



## Enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH via reverse dehydrogenase cascade biocatalysis: Quantitative comparison of efficiencies of immobilized enzyme systems

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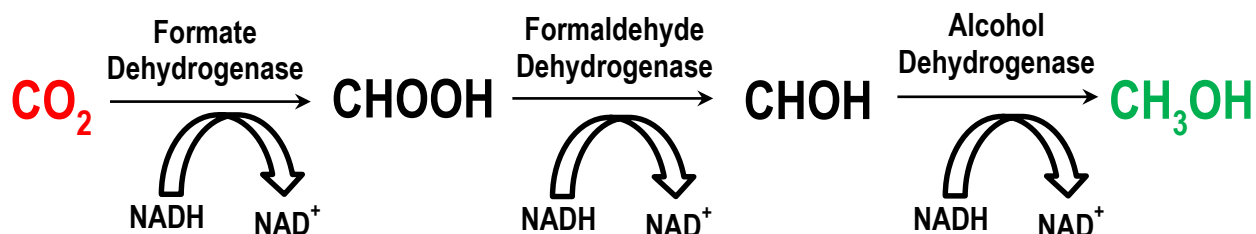
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**Enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH via reverse dehydrogenase cascade biocatalysis:****Quantitative comparison of efficiencies of immobilized enzyme systems**Fauziah Marpani<sup>a,b</sup>, Manuel Pinelo<sup>a</sup>, and Anne S. Meyer<sup>a,\*</sup><sup>a</sup>Department of Chemical and Biochemical Engineering, Center for BioProcess Engineering,  
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## Abstract

A designed biocatalytic cascade system based on reverse enzymatic catalysis by formate dehydrogenase (EC 1.2.1.2), formaldehyde dehydrogenase (EC 1.2.1.46), and alcohol dehydrogenase (EC 1.1.1.1) can convert carbon dioxide (CO<sub>2</sub>) to methanol (CH<sub>3</sub>OH) via formation of formic acid (CHOOH) and formaldehyde (CHOH) during equimolar cofactor oxidation of NADH to NAD<sup>+</sup>. This reaction is appealing because it represents a double gain: (1) reduction of CO<sub>2</sub> and (2) an alternative to fossil fuel based production of CH<sub>3</sub>OH. The present review evaluates the efficiency of different immobilized enzyme systems and reaction designs that have been explored for optimizing this sequential enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH, including multilayer microcapsules, bead scaffolds, cationic nanofibers, and membrane systems. The recent progress within efficient cofactor regeneration, protein engineering of the enzymes for robustness, and advanced uses of membrane systems for enzyme reuse and product separation are assessed for large scale implementation of this biocatalytic reaction cascade. Industrial realization of enzymatic CO<sub>2</sub> to CH<sub>3</sub>OH conversion including the option for reaping of formaldehyde and formate during the reaction warrants innovative development. There is a particular need for development of i) better enzymes; ii) improved understanding of enzyme structure function aspects of reverse catalysis by dehydrogenases, iii) quantitative kinetic models of the enzymatic cascade reaction during simultaneous cofactor regeneration, iv) robust systems for regeneration of reducing equivalents.

**Keywords:** CO<sub>2</sub> conversion, biocatalysis, multi-enzyme, cofactor regeneration, productivity, efficiency

## 1. Introduction

Global anthropogenic carbon dioxide (CO<sub>2</sub>) emissions recently reached a record of high level of 35.7 billion tons per year and is still increasing [1]. According to the Intergovernmental Panel on Climate Change (IPCC), the resulting atmospheric concentrations of CO<sub>2</sub>, along with methane and nitrous oxide, are unprecedented in at least the last 800,000 years and believed to be the dominant cause of global warming. As a consequence, the period from 1983 to 2012 was likely the warmest 30-year period of the last 1400 years in the Northern hemisphere [2]. The emissions of CO<sub>2</sub> are mainly a result of fossil fuel combustion as well as discharges from thermoelectric power plants, refineries, cement plants and steel mills [2]. Recently, a global agreement to reduce such emissions to achieve zero net greenhouse gas emissions and pursue efforts to limit the temperature increase to max. 1.5 °C during the 21<sup>st</sup> century was negotiated at the 2015 United Nations Climate Change Conference, COP 21, in Paris, France [3].

In addition to developing measures for reducing greenhouse gas emissions, utilization of CO<sub>2</sub> as a feedstock for producing chemicals and fuels is an attractive strategy to diminish CO<sub>2</sub> emissions. Hence, options for sustainable conversion of CO<sub>2</sub> into chemicals and fuels with zero or even negative emissions should be considered. CO<sub>2</sub> can indeed be scavenged directly from industrial greenhouse gas emission processes (or with time presumably captured directly from the air) and converted into basic chemicals and fuel chemicals that are otherwise obtained from fossil oil chemistry. Biocatalytic conversion offers a sustainable low temperature approach for such conversions. Several biological processes involve an enzymatic CO<sub>2</sub> fixation or a conversion step with the Calvin cycle being the most dominant for CO<sub>2</sub> conversion in nature [4], but sequential enzymatic reduction of CO<sub>2</sub> to methanol (CH<sub>3</sub>OH) does not occur in nature. However, already in 1993 and 1994 Yoneyama et al. [5,6] demonstrated that CO<sub>2</sub>, in a CO<sub>2</sub>-saturated phosphate buffer solution, could be biocatalytically converted to CH<sub>3</sub>OH. They employed electrolysis and conversion

via formaldehyde in the presence of formate dehydrogenase (EC 1.2.1.2) and methanol dehydrogenase (EC 1.1.99.8) using pyrroloquinoline quinone (or methyl viologen) as electron mediator [5,6]. Subsequently, it was shown that the enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH can also be accomplished in solution in a cascade system set up of three different dehydrogenases in the presence of an electron donor, namely reduced nicotinamide adenine dinucleotide (NADH) [7]. In this designed set-up formate dehydrogenase (FDH, EC 1.2.1.2) catalyses conversion of CO<sub>2</sub> into formate (methanoic acid HCOOH or HCOO<sup>-</sup>), formaldehyde dehydrogenase (F<sub>ald</sub>DH, EC 1.2.1.46) then catalyses reduction of the HCOO<sup>-</sup> to formaldehyde (CHOH), and finally alcohol dehydrogenase (ADH, EC 1.1.1.1) catalyses the conversion of formaldehyde into CH<sub>3</sub>OH (Fig. 1) [7]. Each enzymatic step in this system works in the reverse direction of the natural (reversible) enzymatically catalysed reaction, but the biological reaction equilibrium constants can be shifted by several orders of magnitude to favour CH<sub>3</sub>OH synthesis by optimizing the reaction conditions [7,8]. The requirement for NADH is due to the electron donor selectivity of the currently used microbial enzymes employed in this 3-step enzymatic CO<sub>2</sub> to CH<sub>3</sub>OH reductive cascade system.

CH<sub>3</sub>OH is thus produced as a final product, but it is an important aspect of the process that the intermediate products of the 3-step cascade reaction, formic acid (CHOOH) and formaldehyde (CHOH), are also produced. Currently CH<sub>3</sub>OH is mainly produced from natural gas through syngas (CO and H<sub>2</sub>), and both CH<sub>3</sub>OH and CHOH are among the top 10 petrochemicals produced in the world and are also basic building block chemicals with vast applications as precursors for production of other valuable compounds [9]. Apart from use as a base chemical CH<sub>3</sub>OH can also be used as a fuel itself or as a solvent. CHOOH also has specific separate applications. The enzymatic cascade reaction concept is also highly promising for biorefineries, including bioethanol processes, where CO<sub>2</sub> is an equimolar co-product of the ethanol fermentation.

Due to the significant and increasing interest in CO<sub>2</sub> reduction technology recent reviews have emerged focusing on the principles, redox chemistry, mechanisms, and energy of enzymatic CO<sub>2</sub> conversion, including coverage of the major routes of the metabolic CO<sub>2</sub> processes in cells [4,10-

12], and discussion of methodologies and materials employed for enzyme immobilization [11,12]. However, the challenge with the application of a cascadic dehydrogenase enzyme reaction system for converting CO<sub>2</sub> to CH<sub>3</sub>OH is to employ efficient and robust enzymes and to design a feasible, robust and an efficient reaction set-up having high productivity. The system should thus provide for maximizing both the biocatalytic production rate on the enzymes ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h}$ ) and the cofactor utilization efficiency ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}} \cdot \mu\text{mol}_{\text{NADH}}\cdot\text{h}$ ). Evidently, immobilization of the enzymes to maximize the biocatalytic productivity by ensuring maximal repeated use and confinement of the enzymes has been in focus since Obert and Dave [7] demonstrated enhanced methanol production when the three dehydrogenases were encapsulated in a porous silica sol-gel matrix in a solution with NADH and exposed to CO<sub>2</sub> bubbling. Nevertheless, a detailed assessment of the practically attained enzymatic productivities and conversion efficacies of different enzyme immobilization systems and reaction set-ups is lacking in the literature.

Our aim is to provide an improved knowledge-base for rationally designing reaction systems for efficient enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH. The present treatise will therefore assess and compare the biocatalytic productivity and efficiencies of different immobilized enzyme systems for sequential enzymatic reduction of CO<sub>2</sub> to CH<sub>3</sub>OH without the use of electrolysis. We will also provide an overview of enzymatic cofactor regeneration systems and address their efficacy. Theoretically, the reduction of CO<sub>2</sub> to CH<sub>3</sub>OH requires an electron donor (cofactor) to supply the reducing equivalents in equimolar amounts in each step in the reaction. The cofactor level is an important issue in order to ensure that a high level of reducing equivalents is maintained to balance the forward enzymatic cascade reaction, but cofactor regeneration is also crucially important in the reaction design, particularly with NADH, which is costly. In the longer run, enzymes may be developed to be able to utilize other cofactors, but currently, NADH is the electron donor for the enzymes to catalyze the sequential reduction of CO<sub>2</sub> to CH<sub>3</sub>OH in the forward reaction.

## 2. Enzymatic cascade transformation of CO<sub>2</sub> to CH<sub>3</sub>OH

In the enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH by using three dehydrogenases [7] three moles of NADH are consumed per mole of CH<sub>3</sub>OH produced in the forward cascade reaction (Fig. 1). In general, yields of CH<sub>3</sub>OH have been calculated based on the initial NADH added. For example, to have a yield of 100%, the number of moles of CH<sub>3</sub>OH produced is equal to 1/3 of the initial NADH added. As mentioned, it was shown early that the overall yield from the enzymatic cascade reaction converting CO<sub>2</sub> to CH<sub>3</sub>OH in solution could be significantly enhanced if the reaction was performed in a solution with the enzymes entrapped in a silica sol-gel system [7]. The improvement was presumably due to the increased local concentrations of the reactants within the nanopores of the sol-gel matrix, which apparently resulted in confinement effects and thus improved substrate availability for each of the enzymatic steps involved in the sequence [7]. Since then, a large number of different enzyme immobilization strategies have been attempted in order to enhance the positive impact of the confinement effects and enable maximum enzyme reuse (Table 1). The reported research has involved design and preparation of appropriate immobilization carriers, and analysis of the resulting reaction kinetics and mass transfer limitations [13-17]. Additional improvement was introduced more recently, when the immobilization system also incorporated cofactor regeneration [18–22] (to be discussed later).

In all the reported work employing this enzymatic cascade system, the enzyme immobilization systems and reaction set-ups appear to have been empirically designed. Due to the variety of immobilization systems, process conditions, NADH levels, and enzyme dosages employed, it is difficult to identify the most efficient design. However, a direct comparison of the biocatalytic productivities ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h}$ ) obtained in different immobilized or non-immobilized enzyme systems indicates that the level of NADH added initially may have a significant effect on the biocatalytic productivity (Table 1). Hence, as expected, a high initial NADH level appears to increase the biocatalytic productivity, as e.g. the total biocatalytic productivity of the systems



reported by Xu et al. 2006 [14] and especially the one by Wang et al. 2014 [17] with high NADH addition, appears much higher than those reported by Obert and Dave [7] and Jiang et al. [13] (Table 1). Some differences in biocatalytic productivity that may be particularly ascribable to differences in the immobilization system are obvious, however, since for example different nanoparticle immobilization systems produced fair biocatalytic productivities (e.g. 0.874-1.433), even with very low initial NADH addition and without cofactor regeneration (Table 1 [18,19]) – but at the same time in certain cases the biocatalytic productivities attained without enzyme immobilization was on par or better than the productivity with immobilization (Table 1 [13-15,18]). The interpretation of biocatalytic productivities is equivocal, however, due to the differences in reaction time, enzyme dosages employed in different systems, and whether cofactor regeneration was attempted or not (Table 1).

A comparison of the biocatalytic productivities per initial amount of cofactor added to the cascade reaction, i.e. an expanded efficiency factor term ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h}\cdot\mu\text{mol}_{\text{NADH}}$ ) is therefore a better metric to assess  $\text{CH}_3\text{OH}$  production efficiency, although differences in reaction times and enzyme dosages, and notably immobilized system type and cofactor regeneration obviously influence the biocatalytic productivity and efficiency as well (Table 1). Hence, without cofactor regeneration an immobilized enzyme system using nanobeads, and only low NADH addition [19] gave a better efficiency factor of  $\sim 9.77$  ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h}\cdot\mu\text{mol}_{\text{NADH}}$ ) than a system with alginate beads or protamine-templated titania [15], despite the original biocatalytic productivity being the lowest among the three (Table 1). Also, a system with the enzymes immobilized on a nanofiber support [18], with modest NADH addition, had as high an efficiency factor (42) as the hybrid microcapsules system (41.3) with extremely high initial NADH despite significant differences in NADH addition (Table 1). In general, the attained efficiencies, assessed from  $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h}\cdot\mu\text{mol}_{\text{NADH}}$ , of the reported systems employing the FDH- F<sub>ald</sub>DH-ADH cascade system to convert  $\text{CO}_2$  to  $\text{CH}_3\text{OH}$  have varied almost 10,000 fold from  $\sim 0.006$ -55 (Table 1). With the exception of the efficiencies of 41-42 attained in certain immobilized systems without cofactor

regeneration (Table 1 [17,18]) immobilized enzyme systems including cofactor regeneration have provided the highest efficiency factors, and the use of a nanofiber support or nanoparticles for immobilization seems to give highest efficiencies ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}} \cdot \text{h} \cdot \mu\text{mol}_{\text{NADH}}$ ) of up to 47-55 even with modest NADH addition (Table 1).

The available data also show, as evident from e.g. Cazelles et al.'s work [19], that the obtainment of a maximal  $\text{CH}_3\text{OH}$  yield requires optimization of the enzyme ratio in each step of the multi-cascade catalysis. A compartmentalized scaffold [17] allows for pore adjustment of each layer and hypothetically, the amount of enzyme immobilized on each layer can also be controlled. Co-encapsulation of cofactor with production and regeneration enzymes but tethering of carbonic anhydrase (CA, EC 4.2.1.1 (to be discussed further below)), on a cationic nanofiber system has been reported to give the highest efficiency factor of  $\text{CH}_3\text{OH}$  formation of  $\sim 60$  among all systems reported so far [18]. Tethering of CA is supposed to increase the hydration of  $\text{CO}_2$ , but it is worth noting that the addition of the CA only produced a marginal increase in both the original biocatalytic productivity on the enzymes and of the efficiency factor when compared in the same nanofiber support enzyme immobilization system; hence the CA addition only increased the efficiency factor from 55.2 to  $\sim 60$  [18] (Table 1).

## 2.1 The enzymes

### 2.1.1 Carbonic anhydrase

Carbonic anhydrase (CA, EC 4.2.1.1) catalyzes the solubilization of  $\text{CO}_2$  in water. The catalysis by CA is known to be very fast, with a  $k_{\text{cat}}$  value of up to  $10^6$ , a rate which is almost 10 million times faster than the non-catalyzed natural reaction [23].

Temperature and the presence of certain contaminants can compromise the performance of CA. In fact, the  $\text{CO}_2$  rich exhaust stream from post combustion may reach over  $100^\circ\text{C}$  which can be an extreme temperature for CA. High concentrations of amines, traces and contaminants including

heavy metal, sulfur and nitrogen oxides have also been found to inhibit enzyme activity [24-27]. CA is also sensitive to the highly alkaline environment found in industrial CO<sub>2</sub> sorption columns, where both denaturation and peptide hydrolysis can occur [28]. To overcome these limitations, protein engineering saturated mutagenesis has been used to engineer the  $\beta$ -CA from the extremophile *Desulfovibrio vulgaris* to become highly thermostable, and maintain activity and stability at up to 107°C in 4.2 M amine solvent (pH > 10) [29]. A pilot scale test system employing the engineered CA was able to capture 60% of CO<sub>2</sub> from a continuous stream (30-500 liters per minute) of flue gas with a 12% CO<sub>2</sub> content, and operation for 60 hours in 5 consecutive days gave no enzyme activity loss of CA [29]. In another patent on industrial scale use of CA with real flue gas a liquid membrane system containing enzymes was employed [30]. In this system, a liquid layer was confined between two membranes (gas permeable) operated at different pressures to drive CO<sub>2</sub> across the membranes. CA could be immobilized on the membrane or be free in the solution. The advantage with this system is that the enzyme can facilitate CO<sub>2</sub> uptake by rapid conversion to bicarbonate, while the liquid film restricts the entry of other gases such as nitrogen and oxygen [30-32]. Most importantly the protein engineering work on the *Desulfovibrio vulgaris* CA [29] demonstrates that certain enzymes can be engineered to tolerate temperatures above 100 °C and an alkaline environment and thus tolerate extended use in a harsh environment.

### 2.1.2 Dehydrogenases

There are two types of formate dehydrogenase (FDH, EC 1.2.1.2); (1) Type 1: A metal-independent FDH enzyme which catalyzes the reaction from CHOOH to CO<sub>2</sub> irreversibly, employing nicotinamide adenine dinucleotide (NAD<sup>+</sup>) as cofactor; (2) Type 2: A metal-dependent molybdenum-based (Mo) or tungsten-based (W) FDH enzyme which catalyzes reduction of CO<sub>2</sub> to CHOOH reversibly [33–36]. In FDH type 1 the catalytic step features hydride transfer from the C atom of CHOOH to the C4 atom of the NAD<sup>+</sup> pyridine ring and hydride ion transfer is the rate limiting step in the mechanism. The mechanism of FDH type 2, the type employed in the enzymatic CO<sub>2</sub> to CHOOH conversion (the first report employing the *Candida boidinii* FDH for this reaction

from as early as 1976 [37]) is still debated in particular with respect to how the enzyme reaction with  $\text{CO}_2$  takes place [38–40]. However, it is currently presumed that FDH type 2 catalyzes oxidation of  $\text{CHOOH}$  via transfer of two electrons from the C-H bonds to/from the Mo/W centers collaterally with a proton transfer to the selenocysteine or histidine residue of the enzyme (Fig. 2) (adapted from [41]).

Several protein engineering efforts have been directed towards developing an enzyme with better  $\text{CO}_2$  reductase activity than the available wild type formate dehydrogenases. A selenocysteine-containing, recombinant FDH from *Clostridium carboxidivorans* strain P7T (expressed and purified using an *E. coli* host cell) was reported to efficiently catalyze the conversion of  $\text{CO}_2$  to  $\text{CHOOH}$  [42]. Compared to the FDH from *Candida boidinii*, this FDH from *Clostridium carboxidivorans* thus had a 10-fold-lower binding affinity for  $\text{NAD}^+$  and at least a 30-fold lower binding affinity for  $\text{CHOOH}$  [42]. These properties make this enzyme a more promising FDH candidate for converting  $\text{CO}_2$  than the more widely employed *Candida boidinii* enzyme. Other sources of FDH that have shown to have preference for  $\text{CO}_2$  reduction is summarized in Table 2.

Formaldehyde dehydrogenase ( $F_{\text{ald}}\text{DH}$ , EC 1.2.1.46) and Alcohol dehydrogenase (ADH, EC 1.1.1.1):  $F_{\text{ald}}\text{DH}$  catalyzes the conversion of  $\text{CHOH}$  into  $\text{CHOOH}$ , whereas ADH catalyzes the conversion of alcohol to aldehyde/ketone (with reduction of  $\text{NAD}^+$  to  $\text{NADH}$ ). Table 3 shows an overview of the main microorganisms used for the production of the three enzymes involved in the biocatalytic conversion of  $\text{CO}_2$  to  $\text{CH}_3\text{OH}$ , including FDH,  $F_{\text{ald}}\text{DH}$ , and ADH along with the kinetic parameters of the resulting enzymes. The ADH from *Saccharomyces cerevisiae* has a higher  $K_m$  value for  $\text{CH}_3\text{OH}$  than for  $\text{CHOH}$  (Table 3), indicating that the enzyme prefers the reverse reaction, aldehyde to alcohol. While numerous data have been reported for the kinetics of  $F_{\text{ald}}\text{DH}$  catalyzing the forward reaction ( $\text{CHOH}$  to  $\text{CHOOH}$ ), no data seem available on the kinetics of the reverse reaction.

## 2.2 Immobilization strategies

The most common cross-linking agent, glutaraldehyde, is sometimes disadvantageous due to uncontrollable chemical cross-linking which can affect the active site of the enzyme [43]. Enzyme attachment techniques must be designed in such a way that the enzyme activity and functionality can be maintained. Unfavorable conformational changes or protein folding resulting from improper linkage of the amino acids to the carrier may also limit the accessibility of substrates to the active site, thus affecting biocatalytic activity [44]. As already mentioned a sol-gel method was applied as the earliest immobilization technique for CO<sub>2</sub> to CH<sub>3</sub>OH biocatalysis [7,13] (Table 1). The process of synthesizing the sol-gel involves toxic reagents, which presumably were responsible for the enzymes activity loss and low yield of CH<sub>3</sub>OH (only 43% conversion was attained). Layer by layer assembly of organic-inorganic hybrid microcapsules was later used as a new strategy to create a mild process of an organized immobilization support for the catalysis [17] (Table 1). In addition to enhancing the reuse and thus improving the biocatalytic productivity of the enzymes, a main objective of compartmentalizing the enzymes were to assemble multiple enzymes in nanometer distance as to facilitate substrate/intermediate products diffusion without equilibrating with the bulk solution. It is also a hypothetical way to control the amount of enzyme immobilized in/on the support. This laborious and complex method improved the NADH based yield by up to 72%. It thus seems more simple to facilitate non-covalent immobilization by physical adsorption in membranes [22]. This technique, called “fouling-induced immobilization”, involves sequential immobilization of the three enzymes in three separate membranes, in such a manner that if each of the enzymes used work optimally at different reaction conditions i.e. temperature and pH, the conditions can in theory be adjusted in each of the steps separately. The setup is likely to reduce product inhibition on each step by removing the product once formed, which could also drive the equilibrium towards desired product. Very recently, the enzymes involved in the three different reaction systems (CH<sub>3</sub>OH production, cofactor regeneration and carbonic anhydrase facilitated CO<sub>2</sub> capture) were loaded together on cationic polyelectrolyte-doped hollow nanofibers fabricated by coaxial

electrospinning [18]. Until now, this is the system that has reached the highest biocatalytic productivity of all (Table 1), equivalent to ca. 100% yield [18]. In this system, carbonic anhydrase was immobilized on the outer surface of the nanofibers, which had been pre-loaded with the three dehydrogenases and the cofactor regenerating enzyme. A linear polyelectrolyte (polyallylamine hydrochloride) which penetrated the shell of the nanofibers provided binding sites for specific tethering of the cofactors and helped retain the cofactor inside the lumen via interactions between the oppositely charged polyelectrolytes and the cofactor [18].

Other common methods of immobilization used for the triade dehydrogenase enzyme system include entrapment of enzymes in nanocapsules [19], alginate [45], agarose, cellulose or polyacrylamide gel [46]. These methods may hamper high mass transfer for the enzyme catalysis. The key to achieve an efficient multicascade biocatalysis with immobilized enzymes thus appears to be maintenance of active interactions between enzymes and cofactors enabling so-called pseudo-dynamic biocatalysis [47].

In theory, immobilizing the three dehydrogenases within a defined small area would shorten the diffusion path of the intermediate products (CHOOH and CHOH) to the next enzyme's active site, which in theory would increase the conversion rate. On the other hand, such immediate conversion also maintains the substrate levels at a very low concentration, which lowers the individual enzymatic rates. It is usually difficult to assess the absolute amount of enzyme available for the substrates when several enzymes have been immobilized simultaneously, which in turn makes difficult to determine the actual biocatalytic productivity. For example, when constructing a spatial multi-enzyme support [16], the real amount of enzyme (FDH) entrapped during construction of the support material (precipitation of titania nanoparticles) could not be quantified, and precise assessment of the amount of each immobilize enzyme became even more complicated as an oligodopa solution was subsequently added to the aqueous suspension of FDH-bearing titania nanoparticles, to functionalize the particles with a catechol group. During this step FDH could leak out and be either free in the system (risk of being discarded) or conjugated with titanium

nanoparticles via the available catechol group (which should conjugate F<sub>ald</sub>DH instead). From the data reported [16] it is unclear to what extent the biocatalytic efficiency improvement was due to co-localization effects (FDH and F<sub>ald</sub>DH conjugated together on the titanium nanoparticles) or due to more efficient catalysis of FDH when entrapped in the titanium nanoparticles.

### 2.3 Main challenges for large scale implementation

Currently, there are three main challenges that prevent the exploitation of the multi-enzyme conversion of CO<sub>2</sub> to CH<sub>3</sub>OH at larger scale. Probably the most difficult is to identify microorganisms that produce enzymes that can catalyze the reverse (reduction) efficiently. For example, cultivation of acetogens for expressing FDH with high affinity towards CO<sub>2</sub> uptake is not immediately applicable for implementation at an industrial scale due to the strict anaerobic growth conditions required [48]. Production of CHOOH by using acetogens or methanogens [49] can moreover only be accomplished if further metabolic turnover of CHOOH is arrested by using expensive and toxic additives such as a sodium ionophore or methyl viologen [50]. The major second challenge to make the system work efficiently concerns the cofactor regeneration enzyme system. The kinetics of the reaction has to be adjusted to the needs of the main reaction, and none of the enzymes participating in the regeneration can be inhibited by the intermediate products. Another challenge frequently mentioned in the reported literature relates to the efficient hydration of CO<sub>2</sub> in water. In this case though, it is well known that pressure plays a critical role, and that higher pressure is greatly helpful in solubilization of CO<sub>2</sub> in water [51]. Co-immobilization of CA is also a good option to help hydration of CO<sub>2</sub>.

### 3. Enzymatic cofactor regeneration

Since the whole idea of cofactor regeneration is about reducing the cost associated with the cofactor addition, the enzymes, reagents and equipment used for cofactor regeneration should ideally be inexpensive, easily manipulated and stable under the operational conditions. The intermediate products and unreacted co-substrate from the main reaction should not interfere with

the regenerative system or enzyme, which should be able to regenerate the cofactor without generating intermediates [52,53]. Three methodological principles of enzymatic cofactor regeneration have been used: substrate-coupled, enzyme-coupled and closed loop (Fig. 3).

### **3.1 Substrate-coupled cofactor regeneration**

The substrate-coupled system uses the enzyme already involved in the main production reaction to simultaneously foster the regenerative reaction. Such an enzyme uses both reduced and oxidized forms of the cofactor, and is able to catalyze the target product formation from one substrate to produce a new product from a second substrate, whilst simultaneously regenerating the cofactor (Fig. 3). An example of this type of reaction is the biocatalytic reduction of ketones. The same enzyme, a bacterial ADH, which catalyses the target reduction of the ketone substrate, is thus used for the dehydrogenation of isopropanol under the formation of acetone and regeneration of NADPH [54]. In this context, it is difficult to achieve thermodynamically-favorable reaction conditions for both reactions in the same reaction medium. Therefore, to overcome the limitation, high concentration of substrate is introduced to drive the reaction forward, which can in turn, in some cases, inactivate the enzyme [54].

### **3.2 Enzyme-coupled cofactor regeneration**

Another principle is the enzyme-coupled system, which is the most widely reported method of cofactor regeneration. This methodology employs a second enzyme and a second substrate to accomplish the cofactor regeneration (Fig. 3). In this way, large thermodynamic driving forces for both reactions can be attained, since the second enzymatic regeneration reaction is irreversible or nearly irreversible, providing a strong drive for NADH (or NADPH) regeneration [54-56]. To date, biotransformations coupled with enzymatic cofactor regeneration, i.e. the enzyme-coupled regeneration principle, have shown the best results in terms of attaining high total turnover number TTN (Table 4). Coupling of the FDH reaction with glucose dehydrogenase as a NADH regenerative reaction system is a widely used example of an enzyme-coupled system in CO<sub>2</sub> conversion [52],



but several other examples exist (Table 4). In both batch and continuous enzymatic membrane systems cofactor turnover numbers in the order of magnitude of 100,000 have thus been attained – mostly with glucose dehydrogenase (of different microbial origin) - but a similar high TTN has been reported using ADH from horse liver in a substrate-coupled system (Table 4). In general, yields of the primary reaction and notably the cofactor TTN vary greatly, and no immediately obvious differences or advantages of one system versus another, e.g. continuous versus batch enzymatic membrane reactor systems seem evident from the available data (Table 4).

High productivity rate of the main conversion reaction product, e.g. above  $500\text{-}600\text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ , have been attained with several of the coupled enzymatic systems, but the high productivities have not always been accompanied by high TTN – and vice versa (Table 4). Unfortunately, only limited work has apparently been done on optimizing the systems by balancing the kinetics of the main reaction with the kinetics of the regeneration reaction or on transferring knowledge from one recycling system to another. Some of the common regenerating enzymes and the reactions employed in enzyme-cofactor regeneration systems, beyond FDH and ADH, have been listed in Table 5 [57]. Recently, phosphite dehydrogenase has been reported as a new alternative reaction for NADH generation, besides requiring inexpensive phosphite as the substrate and phosphate as the co-product (that subsequently can be part of the buffer solution), the reaction is also strongly thermodynamically driven [58].

### **3.3 Closed-loop cofactor regeneration**

Reaction-internal closed loop regeneration refers to when the product of the production reaction (intermediate product), is also the substrate for the second reaction (Fig. 3). The feasibility of the method was first demonstrated by the transformation of (L)-lactate via pyruvate to L-alanine [56]. In order to reach a complete conversion at least one step must be irreversible, as the coupling of two reversible enzymatic steps will normally result in an incomplete formation of the product [52]. Although this method is complicated, it can reach zero waste regeneration since the second substrate is not needed.

### 3.4 Regeneration in membrane reactor

An ideal design of a reactor for efficient cofactor regeneration reactor design should avoid mass transfer resistance as to pave access to both “regenerative” and “productive” enzymes. A membrane reactor is a good option because it can function as selective barrier able to retain the enzyme by size exclusion. In a continuous process, product isolation is made simpler with a membrane reactor, which in turn will drive the reaction forward; which has special relevance when the enzymes have a tendency to catalyse the reaction in the reverse way. On the other hand, cofactors are small molecules and it is difficult to retain on the membrane without significant loss. Cofactor and products are normally similar in size (molecular weight), in which case the use of the membrane reactor is not as convenient [59]. In case the products from the main reaction are smaller than the cofactor size, a membrane with small pores will improve retention, but in that case the flux through the membrane will be limited [60]. Efforts have been made to increase the particle size of the cofactor by covalently linking them to large water soluble polymer, such as polyethylene glycol (PEG), polyethylenimine (PEI), polyacrylic acid (PAA), dextran and polylysine, in such a way that the cofactor can be retained by the membrane while the products can pass [61,62]. The native or larger (linked) cofactor can be retained in the reactor either by size exclusion or by charge repulsion [54, 63-65]. However, overall retention effectiveness is very much dependent on molecular weights, structure and charge densities of all species occurring in the solution i.e. products, substrates, salts and other chemicals in the reaction media.

### 3.5 Immobilization of enzymes and cofactors

Another alternative to retain cofactors is by immobilizing them, e.g. in nanoscaffold or other structures [66-69]. Immobilization of the cofactor together with the enzymes involved in the main reaction often leads to easy recycling and allows a more flexible reactor design. Current studies on this line have successfully attempted to immobilize oxidoreductases and the cofactor together on or in a nanoparticles scaffold, within the same or different particles. NAD<sup>+</sup> has been also immobilized in carbodiimide activated silica nanoparticles for L-lactate production using formate

dehydrogenase and keto-reductase [69]. Effective shuttling between covalent enzyme-cofactor bound in nanosized, porous silica glass (Fig. 4), involving lactate dehydrogenase, glucose dehydrogenase and NADH was proven to be effective by tuning the length of the spacer (glutaraldehyde and PEG) and the pore size of the glass [66]. Dynamic particle collisions by Brownian motion resulted in good biocatalytic activity and enhanced reaction rate when a magnetic field was applied [46,67]. Likewise, tethered cofactors (NAD<sup>+</sup> and NADH) on chitosan coated, magnetic nanoparticles platforms were tested with ADH (using benzyl alcohol and acetaldehyde as substrates), and resulted in higher TTN over the free system [68]. Immobilization in nanocarriers is also emerging drastically. Comprehensive reviews on potential and functional nanoparticles carriers have been published recently [69,70]. Advanced development includes silica like dendrimer with hierarchical pores that can accommodate different sizes of enzymes in multi-enzyme cascade [71] and self-assembly of protein-inorganic “nanoflowers” [72].

Contemplation of the available data for the sequential enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH, including the immobilized systems designed to optimize the conversion, clearly show that the concept of using only three enzymes, FDH, F<sub>ald</sub>DH, and ADH (Fig. 1), in sequence for the forward reaction is workable, i.e. even without CA involvement to help bring the CO<sub>2</sub> into solution, but the regeneration of reducing equivalents, or cofactor regeneration, is crucial for feasibility of the system. However, very little research appears to have been done on optimizing the cofactor regeneration in the biocatalytic CO<sub>2</sub> to CH<sub>3</sub>OH cascade, and kinetic models that could help identify the selection of the desirable reaction set-up with high total turnover number of cofactor regeneration are not available, despite the crucial significance of efficient NADH regeneration demonstrated already (e.g. as shown in Table 1). In addition, it appears that more robust enzymes are needed for the concept reaction to be workable to reduce industrial CO<sub>2</sub> emissions – conceptually the workability of molecular evolution of CA to work under harsh conditions of temperature and pH has shown that protein engineering may indeed be possible for obtaining better enzymes for the CO<sub>2</sub> to CH<sub>3</sub>OH biocatalytic cascade to be feasible in industrial settings.

#### 4. Conclusion

This review shows the conceptual workability of enzymatic CO<sub>2</sub> to CH<sub>3</sub>OH conversion, but also highlights that there are bottlenecks to be overcome in order to exploit this type of reaction to convert CO<sub>2</sub> into valuable chemicals at large scale. Development of efficient enzyme immobilization systems, to favor both enzyme stability and reuse, has already been quite extensively investigated, and use of nanocarriers or immobilization on separation membranes have shown promise in this regard. Enzyme immobilization is a key to drive up the biocatalytic productivity of the enzyme cascade. The option for reaping of CHOH and CHOOH during the reaction warrants innovative development as so does the cofactor regeneration which appears crucial for optimal efficiency. However, the quantitative kinetics of the coupling of a reaction for efficient cofactor regeneration has only been scarcely addressed in this regard. In general, the enzymes used in the forward CO<sub>2</sub> to CH<sub>3</sub>OH biocatalytic cascade have been wild-type enzymes (from different microbial sources) and the available data strongly indicate that there is a significant need for identifying or engineering of better enzymes. Notably the evolution of better robustness of the enzymes appears crucial for successful exploitation. However, surprisingly limited knowledge is available on the structure-function of the relevant dehydrogenases to work in the reverse and the kinetics of this type of reactions. Hence, the development of this system for industrial feasibility still holds several biochemical engineering challenges. More efforts to develop better enzymes, kinetic models, and robust enzyme immobilization systems fit for this particular reaction, as well as efficient cofactor regeneration systems are expected in the future.

**Notes**

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## Figures

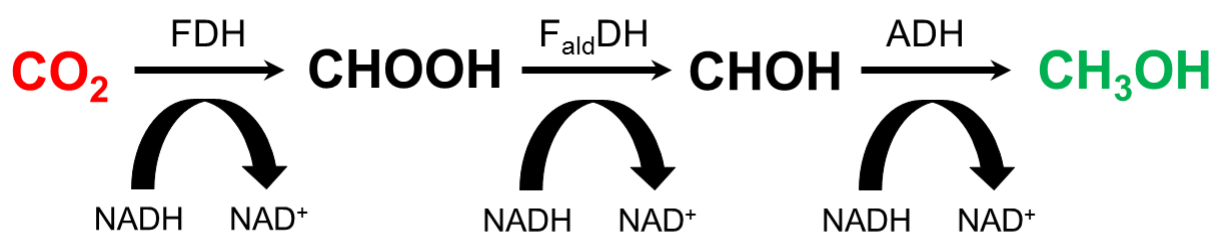


Fig.1 Biocatalytic transformation pathway of CO<sub>2</sub> to CH<sub>3</sub>OH via stepwise reverse enzymatic catalysis by FDH, F<sub>ald</sub>DH and ADH as first introduced by Obert and Dave [7].

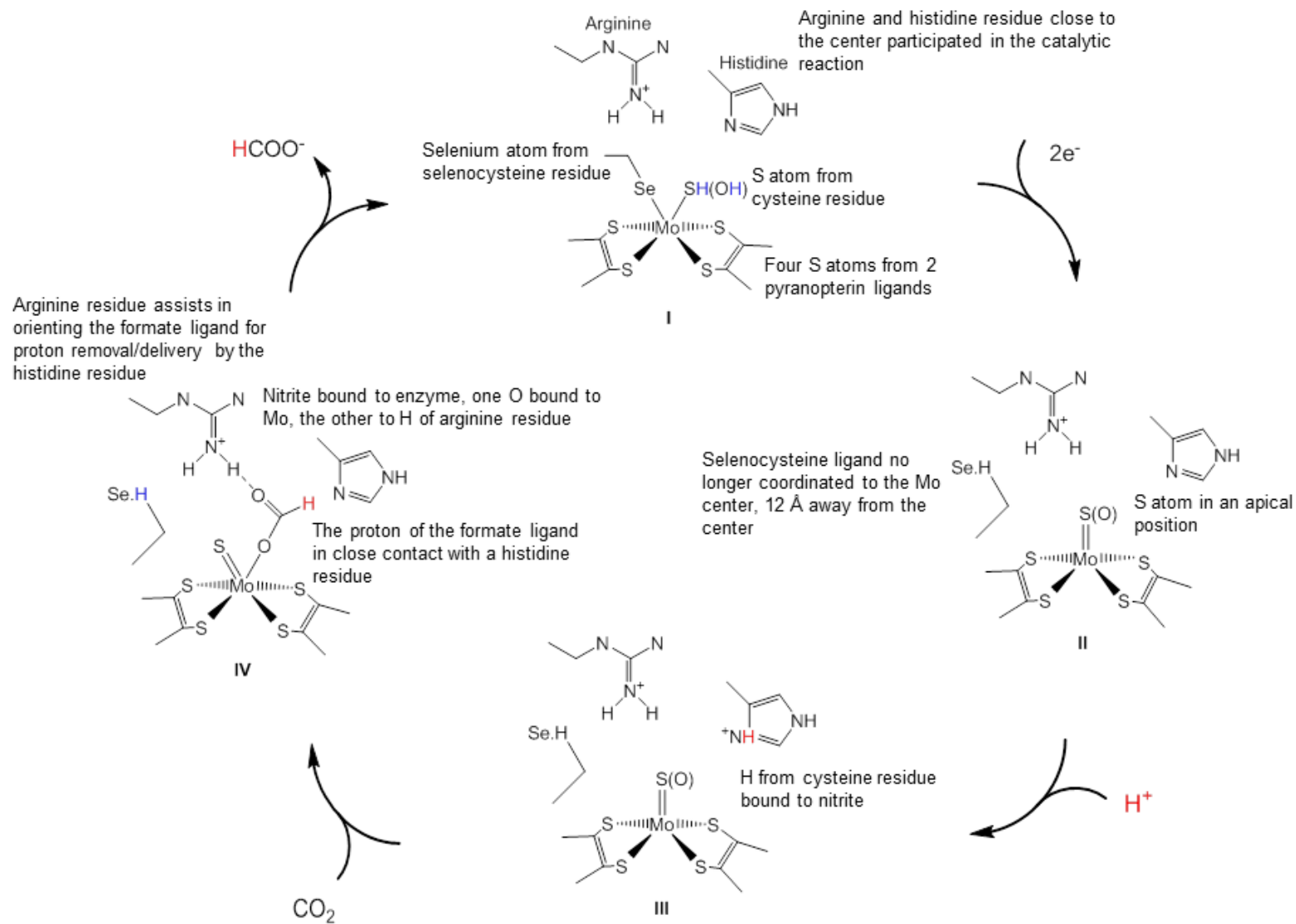


Fig. 2 Reduction of  $\text{CO}_2$  to  $\text{CHOO}^-$  as proposed mechanism adapted from [41].

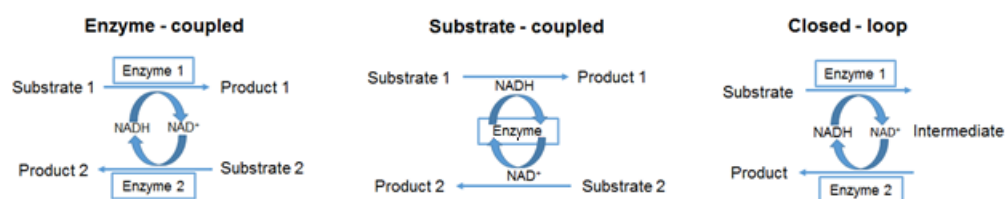


Fig. 3 Principle methods of cofactor regeneration.

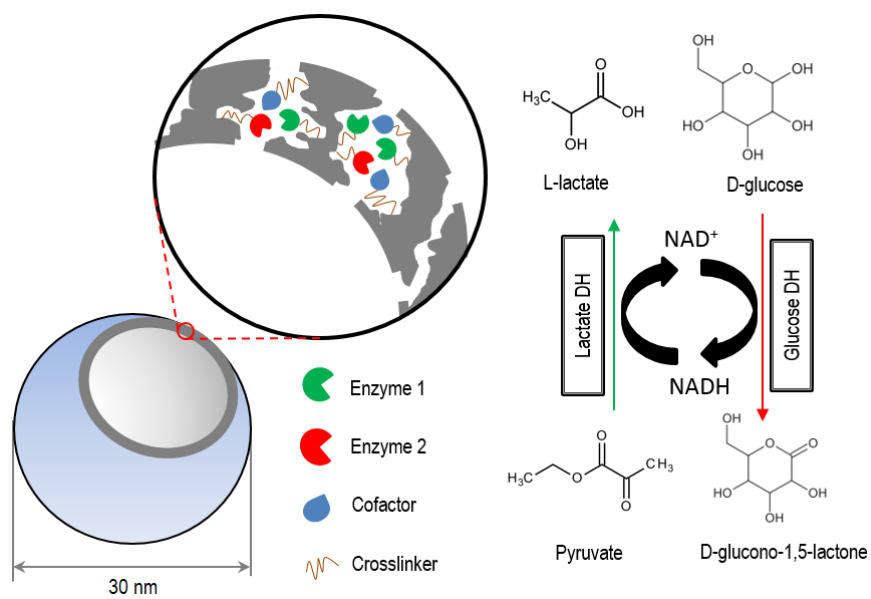


Fig. 4 Covalent enzyme-cofactor bound in nanosized porous silica glass. Adapted from [66].

## Tables

Table 1 Biocatalytic productivity of enzymatic conversion of CO<sub>2</sub> to CH<sub>3</sub>OH from the available literature.

	Optimum reactor conditions	Immobilization matrix	Biocatalytic productivity ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h}$ )	Efficiency factor ( $\mu\text{mol}_{\text{CH}_3\text{OH}}/\text{mg}_{\text{enzyme}}\cdot\text{h} \cdot \mu\text{mol}_{\text{NADH}} \times 10^{-3}$ )	NADH Initial amount ( $\mu\text{mol}$ )	Ref.
Without cofactor regeneration	2 ml, PBS, pH 7	Free system	0.124	0.62	200	[7]
	3 h, 20°C	Sol-gel	0.324	1.62		
	2 ml, PBS, pH 7	Free system	0.375	3.75	100	[13]
	8 h, 37°C	Sol-gel	0.344	3.44		
	28 ml, tris-HCl, pH 7, 8 h, 20°C	Free system	3.870	4.12	940	[14]
		TMOS + alginate (beads)	3.843	4.09		
	tris-HCl, pH 7	Free system	0.060	<sup>a</sup> nd	<sup>b</sup> na	[73]
	8 h, 20°C	Capsule in bead scaffold	0.186			
	2 ml, tris-HCl, pH 7	Free system	0.233	1.16	200	[15]
	8 h, 35°C	Protamine-templated titania	0.256	1.28		
	2 ml, PBS, pH 6.5	Free system	0.020	0.67	30	[19]
	3h, 37°C	Nanoparticle	0.293	9.77		
	18 ml, PBS, pH 7	Free system	1024	20.5	50,000	[17]
	9 h, 20°C	Hybrid microcapsules	2066	41.3		
	18 ml, PBS, pH 7,	Free system	0.339	0.007	50,000	[22]
9 h, 20°C	Co-immobilized	0.315	0.006			
	Sequential immobilization	0.424	0.008			
2 ml, PBS, pH 7	Free system	15.979	15.9	1000	[18]	
10 h, 20°C	Nanofiber support	0.874	42	21		
With cofactor regeneration reaction	20 ml, PBS, pH 7	Free system	1.140	5.7	200	[20]
	0.5 h, 20°C	Microparticles	0.760	3.8		
	2 ml, PBS, pH 6.5	Free system	0.053	1.77	30	[19]
	3 h, 37°C	Nanoparticle	1.433	47.8		
	2 ml, tris-HCl, pH 7	Free system	0.097	0.001	10,000	[22]
	0.5 h, 20°C	Co-immobilized	0.139	0.014		
		Sequential immobilization	0.164	0.016		
2 ml, PBS, pH 7	Free system	19.163	19.2	1000	[18]	
10 h, 20°C	Nanofiber support	1.159	55.2	21		
With cofactor regeneration reaction+CA	2 ml, PBS, pH 7,	Free system	20.958	20.9	1000	[18]
	10 h, 20°C	Nanofiber support	1.254	59.7	21	

<sup>a</sup>nd – not determined; <sup>b</sup>na – not available



Table 2 FDH from different sources that has high affinity for taking up CO<sub>2</sub> as substrate.

Source	Method of expression	Yield	Ref.
<i>Syntrophobacter fumaroxidans</i>	Purified from <i>S. fumaroxidans</i> cells under anaerobic conditions	282 s <sup>-1</sup> for CO <sub>2</sub> reduction	[37]
<i>Thiobacillus sp.</i>	Expressed using an additional C-terminal hexa-histidine sequence	0.3 s <sup>-1</sup> for CO <sub>2</sub> reduction	[74]
<i>Rhodobacter capsulatus</i>	Heterologous expression system in <i>E. coli</i>	1.5 s <sup>-1</sup> for CO <sub>2</sub> reduction	[75]
<i>Syntrophobacter fumaroxidans</i>	Produced in axenic fumarate-grown cells as well as in cells which were grown syntrophically on propionate with <i>Methanospirillum hungatei</i> as the H <sub>2</sub> and formate scavenger	900 μmol CO <sub>2</sub> oxidized min <sup>-1</sup> mg <sup>-1</sup> enzyme	[33]

Table 3 Apparent Michaelis Menten constant,  $K_m$ , of Formate dehydrogenase, Formaldehyde dehydrogenase, and Alcohol dehydrogenase for their respective substrates.

Enzyme	Reaction	$K_m$ (mM)	$V_{max}$ ( $\mu\text{mol}/\text{min}\cdot\text{mg}$ )	Source	Ref
Formate dehydrogenase	$\text{CO}_2 \rightarrow \text{CHOOH}$	40	na	<i>Pseudomonas oxalaticus</i>	[76]
	$\text{CHOOH} \rightarrow \text{CO}_2$	13	2.2	<i>Candida boidinii</i>	[38]
Formaldehyde dehydrogenase	$\text{CHOOH} \rightarrow \text{CHOH}$			nd	
	$\text{CHOH} \rightarrow \text{CHOOH}$	0.2	8.3	<i>Pseudomonas putida</i>	[77]
Alcohol dehydrogenase	$\text{CHOH} \rightarrow \text{CH}_3\text{OH}$	6	6	<i>Saccharomyces cerevisiae</i>	measured
	$\text{CH}_3\text{OH} \rightarrow \text{CHOH}$	100	0.019		

Table 4 Coupled biotransformations with high total turnover (TTN) for cofactor regeneration.

Main reaction	Regeneration reaction	Reactor/capacity	Yield (g·L <sup>-1</sup> ·d <sup>-1</sup> )	Cofactor TTN	References
Mannitol dehydrogenase ( <i>Saccharomyces cerevisiae</i> ) Fructose → Mannitol	Glucose dehydrogenase (NADH) ( <i>Bacillus megaterium</i> ) Glucose → Gluconic acid	Continuous *EMR	nd	150,000	[54]
Alcohol dehydrogenase ( <i>Thermoanaerobium brockii</i> ) Sulcatone → Sulcatol	Alcohol dehydrogenase (NADPH) ( <i>Thermoanaerobium brockii</i> ) Isopropanol → Acetone	Continuous EMR	nd	4,400	[54]
Aldose reductase ( <i>Candida tropicalis</i> ) Glucose → Sorbitol	Glucose dehydrogenase (NADPH) na Glucose → Gluconic acid	Continuous EMR 0.05L	3	106,000	[63]
Lactate dehydrogenase ( <i>Staphylococcus epidermidis</i> ) 2-Oxo-4-phenyl-butyric acid → 2-Hydroxy-4-phenyl-butyric acid	Formate dehydrogenase (NADH) ( <i>Candida boidinii</i> ) Formate → CO <sub>2</sub>	Continuous EMR 0.2L	165	900	[78]
Alcohol dehydrogenase ( <i>Horse liver</i> ) Cyclohexanone → Cyclohexanol	Alcohol dehydrogenase (NADH) ( <i>Horse liver</i> ) Cyclopentanol → Cyclopentanone	Batch EMR	nd	100,000	[79]
Alcohol dehydrogenase ( <i>Rhodococcus erythropolis</i> ) 1-Phenyl-2-propanone → 1-Phenyl-2-propanol	Formate dehydrogenase (NADH) ( <i>Candida boidinii</i> ) Formate → CO <sub>2</sub>	Continuous EMR	100	1350	[80]
Leucine dehydrogenase na Trimethyl pyruvic acid → L-tert-Leucine	Formate dehydrogenase (NADH) na Formate → CO <sub>2</sub>	Continuous EMR 0.01L	373	7920	[60]
Glutamate Dehydrogenase na α-Ketoglutarate → L-Glutamate	Glucose dehydrogenase (NADH) ( <i>Bacillus sp.</i> ) Glucose → Gluconic acid	Continuous EMR 0.25L	120	10,000	[81]
Alcohol dehydrogenase ( <i>Rhodococcus erythropolis</i> ) 1-Phenyl-2-propanone → 1-Phenyl-2-propanol	Formate dehydrogenase (NADH) ( <i>Candida boidinii</i> ) Formate → CO <sub>2</sub>	Continuous EMR 0.05L	64.3	1361	[82]
Glutamate dehydrogenase ( <i>beef liver</i> ) 2-keto-6-Hydroxyhexanoic acid → L-6-Hydroxynorleucine	Glucose dehydrogenase (NADH) ( <i>Bacillus megaterium</i> ) Glucose → Gluconic acid	Batch EMR 0.03L	507	387	[83]
Lactate dehydrogenase ( <i>Leuconostoc mesenteroids</i> ) (R)-3-(4-Fluorophenyl)-2 hydroxy propionic acid → (R)-Methyl 3-(4-fluorophenyl)-2-hydroxypropanoate	Formate dehydrogenase (NADH) ( <i>Candida boidinii</i> ) Formate → CO <sub>2</sub>	Continuous EMR 2.2L	560	2050	[84]

Leucine dehydrogenase ( <i>Bacillus sphaericus</i> ) Trimethylpyruvic acid → L-tert-Leucine	Formate dehydrogenase (NADH) ( <i>Candida boidinii</i> ) Formate → CO <sub>2</sub>	Batch EMR	638	nd	[85]
Lactate dehydrogenase ( <i>Rabbit muscle</i> ) Pyruvate → L-Lactate	Glutamate dehydrogenase (NADH) ( <i>Bovine liver</i> ) L-Glutamate → α-Ketoglutarate	Batch Test tube	nd	20,000	[86]
Carbonyl reductase ( <i>Streptomyces coelicolor</i> ) Ethyl 4-chloro-3-oxobutanoate → Ethyl (S)-4-chloro-3-hydroxybutanoate	Carbonyl reductase (NADH) ( <i>Streptomyces coelicolor</i> ) 2-Propanol → Acetone	Batch Stirred reactor 0.5L	655	12,100	[87]
Xylose reductase ( <i>Pichia stipitis</i> ) Xylose → Xylitol	Glycerol dehydrogenase (NADH) ( <i>Cellulomonas sp</i> ) Glycerol → Dihydroxyacetone	Batch Test tube	160	82	[47]
Alcohol dehydrogenase ( <i>Lactobacillus brevis</i> ) 2-Octanone → 2-Octanol	Glucose dehydrogenase (NADPH) na Glucose → Gluconic acid	Continuous EMR 0.02L	10	245	[88]
Alcohol dehydrogenase ( <i>Thermus thermophilus</i> ) 2,2,2-Trifluoroacetophenone → (S)-α-(Trifluoromethyl)benzyl alcohol	Glutamate dehydrogenase (NADH) ( <i>Thermus thermophilus</i> ) L-Glutamate → α-Ketoglutarate	Batch Test tube	nd	9000	[89]
Alcohol dehydrogenase ( <i>Lactobacillus brevis</i> ) 2-Octanone → 2-Octanol	Alcohol dehydrogenase (NADPH) ( <i>Lactobacillus brevis</i> ) 2-Propanol → Acetone	Continuous Stirred reactor	12	26,000	[90]
Carbonyl reductase ( <i>Bacillus subtilis</i> ) Ethyl 2-oxo-4-Phenylbutyrate → (R)-2- hydroxy-4-Phenylbutyrate	Glucose dehydrogenase (NADPH) ( <i>Bacillus subtilis</i> ) Glucose → Gluconic acid	Batch Stirred reactor 1L	660	32,039	[91]
Carbonyl reductase ( <i>Streptomyces coelicolor</i> ) Ethyl 4-chloro-3-oxobutanoate → Ethyl (S)-4-chloro-3-hydroxybutanoate	Carbonyl reductase (NADH) ( <i>Streptomyces coelicolor</i> ) Isopropanol → Ketone	Batch Stirred reactor 50L	86	6060	[92]
Carbonyl reductase ( <i>Candida glabrata</i> ) Ethyl 4-chloro-3-oxobutanoate → (R)-3- Hydroxy-4-chlorobutyrate	Glucose dehydrogenase (NADPH) ( <i>Bacillus megaterium</i> ) Glucose → Gluconic acid	Batch Stirred reactor 1L	660	108,000	[93]
Alcohol dehydrogenase na Acetaldehyde → Ethanol	Alcohol dehydrogenase (NADH) na Benzyl alcohol → Benzaldehyde	Batch Stirred reactor	nd	3904	[68]
Carbonyl reductase ( <i>Yarrowia lipolytica</i> ) Ethyl 4-chloro-3-oxobutanoate → Ethyl (S)-4-chloro-3-hydroxybutanoate	Carbonyl reductase (NADPH) ( <i>Yarrowia lipolytica</i> ) Mannitol/Sorbitol → Sugar	Batch Stirred reactor 0.05L	600	13,500	[94]

\*EMR – Enzymatic membrane reactor

Table 5 Cofactor regeneration enzyme reaction systems for NADH and NADPH (beyond FDH and ADH).

Regeneration enzyme	Cofactor	Cosubstrate	Coproduct
<b>Glucose dehydrogenase (GDH)</b>	NADP <sup>+</sup> → NADPH NAD <sup>+</sup> → NADH	Glucose	D-Glucono-1,5-lactone
<b>Phosphite dehydrogenase (PDH)</b>	NAD <sup>+</sup> → NADH	Phosphorus acid	Phosphates
<b>Hydrogenase (Hase)</b>	NADP <sup>+</sup> → NADPH	H <sub>2</sub>	-