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Immobilization of Carbonic Anhydrase for Biomimetic CO_2 Capture

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Novel post-combustion treatments include carbon capture and sequestration processes based on biomimetic strategies. These strategies include CO_2 absorption into aqueous solution assisted by enzyme catalysis. Carbonic anhydrase catalyses CO_2 hydration and it has been proposed as industrial biocatalyst for Carbon Capture and Storage (CCS) post-combustion processes. The recombinant enzyme SspCA, isolated from the thermophile bacterium *Sulfurhydrogenibium* sp. was characterized as potential biocatalyst for CO_2 capture processes based on regenerative absorption into alkaline solutions.
This paper reports results of a preliminary study focused on the immobilization of carbonic anhydrase on granular solids to improve biocatalyst stability at the typical operating conditions of the CO_2 absorption processes. This study included the selection of solid supports and of the immobilization technique. Granular fine silica particles were adopted as enzyme carriers. Two classes of solids were investigated: 120 and 9 μm d50 diameter. Bovine carbonic anhydrase was used as enzyme model in order to optimize immobilization procedure and activity assay for immobilized carbonic anhydrase. Enzyme-carrier covalent bonding was adopted as immobilization technique. In particular, solids were silanized and activated with respect to the enzyme by means of glutaraldehyde branches.
For 120 μm particles, the maximum enzyme loading resulted 40±3 mg of bovine CA per g of solids and the maximum yield resulted about 66±5% for initial CA concentration between 1.4 and 4 mg/mL.

1. Introduction

Carbon Capture and Storage (CCS) treatments aimed at the capture of carbon dioxide in flue gases can be adopted as last step of post-combustion treatment train. Carbon dioxide absorption in alkanolamine aqueous solutions is a quite well established process and it is proposed as the benchmark of CCS post-combustion treatments (Metz et al., 2005; Wang et al., 2011). The process is based on a regenerative absorption aimed at the recovery of CO_2 gaseous streams addressed to storage. Drawbacks of this process are the amine oxidation and the production of toxic volatile compounds, e.g. ammonia. Therefore, CO_2 absorption into alkanolamines would require additional treatments to remove toxic products from both the aqueous and gaseous streams.
An alternative process to CO_2 absorption in alkanolamine solutions has been recently proposed to replace the organic promoter (amines) with an environmentally friendly biocatalyst. The proposed biomimetic strategy is based on the use of carbonic anhydrase (E.C. 4.2.1.1) to increase the CO_2 absorption rate in aqueous solutions (Russo et al., 2013). Carbonic anhydrase (CA) is a ubiquitous enzyme that catalyses the CO_2 hydration reaction (Steiner et al., 1975; Tripp eta al., 2001).
The CO_2 absorbed into aqueous solution may be converted according to both hydration and hydroxylation reaction. The hydration reaction is catalysed by CA and the reaction rate depends on both enzyme form and operating conditions. In particular, the turnover number depends on the class which CA belongs to and it may be as high as the human isoform II (hCA II), whose turnover number is about $1.40 \cdot 10^6$ s^{-1} (Vullo

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et al., 2012). Extensive CO_2 capture by absorption in aqueous phase can be successfully carried out only if alkaline solutions are adopted. Indeed, these solutions are characterized by a satisfactory CO_2 absorption capacity.

The design and optimization of biomimetic CCS processes require CAs forms able to operate under conditions close to those adopted in industrial processes (Lacroix and Larachi, 2008; Russo et al., 2013). The temperature of the absorption unit typically ranges between 40 and 60°C. The temperature of the desorption unit is typically about 100°C but it can be reduced whenever the process is carried out under vacuum conditions (about 0.3 bar) (Chen et al., 2007).

Capasso et al. (2012) reported a recombinant α-CA (SspCA) identified and characterized in the thermophilic bacterium *Sulfurihydrogenibium yellowstonense* sp. YO3AOP1. SspCA was characterized by an exceptional thermal stability: high catalytic activity for the CO_2 hydration reaction was recorded after incubation at 70 °C for several hours. De Luca et al. (2012) reported the effects of aqueous species from nitrogen and sulphur oxides, typically contained in flue gas streams, on SspCA activity. The assessed inhibition constants were in the range of 0.58 – 0.86 mM for the anions NO_2^-, NO_3^- and SO_4^{2-}. The kinetics of SspCA was characterized in terms of first order kinetic coefficient by Russo et al. (2012), as well as long term stability at high temperature. The half-life of the recombinant enzyme was about 53 days at 40°C and about 8 days at 70°C. These results encourage further investigation for the development of an industrial biocatalyst for CO_2 capture assisted by SspCA because the enzyme half-life values seem to be suitable for industrial cycles.

Enzyme stability may be increased by enzyme immobilization on solid supports. Recently, several efforts have been carried out to develop immobilization techniques able to confine carbonic anhydrase on solid phase (Russo et al., 2013). Indeed, this technology has twofold advantage: it is one of the most successful strategies adopted to increase enzyme stability, and to operate enzyme bioreactors under continuous conditions without enzyme lost (Russo et al., 2008).

The present study reports the development of an immobilization procedure for the production of a biocatalyst based on recombinant carbonic anhydrase. Granular silica was selected as carrier and bovine CA was adopted as reference enzyme for the process development. The attention was also focused on the particle size distribution of solid supports because this feature strongly affects absorption enhancement by heterogeneous biocatalysts.

2. Materials and methods

The support for enzyme immobilization (Sipernat® 22, Evonik) was made of silica particles (data from supplier SiO_2 ≥ 97%, d_{50}=120 μm). The solids were sieved in the range 90-150 μm. Particle size distribution was assessed by laser scatter particle size analyzer (Mastersizer 2000, Malvern Instruments). Sipernat® 22 pore size was assessed through mercury porosimetry (Autopore 4, Micromeritics).

Sipernat 22LS (data from supplier SiO_2 ≥ 97%, d_{50}=9 μm) was also characterized as possible fine solids for future enzyme immobilization. Bovine CA was supplied by Sigma Aldrich® as lyophilized powder extracted from bovine erythrocytes. Mono- and di-basic Sodium phosphate (purity≥99.8%) were supplied by BDH Prolabo. 98% Glycine was supplied by AppliChem. Chemicals supplied by Sigma-Aldrich® were: (3-Aminopropyl)triethoxysilane (APTES) (purity≥98%), 25% vol glutaraldehyde grade I, 70% nitric acid, tris(hydroxymethyl)aminomethane (purity≥99.9%), and sulfuric acid (95-98%).

2.1 Immobilization procedure

Sipernat® 22 was activated in 5%vol HNO_3 at 50°C for 5 h, subsequently the solids were repeatedly rinsed with bi-distilled water and dried at 120°C overnight. Activated dry silica particles were stored until use.

The immobilization procedure included the following steps. Typically, 0.2g solids batches were used in 5mL liquid volume:

- Silanization of acid activated silica particles in 4%$_V$ APTES, 0.02M phosphate buffer pH 7.6 at 80°C for 2 h.
- Incubation of silanized particles in 0.5%$_V$ glutaraldehyde, 0.02M phosphate buffer pH 7.6 at room temperature for 2h.
- Bovine CA immobilization on silanized particles in 0.02M phosphate buffer pH 7.6 at 4°C over-night. Initial CA concentration set according to the test schedule.
- Blocking of unreacted glutaraldehyde groups with 0.1M glycine in 0.02M phosphate buffer pH 7 at room temperature for 1.5h

Each step was carried out using rotary shaker at mild mixing speed. Solids were repeatedly rinsed with 0.02M phosphate buffer pH 7 after each step.

Total protein concentration was assessed according to the Bradford assay on CA solution before and after immobilization. The protein content of the recovered fractions of washing buffer was also assayed. The total amount of immobilized enzyme was calculated as the sum of the residual enzyme in the liquid after immobilization step and the enzyme recovered in the washing buffer. Immobilization yield was calculated as the ratio between the total amount of immobilized CA and the initial amount of CA dissolved in liquid phase.

Two types of tests were performed: i) single step immobilization with CA concentration set at 1.4, 2.8, and 4 mg/mL; ii) multi-step immobilization. In the latter tests, solids were immersed in successive solutions at initial CA concentration set at 0.4, 1.2 and 2.4 mg/mL. From the point of view of the total amount of incubated enzyme, the multi-step immobilization operated with the same amount of CA adopted for the single step immobilization carried out at 4 mg/mL.

2.2 Activity assays

Carbonic anhydrase. Activity assay of CA in aqueous solutions was carried out according to the titrimetric method reported by Worthington (1988). CA activity was measured as the rate of CO_2 conversion in a CO_2 saturated solution containing 10 mM tris(hydroxymethyl)aminomethane sulfate (TRIS sulfate) buffer pH 8.3 at 0°C. In particular, the time interval necessary for pH decrease from 8.3 to 6.3 (due to CO_2 hydration) was measured. The enzyme concentration was properly tuned to keep hydration time larger than 20 s. The activity *A* was expressed as Wilbur-Anderson units per unit volume of incubated solution according to eq. (1) (Worthington, 1988)

$$A = \frac{t_{blank} - t_{CA}}{t_{CA}} \frac{df}{v_{CA}} \qquad (1)$$

where t_{blank} and t_{CA} are hydration times measured during tests carried out with the buffer and with the CA supplemented buffer, respectively, *df* is the dilution factor of the CA sample, and v_{CA} the added volume of the CA solution. Assays were repeated at least three times.

Immobilized carbonic anhydrase. The activity of CA immobilized on Sipernat ® 22 was assessed according to a modified procedure based on the method adopted for free CA. Under the same conditions adopted for free CA, a known volume (between 100 and 200μL) of immobilized enzyme suspension - solids concentration of 0.04g/mL - was added to the reaction mixture. Solids were kept in suspension by means of a magnetic mixer throughout the assay. The blank test was carried out with a suspension of particles bearing an inert protein (glycine).

3. Results

Immobilization tests were performed in order to assess maximum CA loading on silanized Sipernat® 22. Figure 1A shows results of CA immobilization expressed in terms of mass of immobilized enzyme per gram of solids as function of the residual CA concentration in the liquid buffer. Empty triangles are the immobilized enzyme vs. the residual enzyme concentration of the liquid solution used for each step of the multi-step procedure. Therefore, the first data point can be considered as a single step immobilization. The

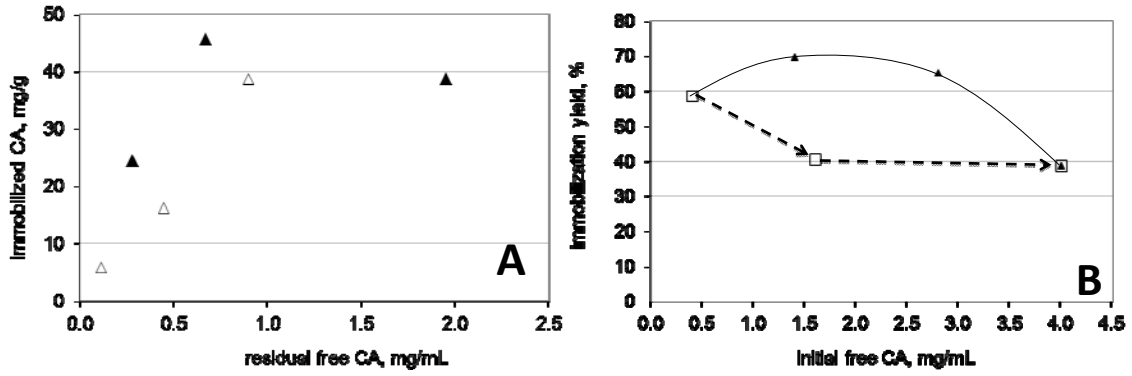

Figure 1: A) Immobilized CA as a function of the residual CA concentration in the liquid buffer, B) immobilization yield as function of initial CA concentration in liquid buffer. Closed symbols) single-step immobilization; open symbols) multi-step immobilization.

maximum loading - 40±3 mg/g - for the silanized Sipernat® 22 was calculated as the average value among the three highest initial concentrations. Figure 1B shows the immobilization yields assessed as reported in section 2.1. As expected, immobilization yield decreased with the initial CA concentration. The continuous line joins the data point related to single step immobilization on bare silanized Sipernat®. The arrows highlight the sequence of data point obtained during multi-step immobilization. It is worth to note that once the total amount of initially available enzyme was set at 4mg/mL, the multi-step and the single step immobilization provided the same amount of immobilized protein (figure 1A) and the same yield (figure 1B). The average yield obtained for initial concentration between 1.4 and 4 mg/mL was 66±5%.

The activity of the immobilized CA was assessed according to the protocol described in the section 2.2. It should be noted that the assay provides the activity related with the apparent rate of the heterogeneous reaction catalysed by immobilized CA (Russo et al., 2008). The apparent rate strictly depends on the operating conditions (e.g. mixing). In other words, the rate depends on phenomena that affect slip velocity and mass transfer rate at the liquid-solid interface. According with the highlighted issues, the assay provides a reliable tool for the optimization of the immobilization conditions but the assessed rate cannot be compared with the activity of free CA (i.e. homogeneous process). Data of activity assayed for immobilized CA are reported in Figure 2. As expected, the activity increases with the amount of immobilized CA. Remarkably, CA immobilized according to the multi-step procedure - total amount of initial CA set at 4 mg/mL (open symbol) - was characterized by the highest activity. This interesting result may be interpreted taking into account the role of the multipoint attachment. The probability of multipoint attachment of enzyme molecules during the single step procedure may be higher than that occurring during the multipoint attachment. In the multi-step immobilization, immobilized enzymes likely prevented the multipoint attachment of enzymes incubated in successive step.

Pore size analysis showed that the Sipernat® 22 was characterized by an average pore diameter of $3.7 \cdot 1^{-8}$m. Since characteristic size of bovine CA is about $5 \cdot 10^{-9}$m (Kumar et al., 1989), it may be assumed that enzymes were immobilized within a peripheral layer of the porous particle. In other words, the enzyme immobilization in the peripheric layer may hinder the diffusion of further enzyme molecules in the inner region of porous particles.

Figure 3A shows size distributions for Sipernat® 22 and Sipernat® 22 with immobilized bovine CA (5.8 mg CA/g Sipernat®). Size distribution of bovine CA-particles and of bare Sipernat® 22 were characterized by: i) a maximum at about 140μm, the maximum for CA-particles was the highest; ii) particles of d_p<100μm of CA-particles less populated than the bare particles within the same size interval. This behaviour may be due to the loss of fines associated with the repeated washings adopted during activation and immobilization procedure.

The analysis on particle size distribution and activity of immobilized enzymes supports the definition of a biocatalyst preparation procedure for large-scale applications. On one hand, the solids should be pretreated in order to obtain a size distribution stable with respect to the immobilization procedure. On the other hand, immobilization procedure should be carried out adopting solids recovering methods characterized by yields as large as possible. These features play a significant role for the enhancement of absorption rate that may be achieved with the aid of granular biocatalyst (Russo et al., 2013).

Sipernat® 22LS was analyzed in terms of particle size distribution because fines could be adopted as

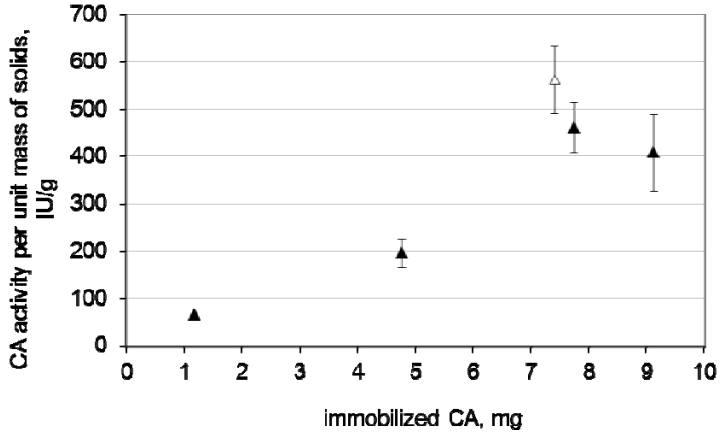

Figure 2: Immobilized CA activity per unit mass of solid supports as function of immobilized CA. Closed symbols) one step immobilization, open symbol) consecutive steps immobilization.

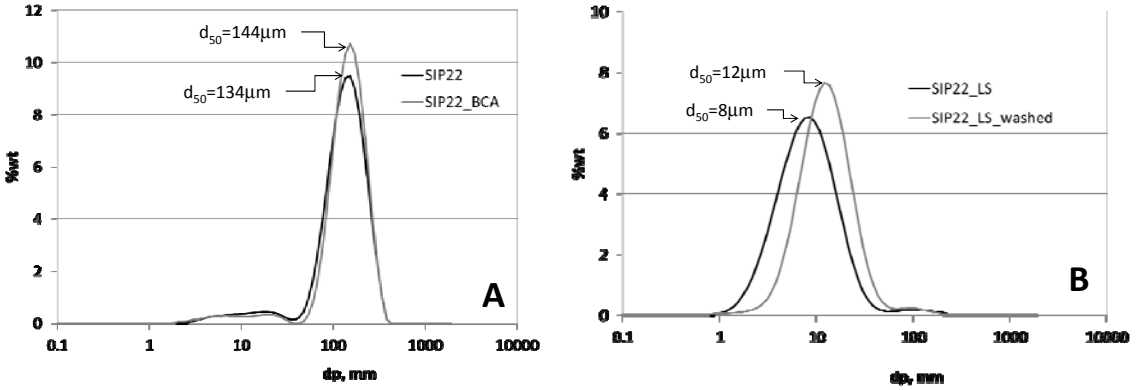

Figure 3: A) Particle size distribution for Sipernat® with and without immobilized bovine CA. B) Particle size distribution for Sipernat® 22LS.

effective supports for biocatalyst: a narrow size distribution of fines can strongly improve catalyst effectiveness and reactor design reliability (Russo et al., 2013). The solids were pretreated: solids were washed and supernatant recovered after 30 min sedimentation. The procedure was repeated 4 times. The particle size distribution measured prior and after the pretreatment is reported in Figure 3B. As expected, the pretreatment gives a more narrow distribution of particles sizes. It may be concluded that a solid pretreatment is advisable and a proper design of the solids recovery during enzyme immobilization is required.
A more comprehensive analysis including further investigation on the effects of protein coating on particle aggregation is in progress.

4. Conclusion

In the framework of the study of biomimetic CO_2 capture process the present contribution focused on the biocatalyst immobilization. Carbonic anhydrase immobilization procedure was developed. Covalent attachment of the enzymes on the carrier surface was adopted: immobilization technique was based on silanization of a siliceous support (Sipernat®) and subsequent activation with glutaraldehyde. The maximum enzyme loading resulted 40±3 mg of bovine CA per g of solids and the maximum yield resulted about 66±5% for initial CA concentration between 1.4 and 4 mg/mL.
A proper procedure for the assessment of immobilized CA activity through titrimetric assay was proposed. The assay provided apparent conversion rates for the heterogeneous reaction catalyzed by the immobilized CA and was adopted as a reliable tool to compare performances of different samples of the solid biocatalyst.

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