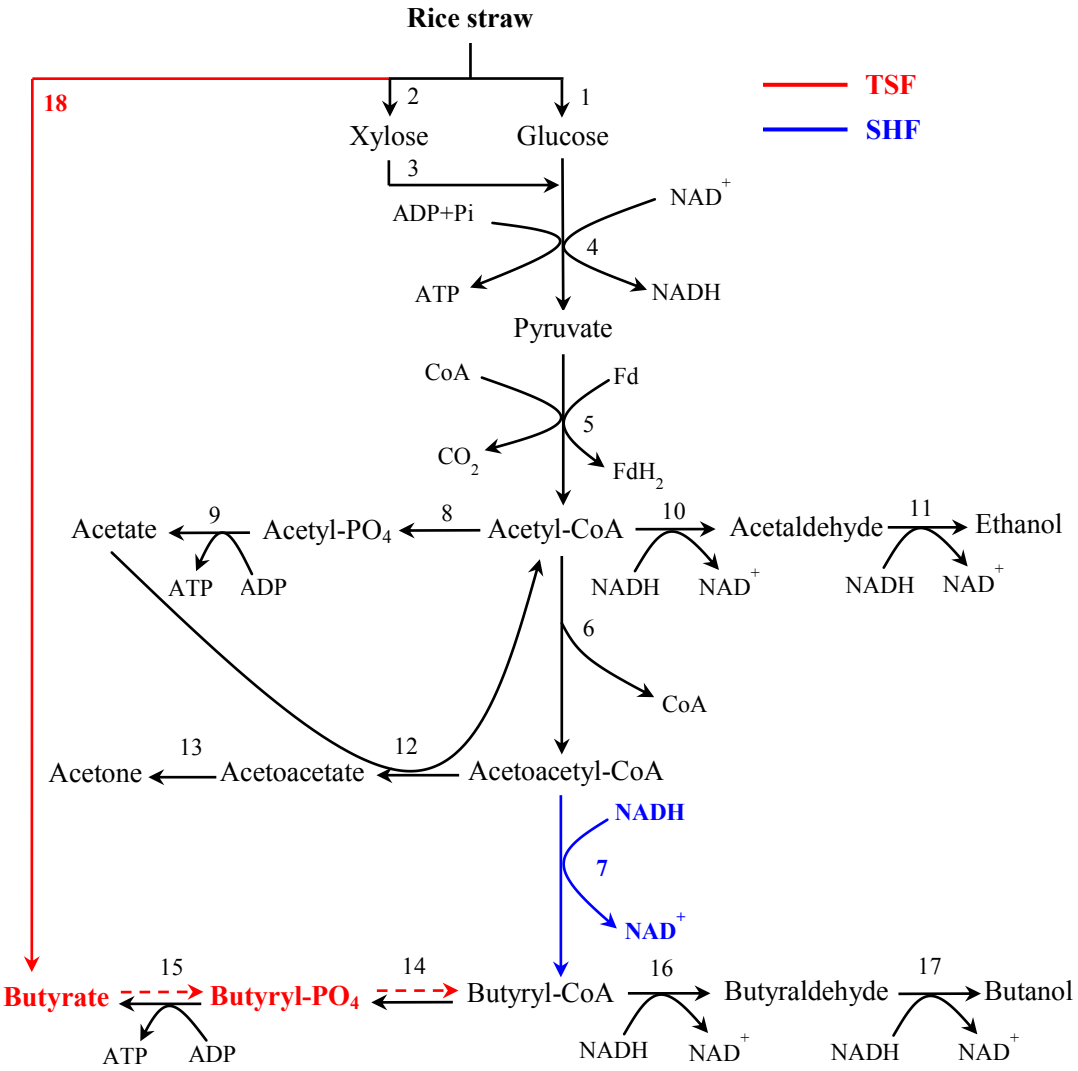
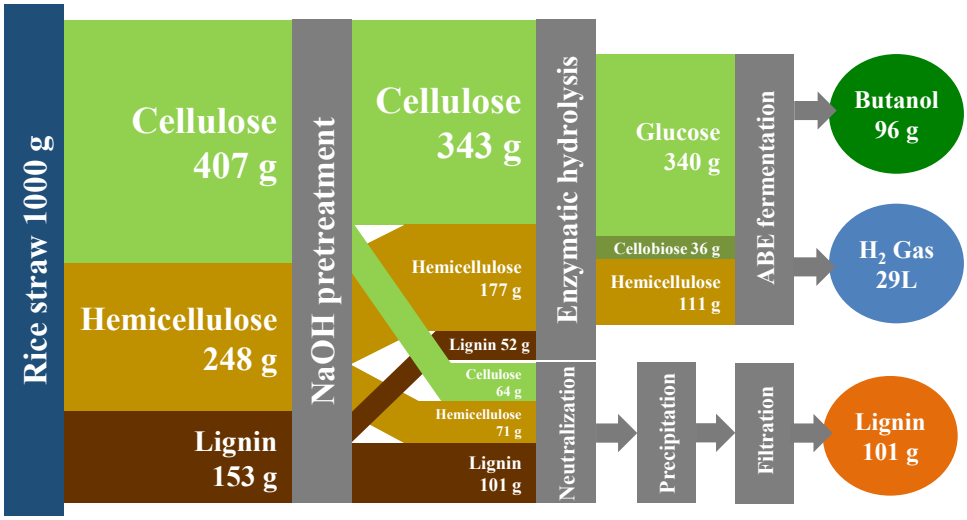

Rice straw
TSF
SHF
18
2
1
Xylose
Glucose
3
ADP+Pi
NAD+
4
ATP
NADH
Pyruvate
CoA
Fd
5
CO2
FdH2
Acetate
9
Acetyl-PO4
8
Acetyl-CoA
10
Acetaldehyde
11
Ethanol
ATP
ADP
NADH
NAD+
NADH
NAD+
6
CoA
13
12
Acetone
Acetoacetate
Acetoacetyl-CoA
NADH
7
NAD+
15
14
16
17
Butyrate
Butyryl-PO4
Butyryl-CoA
Butyraldehyde
Butanol
ATP
ADP
NADH
NAD+
NADH
NAD+

Fig 5.

(A) SHF process



(B) TSF process

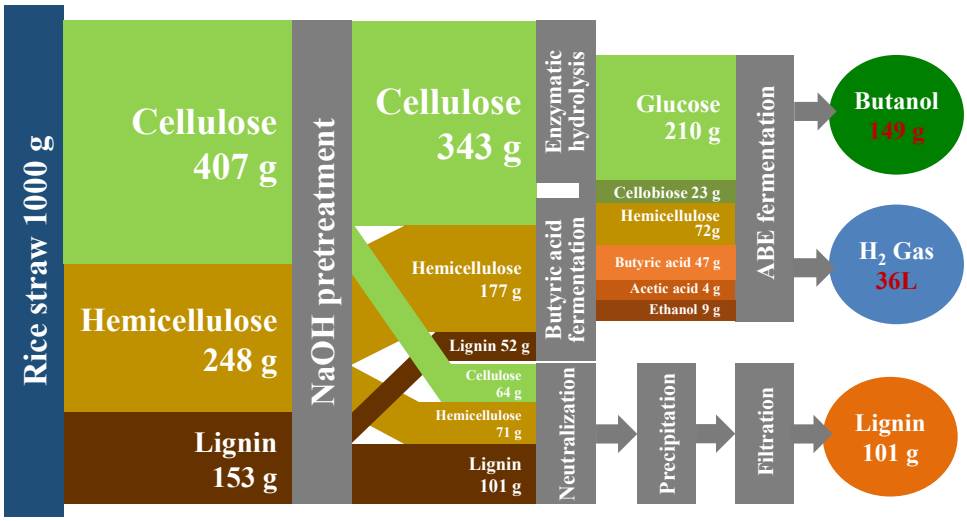


Fig 6.

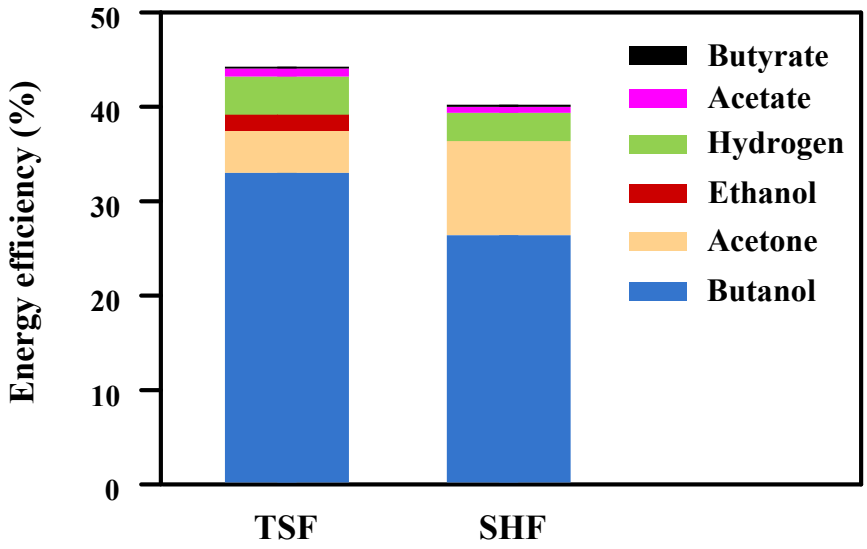

Energy efficiency (%)
50
40
30
20
10
0
TSF
SHF
Butyrate
Acetate
Hydrogen
Ethanol
Acetone
Butanol

Fig 7.

Table 1 Sequences of primers used in PCR

Gene ID	Gene name	Forward primer sequence (5′ - 3′)	Reverse primer sequence (5′ - 3′)
Cbei_0204	butyrate kinase	TATGGGTGGAGGCGTTTCA	CCTTTTCCTACAGCCTTGCC
Cbei_1165	acetate kinase	TCCTAGAGCATTACTTACAA	AAATACAATTGCATCTACAC
Cbei_3278	coenzyme A transferase	TGGAGCATCAATAAACCC	TTCCCTGCTTGTCTACTTCT
Cbei_3835	acetoacetate decarboxylase	CTGCAACAATGGGATATAAGCA	AGTCCAAGCACCGTGAATAGTT
Cbei_1464	alcohol dehydrogenase	CTAAAAGAGCAGGGGCAGAT	AAACGCCACGTCAACTCC
Cbei_2428 [a]	peptidase T	ACAGATGGAACATCATTGCTTG	ATCAAATAAATGCGCTCCAAGT

[a] Cbei_2428 was selected as the endogenous control gene.

Data Statement

Click here to download Data Statement: 04 Supplementary.docx