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Reduction of CO_2 to methanol by a polyenzymatic system encapsulated in phospholipids–silica nanocapsules†

Rémi Cazelles,[a] Jullien Drone,[a] François Fajula,[a] Ovidiu Ersen,[b] Simona Moldovan[b] and Anne Galarneau*[a]

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By reversing the biological metabolic reaction pathway of three dehydrogenases, a formate dehydrogenase, a formaldehyde dehydrogenase and an alcohol dehydrogenase, it was possible to transform CO_2 into methanol by a cascade reaction. The activity of each enzyme was examined separately and then the relative amount of each enzyme for the cascade reaction was optimized. The enzymes consume one molecule of the NADH cofactor each to run which should be regenerated for cost reasons. Three different NAD^+ regenerating systems were compared: 2 enzymes (phosphite dehydrogenase (PTDH) and glycerol dehydrogenase) and a natural photosystem extracted from spinach leaves (chloroplasts). PTDH was proven to be more efficient at neutral pH. The new polyenzymatic system (4 enzymes) was then encapsulated in silica nanocapsules (internal diameter 30 nm) nanostructured by phospholipids (NPS). This hybrid nanobioreactor showed an activity 55 times higher than the free enzymes in solution. A methanol production of 42 μmol g_{NPS}^{-1} corresponding to 4.3 mmol $g_{\text{commercial enzymatic powder}}^{-1}$ in 3 h at room temperature and 5 bar was obtained.

1. Introduction

Considering the need for environmentally friendly carbon sources for chemical, petrochemical and pharmaceutical industries, researchers have developed a process that produces C1 synthetic building blocks from a greenhouse gas such as carbon dioxide. CO_2 is used as feedstock for the synthesis of a broad range of compounds such as urea, salicylic acid, carbonates or an alcohol like methanol.[1–3] Different pathways to produce methanol from CO_2 have been investigated: chemical, electrochemical, photochemical and enzymatic synthesis.[4–8] Among them, a polyenzymatic process using 3 dehydrogenases, firstly described by Obert and Dave,[7] operated at ambient temperature and atmospheric pressure. Its thermodynamic feasibility was proved by Wang and coworkers.[9] This low energy consuming process can be seen as a green and sustainable strategy as compared to the other techniques. The biocatalytic synthesis of methanol from CO_2 involving the 3 dehydrogenases needs a cofactor (reduced Nicotinamide Adenine Dinucleotide, NADH) to achieve the biotransformation. NADH is an expensive molecule that should be regenerated *in situ* in a cost efficient process. This can be achieved through electrochemical and photochemical reactions or by using another enzyme, which converts the oxidized form of the cofactor (NAD^+) into its reduced state (NADH).[10–12] Among these possibilities, the enzymatic pathway is often more selective and provides the highest reaction rates. Formate dehydrogenase and phosphite dehydrogenase are the more efficient recycling systems reported so far.[12,13]

Multi-enzyme cascade catalysis is an attractive alternative to chemical catalysis for the production of a number of fine chemicals, as it allows energy efficient conversion thanks to mild conditions: ambient or near ambient temperature and low pressure. Xue and Woodley already discussed the technology options and strategies that are available for the development of multi-enzymatic processes.[14] Hold and Panke have reproduced *in vitro* the glycolysis of *Escherichia coli* using several enzymes coming from the same bacterium.[15]

In the present study, we used an artificial cascade reaction, in which we combined enzymes from different organisms which may not share either similar optimal pH or have similar reaction rates at similar concentrations of reagents. Under the natural conditions, biological pathways are able to shift the equilibrium of some enzymes by controlling product concentrations, which is complicated to reproduce *in vitro*. Moreover, *in vitro* reactions take place at strong concentration of substrates,

[a] *Institut Charles Gerhardt Montpellier, UMR 5253 CNRS-UM2-ENSCM-UM1, ENSCM, 8 rue de l'Ecole Normale, 34296 Montpellier Cedex 5, France. E-mail: anne.galarneau@enscm.fr*
[b] *Institut de Physique et Chimie des Matériaux de Strasbourg, UMR 7504 CNRS-University of Strasbourg, 23 rue du Loess, 67034, Strasbourg Cedex 2, France*

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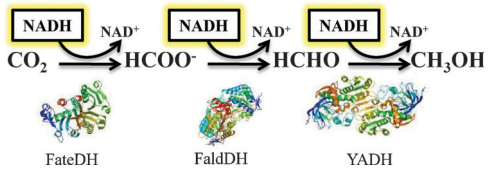


Scheme 1 Representation of the enzymatic pathway of carbon dioxide conversion into methanol by 3 dehydrogenase enzymes. Three moles of the reduced nicotinamide dinucleotide (NADH) cofactor are needed to convert 1 mole of CO_2 into methanol.

the accumulation of the products can then lead to the inactivation of the other enzymes. Furthermore, another limitation for the use of CO_2 is the low solubility of gaseous CO_2 in water and therefore its availability, a limitation which can be partly overcome by increasing the pressure.[16] Indeed CO_2 in water decomposes into carbonate species depending on the pH of the solution and an equilibrium between carbonates and gaseous CO_2 takes place. In the artificial polyenzymatic cascade reaction we studied, the formate dehydrogenase (FateDH) converts gaseous CO_2 into formate, then the formaldehyde dehydrogenase (FaldDH) converts formate into formaldehyde and finally alcohol dehydrogenase (YADH) converts formaldehyde into methanol (Scheme 1). Previous studies have highlighted the crucial role of adding a regenerating system for NADH recycling and of using an appropriate enzyme immobilization technique to improve the reaction activity.[7,17,18]

Immobilization of the enzymes is a critical concern for biochemical processes as it provides stabilization and easier use, but also in the present case, improved activity. Dave and coworkers found that these enzymes encapsulated in a silica matrix by sol–gel show higher productivity of methanol compared to free enzymes in solution.[7,17] Jiang and coworkers investigated the influence of enzyme immobilization into silica sol–gel and alginate–silica nanoparticles as well as an encapsulation in multi-compartmented titanate systems.[8,19]

Herein, we performed a systematic study of the polyenzymatic system and searched for the most effective conditions to reach the highest activity: pH, the ratio between the three enzymes, the nature of the NADH regeneration system and initial NADH concentration. Finally, we encapsulated the polyenzymatic system by an optimized silica sol–gel technique using natural phospholipids (egg lecithin) and lactose to protect the enzymes leading to phospholipids–silica nanocapsules (NPS). NPS have previously proven their efficiency for the encapsulation of bienzymatic systems such as glucose oxidase (GOx) and horseradish peroxidase (HRP) or GOx and bovine hemoglobin (Hb) to generate *in situ* H_2O_2 and oxidize polycyclic aromatic hydrocarbon pollutants in water using O_2 as an oxidant.[20,21]

2. Experimental section

2.1. Materials

Formate dehydrogenase (Homo-dimer, 80.7 kDa) from *Candida boidinii* (FateDH), formaldehyde dehydrogenase (Homo-tetramer, 168 kDa) from *Pseudomonas putida* (FaldDH) and alcohol dehydrogenase (Homo-tetramer, 141 kDa) from *Saccharomyces cerevisiae* (YADH) enzymes were purchased from Sigma-Aldrich. These commercial powders are not pure enzymes and the effective enzyme content needs to be determined prior to use. The genetic construct encoding the phosphite dehydrogenase (PTDH) used in NADH recycling experiments was obtained by courtesy of Prof. H. M. Zhao.[13] In brief, *Escherichia coli* BL21*(DE3) (Life Technologies) was transformed by the plasmid pET15b-PTDH12x[13] and used to overexpress the enzyme as previously described with slight modifications. Homogeneous enzyme preparation was obtained after purification using IMAC Ni^{2+} (HisTrap 1 mL, GE Healthcare) followed by desalting (HiTrap 5 mL, GE Healthcare) with conservation buffer (50 mM MOPS, 10% glycerol, pH 7.25) under standard conditions. Aliquots of the enzyme were stored at −80 °C until use. Oxidized and reduced nicotinamide adenine dinucleotide (NAD^+, NADH), mono- and di-hydrogenated potassium phosphate used for buffers and phosphite (Na_2HPO_3) were obtained from Sigma-Aldrich. For the synthesis of silica nanocapsules for enzymes encapsulation, tetraethoxysilane (TEOS), egg lecithin (60%), dodecylamine, and β-D-lactose were purchased from Sigma. Potassium hydrogen carbonate ($KHCO_3$) and Na_2CO_3 were used as gaseous CO_2 sources ($CO_2(g)$) and were obtained from Sigma. At pH 6.5 and 37 °C, dehydrogenated carbonate (CO_3^{2-}) is likely nonexistent (<1%) and soluble hydrogenated carbonates coexist with gaseous CO_2 in equilibrium with 45% of $CO_2(g)$ and 55% of $K^+HCO_3^-$ (Fig. S1, ESI†), which are enough to carry out enzymatic reactions with dissolved gaseous CO_2.[22,23]

2.2. Enzyme activity measurement

2.2.1. Standard assays for commercial powder characterization. To determine the specific activity of the 3 commercial dehydrogenases, it is first necessary to quantify the real amount of enzymes contained in the commercial powders and then to quantify the activity of each enzyme by specific assays. The enzyme content was determined using the Thermo Scientific Pierce BCA Protein Assay Kit. The catalytic activities of the enzymes were determined under fixed conditions (V = 100 μL, T = 37 °C) in potassium phosphate buffer (0.1 M, pH 6.5). To characterize the activity of the formate dehydrogenase (FateDH), a standard assay was conducted as follows: NAD^+ (1 mM) and formate ($HCOO^-$ 500 mM) were mixed with FateDH (10 mg L^{-1}). For the formaldehyde dehydrogenase (FaldDH), the standard assay was carried out using NAD^+ (0.5 mM), formaldehyde (HCHO 6 mM) and FaldDH (10 mg L^{-1}). For the alcohol dehydrogenase (YADH), the standard assay was performed with NADH (0.025 mM), formaldehyde (HCHO 100 mM) and YADH (1 mg L^{-1}). The activity of the enzymes was determined by following the increase (for FateDH and FaldDH) or the decrease (for YADH) of the absorbance band of NADH at 340 nm by UV-Vis spectroscopy. The pH meter used to adjust pH stock solutions was from Eutech instrument, model pH510. A mini hybridization oven from Appligen was used for all the thermostated reactions and synthesis.

2.2.2. Optimization of the polyenzymatic system used to reduce CO_2 into methanol. All reactions were carried out under a N_2 or CO_2 atmosphere in order to avoid oxidation of NADH.

Solutions of enzymes and chemicals were prepared in sonicated, demineralized H_2O. The optimum pH for each enzyme under their reductive reaction pathway was investigated using different buffer solutions. Solutions of pH 4.5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.3 were prepared by mixing 1 M K_2HPO_4 solution with 1 M KH_2PO_4 solution, pH was adjusted using a pH meter at 25 °C. The activity of FateDH (0.1 g L^{-1}) was measured at 25 °C in 100 μL of 96-well plates with a 0.1 M phosphate buffer solution, NADH (1.5 mM) and Na_2CO_3 (1 mM) used as the CO_2 source. The activity of FaldDH (0.1 g L^{-1}) was measured at 25 °C in 100 μL of 96-well plates with a 0.1 M phosphate buffer solution, NADH (1 mM) and $HCOO^-$ (2 mM) as substrates. The activity of YADH (0.01 g L^{-1}) was measured at 25 °C in 100 μL of 96-well plates with a 0.1 M phosphate buffer solution, NADH (1 mM) and HCHO (3 mM) as substrates. The reactions were carried out in a 0.6 mL Eppendorf tube placed in an incubator at 37 °C with stirring at 250 rpm, for 72 h under a CO_2 atmosphere.

To determine the optimal FaldDH/FateDH ratio, different solutions were prepared using a fixed FateDH concentration (0.1 g L^{-1}) and a variable FaldDH concentration (from 0 to 1.5 g L^{-1}) in a pH 6.5 buffer solution (KH_2PO_4–K_2HPO_4 at 0.1 M) with the following amounts of cofactor and substrate: cofactor [NADH] = 10 mM and [$KHCO_3$] = 100 mM. To determine the best YADH/FaldDH ratio, different solutions were prepared using a fixed FaldDH concentration (0.1 g L^{-1}) and a variable YADH concentration (from 0 to 1 g L^{-1}) in a pH 6.5 buffer solution (KH_2PO_4–K_2HPO_4 at 0.1 M) with the following amounts of cofactor and substrate: cofactor [NADH] = 10 mM and [$HCOO^-$] = 100 mM. The reactions were carried out in a 0.6 mL Eppendorf tube placed in an incubator at 37 °C with stirring at 250 rpm for 22 h under a N_2 atmosphere.

To investigate the NADH recycling system, two enzymes were tested: the phosphite dehydrogenase (PTDH, 10 mg L^{-1}) using phosphite (Na_2HPO_3, 0.5 M) as a substrate and the glycerol dehydrogenase (GlyDH, 10 mg L^{-1}) using glycerol (0.5 M) as a substrate. The two mixtures were incubated in potassium phosphate buffer (0.1 M) with pH varying from 4.5 to 9.3. The reaction took place in a microplate spectrophotometer at 37 °C after adding the oxidized form of the cofactor NAD^+ (1 mM). Another NADH regenerating system was studied, a natural photosystem of chloroplast suspension prepared from spinach leaves as described by Berthold *et al.*[24] A solution containing a chloroplast suspension (0.3 $g_{chlorophylls}$ L^{-1}) in a 0.1 M phosphate buffer solution pH 6 was activated using a 40 W Neon desk lamp in the presence of the oxidized form of the cofactor NAD^+ (1 mM). The NADH regenerating capacity of the 3 systems was followed by monitoring the appearance of an NADH band at the absorbance of 340 nm by UV-Vis spectroscopy.

2.2.3. Activity of the polyenzymatic system used to reduce CO_2 to methanol. The 3 enzymes, FateDH, FaldH and YADH in optimized ratios of commercial powder (0.01, 0.15 and 0.75 g L^{-1}, respectively) were incubated in phosphate buffer solution (0.05 M, pH 6.5) containing $KHCO_3$ (0.05 M) and NADH with a variable concentration from 0 to 0.2 M to identify the most suitable ratio of NADH needed for the reaction. The Eppendorfs used for the enzymatic reaction were flushed with CO_2 prior to incubation at 37 °C for 65 hours. The optimization of the amount of regenerating system (PTDH) was investigated by adding NADH (0.01 M), Na_2HPO_3 (0.05 M) and a variable amount of PTDH from 0 to 6.1 g L^{-1} of pure enzyme. The influence of CO_2 pressure to increase CO_2 gaseous concentration in the aqueous reaction (Fig. S1, ESI†) was investigated for 3 h at 37 °C under 0.5 MPa (5 bar) of CO_2 by using the optimized ratio of the polyenzymatic system and NADH: FateDH, FaldDH, and YADH (0.01, 0.15, and 0.75 g L^{-1}, of commercial enzymatic powder, respectively) and PTDH (3.5 g L^{-1} of pure enzyme) in a phosphate buffer solution (0.05 M, pH 6.5) containing NADH (0.1 M).

2.3. Enzymes encapsulation in silica nanocapsules (NPS) and bioactivity

Silica nanocapsules (NPS) were synthesized as reported by Galarneau *et al.*[21] in a 1.5 mL Eppendorf tube. An organic solution of lecithin (0.1 g), dodecylamine (7.5 mg) and ethanol (0.290 mL) was first prepared. An aqueous solution was prepared containing 0.15 mL of potassium phosphate buffer (0.1 M, pH 6.5), β-D-lactose (1.25 mg), NADH (0.03 mM) and the optimized polyenzymatic system FateDH (0.05 g L^{-1}), FaldDH (0.75 g L^{-1}), and YADH (3.75 g L^{-1}). Another aqueous solution was also prepared by adding the NADH regenerating enzyme PTDH in the following ratio: FateDH (0.0066 g L^{-1}), FaldDH (0.10 g L^{-1}), YADH (0.50 g L^{-1}) of enzymatic commercial powder and PTDH (2.31 g L^{-1}) of pure enzyme. A volume of 43 μL of the organic solution was stirred magnetically at 1300 rpm at 37 °C and then 150 μL of the aqueous solution was added dropwise. After complete addition and 3 min stirring, a homogeneous emulsion was obtained. Then TEOS (25 μL) was added dropwise always under magnetic stirring at 1300 rpm and homogenized for 60 minutes until the silica gelification occurs and prevents the magnetic stirrer to rotate. The mixture was then aged in a static environment for 22 h at 37 °C. A volume of 0.5 mL of potassium buffer (0.1 M, pH 7) was added to the resulting gel and was then centrifuged for 30 min at 4 °C and 10000 rpm. Then the supernatant was filtered using 0.2 μm filters and passed through a 10 mL Sephadex G25 column to separate enzymes from chemicals used for the synthesis. The Bradford assay was used to determine the protein concentration in the supernatant. The washing step was repeated 4 more times. After washing the NPS material was dried under vacuum with P_2O_5 for 72 h at 20 °C. The resulting as-synthesized NPS powders were stored at 4 °C.

2.4. Characterization

2.4.1. Reaction product characterization. Methanol amount was determined using a Varian 3900 gas chromatograph with a polar column (DB 52-CB column 25 × 0.32 × 0.25) and a FID detector by using 1-pentanol as internal standard. The injector was set at 220 °C with a column temperature of 55 °C for 3 min, then from 55 °C to 220 °C at 20 °C min^{-1} under a hydrogen flow of 1.2 mL min^{-1}. The N_2 flux was fixed based on the Van Deemter curve of the methanol chromatographic peak. Chromatograms show the separation of methanol from formaldehyde (Fig. S2, ESI†). The quantification of formaldehyde by gas chromatography was inefficient because of its

low coefficient response with a FID detector, that is why the Nash method[25] was preferred.

2.4.2. Silica nanocapsule (NPS) characterization. XRD patterns were recorded using a Bruker D8 Advance diffractometer with Bragg–Brentano geometry and equipped with a Bruker Lynx Eye detector. XRD patterns were recorded in the range 0.5–6° (2θ) with an angular step size of 0.0197° and a counting time of 0.2 s per step. SEM images were obtained using a Hitachi S4800 microscope. Thermogravimetric analyses (TGA) were performed using a Perkin Elmer STA6000 thermogravimeter. The materials were heated in a N_2-flow at 900 °C with a temperature ramp of 5 °C min^{-1}. Textural properties of the as-synthesized nanocapsules were determined by N_2 adsorption–desorption at 77 K on a Micromeritics Tristar 3000 apparatus. Samples were previously outgassed in a vacuum at 50 °C for 12 h. To characterize their particle size, the silica nanocapsules were first sonicated for 30 min in water before performing size measurement on a Zeta sizer Nano ZS90 instrument.

The electron tomography experiments were carried out on a JEOL 2100F transmission electron microscope (TEM) with a field emission gun operating at 200 kV, equipped with a probe corrector and a GATAN Tridiem energy filter. Prior to observation, the silica nanocapsules were dispersed in deionized water and then sonicated for several minutes. Up to 5 droplets were further deposited onto a copper grid covered by a holey carbon membrane rendered hydrophobic by H_2/Ar plasma cleaning. Afterwards, the specimen was plunged into liquid ethane and mounted on the cryo holder. The latest manipulations were carried out in liquid nitrogen (77 K) in a similar manner as for the biological specimens. Even so, the specimen morphology considerably changed under the electron beam. One should keep in mind as well that during the acquisition of a complete tomography series the specimen is to be exposed to the electron flux for durations of one hour or more. Therefore, we have tested several representative regions in terms of electron beam induced damage, by employing different electron doses and irradiation durations. Once these conditions were established, two tomography series were acquired by tilting the specimen over a range of ±60°, with an image recorded for every 2°. The images were aligned by using the cross-correlation algorithm implemented in the IMODod software. The reconstructions have been computed using 10 iterations within the algorithms based on algebraic reconstruction techniques (ART) implemented in the TOMOJ software. The visualization and quantitative analysis of the final volumes have been done by using the ImageJ software. The statistic distribution of the particle sizes has been built up by direct measurement of 50 nanocapsules observed in the 2D slices extracted from reconstructed volume.

3. Results and discussion

3.1. Optimization of the polyenzymatic system FateDH–FaldDH–YADH

Formate dehydrogenase (FateDH) from *Candida boidinii*, formaldehyde dehydrogenase (FaldDH) from *Pseudomonas putida* and alcohol dehydrogenase (YADH) from *Saccharomyces cerevisiae* are the enzymes originally used for the overall enzymatic conversion of CO_2 to methanol.[7] However, to the best of our knowledge, no systematic study has been performed to determine the amount of active enzyme contained in the commercial enzymatic powders and the optimal conditions of reaction (pH and the relative amount of each enzyme in the polyenzymatic system) to run efficiently the cascade reaction. Here we report a systematic study of the combination of the three different enzymes to get an effective catalytic system. Firstly we investigated the enzyme activity under standard conditions to determine the amount of active enzymes in the commercial powder. In terms of protein or enzyme amount, we found that 160 mg, 140 mg and 630 mg of protein per g of commercial powders were effectively present in FateDH, FaldDH and YADH commercial enzymatic powders, respectively. This confirms that enzyme suppliers add a lot of additives (*e.g.* sucrose, polyethylene glycol, NaCl) before lyophilization to stabilize the enzymes into their commercial forms and therefore the exact amount of active enzyme has to be determined for each batch. The content of active enzymes may vary from batch to batch of commercial enzymatic powders and rather than mass, one should use the activity expressed in Unit (U: micromoles of product formed per min) to be accurate. For the enzymatic commercial powders we used, we found specifics activities of 55 U mg_{FateDH}^{-1}, 14 U mg_{FaldDH}^{-1} and 2300 U mg_{YADH}^{-1} expressed by mg of commercial enzymatic powder (corresponding to a Unit ratio of 1/3.8/3136 U/U/U for FateDH, FaldDH and YADH, respectively). However all of the following weights of commercial enzymatic powder described in this paper should be recalculated from the standard units when a new commercial enzymatic powder is used. The activity of the 3 enzymes in the reductive reactions (Scheme 1) was studied at different pH to determine the optimum pH for the cascade reaction. FateDH and FaldDH cannot reduce CO_2 and $HCOO^-$, respectively, at pH > 8.5 (Fig. 1). The overall reduction of CO_2 to methanol should be carried out at pH values between 6 and 7 to allow at least 80% efficiency of each enzyme, this is concordant with the literature.[26] It appears that highest activities for all the enzymes were obtained at pH 6.5 (Fig. 1). As CO_2 bubbled into water solution gives immediately an equilibrium between soluble gaseous CO_2

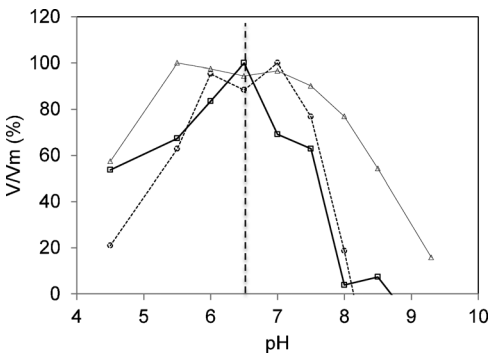


Fig. 1 Relative activity as a function of pH for the 3 enzymes FateDH, (○), FaldDH (□) and YADH (Δ) in 0.1 M potassium phosphate buffer at 25 °C for their specific reductive reactions.

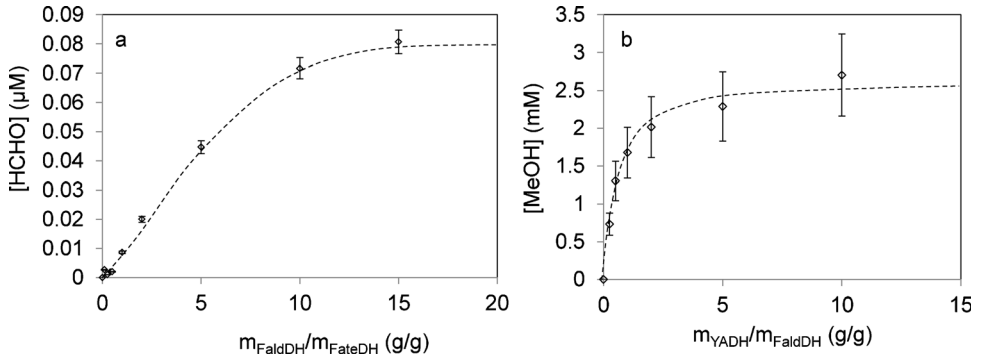


Fig. 2 (a) Influence of the relative amount of FateDH and FaldDH for the conversion of CO_2(g) into formaldehyde at a fixed concentration of FateDH (0.1 g L^{-1}) (72 h with $KHCO_3$ (100 mM) as the substrate). (b) Influence of the relative amount of FaldDH and YADH for the conversion of formate into methanol at a fixed concentration of FaldDH (0.1 g L^{-1}) (22 h with $HCOO^-$ (100 mM) as the substrate). All the reactions were carried out in phosphate buffer (0.1 M, pH 6.5) at 37 °C under a saturated N_2 atmosphere with NADH (10 mM).

(CO_2(g)) and hydrogen-carbonate, with a proportion of 45% of CO_2(g) and 55% of HCO_3^- at pH 6.5, we added $KHCO_3$ instead of bubbling CO_2 for a better reproducibility of the reaction (Fig. S1, ESI†). The affinity constant of FateDH and FaldDH for CO_2 is nearly two orders of magnitude lower than for $HCOO^-$. The Michaelis constants found for the oxidative and reductive reactions of FateDH are K_{mCO_2} = 30–50 mM ≫ K_{mHCOO-} = 0.5 mM,[23,27] and for FaldDH K_{mHCOO-} has never been described in the literature and K_{mHCHO} = 0.09 mM.[28] However the enzymes can be forced to catalyze their unfavorable reduction reaction by adding an excess of the NADH cofactor ($NADH/NAD^+$ molar ratio ≥ 2000).[29] Moreover care should be taken to measure the activity of the enzymes, which has been usually done by following the disappearance of the NADH band in the UV-Vis spectra at 340 nm, as we found that NADH interacts with CO_2 by most probably forming a complex between NH_2 groups of NADH and CO_2 (as CO_2 is very well known to interact preferentially with amines),[30] which displaces the band at 340 nm (Fig. S3, ESI†). This complexation artificially increases the rate of the reaction. However another UV-Vis band located at 290 nm characteristic of the probable NADH–CO_2 complex could be used to follow the consumption of NADH. To be more accurate another analytic method (gas chromatography or the Nash reagent) should be preferred for this kind of reaction when using at the same time NADH and CO_2. In order to displace the reaction in favor of the reduction, the addition of an excess of the second enzyme in the bienzymatic system is necessary. For the FaldDH–FateDH bienzymatic system, an optimum activity corresponding to a productivity of 60 μM formaldehyde in 72 h was reached for an excess of 15 g L^{-1} of FaldDH for 1 g L^{-1} of FateDH (Fig. 2a). The transformation of formaldehyde into methanol is more favorable as methanol is not a suitable substrate for YADH (K_{mMeOH} = 130 mM). The equilibrium between MeOH and HCHO is therefore naturally displaced toward the production of MeOH. The optimization of the bienzymatic system YADH–FaldDH has been performed. An excess of 5 g L^{-1} of YADH for 1 g L^{-1} of FaldDH was necessary to get the highest activity corresponding to a productivity of 2.3 mM methanol in 22 h from formate ($HCOO^-$) (Fig. 2b). The optimum ratio of the three polyenzymatic systems FateDH, FaldH and YADH is therefore 0.01, 0.15 and 0.75 g L^{-1} of commercial enzymatic powder, respectively (the conversion factor between commercial enzymatic powder and pure enzymes is 0.16, 0.14 and 0.63 $g_{pure\ enzyme}\ g_{commercial\ enzymatic\ powder}^{-1}$ for FateDH, FaldDH and YADH, respectively). However, this optimum ratio differs from the weight ratio previously used in the literature for this polyenzymatic system, where for example the same mass of each commercial powder was used.[7,17,31] The reduction of CO_2 into methanol was performed in the previous experiments in the presence of a concentration of NADH of 10 mM. We found that the optimum concentration of NADH for this cascade reaction was 100 mM (Fig. 3) and corresponds to a productivity in methanol of 0.05 mM in 65 h.

3.2. NADH cofactor regeneration systems

NADH is an expensive cofactor, which is consumed stoichiometrically during the enzymatic reaction. Thus, for one molecule of MeOH produced, 3 molecules of NADH will have to be consumed, rendering the whole process prohibitive without recycling.[7] Interestingly, a photosystem PSII was attempted giving no by-products except O_2 produced from H_2O.[17] The authors claimed that the sole

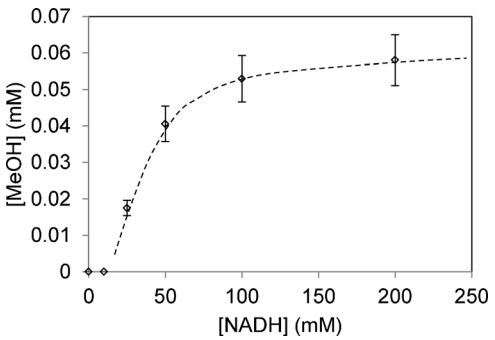
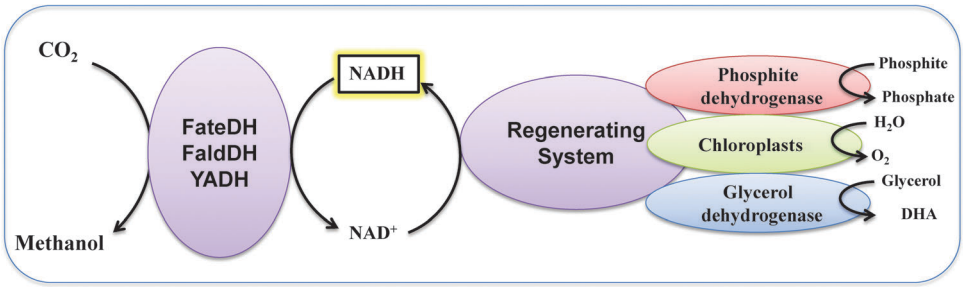


Fig. 3 Influence of initial NADH concentration for CO_2 conversion into methanol with the optimized amount of the trienzymatic system: FateDH, FaldDH, and YADH of 0.01, 0.15, and 0.75 g L^{-1}, respectively (65 h at 37 °C in a phosphate buffer (0.05 M, pH 6.5) with $KHCO_3$ (0.05 M) as the substrate).



Scheme 2 Schematic representation of CO_2 valorization into methanol using different NADH regenerating systems.

photosystem PSII was able to regenerate NADH with sunlight. However the real photosystem able to regenerate NADH is more complicated. The active photosystem is in fact a chloroplast suspension containing five different proteins, which are present on thylakoid membranes within the cell and can be extracted from spinach leaves.[32,33] Several light harvesting complexes, an oxygen evolving complex, two photosystems (PSI and PSII), a plastoquinone pool, cytochrome bf6 complex and a ferrodoxin reductase are required. The plastoquinone transfers electrons from one photosystem to the other and the two essential macromolecular proteins are carrying organometallic complexes bounded on each photosystem. The first protein bounded to PSII contains an oxygen evolving complex (OEC), which can extract electrons from water and the second protein called ferrodoxin, bounded to PSI allows the cofactor regeneration with the concomitant use of a ferrodoxin reductase enzyme (EC 1-18-1-2).[33,34] In our study, we choose to compare this complex photosystem with two enzymes (Scheme 2): the phosphite dehydrogenase (PTDH), one of the most efficient enzymatic recycling systems reported so far (together with formate dehydrogenase[12,13] but already used here in the opposite direction) which converts phosphite into phosphate, a chemical used to buffer the solution, and the glycerol dehydrogenase (GlyDH), which transforms glycerol into dihydroxyacetone (DHA, a tan cosmetics). Glycerol is known to stabilize enzymes and could also help to solubilize gaseous CO_2 into water as CO_2 is 11 times more soluble in glycerol than in water (0.36 mol L^{-1} at 25 °C).[35] The pH dependency of the two regenerating enzymes has been investigated (Fig. 4a). PTDH has an optimum activity at $6 < pH < 8$ and GlyDH is more active at pH 8 to 9. GlyDH presents only 20% of its activity at pH 6.5. Therefore, under the catalytic conditions needed for the CO_2 reduction into methanol (pH 6.5, 37 °C) PTDH is 4 times more active than GlyDH (Fig. 4b). For the chloroplast photosystem, only 0.25 mM NADH was obtained in 30 minutes, which is below the NADH regenerating activity of PTDH. The low activity of the chloroplast could be explained by the poor stability of chloroplasts with time in water and the oxidation of the NADH cofactor induced by the production of oxygen from the parallel reaction. An increase in the natural photosystem stability could be achieved by immobilization of the photosystem and/or the thylakoid membrane into a silica host.[17] Recently, some stable inorganic photosystems based on hierarchical nanostructured carbon nitride materials (g-C_3N_4) prepared by the Antonietti group[11] have shown some efficiency to recycle NADH, but this system was active at pH higher than 8 and formed by-products (oxidized triethanolamine). To be efficient at pH 7, a mediator ([CpRh(bpy)(H_2O)]) should be added, which is known to be an inhibitor of some enzymes.[36] Even if photosystems are very attractive, it is obvious that at this point, the most suitable system for NADH regeneration is based on phosphite dehydrogenase PTDH plus phosphite.

3.3. Reduction of CO_2 to methanol by the optimized polyenzymatic system (FateDH, FaldDH, YADH, and PTDH) free in solution

The regenerating system composed of PTDH plus phosphite was added in different amounts to the optimized polyenzymatic system of FateDH, FaldDH and YADH in the presence of an

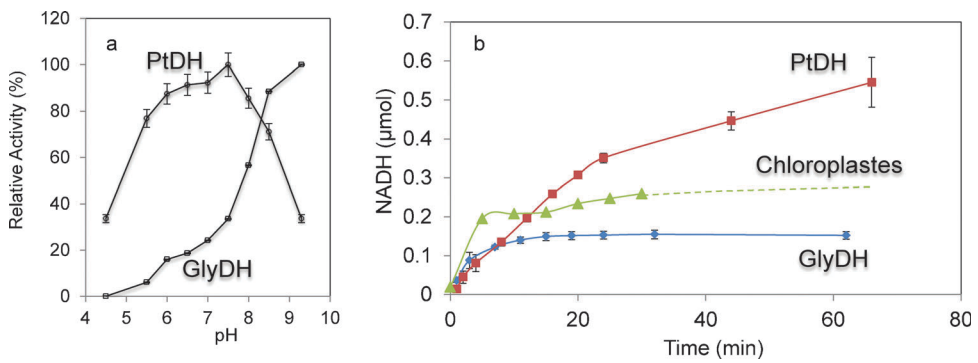


Fig. 4 (a) Relative activity of NADH formation from NAD^+ with PTDH (○) and GlyDH (□) as a function of pH in 0.1 M potassium phosphate buffer at 37 °C at saturated substrate concentration ([Na_2HPO_3] or [Glycerol] = 0.5 M). At pH 6.5, V_{PTDH} = 243 U $mg_{pure\ enzyme}^{-1}$ and V_{GlyDH} = 42 U $mg_{pure\ enzyme}^{-1}$; U = mmol min^{-1}. (b) NADH production from 5 mmol NAD^+ for the two regenerating enzymes (pH 7, 18 °C) corresponding to 50 mmol NAD^+ per $g_{pure\ enzyme}$ and for the photosystem to 3.3 mmol NAD^+ per g of equivalent chlorophylls at pH 6.

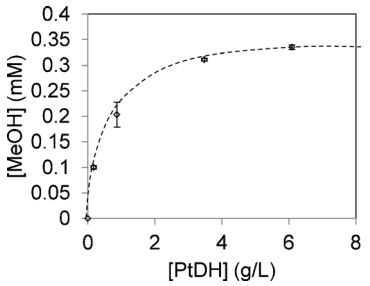


Fig. 5 Influence of phosphite dehydrogenase (PTDH) amount for CO_2 conversion into methanol with the optimized trienzymatic system: FateDH, FaldDH, and YADH of 0.01, 0.15, and 0.75 g L^{-1}, respectively. Reactions were carried out for 65 h at 37 °C in phosphate buffer (0.05 M, pH 6.5) with $KHCO_3$ (0.05 M), NADH (10 mM) and Na_2HPO_3 (0.05 M).

initial amount of 10 mM NADH. The conversion of CO_2 into methanol reaches a plateau of highest methanol productivity for a PTDH content of 3.5 g L^{-1} (Fig. 5). The optimized composition of the polyenzymatic system is: FateDH, FaldDH, YADH, and PTDH of 0.01, 0.15, and 0.75 g L^{-1} of commercial enzymatic powder, respectively, and 3.5 g L^{-1} of pure PTDH. The most suitable NADH concentration was then investigated. In the polyenzymatic system without a NADH regenerating system, the maximum conversion was obtained for 100 mM NADH (Fig. 3). In the presence of PTDH (Fig. 6), an excess of NADH increases drastically the rate of reaction (Fig. 6a) and the same optimum 100 mM NADH was found (Fig. 6b). However under these conditions, the reaction ends after 48 h. Since formate and formaldehyde were not detected, the accumulation of methanol is likely the reason for the process to be inactivated. The process could be therefore improved if continuous removal of methanol could be performed.

3.4. Encapsulation of the polyenzymatic system in phospholipid–silica nanocapsules (NPS) and their bioactivity

Previous studies by Dave and co-workers[7,17] and Jiang and co-workers[8,19] have demonstrated the importance to immobilize the polyenzymatic system FateDH, FaldDH and YADH in inorganic supports as immobilization increases the conversion of CO_2 to methanol in comparison to free polyenzymatic systems. This activity enhancement could arise from a better stability of the enzymes or a better conformation of the enzymes inside the inorganic support or from a better adsorption of CO_2(g) around the inorganic particles and inside the pores increasing the availability of CO_2(g) for the enzymes. Dave[17] used a classical silica sol–gel encapsulation procedure and Jiang and co-workers[8] found that titania oxide nanoparticles templated by protamine were most suited for CO_2 conversion. On our side, we have developed for several years a new way of enzyme encapsulation using a modified silica sol–gel procedure combined with a double protection of the enzymes by the phospholipid bilayer and lactose to avoid direct contact with the silica. This encapsulation process has led to high activity for several enzymes (lipases, GOx, HRP, and Hb) and to the discovery of phospholipid–silica nanocapsules, which were very efficient for bienzymatic systems encapsulation (GOx–HRP, GOx–Hb).[20,21,37–40] The nanocapsule formation is explained by, first the stacking of adjacent phospholipid bilayers and then the hemifusion of the bilayers to form interconnected phospholipid nanocapsules. After TEOS addition, a silica shell is formed around the nanocapsules (Fig. 7). Electron tomography image slices with thicknesses of 0.7 nm extracted from reconstructed volume (Fig. 7) reveal diameters of nanocapsules between 20 and 40 nm, which are interconnected by phospholipid bilayer bridges. The silica shell thickness is between 3 and 5 nm around the nanocapsules and the silica shell thickness decreases at the vicinity of the interconnection and then vanishes. The phospholipid thickness inside the silica nanocapsules is estimated between 3 and 6 nm. In the SEM image, NPS appear as an aggregation of nanoparticles (Fig. S5, ESI†) and the nitrogen adsorption isotherm shows an interparticular porosity of *ca.* 20 nm (Fig. S6, ESI†). After sonication in water, DLS measurements show that NPS have an average size of 30 nm (Fig. 8) in accordance with the statistic size of the nanocapsules determined from the tomography analysis, meaning that the phospholipid interconnections

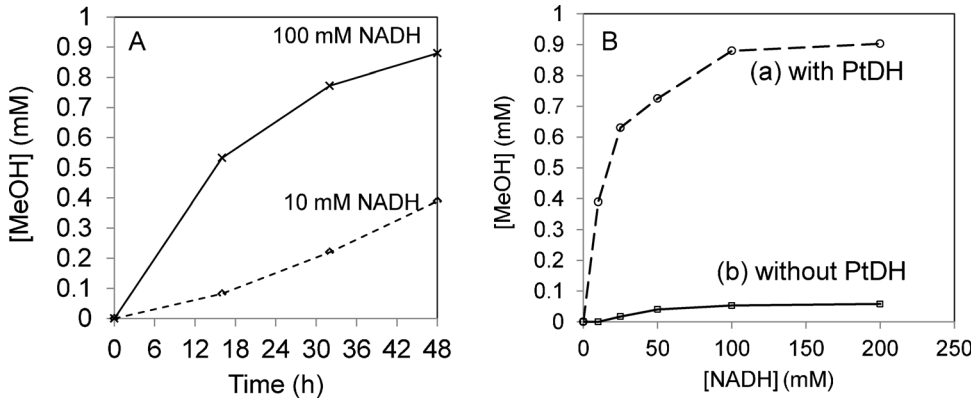


Fig. 6 (A) Methanol production as a function of time for two different initial NADH amounts: 10 mM and 100 mM, for the polyenzymatic system: FateDH/FaldDH/YADH: 0.01/0.15/0.75 g L^{-1} of commercial enzymatic powder and 3.5 g L^{-1} of pure PTDH with Na_2HPO_3 (0.05 M). (B) Methanol production as a function of initial NADH amounts: (a) for the system: FateDH/FaldDH/YADH: 0.01/0.15/0.75 g L^{-1} of commercial enzymatic powder and 3.5 g L^{-1} of pure PTDH, after 48 h of reactions using Na_2HPO_3 (0.05 M); (b) after 65 h of reaction. All the reactions were carried out at 37 °C in phosphate buffer (0.05 M, pH 6.5) with $KHCO_3$ (0.05 M).

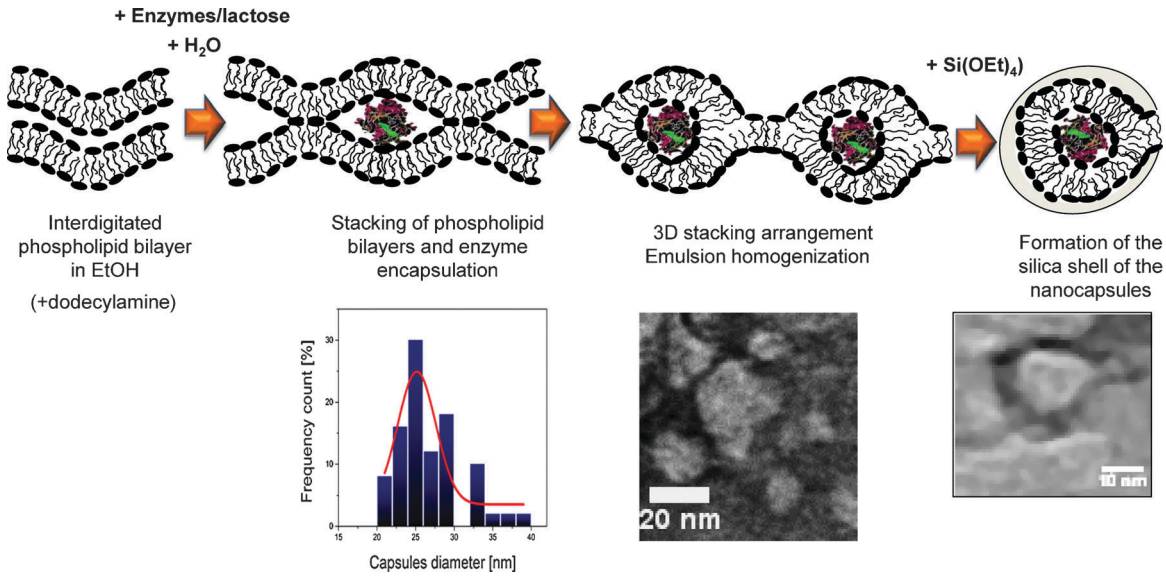


Fig. 7 (up) Proposed mechanisms for the formation of phospholipids-templated silica nanocapsule (NPS) enzymes. (down) Electron tomography: slices redrawn from the reconstructed volume of FateDH–FaldDH–YADH phospholipid–silica nanocapsules (NPS) and the observed statistic size distribution of the nanocapsules as identified from the sections within the volume.

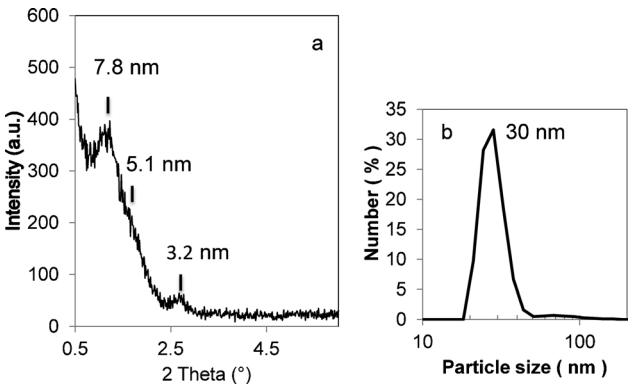


Fig. 8 (a) XRD of FateDH–FaldDH–YADH phospholipid–silica nanocapsules (NPS) and (b) the particle size distribution of NPS by DLS after sonication.

between nanocapsules are fragile and can be disconnected. By XRD a characteristic distance at *ca.* 8 nm, most probably corresponding to the repeated distance of phospholipid bilayer and silica shell, would correspond to a phospholipid bilayer thickness of 4 nm if an average of 4 nm for the silica shell thickness is taken, in accordance with tomography observation. Some characteristic distances at about 5.1 and 3.2 nm are detected and remain to be understood, which could result from the impurities present in the lecithin.

The procedure was applied to encapsulate the optimized polyenzymatic system FateDH, FaldDH and YADH in the phospholipid–silica nanocapsules (NPS). TGA (Fig. S4, ESI†) shows that NPS contains 470 mg of organics (mainly phospholipids) per gram of NPS. The Bradford assay used to quantify the enzymes in the supernatant showed no significant absorbance at 595 nm after enzyme separation on a Sephadex-G25 exclusion column. All enzymes used in the synthesis were therefore encapsulated in the NPS. The loading of enzymes in NPS containing the three dehydrogenases without the regenerating system was 27.5 $mg_{pure\ enzymes}\ g_{NPS}^{-1}$. The encapsulation of the 3 dehydrogenases plus the regenerating enzyme PTDH has been performed and the resulting NPS nanocapsules contain 43.9 $mg_{pure\ enzymes}\ g_{NPS}^{-1}$. The catalytic activity of the polyenzymatic system, used in the presence or absence of the regenerating enzyme PTDH, is reported in Fig. 9. Addition of the regenerating system increases in all cases the productivity in MeOH by a factor of 2 for the free enzymes or a factor of 5 in the case of encapsulated enzymes. The encapsulation in NPS, in the presence or absence of the regenerating enzyme PTDH, increases the bioactivity of the polyenzymatic system by a factor of 10 or 27, respectively. The highest productivity in methanol was obtained for the polyenzymatic

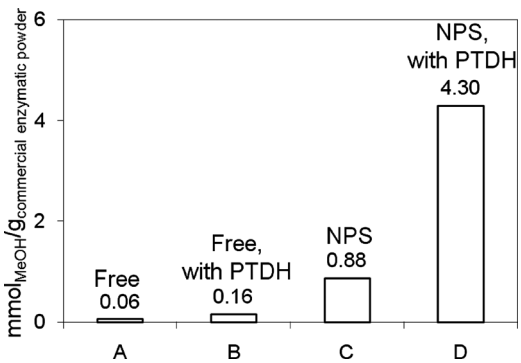


Fig. 9 Production of methanol from CO_2 after 3 h per mass of total commercial enzymatic powders for FateDH, FaldDH, YADH trienzymatic system (0.01, 0.15, 0.75 g L^{-1}, respectively) at 5 bar. (A) Free enzymes; (B) free enzymes with PTDH addition (3.5 g L^{-1} of pure enzyme) to regenerate NADH; (C) enzymes encapsulated in NPS, without PTDH; (D) enzymes and PTDH (3.5 g L^{-1} of pure enzyme) encapsulated in NPS. Conditions: phosphate buffer (0.05 M, pH 6.5), NADH (0.1 M) and Na_2HPO_3 (0.05 M) when PTDH is added.

system plus the regenerating enzyme PTDH encapsulated in NPS and leads to a productivity in MeOH of 42 μmol g_{NPS}^{-1} corresponding to 4.3 mmol $g_{\text{commercial enzymatic powder}}^{-1}$ in 3 h under 5 bar pressure.

These results were compared to those reported earlier by other groups. In terms of process, Dave[17] and Jiang and co-workers[19] used a continuous bubbling of CO_2 into the reaction vessel. Such a design allows to increase CO_2 availability in water, but necessitates high recycle rates to get significant CO_2 reduction yields and costs in the case of use of purified carbon dioxide. CO_2 bubbling was not possible to achieve in our case for the free polyenzymatic system because a dense foam formed in the presence of YADH, flushing away the solution from the reactor. We therefore used a static process with a fixed initial CO_2 amount. Jiang and coworkers[8,19,41] have also tried bubbling under a pressure of 3 bar for a silica sol–gel encapsulation and of 5 bar for alginate encapsulation. However the best results they report in terms of activity were obtained for a polyenzymatic system encapsulated in titanate/protamine particles with a constant bubbling of CO_2 at 1 bar. These authors demonstrated that the type of encapsulation is more important than pressure. In Fig. 10, we compared our results with those reported by the above two groups. Jiang and coworkers[8] in the absence of a regenerating system with the polyenzymatic system encapsulated in protamine templated titanate particles obtained a methanol yield of 0.83 $mmol_{MeOH}$ $g_{\text{commercial enzymatic powder}}^{-1}$ close to that reached with the NPS encapsulated system in the absence of NADH regeneration (0.88 $mmol_{MeOH}$ $g_{\text{commercial enzymatic powder}}^{-1}$). Dave,[17] using a regenerating system composed of a 'PSII' suspension, obtained a MeOH yield of 3.3 $mmol_{MeOH}$ $g_{\text{commercial enzymatic powder}}^{-1}$ slightly lower than the 4.3 $mmol_{MeOH}$ $g_{\text{commercial enzymatic powder}}^{-1}$ obtained using NPS with PTDH as the NADH regenerating system. In terms of CO_2 consumed, however, the productivity achieved in the static system under 5 bar pressure is nearly three orders of magnitude higher than in the open system with CO_2 bubbling.

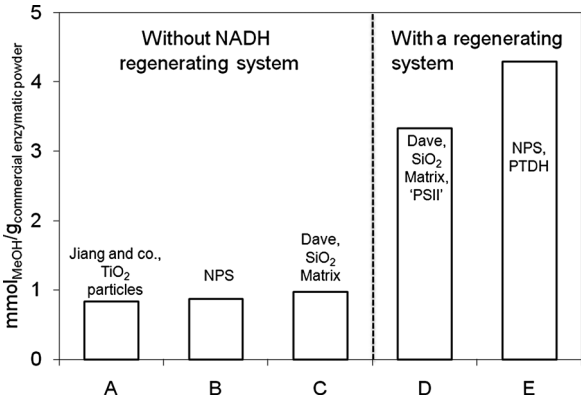


Fig. 10 Comparison with literature results of the production of methanol from CO_2 after 3 h per mass of total commercial enzymatic powder for FateDH, FaldDH, YADH trienzymatic systems encapsulated with and without a NADH regenerating system. (A) Jiang and co-workers:[18] FateDH/FaldDH/YADH (1.35, 1.35, 0.3 g L^{-1}) encapsulated in protamine templated-TiO_2 particles and continuous bubbling of CO_2; (B) enzymes encapsulated in NPS (this work) at 5 bar of CO_2; (C) Dave:[17] FateDH/FaldDH/YADH (5, 5, 5 g L^{-1}) encapsulated in silica sol–gel and continuous bubbling of CO_2; (D) Dave:[17] FateDH/FaldDH/YADH (5, 5, 5 g L^{-1}) and a regenerating system (PSII) encapsulated in silica sol–gel and continuous bubbling of CO_2; (E) enzymes and PTDH regeneration system encapsulated in NPS (this work) at 5 bar. Our conditions: phosphate buffer (0.05 M, pH 6.5), NADH (0.1 M) and with Na_2HPO_3 (0.05 M) when PTDH is added, FateDH/FaldDH/YADH: (0.01, 0.15, 0.75 g L^{-1}) (g of commercial enzymatic powder) and PTDH of 3.5 g L^{-1} (g of pure enzyme).

4. Conclusions

The biocatalytic conversion of CO_2 into methanol was investigated using a cascade reaction of 3 dehydrogenases (formate dehydrogenase (FateDH), formaldehyde dehydrogenase (FaldDH), and alcohol dehydrogenase (YADH)). To avoid any accumulation of the different successive products, which can denature the other enzymes, the rate of conversion of the third enzyme has to be higher than the rate of conversion of the second one, which has to be higher than the rate of conversion of the first one. The optimized ratio for this polyenzymatic system FateDH, FaldDH, and YADH was 0.01, 0.15, and 0.75 g (commercial enzymatic powder) per L or to be more precise if different batches or suppliers are used a composition corresponding to a ratio of 1, 4.4, and 790 Units per L. Units (μmol min^{-1}) have been calculated for each commercial powder by defined standard assays. Different regeneration systems of the NADH cofactor, among which enzymes and photosystems, have been studied and the most efficient system was an enzyme, the phosphite dehydrogenase (PTDH) producing phosphate from phosphite as side reaction, which is very convenient in a phosphate buffer medium. Furthermore PTDH is very active at the optimum pH of the reaction (pH 6.5). Adding an efficient regenerating system allows us to increase the activity of the polyenzymatic system by a factor of 5. However the most important improvement comes from the immobilization of the polyenzymatic system into an inorganic matrix. By using an efficient enzymatic encapsulation host such as phospholipids–silica nanocapsules (NPS), the activity of the polyenzymatic system was increased by a factor close to 30, leading to a productivity in methanol (in static at 5 bar for 3 h) of 4.3 mmol $g_{\text{commercial enzymatic powder}}^{-1}$ equivalent to what was previously reported using a continuous CO_2 bubbling. The productivity in methanol per mass of catalyst corresponds to 42 μmol methanol produced per g_{NPS} in 3 h. The reaction rate decreases after some time, due most probably to the accumulation of methanol. To improve this biocatalytic conversion process, continuous flow catalysis should be performed to extract methanol in order to avoid the denaturing effect of methanol on the enzymes. The limitation associated with the low solubility of CO_2 in water should be overcome by using other media than water, such as ionic liquids, glycerol, polyethylene glycol, supercritical CO_2 or to run the bioconversion in a continuous gas phase.

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