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ORIGINAL PAPER

Ammonium acetate enhances solvent production by *Clostridium acetobutylicum* EA 2018 using cassava as a fermentation medium

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Abstract Cassava, due to its high starch content and low cost, is a promising candidate substrate for large-scale fermentation processes aimed at producing the solvents acetone, butanol and ethanol (ABE). However, the solvent yield from the fermentation of cassava reaches only 60% of that achieved by fermenting corn. We have found that the addition of ammonium acetate (CH_3COONH_4) to the cassava medium significantly promotes solvent production from cassava fermented by *Clostridium acetobutylicum* EA 2018, a mutant with a high butanol ratio. When cassava medium was supplemented with 30 mM ammonium acetate, the acetone, butanol and total solvent production reached 5.0, 13.0 and 19.4 g/l, respectively, after 48 h of fermentation. This level of solvent production is comparable to that obtained from corn medium. Both ammonium (NH_4^+) and acetate (CH_3COO^-) were required for increased solvent synthesis. We also demonstrated substantially increased acetic and butyric acid accumulation during the acidogenesis phase as well as greater acid re-assimilation during the solventogenesis period in ammonium acetate-supplemented cassava medium. Reverse transcription-polymerase chain reaction analysis indicated that the transcription of several genes encoding enzymes related to acidogenesis and solventogenesis in *C. acetobutylicum* EA 2018 were enhanced by the addition of ammonium acetate to the cassava medium.

Keywords Acetone–butanol–ethanol production · Ammonium acetate · Cassava · *Clostridium acetobutylicum* EA 2018

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Introduction

The industrial scale production of acetone, butanol and ethanol (ABE) through fermentation by solventogenic clostridia was carried out throughout China until the early 1990s. This changed with the development of the petrochemical industry and increasing prices of the agricultural products used as substrates for fermentation, with the result that fermentative ABE production could not compete economically with chemical synthetic production processes [6]. However, with the more recent fluctuations in petroleum prices, ABE production by fermentation has provoked renewed interest [9, 18]. Among the fermentation products, butanol is not only a widely used feedstock chemical but also an excellent biofuel that is expected to play an important role in future fuel systems [13, 25]. Most of the major fermentation substrates currently used are of agricultural origin, such as corn and molasses. As the cost of the substrate is one of the most influential factors impacting the economics of fermentation products [12, 20], the increasing price of agriculturally suitable substrates has restricted the competitive power and long-term development of industrial-scale ABE fermentation. Moreover, the National Development and Reform Commission of China suspended approval and/or filing of new corn processing projects at the end of 2006 because of the rapid increase in corn prices. As a result, researchers are now focusing on the use of

lower cost, renewable and abundant agricultural resources for ABE fermentation [8, 10, 19].

Cassava (*Manihot esculenta* 'Crantz'), or manioc, is a non-cereal starchy crop that has been widely planted in the tropical regions of Africa, Asia and Latin America. The cassava plant grows better in poor soils and harsh climates than any other major food plant [4, 14]. Due to its low cost and high yield of starch [7], cassava is considered to be a potential candidate substrate for producing ABE.

Our laboratory previously obtained a hyper-butanolagenic strain of *Clostridium acetobutylicum* EA 2018. When grown on corn medium, this strain's solvent yield, butanol ratio, and productivity were all superior to the traditional species [3, 24]. However, when cassava was used as the substrate for ABE production by strain EA 2018, the titer reached only 60% level of that of the corn fermentation under identical conditions, indicating that optimization of the medium is needed for improved cassava fermentation. We have therefore carried out a study aimed at optimizing ABE production by strain EA 2018 on a cassava substrate. We report here that the addition of ammonium acetate improves the solvent yield of cassava fermented by *C. acetobutylicum* EA 2018 and that this improvement was consistent with the transcriptional upregulation of genes required for acid and solvent synthesis.

Materials and methods

Strain, media, and growth conditions

Clostridium acetobutylicum EA 2018 (China Center for Type Culture Collection—CCTCC number M 94061), the strain used in our study, is a mutant that has hyper-butanolagenic production when grown on corn medium. It was obtained by multiple rounds of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) and ethyl methane sulfonate (EMS) mutagenesis together with selective enrichment on butanol plates as previously described [24]. Strain EA 2018 was maintained in 75% glycerol and stored at −70°C.

As substrates for ABE fermentation, cassava was collected from Chengmai County in Hainan Province of China and corn was kindly provided by the Tianguan Group Co., Henan Province, China. Cassava and corn flour (ground dry matter) media were prepared by adding the appropriate amount of distilled water to the flour to achieve a final starch content of 60 g/l (the most commonly used concentration in industrialized ABE fermentation processes) without the addition of extra nutrients. To examine the effect of ammonium acetate on solvent production by *C. acetobutylicum* EA 2018 grown on cassava medium, we added various amounts of ammonium acetate (7.5, 15, 30, 45, 60 or 75 mM) to the media before sterilization. In experiments examining the effect of either ammonium or acetate ions in isolation on solvent production from cassava, ammonium acetate was substituted with ammonium sulfate or sodium acetate, always maintaining the molarity of ammonium or acetate ions as described above.

The inoculum was prepared by adding strain EA 2018 maintained in 75% glycerol to 50 ml of liquid medium [23] that contained (per liter) 0.75 g KH_2PO_4, 0.75 g K_2HPO_4, 0.4 g $MgSO_4$, 0.01 g $MnSO_4$, 0.01 g $FeSO_4{\cdot}7H_2O$, 1.0 g NaCl, 2.0 g asparagine, 5.0 g yeast extract, 2.0 g $(NH_4)_2SO_4$ and 50 g glucose. Cells were grown at 37°C for approximately 12 h.

All fermentation experiments were conducted in 500-ml Erlenmeyer flasks containing 250 ml of sterilized medium. Batch fermentation was initiated by inoculating 5 ml of the above-mentioned activated inoculum (OD_{600} ~1) onto medium. Batch cultures were incubated at 37°C for 48–64 h inside an anaerobic chamber. All experiments were performed at least in duplicate.

Analytical methods

For solvent (ABE) and acid (acetic and butyric) analysis, samples were taken at appropriate time intervals pre-determined based on the experience of the author and centrifuged at 7000*g* for 10 min at 4°C. The supernatant was frozen until analyzed. Acids and solvents present in culture supernatants were identified and quantified by gas chromatography on a GC961-A instrument (Shanghai Jinda Biochemical Instruments, China) equipped with a capillary column [EC-WAX (Alltech, Lexington, KY), 3.0 m × 0.32 mm packed with fused silica] and a flame ionization detector. The analysis was carried out under the following conditions: oven temperature, programmed from 80 to 140°C at a rate of 25°C/min; injector temperature, 200°C; detector temperature, 200°C; nitrogen (carrier gas) flow rate, 13 ml/min; hydrogen flow rate, 20 ml/min; air flow rate, 140 ml/min. Total solvent was defined as the sum of ABE.

The composition of cassava and corn were determined by Beijing Pony Center for Physical and Chemical Analysis according to Chinese National Standards (GB/T5009.5-2003, GB/T5009.6-2003, GB/T5009.9-2003).

RNA isolation and reverse transcription-PCR analysis

Clostridium acetobutylicum EA 2018 cells were incubated in cassava medium with or without ammonium acetate. Samples for total RNA extraction were withdrawn at 18 (mid-acidogenesis as well as early-solventogenesis phase) and 37 h (mid-solventogenesis phase). The sampling cultures were percolated through filter paper to remove cassava residue. Cell pellets were collected by centrifugation

Table 1 Oligonucleotide primers used in the reverse transcription-PCR

Primer	Sequence (5′–3′)	Fragment size (bp)	Gene name[a]	Number of cycles
ask(F)	TGGGATTTACTCCTCTTG	166	*CAC1743*	27
ask(R)	TCGCTGCTTATTCCACTTA			
buk(F)	ATGGGTGGAGGAGTTTCT	146	*CAC1660*	27
buk(R)	TTCCCACTAAAGCACAAT			
adc(F)	ACTTCGCCTGCATTTCCT	198	*CAP0165*	20
adc(R)	AGCCTGTCCGCTTTCTGT			
ctfAB(F)	AAATGAGGCAGATAAAGATG	112	*CAP0164*	20
ctfAB(R)	GTGACCACCACGGATTAG			
adhE(F)	GATGCAGCCGTTAAGAGT	125	*CAP0162*	22
adhE(R)	AGTGAGGGACCACCAGTT			
bdhB(F)	CAGGATAGAATGGCAGAA	114	*CAC3298*	26
bdhB(R)	AAGACTTGAAGCCCACAT			
16s(F)	AGCCAAAGGATTTATTCG	142	*16s rRNA*	12
16s(R)	GTAGGAGTCTGGACCGTGT			

F, Forward; R, reverse

[a] The definition of the genes are as follows: CAC1743, acetate kinase; CAC1660, butyrate kinase; CAP0165, acetoacetate decarboxylase; CAP0164, acetoacetyl-CoA:acyl-CoA transferase; CAP0162, butyraldehyde dehydrogenase; CAC3298, butanol dehydrogenase; 16s rRNA, inner control

at 7000*g* for 20 min at 4°C, frozen rapidly in liquid nitrogen and then ground into powder. RNA isolation and reverse transcription (RT) were performed according to a previously described method [16]. The following PCR program was used for amplification: an initial 5-min denaturation at 95°C, followed by a specific number of cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final incubation at 72°C for 10 min. The primer sequences used for PCR are listed in Table 1. *Clostridium acetobutylicum* 16S rRNA was used as internal control for the RT-PCR assay. The RT-PCR reaction products were subjected to 2% agarose gel electrophoresis for visualization.

Statistical analysis

All experiments were performed at least in duplicate. The data presented are the mean values of the repeated experiments. One-way analysis of variance (ANOVA) was performed. Differences between means below 5% ($P < 0.05$) were considered to be significant.

Results

Solvent productivity of *C. acetobutylicum* EA 2018 grown on corn and cassava medium

Cassava and corn are both crops that are rich in starch. The starch, fat, and protein content of these fermentation substrates are listed in Table 2; starch content in cassava is relatively higher than that in corn, while the content of fat and protein is lower. In our previous study, *C. acetobutylicum* EA 2018 was shown to be an excellent starch-fermenting strain that can grow and ferment in corn mash, yielding a total solvent concentration of 20 g/l with a butanol ratio of 70% [24]. In order to determine the difference between cassava and corn media in terms of ABE production by *C. acetobutylicum* EA 2018, batch fermentation from these two substrates was carried out. The results are shown in Table 3. Corn and cassava were fermented for 48 and 60 h, respectively. The fermentation of cassava for this duration resulted in solvent yields that were significantly lower than those obtained by the fermentation of corn. In particular, acetone and butanol yields were 43 and 30% lower than those in the corn controls. Fermentation in cassava medium produced 22.6% less total solvents than that in corn medium. When

Table 2 Comparison of several components of corn or cassava flour in terms of content

Samples[a]	Main components (%, w/w)			
	Water	Starch	Fat	Protein
Corn flour	11.23 ± 0.24	69.89 ± 1.61	6.69 ± 0.05	7.75 ± 0.09
Cassava flour	12.51 ± 0.37	80.44 ± 0.92	1.08 ± 0.02	2.38 ± 0.08

Values represent the mean of duplicate experiments ± the standard deviation (SD) between samples

[a] Both samples were stabilized at 25°C before testing

Table 3 Comparison of acetone–butanol–ethanol production in batch fermentation by *Clostridium acetobutylicum* EA 2018 grown in corn and cassava medium

Characteristics and product	Substrates[a]	
	Corn	Cassava
Fermentation time (h)	48	60
Yield (g/l)		
Ethanol	1.3 ± 0.1	2.9 ± 0.2
Acetone	4.6 ± 0.1	2.6 ± 0.1
Butanol	14.1 ± 0.3	9.9 ± 0.4
Total solvent	19.9 ± 0.5	15.4 ± 0.7
Butanol ratio (%)	70.9 ± 0.3	64.3 ± 0.3
Batch productivity [g/(l h)]		
Ethanol	0.027 ± 0.002	0.048 ± 0.003
Acetone	0.096 ± 0.002	0.043 ± 0.002
Butanol	0.294 ± 0.006	0.165 ± 0.007
Total solvent	0.415 ± 0.006	0.257 ± 0.005

Values represent the mean of duplicate experiments ± SD between samples

[a] Corn and cassava media both contain starch of 60 g/l

the fermentation time was reduced to 48 h to mimic the fermentation of corn medium, the ABE titer in the cassava medium was even further reduced to 12.1 g/l, which was 39.2% lower than that in the corn medium (Fig. 1a).

Increased solvent production in cassava medium supplemented with ammonium acetate

In order to improve cassava medium for solvent production, we initially supplemented the medium with a mixture of ingredients (except glucose) which would enhance the production of ABE. In cassava medium fully supplemented in this way, the production of solvent increased markedly, almost achieving the same level as that obtained from corn medium (data not shown). However, the cost of these supplemental ingredients would be prohibitive in industrial scale applications. Interestingly, a series of screening tests carried out to determine the ingredient(s) required for increased ABE production identified ammonium acetate as the only effective ingredient in the supplement mixture.

When ammonium acetate was introduced into cassava medium to a final concentration of 30 mM, the acetone, butanol and total solvent yield increased to 5.1, 13.0 and 19.4 g/l, respectively, after 48 h of fermentation (Fig. 1b). When the incubation was prolonged to 60 h, there was no obvious further enhancement of solvent production. The solvent yield from ammonium acetate-supplemented cassava medium almost reached the maximum level within 48 h, while the yield for the unsupplemented control was much lower, with 2.5 g/l acetone and 8.3 g/l butanol. These results demonstrate that the addition of ammonium acetate to the cassava medium not only significantly ($P < 0.001$) increases solvent production by *C. acetobutylicum* EA 2018 within 48 h of fermentation but that it also shortens the fermentation time by about 12 h.

Clostridium acetobutylicum starts to produce solvents at the end of the exponential growth phase, which coincides with a switch from acidogenesis to solventogenesis [22]. We discovered that acid production by *C. acetobutylicum* EA 2018 grown in cassava medium with or without ammonium acetate differed greatly (Fig. 2). After 20 h of fermentation, the levels of both butyric acid and acetic acid in ammonium acetate-supplemented medium had increased significantly, and their maximum levels were 0.8- and 2.6-fold higher, respectively, than that of the unsupplemented control during the acidogenesis period. Solventogenesis was initiated thereafter, and about 230% more acetic acid and 61% more butyric acid were reassimilated in ammonium acetate-supplemented medium as compared to the control.

The impact of ammonium acetate concentration on solvent production by *C. acetobutylicum* EA 2018 on

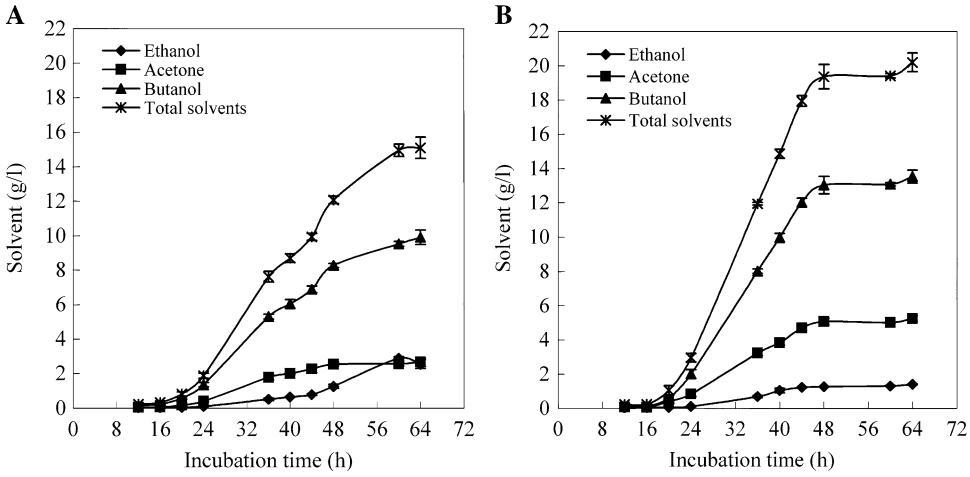

Fig. 1 Comparison of solvent production by *Clostridium acetobutylicum* strain EA 2018 grown in cassava medium either lacking ammonium acetate (**a**) or supplemented with 30 mM ammonium acetate (**b**). The fermentation was performed at 37°C for 64 h. Data represent the mean ± standard deviation (SD) of triplicate individual experiments

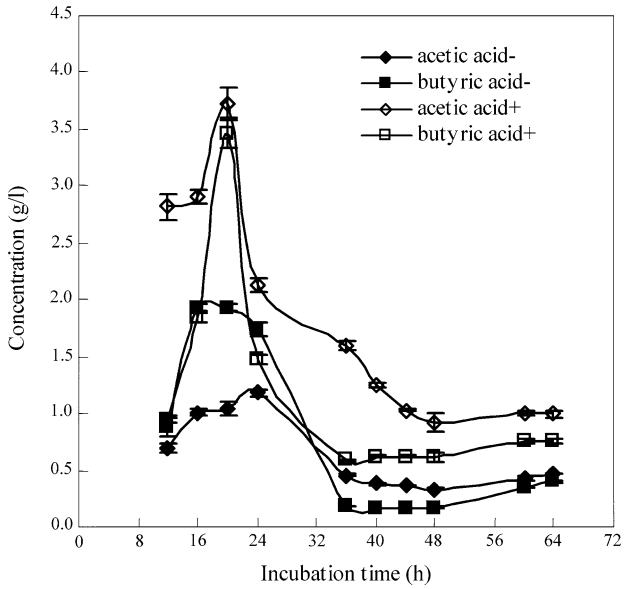

Fig. 2 Comparison of acid production by *C. acetobutylicum* strain EA 2018 grown in cassava medium either lacking ammonium acetate (−) or supplemented with 30 mM ammonium acetate (+). The fermentation was performed at 37°C for 64 h. Data represent the mean ± SD of triplicate individual experiments

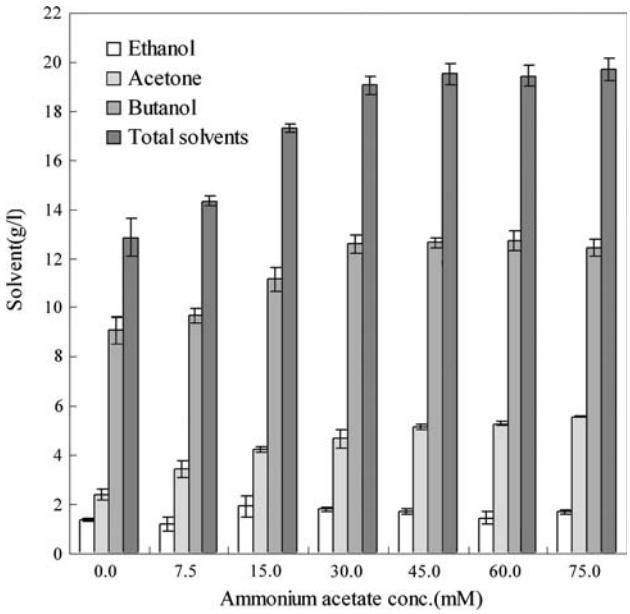

Fig. 3 Solvent production by *C. acetobutylicum* strain EA 2018 grown in cassava medium containing increasing concentrations of ammonium acetate. The fermentation was performed at 37°C for 48 h. Data represent the mean ± SD of triplicate individual experiments

cassava medium was also investigated (Fig. 3). We observed a concentration-dependent increase in solvent production up to 30 mM ammonium acetate. At this molarity, acetone and butanol concentration reached 4.7 and 12.6 g/l, respectively, almost matching the maximum level of solvent concentration achieved by fermentation in the corn medium. The further increase of ammonium acetate concentration did not result in a higher yield of solvent (Fig. 3).

Ammonium and acetate are both required for enhancing solvent production from cassava

Having demonstrated that ammonium acetate enhanced solvent production, we next investigated whether it was the ammonium (NH_4^+) or acetate (CH_3COO^-) that was required for this effect. *Clostridium acetobutylicum* EA 2018 was grown on cassava medium supplemented with either an ammonium source, ammonium sulfate ($(NH_4)_2SO_4$) or an acetate source, sodium acetate (CH_3COONa). As shown in Fig. 4, solvent production was not enhanced when either ammonium sulfate or sodium acetate was added singly at the tested concentration. However, when both sodium acetate (30 mM) and ammonium sulfate (15 mM) were included in cassava medium, the levels of acetone, butanol and total solvents reached 4.8, 13.2 and 19.4 g/l, respectively, which were approximately the same as those achieved by adding 30 mM ammonium acetate.

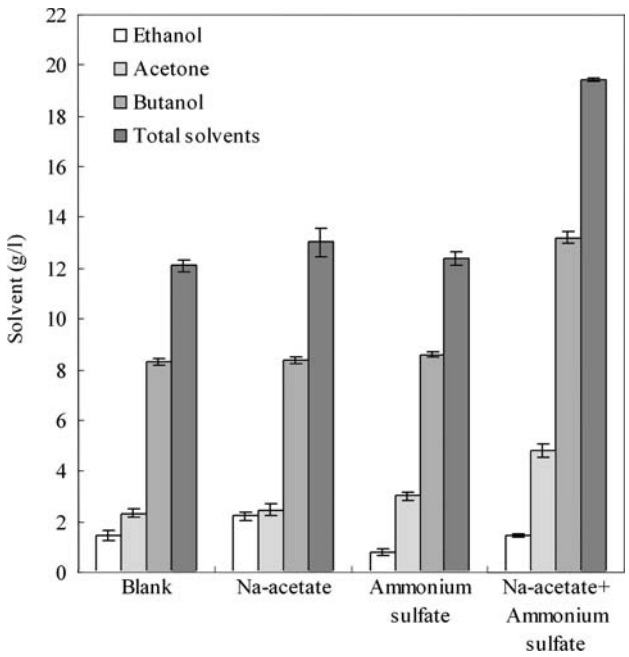

Fig. 4 Role of ammonium and acetate ions on solvent production by *C. acetobutylicum* strain EA 2018 grown in cassava medium. *Blank* No CH_3COONa, *Na-acetate* 30 mM CH_3COONa, *ammonium sulfate* 15 mM $(NH_4)_2SO_4$, *Na-acetate + ammonium sulfate* 30 mM CH_3COONa and 15 mM $(NH_4)_2SO_4$. The fermentation was performed at 37°C for 48 h. Data represent the mean ± SD of triplicate individual experiments

We therefore conclude that both the nitrogen source provided by ammonium as well as acetate were required for enhancing solvent production from the cassava substrate.

Effect of ammonium acetate on gene transcription of enzymes associated with acidogenesis and solventogenesis

In order to decipher the mechanism of enhanced solvent production by ammonium acetate at the molecular level, we employed an RT-PCR approach to compare gene expression in *C. acetobutylicum* strain EA 2018 fermenting cassava medium that lacked ammonium acetate or was supplemented with ammonium acetate, respectively. For this analysis, we chose genes that had been previously shown to be involved in acidogenesis and solventogenesis in clostridia [17]. The transcripts measured were *ask* (encoding acetate kinase), *buk* (butyrate kinase), *adc* (acetoacetate decarboxylase), *ctfAB* (acetoacetyl-CoA/acyl-CoA transferase), *adhE* (butyraldehyde dehydrogenase) and *bdhB* (butanol dehydrogenase). Cells were sampled at two time points following the initiation of fermentation. The 18-h point represented the mid-acidogenesis as well as the early-solventogenesis phase, and the 37-h point represented the mid-solventogenesis phase. The sequences of gene-specific primers used in the RT-PCR analysis are presented in Table 1.

Clostridium acetobutylicum strain EA 2018 cells sampled at 18 h in the presence of ammonium acetate demonstrated an increased transcription of acidogenic (*ask* and *buk*) and solventogenic genes (*adc*, *ctfAB*, *adhE* and *bdhB*) (Fig. 5a). The transcriptional upregulation of *ask* and *buk* was consistent with our observation of higher acetic and butyric acid accumulation during the acidogenesis phase of cassava fermentation (Fig. 2). Furthermore, when fermentation proceeded for 37 h, until the mid-solventogenesis phase, the transcript level of *ctfAB*, *adhE* and *bdhB* was still maintained at higher levels in cells grown on cassava medium supplemented with ammonium acetate. At 37 h, no obvious difference could be detected in *ask*, *buk* and *adc* gene expression in cells sampled from both media (Fig. 5b).

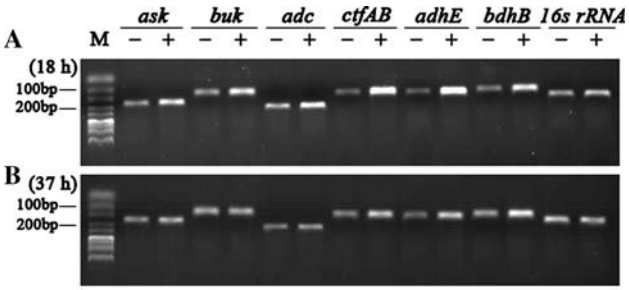

Fig. 5 Reverse transcription-PCR analysis of the expression of genes involved in acid and solvent synthesis by *C. acetobutylicum* strain EA 2018. *ask* acetate kinase, *buk* butyrate kinase, *adc* acetoacetate decarboxylase, *ctfAB* acetoacetyl-CoA/acyl-CoA transferase, *adhE* butyraldehyde dehydrogenase, *bdhB* butanol dehydrogenase, *M* DNA marker. + and − denote cassava medium with or without the addition of 30 mM ammonium acetate, respectively. *16s rRNA* was used as a loading control

Discussion

Cassava is a cost-effective source of starch which could be used as a fermentation substrate. In our previous study, the level of solvent produced by *C. acetobutylicum* EA 2018 using cassava as a substrate was sub-optimal. Our initial hypothesis was that the abundant amylopectin contained in cassava may limit solvent production because amylopectin is highly branched and therefore more difficult to degrade than amylose [21]. However, this hypothesis was incorrect because we observed no increase in solvent production when pullulanase, a starch debranching enzyme [5], was added to the cassava medium with the aim of enhancing the digestibility of amylopectin (data not shown). Therefore, we switched our research focus towards improving the cassava medium. The goal of the study reported here was to optimize cassava medium to become a reliable, low-cost producer of ABE. To the best of our knowledge, this is the first report that cassava can be efficiently used in ABE production.

It has been reported that ammonium acetate is a necessary compound in synthetic medium for the growth of *C. acetobutylicum* and for solvent synthesis [15]. In this study, by screening necessary additives for solvent formation in cassava medium, we were able to identify ammonium acetate as a key component for improving solvent titer and reducing the fermentation time of *C. acetobutylicum* EA 2018. We observed that ammonium acetate-supplemented cassava medium was comparable to the commonly used corn medium in terms of ABE production. Although the level of total solvents obtained through cassava fermentation is not as high as that (24.7 g/l) obtained using starch-containing synthetic P2 medium [11], cassava medium has a significant potential as an industrial medium because the cassava plant is inexpensive to grow and performs well in poor soils.

Modifying cassava medium through the addition of ammonium acetate provides a feasible fermentation medium for producing ABE. We were therefore interested in elucidating the mechanism of ammonium acetate activity during the fermentation of cassava. Chemical analysis of cassava and corn demonstrated that the total fat and protein content of cassava are much lower than those of corn (Table 2). Protein and fat provide a nitrogen source as well as volatile fatty acids, such as acetate and butyrate, which are key factors ensuring the expression of enzymes associated with acidogenesis and solventogenesis in ABE fermentation [1, 2]. Consequently, we suggest that the low solvent production from cassava fermentation is due to a

lack of nitrogen and volatile fatty acids. Our results clearly demonstrate that both nitrogen-containing ammonium ions in addition to acetate were necessary for increased solvent synthesis (Fig. 4). This hypothesis is also supported by the observation that acid accumulation and reassimilation were much higher in cassava media containing ammonium acetate than in the control (Fig. 2). Although the increased acetic acid yield may partially be attributed to the extra acetate provided by ammonium acetate, the increased accumulation and reassimilation of butyric acid is likely due to ammonium acetate-activated metabolism. We conclude that in cassava medium supplemented with ammonium acetate, acetic acid and butyric acid are generated in abundance and then efficiently re-utilized by *C. acetobutylicum* EA 2018 to produce more acetone and butanol.

The gene expression analysis also suggested that ammonium acetate upregulated the expression of enzymes involved in acidogenesis and solventogenesis in the cassava medium. During the early solventogenesis phase (18 h), the increased transcription of several genes (*ask*, *buk*, *adc*, *ctfAB*, *adhE*, *bdhB*) may contribute to enhanced solvent production. Furthermore, *ask* and *buk* may also be responsible for the higher acetic and butyric acid accumulation that occurred during the acidogenesis phase (Fig. 2). At mid-solventogenesis (37 h post fermentation), there were no significant differences in the transcription levels of *ask*, *buk* and *adc*, but other genes (*ctfAB*, *adhE*, *bdhB*) maintained higher levels of expression in cassava medium supplemented with ammonium acetate. Among these genes, expression of *adhE* and *bdhB* is tightly associated with butanol production [17]. Therefore, their increased expression in ammonium acetate-supplemented medium from the early- to the mid-solventogenesis period may be responsible for increased butanol yield. Finally, acetoacetyl-CoA/acyl-CoA transferase, the product of *ctfAB*, is a key enzyme required for acid reassimilation in solventogenic clostridia [2]. Any substantially increased expression of *ctfAB* from the early- to the mid-solventogenesis phase would result in heightened acid uptake and increased solvent yield.

In summary, we conclude that ammonium acetate enhances solvent production due to the fermentation of cassava by supplying an additional source of nitrogen as well as acetate. This supplementation in effect replaces essential compounds present in corn but lacking in cassava. The likely function of acetate, an important compound for ABE fermentation, is to maintain the robust expression of enzymes associated with acidogenesis and solventogenesis in *C. acetobutylicum* growing in cassava medium. Therefore, with the addition of 30 mM ammonium acetate to the medium, cassava can be used to produce levels of solvent that are nearly equal to those produced with corn. Due to cassava being a non-cereal and its ease of cultivation and relatively cheaper price, its application in industrial level ABE fermentation will yield substantial economic and social benefits.

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