BIOTECHNOLOGY LETTERS
Volume 16 No.3 (March 1994) pp.269-274
Received 28th January

ENHANCED ALCOHOL YIELDS IN BATCH CULTURES OF *CLOSTRIDIUM ACETOBUTYLICUM* USING A THREE-ELECTRODE POTENTIOMETRIC SYSTEM WITH METHYL VIOLOGEN AS ELECTRON CARRIER

S. PEGUIN, P. DELORME, G. GOMA and P. SOUCAILLE*

Institut National des Sciences Appliquées, Centre de Bioingénierie Gilbert Durand, URA CNRS 544, L.A. INRA, Complexe Scientifique de Rangueil, 31077 Toulouse Cédex, FRANCE.

SUMMARY

Batch cultures of *Clostridium acetobutylicum* at controlled pH values of 5 and 5.5 were carried out in a three-electrode potentiometric system with methyl viologen (1 mM) as electron carrier. Although an irreversible loss of methyl viologen at the electrode surface was observed, a significant increase in alcohol yield was obtained. In comparison to control fermentation with or without methyl viologen addition, the butanol yield improvements were respectively of 7 or 51% at pH 5, and 56 or 467% at pH 5.5.

INTRODUCTION

The first report which attempted to improve the yield of fermentation products by electrical current was realized by Hongo and Iwahara for glutamate production by *Brevibacterium flavum* (1979a,b). The authors introduced the term "electroenergizing method" and reported a 13% enhancement of glutamic acid yields when neutral red was added to the fermentation broth and reduced at an electrode. The authors assumed that electrons were transferred from the cathode to the bacterial electron transport systems. Little attention has been given to this method of modulating product profiles and/or yields. In an amperometric poised-potential culture system with anthraquinone 2,6 disulphonic acid and cobalt sepulchrate as mediator, the fermentation balance of *Propionibacterium freudenreichii* subsp. *freudenreichii* was shifted towards enhanced propionic acid formation (Emde et al., 1990). However, as the fermentation was carried out with only 0.1 g/l glucose, low product concentrations were obtained. A growing culture of *Propionibacterium acidi-propionici* with cobalt sepulchrate and 6.5 g/l of lactose as substrate (Schuppert et al., 1992), produced propionate as the sole fermentation product but at higher substrate concentrations the propionate to acetate ratio decreased. In these two investigations, it was reported that all the electrons transferred by the cathode were taken up by the cells. Electroenergizing method was also applied to *Clostridium acetobutylicum* with neutral red as mediator (Ghosh and Zeikus, 1987). The butanol yield increased from 0.42 to 0.47 mole / mole of glucose consumed and the authors reported that electrochemical reduction of neutral red stimulated hydrogen uptake activity. On the other hand Kim and Kim (1988) showed that *in vitro* the $NAD(P)^+$ ferredoxin oxidoreductase of *C. acetobutylicum* can mediate the electron transfer from electrochemically reduced methyl viologen to $NAD(P)^+$. In the presence of 2

mM methyl viologen, a controlled potential of -2.5 V applied to a growing culture, increased butanol production to 6.9 g/l compared to 6.4 g/l when methyl viologen was added without electrochemical reduction.

Investigations have shown that one of the most important factors that control the alcohol yield in *C. acetobutylicum* is the regulation of the NADH/NAD^+ ratio (Rao and Mutharasan, 1989; Vasconcelos et al., 1994) by the electron distribution system. If reducing equivalents are electrochemically supplied, a change of the NADH/NAD^+ ratio and a modification of the metabolism might be expected. To apply the electroenergizing concept to *C. acetobutylicum*, methyl viologen was chosen as electron carrier for the following reasons : i) methyl viologen can replace ferredoxin in all the reaction where this protein is involved (Girbal, 1994); ii) the ferredoxin-$NAD(P)^+$reductases can mediate the electron transfer from electrochemically reduced methyl viologen to $NAD(P)^+$ (Kim and Kim, 1988); iii) methyl viologen satisfies with a number of requirements as a good electron mediator (Allen and Bowden, 1985)

MATERIALS AND METHODS

Organism and growth conditions. *Clostridium acetobutylicum* strain ATCC 824 was maintained at 35°C, in the following culture media (per litre of distilled water): glucose, 66g; NH_4Cl, 1.5g; KH_2PO_4, 0.5g; $K_2HPO_4.3H_2O$, 0.5g; $MgSO_4.7H_2O$, 0.2g; $FeSO_4.7H_2O$, O.O1g; p-aminobenzoic acid, 8mg; biotin, 0.04mg; structol (antifoam), 0.05g. Control fermentations were conducted in 2-litre fermentors (Setric, France) while three-electrode potentiometric culture system were performed in a glass reactor (250 ml) similar to that designed by Schuppert et al. (1992). Redox potential was monitored using a redox Fermprobe (Broadley-James corporation) with a $Ag/AgCl/Cl^-_{sat}$ reference electrode. The working electrode was a carbon rod (40 cm^2), the auxiliary electrode was a platinum wire, while a saturated calomel reference electrode was used. The electrodes were connected to a potentiostat (model 362, EG&G Princeton Applied Research, USA) and current intensity was continuously recorded (Type BD40, Kipp & Zonen, Holland). All potential are here referred to the normal hydrogen electrode (NHE). Current-potential curves for the reduction of MV was studied with a rotating carbon or platinum disk electrode (Type EDI, Tacussel, France) in an electrochemical cell containing the fermentation broth (pH 5.5). After sterilization (20 min, 120°C), the medium was sparged with oxygen free nitrogen during cooling until a temperature of 35°C was reached. Simultaneously, vitamins, iron (II) sulfate and methyl viologen were added to the fermentor by filtration through Millipore Millex^R-GV membrane. The fermentor was inoculated with 1/10 volume of an early exponential phase culture, previously subcultured in the same medium. The culture pH was controlled by automatic addition of 6N NH_4OH or sulfuric acid.

Analytical methods. Cell concentration was measured by optical density at 620 nm and by cell dry weight (Minier et al., 1990). Concentration of glucose, glycerol, butanol, acetone, ethanol, acetoin, acetic, butyric and lactic acids were determined by High Pressure Liquid Chromatography as previously described (Buday et al., 1990).

As significant discrepancies are seen in the literature regarding the absorption coefficient of reduced methyl viologen it was redetermined using an anaerobic spectrophotometric device. Reduction of oxidized methyl viologen was realized at 30°C in Tris-acetate buffer at pH 7.5 with standard solutions of sodium dithionite; the absorbance was measured at 600 nm. All assays were realized in an anaerobic work-station (Girbal, 1994) and with deoxygenated solutions. The detection threshold was 4.10^{-6}M and Beer-Lambers law was verified up to a concentration of 10^{-4}M with an absorption coefficient at $1.09.10^4 M^{-1}cm^{-1}$.

RESULTS AND DISCUSSION

Methyl viologen (MV, 1,1'-dimethyl-4,4'-bipyridinium dichloride) exists in three main oxidation states. The parent dication (MV^{2+}) can be reduced in two successive one-electron steps to produce a blue monocation radical ($MV^{+\cdot}$) and a colorless uncharged form (MV°). The apparent standard redox

potential (E°') of these two reduction processes are respectively -449 and -772 mV vs NHE (Stargart and Hawkridge, 1983). The first reduction is reversible and independent of pH over a wide range, while the second reduction exhibits an essentially irreversible character. The uncharged MV° adsorbs strongly on electrode surfaces in aqueous solution (Bowden and Hawkridge, 1981). The small potential gap between first and second MV reduction causes problems on account of the relative irreversibility of the second reduction (Bird and Kuhn, 1981). As the half-wave potentials of the $MV^{2+}/MV^{+\cdot}$ and $MV^{+\cdot}/MV^{\circ}$ couples were found to be dependent on the medium composition (Kaifer and Bard, 1985), the current-potential curves for the reduction of MV was studied in the fermentation medium without cells (fig. 1). Similar curves were obtained with carbon or platinum electrodes and the current was proportional to MV concentrations. According to these results, the best working electrode potential to reduce MV was about -560 mV vs NHE.

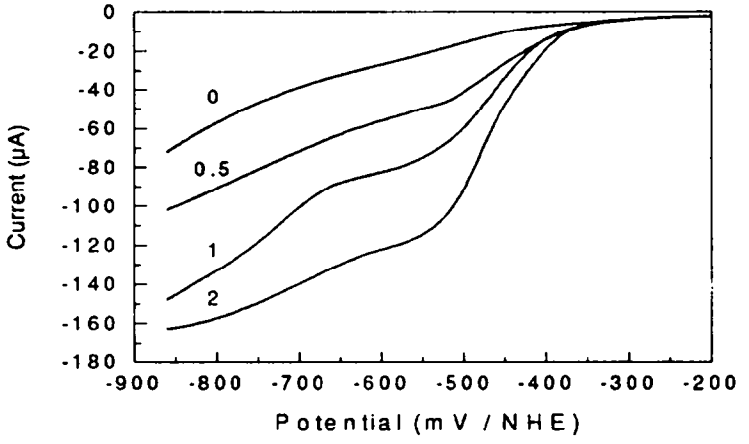

Figure 1. Current-potential curves obtained with a carbon electrode for solutions containing 0, 0.5, 1 and 2 mM methyl viologen. The potential was scan at 0.5 mV s^{-1}.

Effects of a three-electrode potentiometric system were investigated in batch culture at a controlled pH of 5. For this study, two fermentation experiments were realized, one at a constant poised potential of -560 mV vs NHE, and the other at three different applied potentials, modified during the course of the fermentation: -460, -560 and -660 mV vs NHE. Only the second is presented (fig. 2), but a similar metabolism was observed for the two experiments. Surprisingly, the current rapidly decreased during the course of the fermentation (fig. 2a). Since current is dependent on MV concentration (fig. 1), the amount of MV was determined as a function of time. MV concentration was shown to decrease as soon as the potential was applied and after 100 hours a complete disappearance of MV was observed (fig 2a). We have demonstrated that MV is chemically modified by the pyruvate:ferredoxin oxidoreductase (unpublished observations), however, this only occurred when glucose was assimilated and, in any case, very slowly at this pH (only 8% of MV disappeared at the end of a batch culture). When three different potentials were applied during the

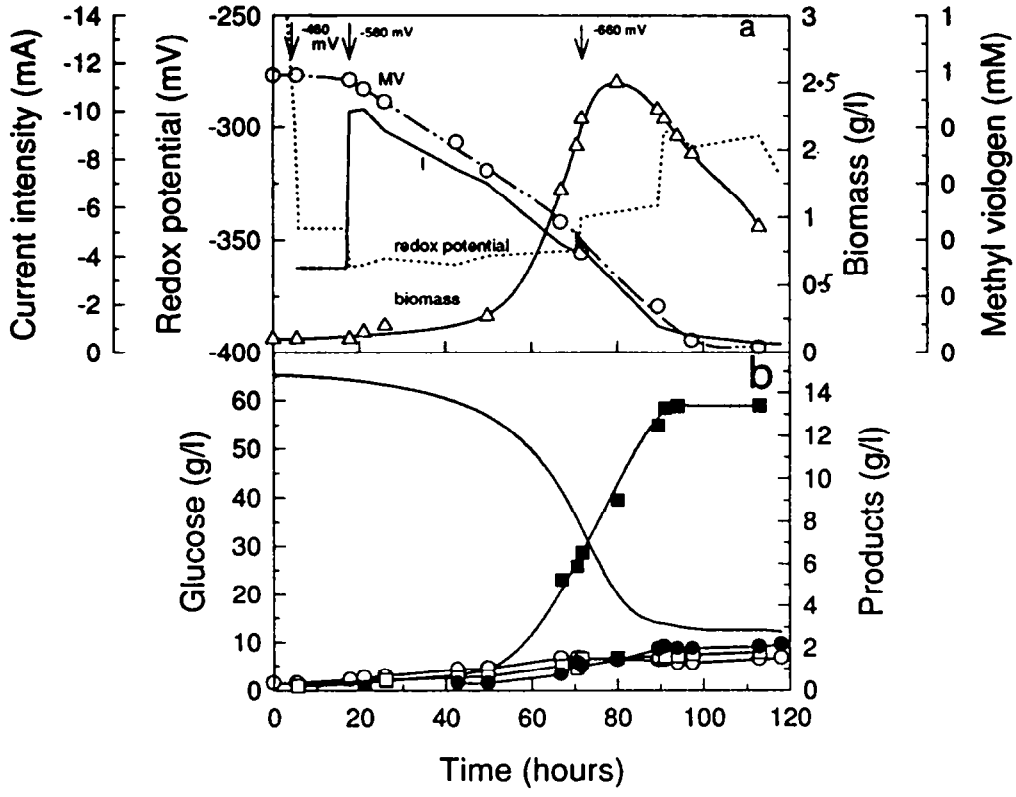

Figure 2a,b. Biomass, substrates, products, methyl viologen, redox potential, and current intensity profiles in a three-electrode potentiometric batch culture of *Clostridium acetobutylicum* at pH 5. —, glucose;■, butanol; ●, ethanol; □, butyric acid; ○, acetic acid.

fermentation (fig 2), for each one, the irreversible loss of MV followed a linear zero-order behavior which increased when poised potential decreased. Such a loss of MV has already been reported by investigators who studied hydrogen production from water using viologen-dye as a reducible relay (Keller et al., 1980; Bowden and Hawkridge, 1981; Meisel et al., 1981, Tamanushi and Tanaka, 1987). During the electrochemical reduction of MV, molecular hydrogen can be generated through matching of the hydrogen overpotential of the electrode material and solution pH, and then, an irreversible loss of MV was indicated. Atomic hydrogen was believed to be the reaction partner of $MV^{+\cdot}$ (Bowden and Hawkridge, 1981) but the products of the reaction were not identified; previous work had just shown that they were complex (Hyde and Ledwith, 1974). This MV hydrogenation was observed with different catalysts: gold and nickel electrode (Bowden and Hawkridge, 1981; Meisel et al., 1981), mercury electrode (Tamamushi and Tanaka, 1987) or colloidal platinum (Keller et al., 1980). Our results show that it can occur at carbon electrode.

The three-electrode potentiometric culture system, provoked a significant change in the metabolism of *C. acetobutylicum* (fig 2b). Growth stopped prior to total glucose exhaustion, probably because butanol production begun very early in the course of the fermentation and a high inhibitory butanol concentration was rapidly obtained. Stoichiometric analysis and yield comparison with

Table 1. Stoichiometric analysis of *C. acetobutylicum* fermentation at a controlled pH of 5

	redox	molar yields relative to glucose consumption				
	potential (mV)	NAD(P)H	butanol	ethanol	acetone	butyrate	acetate
Control	- 305	0.18	0.41	0.06	0.26	0.08	0.16
MV 1 mM	-305	1.05	0.58	0.17	0.04	0.13	0.12
MV+Electrodes	-365	1.23	0.62	0.2	0.02	0.08	0.08

control experiments (with or without MV) are reported in table 1. Metabolism of *C. acetobutylicum* was strongly altered by MV addition: butanol and ethanol formation increased at the expense of acetone and acetate. Final butyrate concentration was slightly increased by MV addition because less butyrate was reassimilated at the end of the fermentation, but maximal butyrate concentration was higher without MV. The electrochemical device further increased the butanol yield by 7%. Compared to a control experiment at pH 5 or at the optimal pH (4.8) for solvent production (Minier et al., 1990), this improvement is respectively of 51 and 38%. The molar ratio of NAD(P)H / glucose reported in table 1 represents the moles of $NAD(P)^+$ reduced by the ferredoxin-$NAD(P)^+$ reductases per mole of glucose consumed. This value was calculated from the difference between the moles of NAD(P)H consumed in products formation and the moles of NADH produced by the EMP pathway and it was seen to increase from 0.18 in the control, to 1.05 with MV addition and 1.23 with MV plus the electrochemical device. During growth, approximately 15 mM of electrons were transferred from the cathode to MV. If all this reduced MV was oxidized by the ferredoxin-$NAD(P)^+$ reductases, 7.5 mM of NAD(P)H would be produced. A stoichiometric analysis showed that the amount of NAD(P)H produced by the ferredoxin-$NAD(P)^+$ reductases increased by 13 mM. As already reported, part of $MV^{+\cdot}$ was used to produce hydrogen at the electrode surface and was also irreversibly loss. So, the changes induced in bacterial metabolism cannot be explained simply by an electron transfer from the cathode to $NAD(P)^+$, as previously reported (Emde et al., 1990; Kim and Kim, 1988; Schuppert et al., 1992).

A batch fermentation, with a three-electrode potentiometric system, was also studied at controlled pH 5.5. When poised potential was applied (-560 mV), a current of 3 mA occurred and decreased until 1.5 mA at the end of the fermentation. In this case, MV hydrogenation reaction was slower and only half of the MV disappeared. This result was in accordance with Bowden and Hawkridge (1981) who showed that hydrogen evolution and MV decay was pH dependent. 11.75 mM of electrons were transferred by the cathode, a lower value than at pH 5. However, the stoichiometric analysis (table 2), showed a drastic effect on the bacterial metabolism.

Table 2. Stoichiometric analysis of *C. acetobutylicum* fermentation at a controlled pH of 5.5

	redox	molar yields relative to glucose consumption					
	potential (mV)	NAD(P)H	butanol	ethanol	lactate	glycerol	butyrate	acetate
Control	- 335	-0.17	0.09	0.03	0	0	0.54	0.3
MV 1 mM	-335	0.38	0.27	0.08	0.04	0.02	0.41	0.19
MV+Electrodes	-395	0.70	0.42	0.06	0.07	0.04	0.32	0.06

When MV was added, the molar NAD(P)H / glucose ratio was altered from a negative to a positive value (table 2), hence, the net electron flow through the ferredoxin-NAD(P)H oxidoreductases was reversed. When an electrochemical MV reduction was realized, the $NAD(P)^+$ reduced by the ferredoxin-$NAD(P)^+$ reductases was two-fold higher. The butanol yield was three times higher with MV addition in comparison with the control, and the electrochemical device further increased this production by 56% (467% in comparison with control) at the expense of acetate and butyrate production. At this pH, acetone was never produced in culture supplemented with MV. In previous work, we have reported production of lactate and glycerol by *C. acetobutylicum* when a high NADH / NAD^+ ratio was induced by MV at this pH. When the electrochemical device was employed, lactate and glycerol production were further stimulated.

Cathodic reduction of MV induced an increase in the $MV^{+\cdot}$/ MV^{2+} ratio, observed by a change of the broth color and by a decrease of redox potential (table 1 and 2). Since $MV^{+\cdot}$ has been shown to be a good substrate for the ferredoxin-$NAD(P)^+$ reductases (Girbal, 1994), an increase in the $MV^{+\cdot}$/ MV^{2+} ratio, could alter the equilibrium of the oxidoreduction reaction between this electron carrier and the NADH / NAD^+ couple, in favor of NADH formation. An essential role of the NADH pool size for increased alcohol production has already been shown (Srivastava and Volesky, 1991; Grupe and Gottschalk, 1992; Vasconcelos et al., 1994). Hence, the effect of this three-electrode potentiometric system would be dependent on $MV^{+\cdot}$/ MV^{2+} ratio, this one being in direct relation to the redox potential of the broth. Although these results are promising, further investigations are necessary to characterize the influence of redox potential (controlled electrochemically) on the metabolism of *C. acetobutylicum*. A continuous culture device that enables both the MV concentration and the broth redox potential to be maintained constant is under investigation in our laboratory.

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