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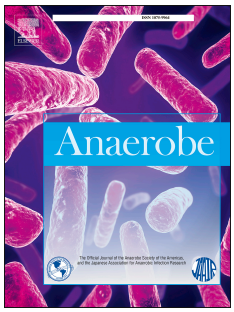

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Improvement of the butanol production selectivity and butanol to acetone ratio (B:A) by addition of electron carriers in the batch culture of a new local isolate of *Clostridium acetobutylicum* YM1

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Abstract

Improvement in the butanol production selectivity or enhanced butanol: acetone ratio (B:A) is desirable in acetone-butanol-ethanol (ABE) fermentation by *Clostridium* strains. In this study, artificial electron carriers were added to the fermentation medium of a new isolate of *Clostridium acetobutylicum* YM1 in order to improve the butanol yield and B:A ratio. The results revealed that medium supplementation with electron carriers changed the metabolism flux of electron and carbon in ABE fermentation by YM1. A decrease in acetone production, which subsequently improved the B:A ratio, was observed. Further improvement in the butanol production and B:A ratios were obtained when the fermentation medium was supplemented with butyric acid. The maximum butanol production (18.20±1.38 g/L) was gained when a combination of methyl red and butyric acid was added. Although the addition of benzyl viologen (0.1 mM) and butyric acid resulted in high a B:A ratio of 16:1 (800% increment compared with the conventional 2:1 ratio), the addition of benzyl viologen to the culture after 4 h resulted in the production of 18.05 g/L butanol. Manipulating the metabolic flux to butanol through the addition of electron carriers could become an alternative strategy to achieve higher butanol productivity and improve the B:A ratio.

Keywords*:* Renewable energy; Butanol; B:A ratio; Electron carriers; *Clostridium acetobutylicum* YM1.

1. Introduction

Fuel production from alternative renewable resources has attracted increased attention in the last few years principally due to escalating oil prices, increasing environmental concerns, and the need for substitutes for fossil fuel resources, which are exhaustible [1, 2]. Thus, there is the need for a gradual shift from fossil fuel resources to renewable resources such as feedstock. The most preferred liquid biofuel candidate that potentially substitutes gasoline is butanol. Typically, n-butanol is biologically produced in a mixed product, acetone-butanol-ethanol (ABE), by fermentation by *Clostridium* [3].

The production of fuels from renewable resources must meet two desired criteria to be applicable, such as high energy content, high octane value, and compatibility with the current fuel supply, distribution systems and existing engines [4, 5]. Butanol meets these required criteria. Furthermore, compared with ethanol, butanol is a better gasoline substitute because of its higher energy content, less corrosive nature, higher octane number, higher hydrophobicity and compatibility when blended with gasoline or directly used in existing engines without modifications [6-8].

Clostridium acetobutylicum YM1 is a solvent-producing *Clostridium* that was recently isolated from local agricultural soil in Malaysia and has been demonstrated to produce butanol and biohydrogen [9-11].

The normal ratio of ABE in ABE fermentation is 3:6:1 (butanol/acetone ratio of 2:1) [12]. In order to make ABE fermentation economically viable, improvement of the butanol/acetone ratio to greater than 2:1 (B:A) is an ideal requirement. Acetone is not favorable in ABE fermentation due to its recovery cost, corrosiveness to rubber engine parts and poor performance in terms of

fuel properties. Hence, reducing acetone production and increasing butanol concentration, which invariably increases the butanol/acetone ratio, are key factors in improving the economic efficacy of ABE fermentation [13]. Metabolic engineering has been recognized as the best method to enhance the process productivity and yield by targeting certain pathways [14].

Previous efforts have been applied to enhance the butanol/acetone ratio by implementing oxidative-reductive-potential control [15], redistributing the carbon flux to butanol by the addition of precursor of cofactors [14], using mixed substrates [16], employing metabolic engineering methods [3, 13, 17] and inhibiting the hydrogenase enzyme by the addition of electron carriers such as methyl viologen and neutral red [18-20]. The production of butanol without acetone and acids using solvent-producing *Clostridia* is difficult by metabolic engineering due to the inability to control the electron flow, which may be affected by unknown pSOL1 genes [21]. However, in studies where this approach has been attempted, achievements of higher butanol/acetone ratios were made at the expense of total solvents/butanol productivities or increased purification loads [22].

The main point for the successful industrial revival of ABE fermentation by *Clostridia* is to generate a single product, which is butanol. However, it is difficult to direct the carbon flow only to butanol because *C. acetobutylicum,* as well as all other related bacteria, possess complex metabolic pathways with many branching points [23]. In this study, an attempt was made to improve the yield of butanol and the B:A ratio in a batch culture of a new local isolate of *Clostridium acetobutylicum* YM1. For this purpose, various artificial electron carriers were introduced to the fermentation medium of *C. acetobutylicum* YM1 to improve the butanol production and decrease the acetone production. To further enhance the production of butanol without acetone, butyric acid was added with electron carriers to the medium of *C.*

acetobutylicum YM1. Moreover, the effect of the addition time of benzyl viologen was also investigated. This study verifies the significance of using electron carriers in improving the butanol production and B:A ratio based on *C. acetobutylicum* strain YM1 fermentation.

2. Materials and Methods

2.1. Microorganism and inoculum preparation

In this study, a newly isolated strain of *Clostridium acetobutylicum* YM1, which was isolated from local soil in Malaysia, was used. The culture was maintained in 50% glycerol at -30 °C as a spore suspension. In order to prepare the inoculum, the spores were first activated by transferring 1 mL of the spore suspension into 10 mL of tryptone yeast-extract acetate medium (TYA) followed by a 1 min period of heat shock in boiling water. The resulting suspension was immediately cooled in ice and incubated for 1-2 days at 30°C under anaerobic conditions. This culture was then transferred to TYA medium and incubated for 20 hours at 30°C.

2.2. Preparation of medium

TYA medium was used to prepare the inoculum and as a fermentation medium. The TYA medium consisted of 6 g/L tryptone, 3 g/L ammonium acetate, 2 g/L yeast extract, 0.3 g/L $MgSO_4.7H_2O$, 0.5 g/L KH_2PO_4, and 0.01 g/L $FeSO_4.7H_2O$. The glucose concentration was 20 g/L in the inoculum preparation medium and 50 g/L in the fermentation medium. After mixing these components, the medium was then sterilized at 121°C for 15 min.

2.3. Fermentation conditions

The fermentation medium used in this study was TYA with 50 g/L glucose, and the conditions were as follows: inoculum size of 10% (v/v), incubation temperature of 30 °C, and an initial pH of 6.2 for 120 h. All of the fermentation experiments were carried out in 100-mL serum bottles equipped with rubber caps and crimped with aluminum seals. The cultures in serum bottles were agitated by hand once per day in the first 48 h to maintain culture homogeneity. Anaerobic conditions were achieved by flushing the medium with nitrogen gas for 1 min before inoculation. The electron carriers used in this study were prepared and sterilized by filtration using a 0.2-μm filter membrane. Only in the cases stated, the electron carriers were initially added to the fermentation medium before inoculation under aseptic conditions.

2.4. Analytical methods

Fermentation samples were collected and centrifuged at 7000 rpm for 5 min, and the supernatant was used for the analysis of solvents, acids and glucose. The analyses of acetone, butanol, ethanol and acids (butyric acid and acetic acid) were carried out using a gas chromatography system (7890A GC-System, Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a 30-m capillary column (Equity1™; 30 m × 0.32 mm× 1.0 μm film thickness; Supelco Co, Bellefonate, PA, USA). The temperature of the injector was set at 250 °C, and the detector was set at 280 °C. The carrier gas was helium at a flow rate of 1.5 mL/min. Growth was detected based on the optical density measured at 600 nm using a UV-Vis spectrophotometer (Shimadzu, UV mini-1240, Kyoto, Japan). The glucose concentration was detected using a glucose oxidase kit [GOD, (E.C. 1.1.34), Roche Ltd., Swiss] following the manufacturer's procedure.

The butanol yield was calculated based on the consumed substrate (glucose and butyric acid) using the following equation:

$Y_{butanol/substrate} = B/S_c \ (C-mol/C-\ mol)$

Where B/S_c is the yield of butanol to the substrate consumed (C-mol/C-mol), B is the numbers of carbon moles of butanol produced and S_c is the substrate consumed (S_c=carbon moles of butyric acid consumed + carbon moles of glucose consumed).

3. Results and discussion

3.1. Effect of electron carriers on the butanol production and B:A ratio

The effect of the supplementation of the fermentation medium with artificial electron carriers including methyl viologen, benzyl viologen, methyl red, neutral red, Tiron, 2,2-bipyridyl, phenosafranin, menadione, methylene blue, trimethylamine N-oxide (TMAO), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO) and bromophenol blue on the butanol production and B:A ratio by *C. acetobutylicum* YM1 was studied.

Different concentration ranges for each electron carrier were individually applied to investigate the optimal concentration that effectively improved the B:A ratio (data not shown). Table 1 illustrates the effect of various electron carriers on the production of solvents and acids as well as the butanol yield and B:A ratio when they were used as additives to the growth media in batch cultures of *C. acetobutylicum* YM1. Generally, the addition of artificial electron carriers in this study resulted in higher ABE and butanol concentrations, as well as improved butanol yield when compared with the control culture, which was not supplemented with electron carriers.

Among the tested electron carriers, benzyl viologen was found to be the most efficient in improving the butanol productivity and yield in batch cultures of *C. acetobutylicum* YM1. The highest butanol yield (0.504 C-mol/C-mol) and butanol concentration (15.57 g/L) were observed when the culture was supplemented with 0.1 mM benzyl viologen. The presence of benzyl viologen improved the butanol concentration to 15.57 g/L, whereas the acetone concentration was 1.74 g/L. The butanol yield (0.504 C-mol/C-mol) was also notably higher (by 48.23 %) than that obtained from the control culture. Moreover, the highest B:A ratio (8.95:1) was obtained when 0.1 mM of benzyl viologen was added to the culture medium.

Methyl red addition at a concentration of 0.01 mM improved the ABE and butanol production to 14.34 and 19.58 g/L, respectively, whereas no improvement in B:A ratio was observed with the addition of methyl red to the fermentation medium of *C. acetobutylicum* YM1.

Although no significant improvement in the B:A ratio was observed, Tiron addition to the fermentation medium caused an increase in the butanol and ABE production. Menadione increased the production of acetone, butanol and ethanol to yields of 4.67, 13.07 and 1.2 g/L, respectively, whereas the B:A ratio decreased to 2.8:1 compared with the control culture (3.22:1).

The use of DMSO, 2,2 bipyridyl, bromophenol blue, DTNB and phenosafranin, methylene blue, and menadione as electron carriers led to enhanced production of butanol and ABE, as well as butanol yield, whereas the B:A ratios obtained were less than those of the control culture, which lacked any electron acceptor (Table 1).

Remarkably, when neutral red, methyl viologen or benzyl viologen were incorporated as electron carriers, the production of butanol, the butanol yields and the B:A ratios were enhanced. The addition of methyl viologen (0.1 mM) produced 12.49 g/L of butanol with a butanol yield of

0.405 C-mol/C-mol and a B:A ratio of 6.09:1. The improvement of the B:A ratio is ascribed to the ability of methyl viologen to modify the distribution of carbon and electrons in the ABE fermentation pathway. It was previously reported that the addition of 1 mM methyl viologen to a continuous culture medium of *C. butyricum* DSM 5431 caused a strong decline in the hydrogen (H_2) production and an increase in the production of 1,3-propanediol and butyrate [24]. Furthermore, the addition of methyl viologen to a *C. butyricu*m culture medium significantly reduced the biomass growth, which was attributed to the toxicity of methyl viologen [24, 25].

Similar butanol and ABE concentrations were produced with the use of methylene blue and TMAO as electron acceptors. In the presence of 0.5 mM methylene blue in the medium, a B:A ratio of 2.96:1 was recorded. The butanol and ABE concentrations obtained were 11.73 and 16.78 g/L, respectively. This observation indicated that methylene blue improved the ABE and butanol productivity but failed to improve the B:A ratio. In a similar study [26], methylene blue addition in ABE fermentation by *Clostridium acetobutylicum* was found to enhance the ABE, acetone and butanol production by 70%, 88% and 43%, respectively. However, a B:A ratio of 2.2:1 was obtained.

Ballongue et al. [26] showed that methylene blue could shift the carbon flow towards acetone production by eliminating reduced compounds such as $NADH+H^+$. A similar observation was noted in our study because the B:A ratio decreased to 2.96:1 compared with the control culture. This result suggested that methylene blue generally enhanced the overall solvent production without selectivity to butanol.

Based on these results, it was clear that the addition of artificial electron carriers to the fermentation medium was of vital importance in butanol fermentations requiring high butanol yields. Because electron carriers can increase the butanol production and decrease the acetone

production, electron carriers tend to be involved in the metabolic pathway. The mechanism of the action of artificial electron carriers on the metabolic flux in ABE fermentation by *C. acetobutylicum* is illustrated in Fig. 1.

It has been reported that methyl viologen has an inhibitory effect on hydrogenase enzyme activity [18, 27]. In the presence of methyl viologen in the culture, an inhibitory competition emerged between methyl viologen and reduced ferredoxin (FdH_2) for the active site of the hydrogenase enzyme, which led to the inhibition of the hydrogenase-mediated oxidation of FdH_2. In order to continue conversion of pyruvate to acetyl-CoA, FdH_2 must be re-oxidized. As a result of this inhibition, another pathway mediated by ferredoxin oxidoreductase was activated to oxidize FdH_2, along with the generation of NADH. Thus, the inhibition of hydrogenase and the activation of ferredoxin oxidoreductase functioned cooperatively, leading to the production of a large amount of NADH. The accumulation of NADH encouraged the alcohol formation pathway, which was the only available pathway to eliminate NADH because reduced electron carriers such as NADH need to be re-oxidized to maintain electron balance [18, 21].

Accordingly, it is clear that efforts aimed at increasing the intracellular NADH/NADPH level by redirecting the electron flow are associated with low hydrogen production and result in a metabolic shift from an acidogenic phase to a solventogenic phase, which consequently improves the butanol yield, as well as the B:A ratio [28, 29].

3.2. Effect of butyric acid addition

In our previous studies [30, 31], it was observed that butyric acid could be efficiently converted to butanol in the presence of a carbon source. These studies showed that supplementation of the

medium with butyric acid as a co-substrate or a butanol precursor led to enhanced production of butanol.

Our strain, *C. acetobutylicum* YM1, showed that the addition of butyric acid could increase the butanol production but not the acetone production (data not shown), which implied that *C. acetobutylicum* YM1 converts butyric acid to butanol through the butyrate kinase pathway. This property is preferred because acetone is considered a by-product in ABE fermentation.

Based on the obtained results, in subsequent fermentations, the medium was supplemented with a combination of electron carriers and butyric acid (8.7 g/L). Fig. 2 shows the comparison of butanol production using TYA medium in a batch culture of *C. acetobutylicum* YM1 supplemented with electron carriers in the presence or absence of butyric acid.

In the cultures supplemented with electron carriers and butyric acid, the B:A ratios, production and yield of butanol significantly increased compared with the control culture (Figs. 2&3). It was previously reported that the addition of butyric acid to the fermentation medium became an additional carbon source in ABE fermentation and resulted in increased butanol production [22, 30].

Further addition of butyric acid to the culture of *C. acetobutylicum* YM1 in the presence of electron acceptors resulted in further increments in the butanol production, as well as the B:A ratios. Figs. 2 and 3 show that the B:A ratios and butanol production improved due to the addition of 8.7 g/L butyric acid with all of the electron carriers tested in this study.

Surprisingly, a B:A ratio of 16.11:1 was obtained when a combination of benzyl viologen and butyric acid was added to the fermentation medium of *C. acetobutylicum* YM1. With this

combination, 16.3 g/L butanol, 1.11 g/L acetone and 0.54 g/L ethanol were produced with a butanol yield of 0.54 C-mol/C-mol.

Methyl red was found to be the most efficient in improving the butanol production in batch cultures of *C. acetobutylicum* YM1. The highest butanol (18.20 g/L) and ABE concentrations (24.42 g/L) were observed when the culture was supplemented with 0.01 mM methyl red with 8.7 g/L butyric acid. The presence of methyl red in the culture medium significantly improved the butanol production and butanol yield. However, the B: A ratio obtained was 3.62:1. Therefore, methyl red did not reduce the acetone production, and it generally improved the overall solvent concentrations.

Viologen compounds (methyl and benzyl) were found to produce high concentrations of butanol when added to the culture with initial butyric acid addition. The addition of methyl and benzyl viologen led to the production of 16.29 and 16.30 g/L butanol, respectively. In the culture with menadione, 15.25 g/L of butanol was produced. DMSO also increased the butanol generation in the presence of butyric acid with an observed butanol yield and concentration of 0.482 C-mol/C-mol and 14.55 g/L, respectively. All of the other election acceptors also improved the butanol production with yields comparatively higher than that observed in the control culture (Fig. 2). The increment in the butanol production when butyric acid was added reached 30.48% with methyl viologen and 22.54 % with DMSO.

The limited butanol production was attributed to the inhibitory effect of butanol on the culture. It was stated that the butanol toxicity directly controlled the butanol production capability of *Clostridium* [32, 33]. It was reported that the butanol production rarely exceeded 13.0 g/L in wild-type strains of *C. acetobutylicum* due to the butanol accumulation toxicity [12, 34].

However, our wild-type strain could produce higher than 13.0 g/L of butanol in some cases (Fig. 2).

3.3. Effect of the addition time of benzyl viologen on the growth, butanol production and B:A ratio

Results from the initial aspects of this study showed that the best electron acceptor was benzyl viologen (0.1 mM) because it significantly improved the B:A ratio as well as the butanol productivity. Hence, the effect of the addition time of benzyl viologen on the ABE production and B:A ratio was investigated. Benzyl viologen was added at different times during the growth of the culture (0, 4, 8, 12 and 24 h).

It was reported that benzyl viologen dye is not able to cross the cytoplasmic membrane of *Escherichia coli* cells in their oxidized form though the reduced forms can easily penetrate the cell membrane [35]. In our experiments, once the medium was inoculated by YM1, the color of the medium containing benzyl viologen was changed to blue, which indicates that the viologen had been reduced. Similar observation was reported by Rao and Mutharasan [36] when benzyl viologen was added to the continuous culture of *C. acetobutylicum*.

With an observed concentration of 18.05 g/L butanol, the results showed that the highest butanol production was obtained when benzyl viologen was added at 4 h (Fig. 4). The addition of benzyl viologen initially produced the lowest concentration of butanol (16.29 g/L) but showed the highest B:A ratio of 16.11. Benzyl viologen had a toxic effect on the *C. acetobutylicum* YM1 culture, which was indicated by the growth of the organism. The results showed that delays in the addition time of benzyl viologen led to a corresponding enhancement of the culture growth

(Fig. 5). The growth of *C. acetobutylicum* YM1 was significantly affected by the addition time of benzyl viologen. The growth increased by increasing the time of addition of benzyl viologen. When the medium was supplemented with benzyl viologen at 0 h (initially), the optical density (OD_{600}) obtained as a measure of the growth was 1.933. A maximum OD_{600} (2.574) was recorded when benzyl viologen was added at 24 h after the inoculation time. The relationship between the addition time of benzyl viologen and the growth of *C. acetobutylicum* YM1 was in direct proportion (Fig. 5).

Increasing the addition time of benzyl viologen resulted in reduced B:A ratios, with an addition time of 0 h as the optimal time for the highest B:A ratio. Meanwhile, an addition time of 4 h resulted in the highest production of butanol (18.04 g/L). No significant differences in ethanol production were observed when the addition time of benzyl viologen was changed. The acetone production increased from 1.01 g/L when benzyl viologen was initially added to 2.6 g/L when benzyl viologen was added 24 h after inoculation.

Fig. 6 shows a time course of ABE fermentation by *C. acetobutylicum*YM1 in TYA medium with butyric acid (8.7 g/L) without benzyl viologen (Fig. a) and with benzyl viologen addition (addition time at 4h (Fig. b)). According to the profiles of the cell growth and products, *C. acetobutylicum* YM1 produced 1.47 g/L acetone, 18.05 g/L butanol and 0.65 g/L ethanol with a total of ABE 20.17 g/L and a B:A ratio of 12.3:1 after 96 h of fermentation (Fig. 6b).

The total ABE and butanol concentrations produced in the medium supplemented with benzyl viologen at 4 h were more than that produced from the medium without benzyl viologen supplementation. However, the fermentation time taken to reach the highest product concentration (96 h) and growth profile was similar in both cultures.

In the control experiment (without benzyl viologen addition), the total concentration of ABE and butanol obtained were 15.49 g/L and11.84 g/L, respectively. Additionally, the B:A ratio was 3.96:1. Thus, observations from the control experiment revealed that glucose consumption was higher in the medium devoid of benzyl viologen compared with the culture supplemented with benzyl viologen (Figs. 6 a & 6b).

The butanol concentration produced in the *C. acetobutylicum* YM1fermentation medium supplemented with benzyl viologen was 34.4 % higher than that produced in the control culture (without benzyl viologen addition). Additionally, a higher B:A ratio (12.3:1) was observed.

The B:A ratio significantly improved, and the solvent production proceeded towards the direction of generating more butanol in the presence of benzyl viologen. The B:A ratio in the medium with benzyl viologen supplementation was approximately four times higher than that observed in the culture without benzyl viologen supplementation. The final concentration of ethanol (0.65±0.01 g/L) was similar in the control culture and the culture supplemented with benzyl viologen.

The residual glucose concentration in the medium with benzyl viologen was 6.0 g/L, whereas the glucose was totally utilized in the control culture. The consumption of initially added butyric acid was better in the control culture compared with the culture supplemented with benzyl viologen. The residual butyric acid concentrations in the control culture and culture supplemented with benzyl viologen were 3.13 and 5.06 g/L, respectively. Accordingly, the presence of 0.1 mM benzyl viologen in the *C. acetobutylicum* YM1 fermentation medium was a key factor in the enhancement of the butanol production selectivity or B:A ratio.

4. Conclusion

The effect of various artificial electron carriers on the butanol production, yield, and B:A ratio in a batch fermentation process using *C. acetobutylicum* YM1 was investigated. The productivity and yield of butanol were enhanced with all of the electron carriers tested in this study. A combination of electron carriers and butyric acid supplementation in the fermentation medium generated higher concentrations of butanol and ABE, as well as B:A ratios, compared with electron carrier supplementation alone. A significantly improved B:A ratio of 16.11 with a butanol concentration of 16.29 g/L was recorded when benzyl viologen was added to the culture. The addition of benzyl viologen (0.1 M) to the culture of YM1 after 4 h resulted in 18.05 g/L butanol. Although 4 h was the optimal time for butanol production, the optimal time for the B:A ratio was 0 h. The data in this study demonstrated that the use of electron carriers and butyric acid as supplementation for butanol production could be an alternative strategy for achieving higher butanol productivity and B:A ratios.

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Figures caption:

Fig. 1. Metabolic pathway of ABE fermentation by *Clostridium acetobutylicum.* The solid and dashed arrows represent the products during the acidogenic phase and solventogenic phase, respectively.

Fig. 2. Effect of electron carriers and butyric acid on the butanol production in batch cultures of *C. acetobutylicum* YM1.

Fig. 3. Effect of electron accepters and butyric acid addition on the B:A ratio in batch cultures of *C. acetobutylicum* YM1.

Fig. 4. Effect of the addition time of benzyl viologen on ABE production

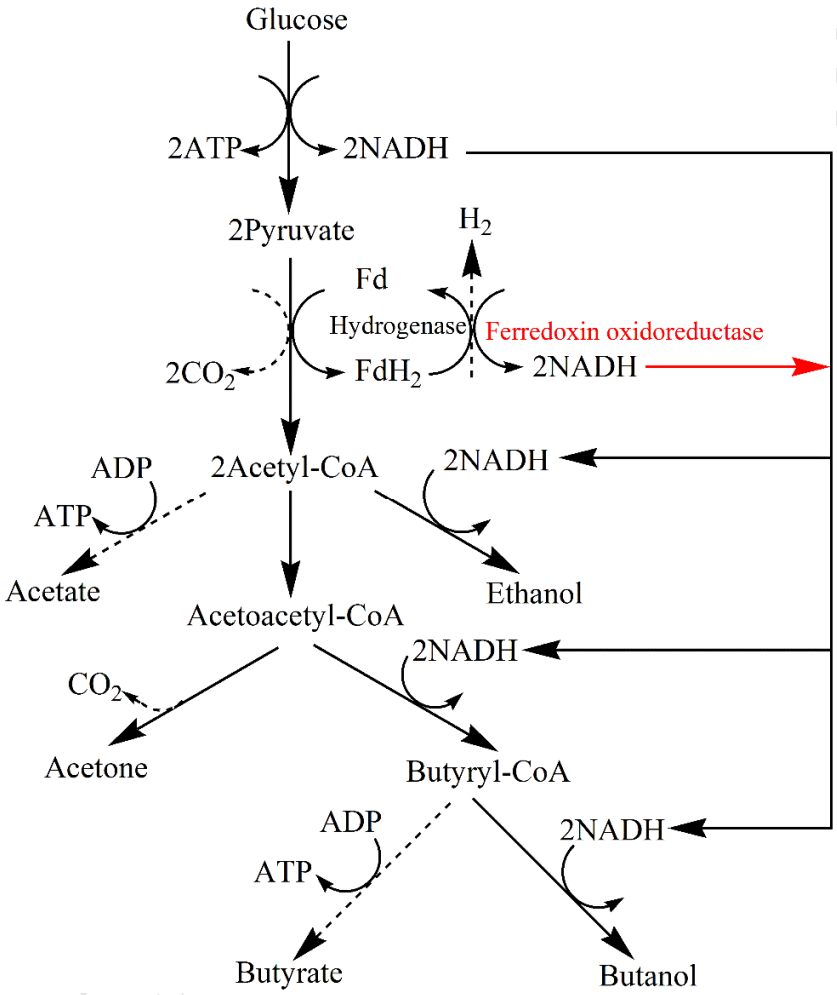
Fig. 5. Effect of the addition time of benzyl viologen on the growth of *C. acetobutylicum* YM1, butanol production and B:A ratio.

Fig. 6. Profile of the ABE fermentation by *C. acetobutylicum* YM1 in TYA medium with butyric acid (8.7 g/L) but without benzyl viologen (a) and that with butyric acid and benzyl viologen (addition time at 4h) (b).

Table 1

The effect of medium supplementation with different electron carriers on the butanol production and B:A ratio in a batch culture of *C. acetobutylicum* YM1

Electron carriers	Concentration (mM)	Solvent Production (g/L)				Acid (g/L)		B: A ratio	Butanol Yield (C-mol/C-mol)
		Acetone	Butanol	Ethanol	ABE	Butyric	Acetic		
Menadione	0.1	4.67±0.11	13.07±0.18	1.20±0.11	18.94±0.04	1.68±0.04	0.30±0.14	2.80	0.427
DMSO	10.00	3.89±0.04	11.88±0.17	1.26±0.08	17.03±0.13	1.04±0.08	0.79±0.35	3.05	0.387
Methylene blue	0.50	3.96±0.06	11.73±0.33	1.10±0.10	16.78±0.28	1.36±0.06	0.82±0.33	2.96	0.384
2,2 -Bipyridyl	0.01	3.81±0.13	11.49±0.33	1.75±0.18	17.05±0.01	1.33±0.07	0.07±0.03	3.02	0.384
Tiron	0.50	4.30±0.33	14.71±0.06	1.13±0.03	20.14±0.24	1.68±0.17	1.06±0.28	3.42	0.483
TMAO	0.10	3.92±0.28	11.79±0.20	1.24±0.06	16.95±0.03	0.99±0.07	2.81±1.05	3.01	0.382
Bromophenol blue	0.001	3.77±0.31	11.45±0.38	1.15±0.08	16.37±0.16	0.68±0.18	2.87±0.27	3.04	0.372
DTNB	0.05	3.90±0.08	11.80±0.35	1.01±0.08	16.71±0.35	0.04±0.03	0.46±0.27	3.03	0.385
Neutral red	0.50	3.31±0.14	12.04±0.18	0.79±0.04	16.15±0.37	1.16±0.08	0.74±0.28	3.63	0.392
Phenosafranin	1.00	4.07±0.11	12.43±0.14	0.55±0.07	17.05±0.10	2.11±0.10	0.60±0.17	3.05	0.406
Methyl red	0.01	4.13±0.10	14.36±0.49	1.09±0.04	19.58±0.35	2.80±0.38	1.59±0.27	3.48	0.479
Benzyl viologen	0.1	1.74±0.06	15.57±0.66	1.43±0.20	18.74±0.41	0.51±0.08	0.19±0.04	8.95	0.504
Methyl viologen	0.1	2.05±0.11	12.49±0.16	0.90±0.03	15.44±0.07	0.26±0.06	0.56±0.20	6.09	0.405
Control	-	3.30±0.13	10.61±0.13	1.27±0.16	15.18±0.41	1.47±0.59	0.69±0.06	3.22	0.340



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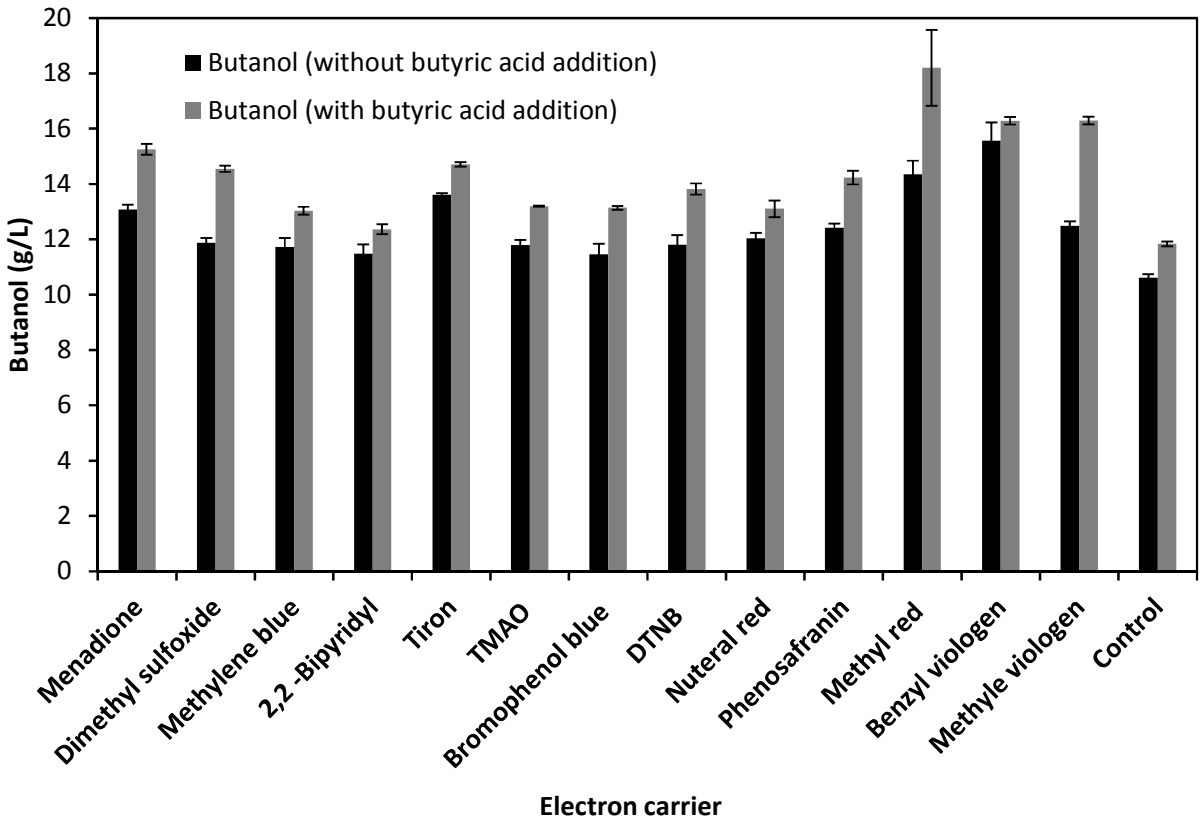


Fig. 2. Effect of electron carriers and butyric acid on the butanol production in batch cultures of *C. acetobutylicum* YM1

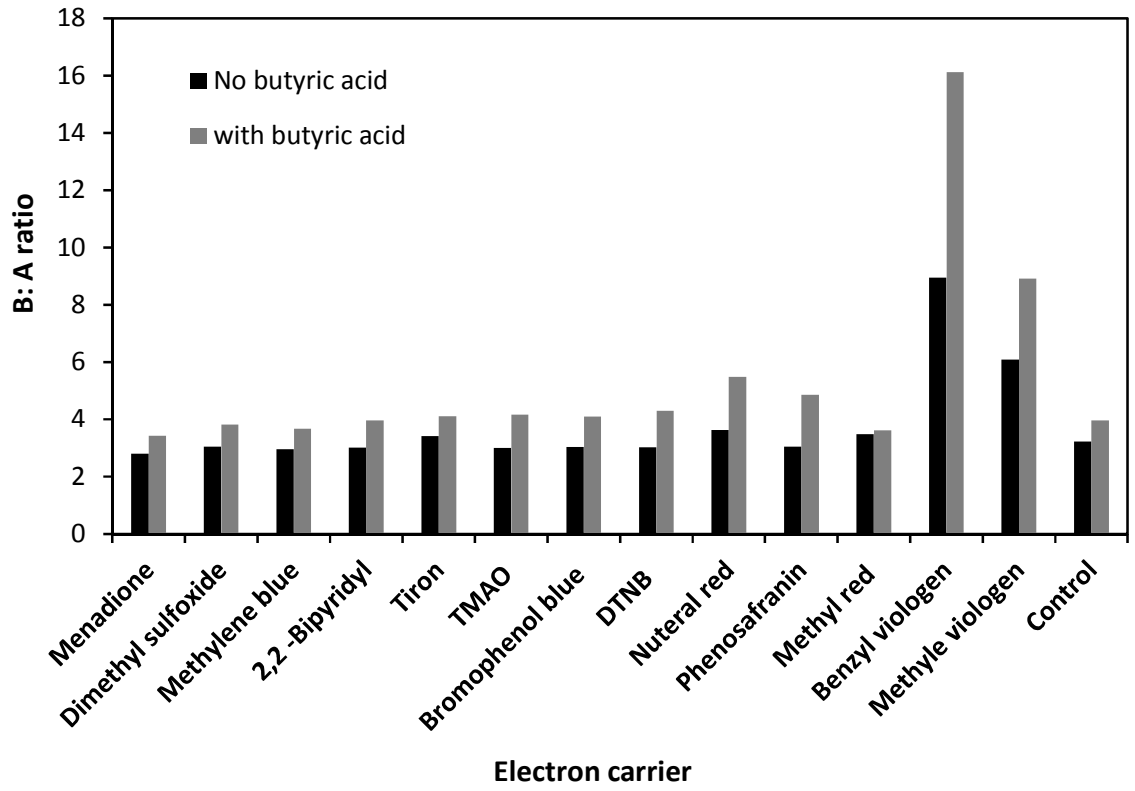


Fig. 3. Effect of electron accepters and butyric acid addition on the B:A ratio in batch cultures of *C. acetobutylicum* YM1

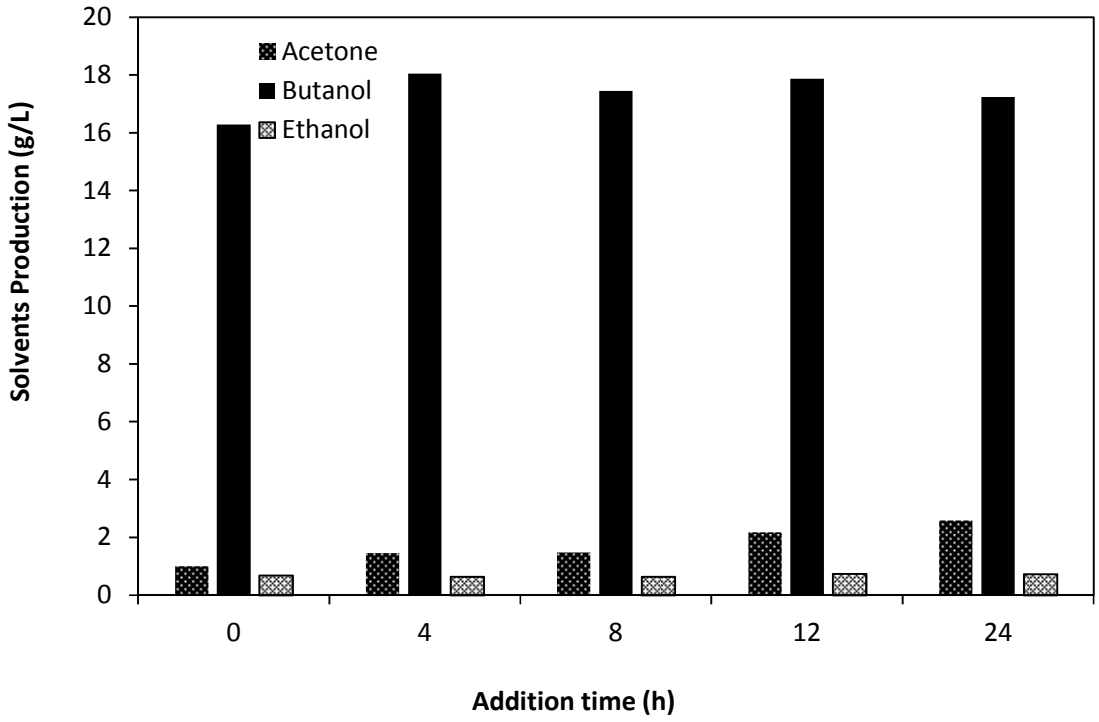


Fig. 4. Effect of the addition time of benzyl viologen on ABE production

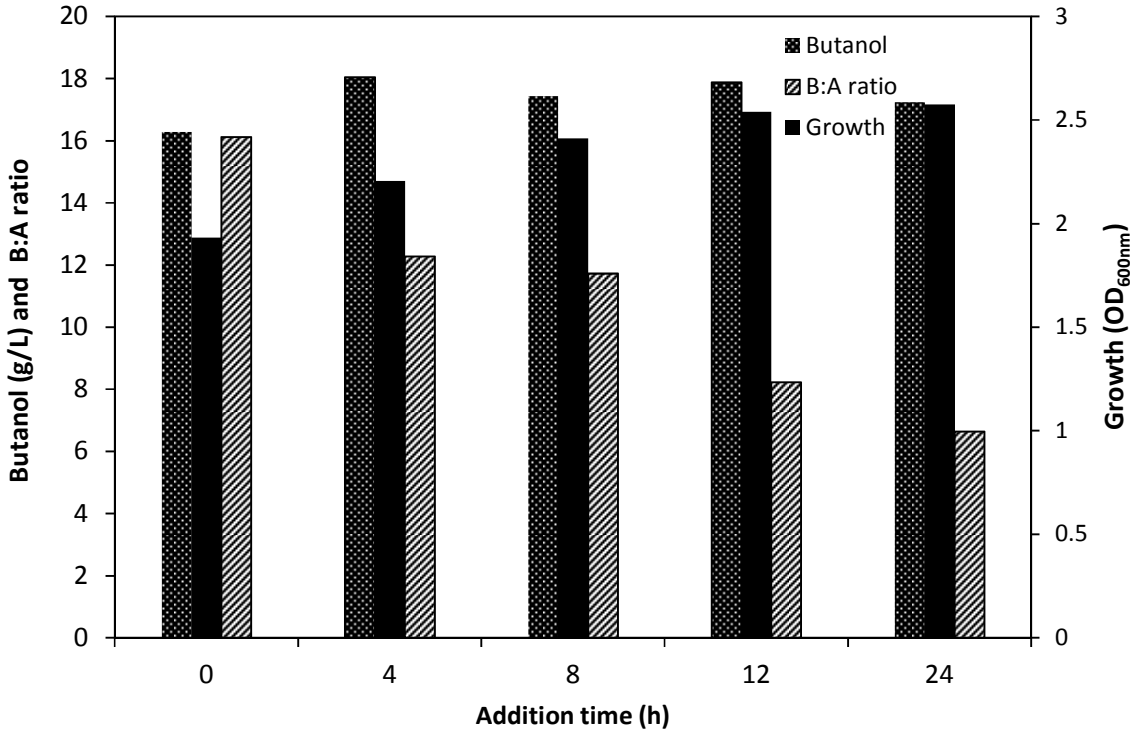
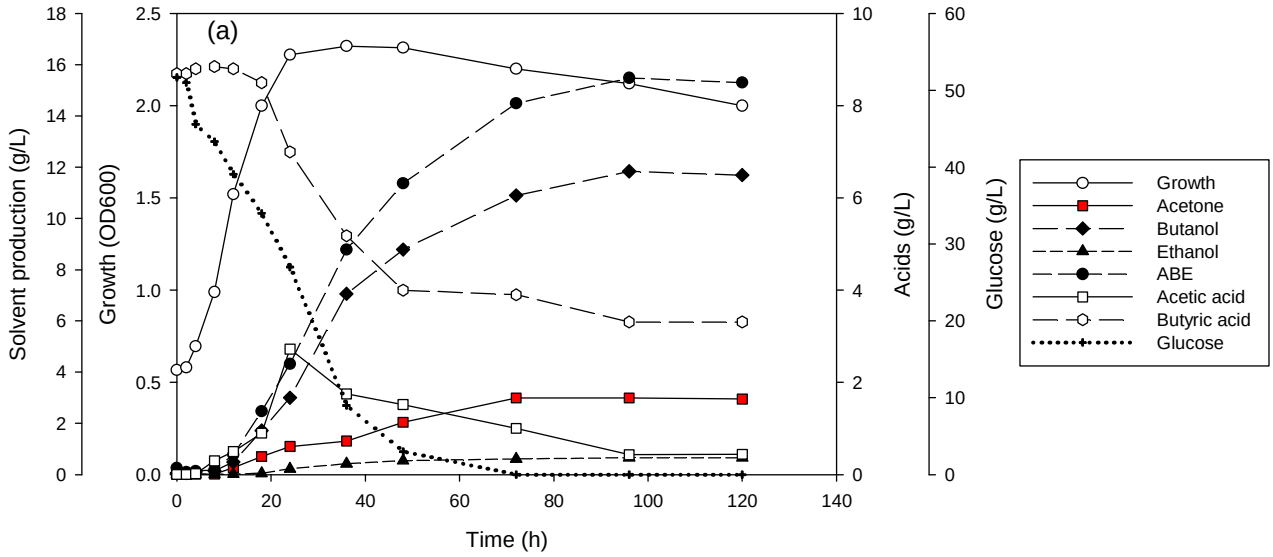


Fig. 5. Effect of the addition time of benzyl viologen on the growth of *C. acetobutylicum*YM1, butanol production and B:A ratio.



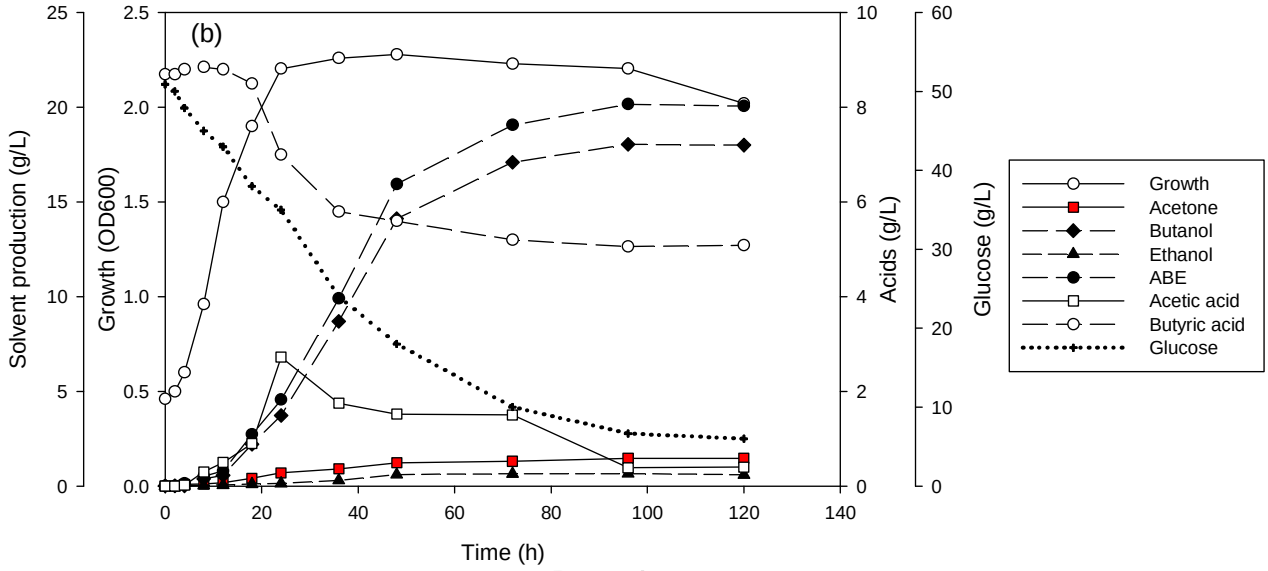


Fig. 6. Profile of the ABE fermentation by *C. acetobutylicum* YM1 in TYA medium with butyric acid (8.7 g/L) but without benzyl viologen (a) and that with butyric acid and benzyl viologen (addition time at 4h) (b).

Highlights

- Combination of artificial electron carriers and butyric acid improved butanol: acetone ratio as well as the yield and butanol productivity.
- Supplementation the YM1 culture with 0.01 mM benzyl viologen after 4 h resulted in 18.02 g/L butanol.
- The highest obtained butanol: acetone ratio was 16:1 which is higher than the conventional B:A ratio by 800%.