INTERNATIONAL CONFERENCE ON BIOREACTORS AND BIOTRANSFORMATIONS
GLENEAGLES, SCOTLAND, UK: 9-12 NOVEMBER 1987

Paper G3

BIOELECTROCHEMICAL TRANSFORMATIONS CATALYSED BY *Clostridium sporogenes*.

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Abstract

One of the major limitations to the use of oxido-reductase enzymes in performing biotransformations is the regeneration of cofactors. One approach has been to use redox mediators indirectly or directly to couple oxido-reductase enzymes to electrochemical regeneration of cofactors. We have used methyl viologen (MV) as a mediator to drive a number of reductive reactions in the proteolytic anaerobe *Clostridium sporogenes*.

Studies have been performed with permeabilised cells. We have investigated the MV-coupled reactions catalysed by 2-oxoacid synthase and MV-NAD reductase using a dropping mercury electrode and a stirred mercury pool electrode to measure and electrochemically to reduce MV. During the course of this work we have developed polarographic assays (based on MV oxidation) for these enzymes and have subsequently characterised the reactions.

2-oxoacid synthase reactions involve the reductive carboxylation of acyl CoA derivatives to form 2-oxoacids. Using acetyl phosphate we have succesfully synthesised pyruvate. It is envisaged that these reactions could be used to synthesise 2-oxoacids, hydroxy-acids and amino-acids from 2-oxoacid derivatives using NADH regenerated via the substantial MV-NAD reductase activity, together with the aminotransferases and glutamate dehydrogenase, that this organism possesses.

INTRODUCTION

The clostridia are a diverse group of spore-forming bacteria that are differentiated on the basis of their ability to ferment different carbon sources. The biochemistry and physiology of the saccharolytic clostridia have been most widely studied, and have recently been re-examined in detail for the ability to produce solvents and organic acids from carbohydrates [1-3]. Similarly the proteolytic clostridia, those which ferment amino acids, have also been the subject of renewed interest, although attention has focussed upon potentially useful enzymes that these organisms produce. Apart from extracellular hydrolases, most notable are enoate [4], nitroaryl [5], linoleate [6], 2-oxoacid [7], proline [8] and glycine reductases [9] (table 1). Another reduction reaction involved in the synthesis of amino acids is that of ferredoxin-linked reductive carboxylation. These reactions

are used by certain bacteria for the synthesis of amino acids and in some cases to drive a reductive tricarboxylic acid cycle [10-12].

TABLE 1
Potentially useful reductive enzymes in the clostridia

Reduction reaction	Electron donor	Organism	Ref
aldehyde/ketone dehydrogenase			
steroids	?	C. paraputrificum	[13]
		C. bifermentans	[14]
methyl ketones	NADPH	C. thermohydrosulfuricum	[15]
ketones	?	C. pasteurianum	[17]
	?	C. tyrobutyricum	[17]
2-oxoacid synthase			
fatty acids	ferredoxin	C. sporogenes	[18]
acetate	"	C. kluyveri	[19]
Linoleic reductase			
linoleic acid	?	C. sporogenes	[6]
Enoate reductase			
cinnamic acid	NADH	C. sporogenes	[20]
crotonic acid	"	C. tyrobutyricum	[21]
2-oxoacid reductase			
phenylpyruvic acid	NADH	C. sporogenes	[20]
Nitroaryl reductase			
chloramphenicol	ferredoxin/flavodoxin	C. acetobutylicum	[22]
metronidazole	"		[23]
p-nitrobenzoate	"		[5]
2 nitrobenzene	"		[5]
Proline reductase			
proline	NADH/MV	clostridia	[8,24]
Glycine reductase			
glycine	ferredoxin	clostridia and other anaerobes	[9]
Lipoamide dehydrogenase			
NAD/lipoamide	lipoamide/NADH	C. kluyveri	[25]
MV-NAD(P) reductase			
NAD(P)/ferredoxin	ferredoxin/NAD(P)H	clostridia and other anaerobes	[23]

Clostridium sporogenes performs the Stickland reaction in which pairs of amino acids are fermented, one amino acid acting an electron donor (e.g. valine,leucine isoleucine) whilst the other acts as an electron acceptor (e.g. proline or glycine). Phenylalanine can act either as donor or acceptor. We have investigated the biochemistry and physiology of these reactions [17,24,27-29]. In this respect C. sporogenes has been shown to synthesise many of its required amino acids from deaminated and decarbonylated fatty acid analogues; the relevant enzymes have been shown to be highly active and to show broad specificity in the case of

oxo-glutarate aminotranferase, 2-oxoacid synthase, acyl-CoA phosphotranferase and acyl kinase reactions [28].

The main carbon and electron flow pathways of Clostridium sporogenes have been investigated by several workers [e.g. 8,9, 27-29] and are shown in figure 1. It should be noted that the reduction of acyl phosphate derivatives proceeds by a series of reductive steps, the derivatives being first reductively carboxylated to 2-oxoacids and further reduced to hydroxyacids and/or amino acids.

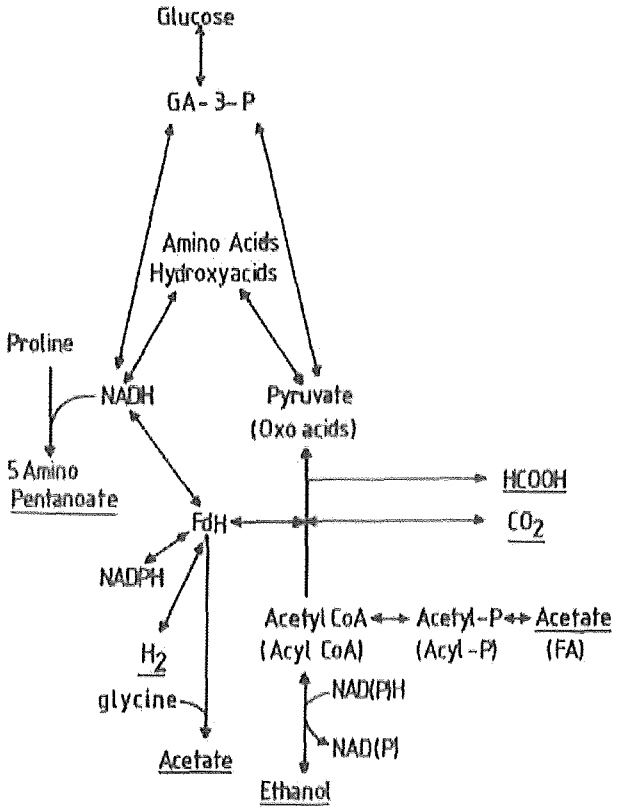

FIGURE1

Carbon and electron flow pathways of C. sporogenes

To harness these reductive reactions for commercial biotransformation processes, a number of approaches are possible: (1) direct incubation of the substrate in the presence of whole cells. (2) incubation of substrate with permeabilised cells, or (3) incubation of substrates with cell-free extracts or purified enzymes.

The major limitation of approach (1) is the access of the substrate to the intracelluar enzymes, whilst the more significant limitation on the use of oxidoreductase enzymes in (2) and (3) is the problem of cofactor recycling. To overcome the problem of cofactor recycling two approaches are possible: (a) recycling using another dehydrogenase reaction, e.g. formate dehydrogenase [30], though it is not possible to reduce ferredoxin by this means, or (b) recycling using direct or indirect electrochemical means as pioneered by Simon and coworkers [4,21,26] . The direct and reversible electrochemical reduction of ferredoxin and other iron-sulphur proteins is also possible [31]; however at present, these proteins are expensive to produce and have slow reduction kinetics at electrodes, due to their low diffusion coefficients.

In this paper we report on our studies of two reductive enzymes in permeabilised cells of C. sporogenes viz. 2-oxoacid synthase and MV-linked NAD reductase. The first is used to synthesise pyruvic acid and other 2-oxo acids from acyl phosphate derivatives, whilst the second is used to recycle NAD using MV as a mediator between an electrode and the enzyme.

EXPERIMENTAL

Microbiological and Biochemical methods

Clostridium sporogenes NCIB 8053 was grown and maintained on a defined medium as outlined elsewhere [18]. Cells were harvested from overnight cultures (12-14 h) in late exponential phase (OD 0.9-1.0), washed once in anaerobic 0.1 M potassium phosphate (pH 7.0), resuspended in the same buffer and stored anaerobically in sealed vials. The organisms were then permeabilised by the addition of 20 µl of 10% v/v toluene in ethanol per ml of cell suspension [18]

The preparation of Acyl phosphates

Apart from Acetyl phosphate (obtained from Sigma), these compounds were synthesised, by the hydrolysis of fatty acid anhydrides in the presence of potassium phosphate, and purified, according to the method of Stadtman [32].

Electrochemical methods

The determination of Methyl Viologen (MV)-linked enzyme activities was performed using a PAR 174A Potentiostat and model 303 dropping mercury electrode cell (in DC sampling mode), by measuring polarographically at

-875 mV (vs Ag/AgCl (3M KCl)) the rate of MV oxidation in a 3-electrode cell completed with a Pt wire counter elctrode. It was demonstrated using these methods that it is possible, after calibration, accurately and continuously to measure the amount of oxidised MV in the system, and thus the activities of 2-oxoacid synthase and MV-NAD reductase.

The assays were performed in a 5 ml volume using permeabilised cells at 20°C. For 2-oxoacid synthase the reaction mixture contained 100mM potassium phosphate pH 7.0, 10 mM Acyl phosphate, 50 μM CoA, 10 mM $KHCO_3$ and 5 U of phosphotransferase (Sigma, optional). The reaction was initiated by the addition of bicarbonate. For MV-NAD reductase the reaction mixture contained 100mM potassium phosphate pH 7.0, 1 mM reduced MV, (prereduced in the preparative cell, see below) and 0.5mM NAD. The reaction was initiated by the addition of NAD.

The preparative reactor cell is illustrated Figure 2. The cell is a glass vessel with a mercury pool electrode at the base, the surface of which is stirred by a glass-coated magnetic follower coupled to a magnetic stirrer. The mercury pool is the working electrode and is the reactive surface at which MV is reduced. The reference electrode, Ag/AgCl (3M KCl) is present in the cell whilst the Pt auxiliary electrode is connected to the cell via a salt bridge so as to isolate it from the highly reducing environment of the reactor cell. The cell is kept anaerobic by constant gassing and additions are made though the gas outlet port. The cell was jacketed and was operated at 20°C with a total reaction volume of 5 to 10 ml.

For preparative work the reaction medium consisted of 200 mM potassium phosphate pH 7.0, 1 to 25 mM Acetyl-PO_4, 50 μM CoA (optional) and 5U phosphotranferase (optional). The system was purged and gassed with CO_2 before cells and other additions are made and the reaction was carried out under a CO_2 headspace. The reaction in dilute cell suspensions (<.1 mg dry wt ml^{-1}) is dependent upon the addition of CoA; however at high cell concentrations (>2 mg dry wt ml^{-1}) the reaction is dependent only upon added Acetyl-PO_4.

RESULTS

Characterisation of the reductive carboxylation reactions of C. sporogenes

The reductive carboxylation reactions of C. sporogenes were first demonstrated by showing that certain fatty acids could substitute for amino

acids required for anabolism and hence for growth. It was shown that acetate, 2-methyl propionate, 2-methyl butyrate and 3-methyl butyrate could replace serine, valine, leucine and isoleucine respectively [18].

The requirements of the reductive carboxylation reaction were investigated using MV as a reducing agent and are shown in Table 2. As can be seen, the reaction was shown to require CO_2, CoA and Acetyl phosphate. Exogenous phosphotranferase stimulated the reaction but was not required.

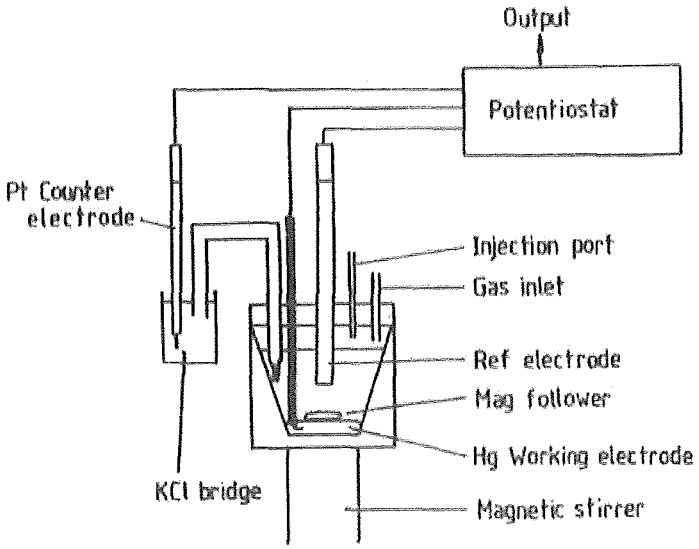

FIGURE 2

Diagram of preparative reactor cell

TABLE 2.

The characteristics of the reductive carboxylation reaction

	Activity μmol. (min mg dry wt)$^{-1}$
Complete system	0.172
- Bicarbonate	0.031
- CoA	0.026
- Phosphotranferase (PTA)	0.133
- Acetyl Phosphate	0.027

The complete system, reactor volume 5ml, contained 200 mM potassium phosphate, (pH 7.0), 10 mM Acetyl phosphate, 1mM MV, 20 mM $KHCO_3$, 50 μM CoA, 5U PTA, and Cells 1-2 mg (dry wt. ml)

The pH optimum for the reaction was in the range 6.7 to 7.0, and tris and "Good"-type buffers were more inhibitory to the reaction than was

phosphate buffer. The CoA requirement for the reaction was small and the reaction was unaffected even when the added CoA was reduced to 30 μ M. The K_m for bicarbonate was approximately 6 mM at pH 7.0.

To test the specificity of 2-oxoacid synthase the activity of permeabilised C. sporogenes cells towards other acyl phosphates was assessed. The observed activity of the cells to acyl phosphates is shown in Table 3. In this assay system no addition of PTA was made, as this commercial enzyme is specific for acetyl phosphate [33]. The activities for these compounds were lower than those observed with for acetyl phosphate, although significant activity was detectable.

TABLE 3

2-oxoacid synthase activity towards various acyl phosphates

substrate	Activity μ mol. (min mg dry wt)$^{-1}$
Acetyl phosphate	0.104
Butyryl phosphate	0.044
2-methyl propionyl phosphate	0.034
Pentyl phosphate	0.042
3-methyl butyryl phosphate	0.060
Hexyl phosphate	0.030

Conditions of Assay: temperature 21°C, 100 mM potassium phosphate pH 7.0, 10 mM Acyl phosphate, 20mM $KHCO_3$ and cells as described in the legend to Table 2.

2. Reductive carboxylation in a preparative cell: the synthesis of pyruvate from acetyl phosphate

Now that the reductive carboxylation reaction had been characterised and optimised, the reaction was investigated on the preparative scale. A 5-10 ml reactor (Figure 2) was used. A typical experiment involved, firstly, the reduction of the MV in the reactor. After the addition of cells, CoA and PTA, the reaction was started by the addition of Acetyl phosphate. Figure 3a shows a typical current-time profile. The current passed in the reactor is proportional to the activity of the MV reductase processes involved and thus its time integral, in a highly coupled reaction, to the amount of substrate added.

In experiments with high cell concentrations where the reaction no longer required additions of CoA or PTA to obtain good activity, it is presumed that the concentration of cell-derived CoA was sufficient to satisfy the requirement for the reaction. Figure 3b shows a current-time profile for a reaction involving a high cell concentration. By integration

of the reaction profiles the amount of charge used can be calculated and is equivalent (within 5%) to the amount of Acetyl phosphate added (assuming a two electron reduction of acetyl phosphate).

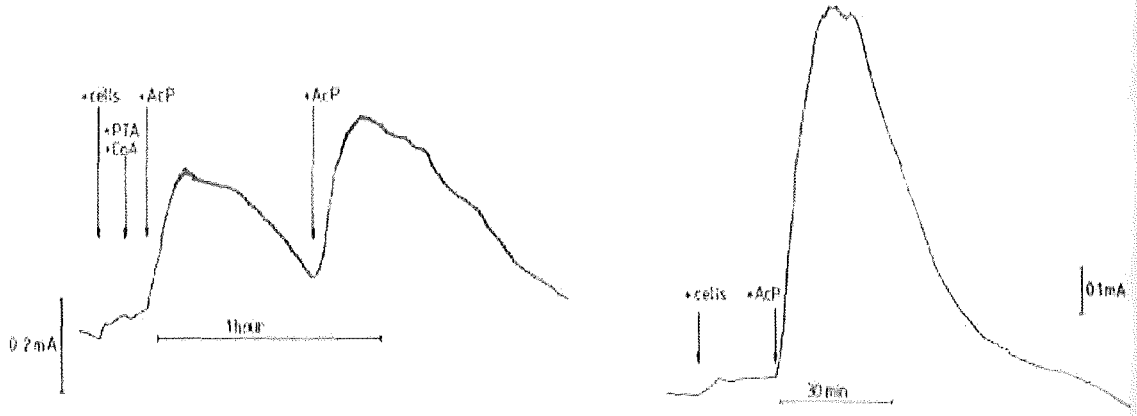

Fig 3a Fig 3b

FIGURE 3
Current-time profiles observed on the addition of acetyl phosphate.

Fig 3a shows 2 additions of acetyl phosphate, of 5 and 15 μ mol respectively at low cell density (0.15 mg dry wt ml^{-1}). Figure 3b shows an experiment ot high cell density (1.5 mg dry wt ml^{-1}); 15 μ mol of Acetyl phosphate were added at the point indicated, note that exogenous phosphotranferase and CoA are not required.

To confirm the rate of pyruvate synthesis and to investigate the coulombic efficiency of the reaction, an experiment was performed to compare the amount of charge passed with the amount of pyruvate formed. This was done by sampling the reaction periodically and measuring the pyruvate concentration, using the standard enzymatic method based upon lactate dehydrogenase [34]. The results of this experiment are shown in Table 4 and Figure 4. The time course of pyruvate production was approximately linear and 7 μ mol of pyruvate were formed in 2.8 h. As mentioned above the amount of charge passed was proportional to the amount

of acetyl phosphate; however as revealed by this experiment, the amount of pyruvate formed from the acetyl phosphate added showed only a 10-28% yield depending upon the method of calculation (Table 4). The reaction appeared to be stable for at least 24 h.

TABLE 4
Coloumbic efficiency of pyruvate formation from acetyl phosphate

time (h)	Pyruvate [mM]	Pyruvate µMole in the reactor	charge (C)	charge -endog (C)	Pyruvate equivalent charge (C)	% conversion -endog	% conversion total
0	0	0	0	0	0	0	0
0.33	-	-	1.74	1.14	-	-	-
0.66	0.256	1.7	3.36	2.28	0.328	14.3	9.7
1.00	0.401	2.8	4.98	3.42	0.540	15.7	10.8
1.33	0.502	3.5	6.42	4.38	0.675	15.2	10.5
1.66	0.550	3.8	7.62	5.06	0.733	14.4	9.6
2.00	0.610	4.2	8.82	5.82	0.829	14.2	9.3
2.80	1.01	7.0	11.97	7.97	1.350	16.9	11.2
11.5	9.00	63.0	55.71	44.19	12.15	27.6	21.8

Conditions. The transformation was performed at 22°C in a reaction volume of 7 ml. The reaction took place in saturating carbon dioxide, 200 mM potassium phosphate pH 7.0, 16 mg dry wt cells. 5 Units PTA, 50 mM Acetyl phosphate, 50 µ mol CoA. The assumption is made that the endogenous rate is constant throughout the experiment an assumption which minimises the calculated yield. 1 µ mol equivalent of electrons is 0.096487 C.

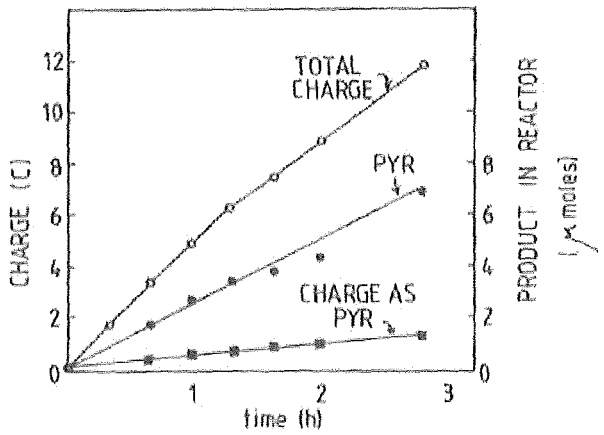

FIGURE 4
Time course of the reductive carboxylation reaction using acetyl phosphate as substrate.

The figure shows the amount of pyruvate produced, the total charge passed during the reaction and the amount of charge incorporated into the pyruvate formed (see also table 4).

3. MV-NAD reductase

MV-NAD reductase is of potential utility in systems where the regeneration of NADH is required. This enzyme system is highly active in many clostridia including C. sporogenes [26]. The enzyme is generally present at an activity of 1 μ mole min^{-1} mg dry wt^{-1} although on occasions up to 2 μ mole min^{-1} mg dry wt^{-1} were measured. The enzyme is expressed thoughout the growth cycle of the organism. The characterisation of this enzyme is now underway. The first and most important characteristic to be assessed was its stability. This was investigated by incubating the toluenised preparations at various temperatures, and assaying the activity at 20°C. The results are presented in figure 5. Incubation at 50°C rapidly inactivated the enzyme; however at 37°C, 21°C and 1°C the enzyme remained stable for many hours. After an initial short period where the activity was stimulated, the activity was then slowly lost. The approximate half-lives for the enzyme were between 65 h (37°C) and 175 h (21°C). Indeed considerable activity remained after 2 weeks. We are now endeavoring to employ this reaction to drive NADH-linked oxidoreductase enzymes.

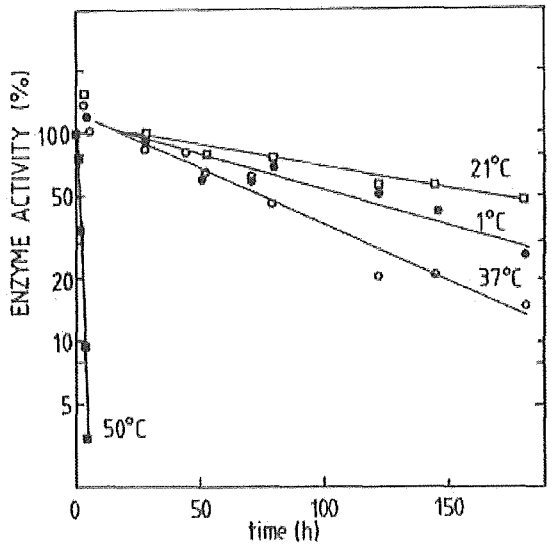

FIGURE 5
The effect of temperature on the stability of MV-NAD reductase of C. sporogenes

DISCUSSION

Pyruvate synthase and 2-oxoacid synthesis

We report here our studies of two reactions performed by C. sporogenes. Using the 2-oxoacid synthase, MV-NAD reductase and additional enzymes present in C. sporogenes it is possible to drive these reactions electrochemically to produce a series of oxo-, and thus hydroxy- and amino-acids. Several important aspects of the 2-oxoacid synthase remain to be investigated; in particular its temperature optimum and stability need to be determined and this work is currently being undertaken.

At present we have no explanation for the apparently low coloumbic yield of pyruvate formation. There are a number of possible routes whereby the Acetyl phosphate may be reduced. Acetyl CoA may be reduced to acetaldehyde by aldehyde dehydrogenase; this would require the presence of NADH, but if this reaction were taking place, acetaldehyde would most probably be further reduced to ethanol using the alcohol dehydrogenase known to be present in this organism (figure 1) [18]. If this were the case, the charge passed would be greater than the two electron equivalents calculated from the current-time profiles (figure 3a and 3b). This argument, and the fact that little or no NAD is present, would tend to militate against such a possibility. The loss of the dependence of the reaction on added CoA when high cell concentrations are used would suggest that under these conditions, other cell-derived cofactors such as NAD(P), FAD and FMN are present in concentrations sufficient to allow significant side reactions to occur. In tranformations using low cell concentrations (and which are CoA-dependent), the pyruvate yield is improved to 30-35% and this may be due to the decreased rate of side reactions. The remaining alternative to explain the low efficiency is the possibility that the reaction is forming pyruvate which is then converted to other products such as acetolactate. Investigations are currently taking place to obtain accurate material balances within the reactor.

MV-NAD reductase and its use in cofactor recycling

The MV-NAD reductase present in C. sporogenes is a suitable enzyme for the recycling of NAD to NADH and is stable at physiological temperatures. An alternative enzyme, pig heart lipoamide dehydrogenase, is obtainable commercially and also shows good stability [35]. However, MV-NAD reductase is obtained from a microbial source in a simple permeabilised cell

preparation and in conjunction with the 2-oxoacid synthase and other enzymes allows a variety of multistep transformations using only one organism.

Reactor design and mediated reactions

The limits to the rates of bioelectrochemical reductions are in fact strongly governed by the electrochemical reaction at the working electrode of the reactor since it is easy to saturate the overall reaction rate by addition of high concentrations of permeabilised cells. For efficient conversion and scale up, the relative area of the working electrode must be increased.

ACKNOWLEDGEMENTS

We are indebted to the SERC Biotechnology Directorate and ICI Bioproducts Business for generous financial support.

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