CARBONIC ANHYDRASE BIOCATALYSTS FOR BIOMIMETIC CO_2 CAPTURE

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RIASSUNTO

Il continuo rilascio della CO_2 dalle intense attività antropiche è tra le principali cause del surriscaldamento globale degli ultimi decenni. L'origine di tali emissioni è dovuta per la maggior parte all'uso dei combustibili in ambito industriale ed al loro impiego per la produzione di energia elettrica, riscaldamento e nel settore dell'autotrasporto. L'entità di tale emissione è destinata ad aumentare a causa dell'installazione di nuovi siti industriali, in particolar modo nel Sud-Est asiatico, prevista entro il 2030 (Kang et al., 2017). Tra le possibili strategie finalizzate a ridurre le emissioni totali di CO_2 si richiamano: i) l'utilizzo di fonti combustibili di origine non fossile (ad es. idrogeno, biomasse), ii) il passaggio ad energie rinnovabili (ad es. solare, eolica), iii) l'implementazione di processi *Carbon Capture and Storage* (CCS). I processi CCS consistono nella cattura della CO_2 nel luogo in di produzione, trasporto ed infine stoccaggio a lungo termine in siti opportunamente selezionati. I potenziali metodi di stoccaggio della CO_2 includono l'alimentazione in particolari siti geologici (stoccaggio geologico), l'iniezione diretta della CO_2 sul fondo degli oceani (stoccaggio oceanico) oppure la fissazione sotto forma di carbonati (sequestro minerale). In alternativa allo stoccaggio a lungo termine, la CO_2 catturata può essere utilizzata in diverse applicazioni, quali uso diretto, produzione di *chemicals* (Schakel et al., 2016; Zhang et al., 2016) ed applicazioni biotecnologiche (Cheah et al., 2016; Oh and Martin, 2016).

Nonostante l'impiego di fonti di energie alternative costituisca una valida risposta al problema delle emissioni di CO_2, bisogna tuttavia prendere atto che i combustibili fossili, e quindi gli impianti di termoconversione attualmente in funzione, saranno ancora utilizzati per lungo tempo a causa del fabbisogno energetico sempre in continua crescita. È necessario, quindi, dar vita ad una politica di sviluppo sostenibile, capace cioè di contenere gli effetti del riscaldamento globale senza sacrificare la crescita economica/sociale. L'adozione di processi CCS costituisce una valida soluzione per il conseguimento di tale obiettivo. I processi CCS possono essere suddivisi in tre categorie: processi di post-combustione; processi di pre-combustione; processi di ossi-combustione. I processi CCS di post-combustione consistono nella rimozione della CO_2 a valle dell'impianto di termoconversione e costituiscono una delle unità preposte alla purificazione dei gas esausti. Essi risultano particolarmente interessanti ogni qual volta è possibile il *retrofitting* degli impianti industriali esistenti, quali impianti di termoconversione, fornaci per la produzione del cemento ed altoforni per la produzione di acciaio e ferro. Esistono diverse tecnologie commercialmente disponibili per la rimozione della CO_2 dai fumi di combustione. Studi di valutazione comparativa hanno dimostrato che l'opzione più conveniente è offerta dai processi di assorbimento chimico, con soluzioni acquose di alcanolammina, in special modo con monoetanolammina (MEA), grazie all'alta efficienza di cattura ed ai minori costi di esercizio richiesti. L'assorbimento rigenerativo della CO_2 in soluzioni acquose di alcanolammine è basato su uno schema di processo ciclico (absorption/desorption closed loop) che prevede: (i) uno stadio di assorbimento della CO_2 in unità di contatto gas-liquido, attraversato dai fumi di combustione in controcorrente rispetto alla corrente di liquido assorbente ed (ii) uno stadio di desorbimento in cui il solvente esausto è rigenerato per desorbimento della CO_2. Tuttavia gli svantaggi connessi all'utilizzo delle ammine sono diversi e tra questi l'elevata energia richiesta per la rigenerazione del solvente, l'alta volatilità delle soluzioni e la formazione di composti tossici dovuti all'ossidazione e/o degradazione termica delle ammine. Alla luce tali inconvenienti, l'obiettivo della

ricerca è volto ad implementare processi di cattura della CO_2 eco-compatibili ed economicamente competitivi come alternativa al processo di assorbimento con ammine. Particolare attenzione è stata rivolta all'assorbimento rigenerativo in soluzioni alcaline di carbonato di potassio ad alta temperatura (*hot potash*), già utilizzate nel processo Benfield, le quali non comportano la produzione di composti inquinanti.
L'assorbimento della CO_2 in soluzioni acquose alcaline è associato ad una rete di reazioni tra cui la reazione di idratazione (I) e la reazione di idrossilazione della CO_2 (II) (Danckwerts and Sharma, 1966):

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad \text{(I)}$$
$$CO_2 + OH^- \rightleftharpoons HCO_3^- \quad \text{(II)}$$

A pH > 8 il contributo della reazione (II) sulla velocità globale di conversione della CO_2 prevale su quello della reazione (I). Nel processo Benfield, la fase di assorbimento è tipicamente condotta alla temperatura di 90-100°C e la rigenerazione è effettuata a temperature al di sopra dei 100°C. In alternativa, la CO_2 può essere strippata sotto vuoto (circa 0.3 bar) consentendo la riduzione della temperatura sotto i 100°C (Chen et al., 2007). Queste condizioni rendono il processo competitivo con quello di assorbimento con la monoetanolammina (MEA) grazie alla minore energia richiesta per lo stripping ed alla gestione con sottoprodotti non tossici. Tuttavia, l'idrossilazione presenta una cinetica relativamente lenta, tre o quattro volte inferiore, rispetto a quella della reazione tra CO_2 e MEA. Per questo motivo, i processi con le soluzioni di carbonato richiedono l'uso di additivi per promuovere la velocità di assorbimento di CO_2. Diversi sono i promotori proposti, tra questi ancora le ammine (MEA, dietanolammina, piperazina, amminoacidi), l'acido arsenico, l'acido borico ed l'anidrasi carbonica (Hu et al., 2016). L'anidrasi carbonica (CA) (E.C. 4.2.1.1) è un enzima in grado di catalizzare la reazione di idratazione della CO_2 ad acido carbonico con un numero di *turnover* che varia tra 10^4 e 10^6 s^{-1} a seconda della classe dell'enzima e dell'organismo di appartenenza (α, β, γ, δ, ε) (Di Fiore et al., 2015). L'uso della CA nei processi di cattura della CO_2 offre il vantaggio di utilizzare un catalizzatore biodegradabile, eliminando gli inconvenienti dovuti alla formazione degli scarti tossici derivanti dalla degradazione delle ammine o dall'impiego di altri tipi di promotori.
La progettazione/ottimizzazione di un'unità di assorbimento biomimetico – assorbimento catalizzato da enzimi – richiede la caratterizzazione cinetica delle forme di CA selezionate come possibili candidate per promuovere la velocità di assorbimento di CO_2. A tale scopo, l'approssimazione lineare dell'equazione di Michaelis e Menten (Eq. 1) costituisce un ottimo strumento per effettuare uno screening cinetico preliminare.

$$r_{AC} = \frac{k_{cat}}{K_M}[CA]([CO_2] - [CO_2]_{eq}) \quad (1)$$

dove k_{cat} è il numero di turnover (s^{-1}) e K_M la costante cinetica di Michaelis-Menten (m^3 kg^{-1}). Le forme di CA selezionate devono presentare non solo un'elevata attività ma anche una buona stabilità a lungo termine alle condizioni tipiche di processo, quali temperature di assorbimento, tra i 40 e 60°C, e di desorbimento intorno ai 100°C, elevata alcalinità (pH > 10), ed elevate concentrazioni di Sali. Inoltre, esse devono mantenere un'attività significativa anche in presenza di tracce di inquinanti che possono trovarsi nei gas combusti effluenti, quali ceneri, NO_x ed SO_2.

Numerose configurazioni reattoristiche sono state proposte in letteratura per lo sviluppo di unità di assorbimento biomimetico. In generale, la scelta della configurazione è strettamente legata alla morfologia del catalizzatore cioè alla possibilità di usare l'enzima in forma libera (catalisi omogenea) o immobilizzata (catalisi eterogenea). L'assorbimento biomimetico può trarre vantaggi se l'anidrasi carbonica è immobilizzata su un supporto solido. L'immobilizzazione infatti: i) permette il confinamento del biocatalizzatore nell'unità di assorbimento, evitando l'esposizione del biocatalizzatore alle più drastiche condizioni di desorbimento; ii) migliora la stabilità dell'enzima alle condizioni di processo; iii) permette la massimizzazione della concentrazione di enzima (≥ 0.1 kg m^{-3}) anche oltre il limite di solubilità dello stesso.
È noto che l'enzima immobilizzato deve essere disponibile ed attivo in prossimità dell'interfaccia gas-liquido al fine di contribuire all'aumento della velocità di assorbimento della CO_2 (Iliuta and Larachi, 2012; Penders-van Elk, 2013; Russo et al., 2013a; Hou et al., 2015; Russo et al., 2016). Gli assorbitori eserciti con *slurry* di catalizzatori sono una valida configurazione reattoristica per soddisfare la richiesta richiamata (Penders-van Elk, 2013; Russo et al., 2013a; 2016). Infatti, la presenza di particelle catalitiche fini, sospese in prossimità dell'interfaccia gas-liquido, efficacemente aumenta la velocità di assorbimento della CO_2. In accordo alla teoria del doppio film sul trasporto di materia, l'efficacia dell'enzima immobilizzato su particelle disperse dipende dalla possibilità del supporto di disperdersi anche nel film liquido stagnante in prossimità dell'interfaccia gas-liquido: ne consegue che le dimensioni delle particelle devono essere mediamente inferiori a quelle film liquido stagnante.
Con riferimento allo sviluppo di biocatalizzatori solidi, esistono numerose tecniche di immobilizzazione degli enzimi. In generale, a seconda della formazione o meno di legami covalenti tra gli enzimi ed il supporto, l'immobilizzazione può essere di tipo fisico o di tipo chimico (Moehlenbrock e Minteer, 2011). La principale tecnica di immobilizzazione di tipo fisico è l'adsorbimento. Essa consiste nella formazione di interazioni di tipo fisico (forze di van der Waals, interazioni elettrostatiche, legami a ponte di idrogeno) tra l'enzima e un supporto solido. L'immobilizzazione si ottiene in condizioni blande, tuttavia è altamente probabile il rilascio dell'enzima nel tempo poiché i legami tra esso ed il supporto solido sono molto labili: tipicamente i legami sono stabili solo in piccoli intervalli di pH e forza ionica. Le tecniche di immobilizzazione di tipo chimico comportano la formazione di legami covalenti tra l'enzima e un supporto solido oppure tra più enzimi (*cross-linking*). Grazie alla formazione del legame chimico si ha un ancoraggio più stabile rispetto alle tecniche di immobilizzazione di tipo fisico e si evita il rilascio dell'enzima durante l'utilizzo. In entrambi i casi, la procedura di immobilizzazione non deve comportare condizioni che denaturino l'enzima e dovrebbe promuovere rese elevate in termini di enzima immobilizzato rispetto a quello originariamente disciolto in fase liquida. Inoltre è opportuno che l'interazione tra l'enzima ed il supporto non coinvolga il sito attivo al fine di minimizzare la perdita di attività a seguito dell'immobilizzazione. I supporti solidi dell'enzima possono essere di natura organica (e.g. polimeri) o inorganica (e.g. materiali di natura silicea).
La formazione di aggregati enzimatici per *cross-linking*, denominati CLEA (*Cross-Linked Enzyme Aggregate*) è una tecnica di immobilizzazione priva di supporto solido. La preparazione dei CLEA prevede in generale due passaggi (Fig. 1): formazione di aggregati enzimatici mediante precipitazione per aggiunta di un agente

precipitante (Sali o solventi) in fase acquosa; *cross-linking* degli aggregati mediante agente bifunzionale (ad es. glutaraldeide).

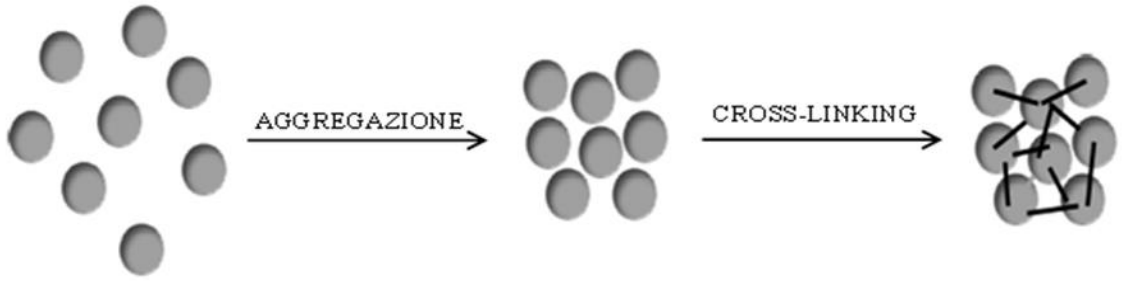

Fig. 1: Rappresentazione schematica dei passaggi per la preparazione dei CLEA.

La precipitazione degli enzimi può essere indotta mediante addizione dell'enzima a soluzioni saline o soluzioni acquose di solventi organici, in modo da minimizzare le modificazioni strutturali della proteina. Gli aggregati, tenuti insieme da interazioni di tipo fisico, sono resi insolubili in assenza dell'agente precipitante mediante *cross-linking*, cioè mediante la formazione di legami covalenti tra le proteine dell'aggregato e l'agente bifunzionale.
L'attività svolta nell'ambito della Tesi di Dottorato si inserisce in un progetto pluriennale volto allo sviluppo di un processo di assorbimento biomimetico della CO_2 e il successivo riutilizzo come fonte di carbonio per coltivazioni algali. L'oggetto del presente lavoro di tesi è lo sviluppo e la caratterizzazione di biocatalizzatori costituiti da anidrasi carbonica da impiegare in un'unità di assorbimento biomimetico trifasico di tipo *slurry*. Lo studio si è articolato in accordo alla seguenti linee:

A) Sviluppo di una procedura affidabile per la caratterizzazione cinetica di una forma di CA ricombinante non commerciale in forma di miscela di proteine in condizioni prossime a quelle del processo di assorbimento industriale. La miscela proteica (320 kg m^{-3}) contiene circa il 60% in peso di CA. In collaborazione con l'istituto di Bioscienze e Biorisorse (CNR), la miscela è stata utilizzata per sviluppare dei protocolli di immobilizzazione covalente su nanoparticelle paramagnetiche, mediante attivazione con carbodiimide. La scelta delle nanoparticelle paramagnetiche è dettata dall'esigenza di guardare ad una tecnica di recupero/confinamento del biocatalizzatore;
B) Sviluppo delle tecniche di immobilizzazione per la preparazione di biocatalizzatori solidi "carrier- free". La tecnica dei CLEA è stata applicata e ottimizzata rispetto all'enzima modello CA bovina (estratto da eritrociti). Anche in questo caso è stato incluso nella preparazione del biocatalizzatore l'utilizzo di nanoparticelle paramagnetiche per offrire ulteriore spunto allo sviluppo di tecniche di confinamento/recupero del catalizzatore dal volume di reazione basate sull'impiego di campi magnetici. Tale attività sull'immobilizzazione della CA è stata supportata anche dalla dall'attività sperimentale condotta con la supervisione del Dott. Roberto Fernández-Lafuente, presso l'Instituto de Catálisis y Petrolquímica di Madrid appartenente al Consejo Superior de Investigaciones Cíentificas. In quest'ambito si sono acquisite tecniche di immobilizzazione di enzimi basate su protocolli innovativi di co-immobilizzazione volti allo sviluppo di biocatalizzatori solidi per applicazioni industriali;
C) Utilizzo del biocatalizzatore, sia in forma libera (catalisi omogenea) che in forma

immobilizzata (catalisi eterogenea), in prove di assorbimento della CO_2 in soluzioni alcaline. In particolare, le prove sono state condotte in condizioni operative rappresentative del processo industriale di cattura della CO_2: l'utilizzo di solventi a base di carbonati (di sodio e potassio) e temperature prossime 40°C. I dati delle prove di assorbimento hanno permesso di caratterizzare l'effetto del biocatalizzatore libero ed immobilizzato sulla velocità di assorbimento della CO_2. Al fine di discernere l'effetto dei molteplici parametri chimico fisici coinvolti nel processo di assorbimento gas-liquido con reazione catalizzata da un enzima si è proceduto alla ulteriore elaborazione dei dati sperimentali secondo le accreditate teorie sull'assorbimento chimico (Danckwerts, 1970; Alper et al., 1980; Ye and Lu, 2014a,b). In questo modo è stato possibile stimare per alcune delle condizioni operative adottate (concentrazioni di enzima circa 10^{-3}kg m^{-3}) il valore della costante cinetica k_{cat}/K_M. L'elaborazione dei dati secondo la teoria non è stata estesa a tutte le condizioni operative applicate, quali ad esempio quelle corrispondenti ad elevate concentrazioni di CA (maggiori di 0.3 kg m^{-3}). Tali prove sono state condotte per indagare il comportamento dell'enzima alle concentrazioni effettivamente utili ai fini dell'implementazione del processo di cattura della CO_2 a valle di lavori sperimentali e teorici riportati in letteratura (Lu et al., 2011; Ye and Lu, 2014b; Zhang and Lu, 2015). In particolare si è investigato l'effetto dei precipitati di enzima che inevitabilmente si formano quando nelle soluzioni saline adottate la concentrazione di proteine supera la soglia di circa 0.3 kg/m^3;

D) Valutazione della stabilità della forma di CA ricombinante in forma libera sul lungo periodo (circa 30 giorni) in condizioni di interesse applicativo (soluzioni di carbonati a 40°C).

SUMMARY

Novel post-combustion treatments include biomimetic CCS processes based on CO_2 absorption into aqueous solutions assisted by enzyme catalysis. Carbonic anhydrase (CA) has been proposed as biocatalyst for biomimetic CCS: CA catalyses CO_2 hydration at a turnover number ranging between 10^4 and 10^6 s^{-1}; it can be produced via fermentation and may be disposed of with minimal detrimental impact on the environment. The design/optimization of biomimetic CCS processes asks for the selection of proper CA form because CA must be active and stable at the typical operating conditions of the CO_2 capture unit (large temperature, pH, and ionic strength, presence of pollutants) and should preserve a remarkable activity in the time. Design of the absorption unit (e.g. the reactor configuration) strongly depends on the use of the dissolved (homogeneous catalysis) or immobilized (heterogeneous catalysis) enzyme. The use of immobilized CA-based biocatalysts provides numerous advantages with respect to the use of the biocatalyst in dissolved form. Indeed, immobilized enzymes are characterized by: i) significant increase of the enzyme stability at the process conditions, ii) the confinement of CA in the absorption unit, avoiding the enzyme exposure to the harsh conditions of the desorption unit, and the recovery and reuse of the enzyme from the liquid solvent; iii) the use of large enzyme loadings even above the limit of enzyme solubility in salty solutions.

Whenever immobilized CA is used as CO_2 absorption rate promoter, it should be active as close as possible to the gas-liquid interface to take advantage from the enzyme catalysis. This prerequisite may be fulfilled by a careful design of the morphology of the solid biocatalyst and selection of reactor configuration. The use of the slurry biocatalyst has been proposed as an optimal strategy. Indeed, the contribution of the catalytic fine particles to absorption rate enhancement is due to their ability to be available close to the gas–liquid interface.

The present PhD thesis was focused on the development and characterization of CA-based biocatalysts to be used in innovative biomimetic absorption units. The activity was articulated as reported hereinafter:

- Characterization of a CA recombinant form and the development of protocols aimed to the production of solid CA-based biocatalysts. Two immobilization techniques were investigated (covalent attachment on fine solids and cross-linked enzyme aggregates) using the recombinant CA and bovine CA as models;
- Kinetic characterization of CA in free (homogeneous catalysis) and immobilized (heterogeneous catalysis) forms. In particular, absorption tests were carried out to assess the enzymatic kinetic constant k_{cat}/K_M according to the protocol proposed by Alper et al. (1980);
- Absorption tests at operating conditions close to those adopted in absorption units, such as temperature up to 313 K and carbonate-based solvents at concentrations ≥ 10%wt;
- The study of CO_2 absorption rate at CA concentrations ≥ 0.3 kg m^{-3} according to conditions reported in the literature for experimental and theoretical models of industrial capture units;
- Long term free CA stability under operating conditions close to the industrial applications;
- Development of innovative enzymes co-immobilization techniques. It was carried out at the Instituto de Catálisis y Petrolquímica (ICP) of the Consejo Superior de Investigaciones Cíentificas (CSIC) in Cantoblanco (Madrid, Spain), with the supervision of Dr. Roberto Fernández-Lafuente.

1. SCIENTIFIC BACKGROUND

1.1 Preface

The continuous emission of greenhouse gases to the atmosphere from anthropic activities is of great concern because they are one the main responsible of the global warming. A large fraction of the emitted CO_2 is produced during the combustion of fossil fuels - as coal, oil and natural gas - in several fields including power generation, industrial processes, and transports (Kang et al., 2017). The International Energy Agency (IEA) claimed that in 2030 the CO_2 emissions will be 63% higher than today. As a direct consequence, the global temperature is destined to increase: it has been predicted that the temperature increase with respect to today will be between 1.4 and 5.8°C in 2100 (De Silva et al., 2015). Another alarming scenario related to the CO_2 concentration raising is the increase of the ocean acidity, which constitutes a serious potential threat to the delicate equilibrium of the sea ecosystems (Farrelly et al., 2013). In order to reduce the amount of CO_2 in the atmosphere and minimize undesirable environmental effects, several solutions have been proposed. They include the use of renewable energy resources, such as solar, wind and biomasses, which are gaining much interest due to their near zero carbon footprint. In particular, solar and wind energy resources are very attractive thanks to the wide availability for free in nature (Tiwari et al., 2016). The production of biofuels from biomasses is also a very promising solution because it provides significant benefits with respect to the use of fossil fuels in terms of (Alaswad et al., 2015):

- CO_2 emissions: the CO_2 emitted in the atmosphere during the combustion is fixed by the chlorophyll synthesis to produce new biomass;
- renewability: the biomass can be re-produced continuously, unlike the fossil fuels, which require several centuries to their re-formation;
- Biodegradability.

Among the wide variety of existent biomasses, feedstocks as lignocellulosic materials derived from agricultural and forestry residues (grass, wood) and organic wastes are gaining much consideration, due to the zero cost of the starting materials. They constitute the second-generation feedstocks. On the contrary, the potential use of food crops such as sugarcane, corn and vegetable oils, initially proposed as biofuel feedstocks and classified as first-generation feedstocks, has been strongly limited by environmental and socio-economic barriers, including the extension of plantation field, which leads to deforestation, and competition between biofuels and food production (Chea et al., 2016). Recently, microalgae have been proposed as feedstock to produce biofuels: they constitute the third-generation biofuel (Alaswad et al., 2015). The spectrum of biofuels from microalgae is quite wide: biogas from anaerobic degradation, biodiesel from the lipid fraction, ethanol/butanol from carbohydrate fraction, hydrogen, etc. The photosynthesis efficiency of microalgae is higher than that of high- plants: it has been reported that photosynthetic efficiencies for algae range from 3% to 8%, compared with 0.5% for many terrestrial crops. Further advantages with respect to the terrestrial crops are the short life cycles and the fast growing without the need of herbicides or pesticides.
Despite the environmental advantages and the promising features of the emerging technologies for energy production from renewable resource, the fossil fuels are still the major energy sources and will still play a significant role in energy supply for

many decades to come (Kang et al., 2017). Moreover, it should be taken into account that the society industrialization - combined with the population growth - is destined to increase and the fossil fuels demand is expected to increase too. Therefore, the reduction of the CO_2 emission into the atmosphere is a crucial challenge.
The Carbon Capture and Storage (CCS) technologies are powerful solutions to reduce the CO_2 concentration in the atmosphere. These technologies include processes aimed to capture the CO_2 from flue gases streams, to concentrate the gaseous CO_2, to condensate the CO_2 and to transport it in long term storage. The CO_2 can be stored underground in particular geological formations - such as deep saline aquifers or disused hydrocarbon reservoirs - or stored in the deep of oceans. A potential alternative is the precipitation as carbonates, which do not pollute the environment (Liu et al., 2005).
The processes aimed to the capture of CO_2 may be grouped in three categories: pre-combustion, post-combustion and oxyfuel (Sanz-Perez et al., 2016). Pre-combustion carbon capture processes consist in the prior fuel gasification (reaction I) followed by the water gas shift reaction (reaction II):

$$C_xH_y + xH_2O \rightarrow xCO + \left(x + \frac{y}{2}\right) H_2 \quad \text{(I)}$$

$$CO + H_2O \rightleftharpoons CO_2 + H_2 \quad \text{(II)}$$

The produced CO_2 is relatively easy to recover because its concentration ranges between 15 and 60%. Definitively, the fuel is converted into a hydrogen-rich gas stream. Post-combustion carbon capture processes are based on the capture of CO_2 from flue gas released after combustion, as last step of the flue gas stream treatments after removal of fly ashes, NO_x, and SO_2. Oxyfuel process aims to the combustion of fossil fuels with pure oxygen instead of air. The resultant flue gas is mainly made by CO_2 (up to 90%), so that an easy recovery is possible. However, because of the high costs to obtain pure oxygen from air, these technology is not yet highly favored.
Among the three process typologies, post-combustion carbon capture is particularly attractive due to the possibility of retrofitting the existing coal fire power plants. In addition to CCS, other technologies have been proposed to re-utilizate the captured CO_2 as alternative to the long term storage. These technologies – known as Carbon Capture and Utilization (CCU) - aim to the CO_2 valorization and require the CO_2 capture and the conversion of the CO_2 into a wide spectrum of organic or inorganic products. Some processes are listed hereinafter:

- The CO_2 can be potentially be used in the production of urea, propylene and ethylene as substitute of the petroleum-derived feedstocks (Patricio et al., 2017);
- The Bauxite residue carbonation process is a potential process for CO_2 exploitation: the CO_2 is supplemented to the highly alkaline bauxite residue slurry (also known as “red mud”), the waste stream of the extraction of alumina from bauxite ore. Products from this process are materials characterized by excellent mechanical properties that can be used in the constructions sector (Patricio et al., 2017);
- The captured CO_2 may be used in the greenhouses to increase the carbon CO_2 in the air and to accelerate the plant photosynthesis (Patricio et al., 2017);
- The CO_2 may be used in the production of methanol though catalytic hydrogenation of carbon dioxide. However, this application is still in study

phase due to the highly stability of CO_2 which leads to very scarce methanol production. Improvements are turned to the research of the appropriate formulation of the catalysts in order to enhance the reaction yield (Alaba et al., 2017).

As mentioned above, the CO_2 can be used as feedstock for microalgal cultivation to produce biofuels. Although the high costs and energy input required for commercial scale of microalgal cultivations, microalgae are intensively studied in order to improve their CO_2 sequestration capabilities. Nevertheless the several advantage from microalgae exploitation, several issues hinder the development of these route. The CO_2 capture – in terms of rate – is still a critical issue to be addressed.

1.2 Post-combustion Carbon Capture Processes

The technologies aimed to post-combustion CO_2 capture include absorption, adsorption, membrane and metal−organic frameworks (MOFs) (Sanz-Perez et al., 2016). The absorption based processes are the most advanced technology. CO_2 absorption processes consists in the selective transfer of CO_2 from the flue gas stream to the liquid phase. In particular, the chemical absorption associates the physical absorption with the reaction between the solvent and the absorbed CO_2: the enhancement of the mass transfer rate is expected. The CO_2 absorption rate is affected by both hydrodynamic conditions - flow-rate, design of the gas-liquid contact system, physical properties of liquid, etc. - and by the physico-chemical features of the gas-liquid system (solubility of the solute in the solvent, diffusivity of dissolving solute and of other species, etc.). Furthermore, the absorption rate is affected by the kinetics of reactions occurring in the liquid phase (Dankwerts and Sharma, 1966). The reactions need to be reasonably fast in order to reduce the equipment size and the associated cost. Typically, the units may be operated at high flue gas flow rate, atmospheric pressure and the CO_2 concentration in the gas phase is very low, therefore the driving force is quite low (Gladis et al., 2017). To enhance the CO_2 mass transport from gas to liquid phase, a lot of solvents have been investigated, such as aqueous solution of monoethanolamine (MEA), diamines and tertiary amines (piperazine and its derivatives), ammonia and amino acid salts (Hu et al., 2016). Nowadays, the regenerative absorption with MEA is the most advanced technology of post-combustion CO_2 capture processes and it is the process benchmark for post-combustion CCS processes. The product of the reaction between CO_2 and MEA is typically the amine salt of the carbamic acids (reaction III - Dankwerts and Sharma, 1966):

$$CO_2 + OH(CH_2)_2NH_2 \rightarrow OH(CH_2)_2NHCOO^- + OH(CH_2)_2NH_3^+ \quad \text{(III)}$$

$$r_{MEA} = k_{MEA}[MEA][CO_2] \quad (1.1)$$

$$k_{MEA} \sim 10^3 \, L \, mol^{-1} s^{-1}$$

Drawbacks related to the MEA-based process are mainly due to the nature of the used solvents: they impact negatively to the environment because of the amines degradation, they easily corrode the equipment and require high energy consumption for their regeneration (Luis, 2016). An environmentally friendly alternative to alkanolamines-based solvents may be achieved by carrying out the CO_2 absorption process in potassium carbonate alkaline solutions. Potassium carbonate solutions have already been widely used in the past to clean the gas streams produced in petroleum treatment processes or synthesis gases from the acid gases, such as CO_2

and H_2S (Benfield process). Recently potassium carbonate solutions have been proposed to carry out CO_2 absorption from flue gases (Chen et al., 2007; Hu et al., 2016). The CO_2 absorbed in aqueous alkali is involved in the reactions of hydration (reaction IV) and hydroxylation (reaction V) reactions (Danckwerts and Sharma, 1966):

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad \text{(IV)}$$

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \quad \text{(V)}$$

The reaction rates are, respectively:

$$r_{H2O} = k_{H2O}([CO_2] - [CO_2]_{eq}) \quad (1.2)$$

$$k_{H2O} \sim 10^{-2} s^{-1}$$

$$r_{OH^-} = k_{OH^-}[OH^-]([CO_2] - [CO_2]_{eq}) \quad (1.3)$$

$$k_{OH} \sim 10^3 L\, mol^{-1} s^{-1}$$

At pH larger than 8 the contribution of reaction V to the overall CO_2 conversion rate is dominant with respect to that of reaction IV. The advantages of this kind of process include low regeneration energy requirement, no salts degradation and low equipment corrosiveness (Anderson et al., 2013). A potential potassium carbonate solvent absorption process flow diagram is shown in Fig. 1.1 (Hu et al., 2016). It is a cyclic absorption process - analogous to the amine absorption configuration - where the principal units are the absorber and the desorber. The flue gas is fed into the absorber counter-current to the solvent stream for absorption of CO_2. The CO_2 rich solvent is sent into the desorption unit where CO_2 is stripped from the solvent by increasing the temperature and/or decreasing the pressure of the unit. The stripped CO_2 is compressed for utilization or storage and the regenerated solvent is sent to the absorption unit.

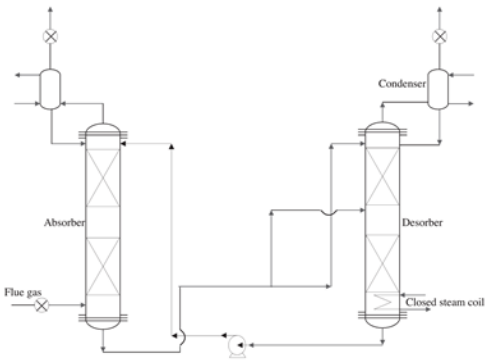

Fig. 1.1: Flow diagram of a potential absorption process with potassium carbonate alkaline solutions (Hu et al., 2016).

The CO_2 absorption rate is relatively slow in carbonate-based solvents, so that large size of the absorption units are required to obtain satisfactory capture efficiency. The presence of an absorption rate promoter may improve significantly the rate of the absorption process. This beneficial effect can lead to economic benefits in terms of capital investments for the reduction of the absorption unit size and process costs. Several researchers have carried out investigation on CO_2 absorption promoters. Promoters can be classified - on the basis of their physical and chemical properties - in three main groups: inorganic, organic, and enzymatic promoters (Hu et al., 2016).

The inorganic promoters include arsenite, boric acid, vanadate, hypochlorite, selenite, and tellurate. The inorganic promoters are characterized by several drawbacks such as the high toxicity and corrosiveness. Moreover, the switch of boric acid and vanadate among different speciation as a function of the temperature and of the pH limits their application under the wide range of industrial operating conditions (Phan et al., 2014 and 2015; Hu et al., 2016). The most promising organic promoter in potassium carbonate solutions is the MEA. The system based on MEA in carbonate solutions is characterized by the same drawbacks reported for the MEA based solvents (high energy requirement for regeneration, species degradation and apparatus corrosion) even though with a less extent. The piperazine has recently been proposed as promoter because it is characterized by low vapor pressure, low degradation and low corrosiveness (Cullinane et al., 2004; Park, 2014) and promoter performance comparable with the MEA (Rahimpour and Kashkooli, 2004). However, the low solubility of piperazine limits its application because it precipitates and forms other salts (Fosbøl et al., 2013). Amino acid salts - such as glycine, L-alanine and L-proline – are also used as organic promoters in potassium carbonate solutions. The solvents made by amino acid salts aqueous solutions successfully accelerate the CO_2 absorption. Moreover, they are commercially available, not toxic and are characterized by low volatility (Aronu et al., 2011; Lim et al., 2012). Disadvantages include high solvent regeneration energy due to the formation of stable carbamates. Therefore, the use of these salts as a promoters in potassium carbonate solutions is an area of active research (Shen et al., 2013; Thee et al., 2014).
The enzyme carbonic anhydrase (CA, E.C. 4.2.1.1) has been proposed as enzymatic promoter. This biological catalyst is characterized by a large spectrum of advantages and in particular it is environmental friendly and catalyzes at large turnover number the CO_2 hydration reaction (Bond et al., 2001; Lu et al., 2011; Vullo et al., 2012; Russo et al., 2013a; Zhang et al., 2013; Ye and Lu, 2014). The use of CA as promoter in potassium carbonate solutions is a promising option for the enhancement of CO_2 absorption rate, providing optimistic perspectives to the development of a high potential biomimetic post-combustion CO_2 capture processes.

1.3 The carbonic anhydrase and the biomimetic CO_2 capture process

The biomimetic CO_2 capture process is based on the use of the enzyme carbonic anhydrase (CA) (EC 4.2.1.1) in aqueous alkaline solutions as catalyst for the enhancement of CO_2 absorption rate. The CA can be produced via fermentation and it may be disposed of with minimum detrimental impact on the environment. CA is typically classified into five different classes: α, β, γ, δ, ε. Although these classes are characterized by small similarities in sequence and structure, they carry out the same function with a divalent zinc ion in the active site. CA is able to catalyze the hydration reaction (IV) with a rate depending on the class of the enzyme and on the operating conditions (temperature, pH, ionic strength). In particular, the CA *turnover number* range between 10^4 and 10^6 s^{-1} (Di Fiore et al., 2015). The reaction mechanism of CA has been deeply studied and the reaction scheme is reported in Fig. 1.2 (Gladis et al., 2017). The enzyme in its active state (A) reacts with the CO_2 molecule via a nucleophilic attack (B) giving a zinc-bound bicarbonate complex (C). The substitution of bicarbonate ion by a water molecule occurs and gives back the enzyme under unreactive state (D). To restore the enzyme active state, the proton has to be removed from the zinc-bound water molecule. The restore occurs via a two-step mechanism where the proton is transferred to an amino acid AM side chain (intramolecular proton transfer) (E) and then released to the proton acceptor B

present in the liquid bulk (intermolecular proton transfer) (F). It has been showed that the regeneration of the enzyme is the rate-limiting step in this cycle. In particular, the intermolecular and the intramolecular proton transfer can be the rate limiting step under conditions of low and high buffer concentration, respectively (Lindskog, 1997; Salmon and House, 2015). Because of the large ionic strength of the solvents used in the CO_2 absorption processes, the rate limiting step in carbon capture application is the intramolecular proton transfer due to the large abundance of proton acceptor species.

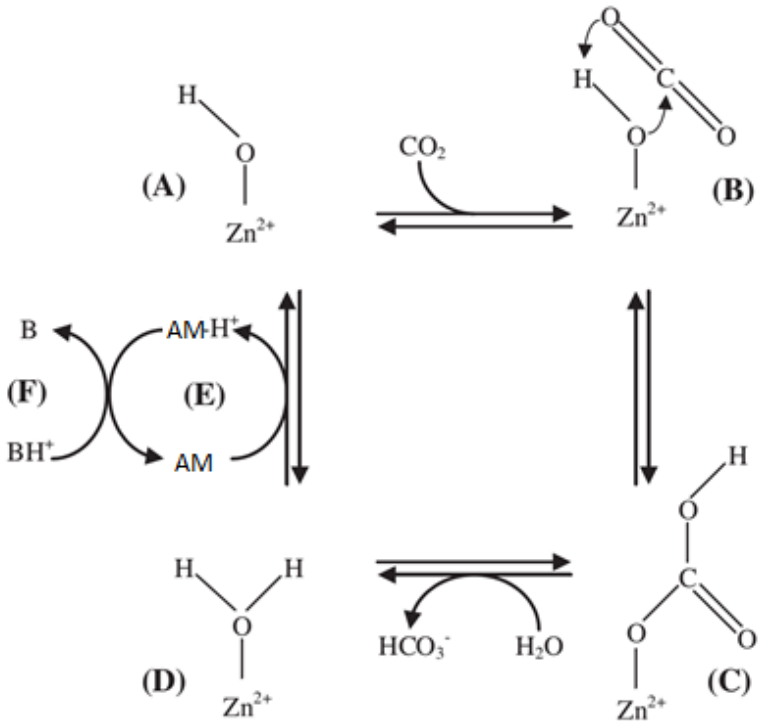

Fig. 1.2: Reaction mechanism of carbonic anhydrase (Gladis et al., 2017).

The design and the optimization of the biomimetic absorption process ask for an accurate kinetic characterization of the CA forms. As a potential model, the linear approximation of the Michaelis-Menten kinetics equation (Eq. 1.4) is a satisfactory tool:

$$r_{AC} = \frac{k_{cat}}{K_M}[CA]([CO_2] - [CO_2]_{eq}) \qquad (1.4)$$

where k_{cat} is the *turnover number* (s^{-1}), and K_M the Michaelis-Menten kinetic constant (L mol^{-1}). The k_{cat}/K_M assessed for the α-class human carbonic anhydrase HCAII is about $10^8 M^{-1}s^{-1}$ (Steiner et al.,1975). It is possible to compare the pseudo-first order rate constant of the hydroxylation reaction $k_{OH}[OH^-]$ at pH 10 (Eq. 1.3) with the same parameter of the CA catalyzed CO_2 hydration rate, $\frac{k_{cat}}{K_M}[CA]$ (Eq. 1.4). At CA concentration of about 3 10^{-6} M (about 0.1 g/L of enzyme having 30 kDa molecular weight), the contribution of the CA-catalyzed hydration reaction is three orders of magnitude larger that the hydroxylation rate. Moreover, it is just one order of magnitude smaller than the product k_{MEA}[MEA] (Eq. 1.1). This analysis shows the high CA potentiality as promoter of the CO_2 absorption rate in alkaline solution at

moderately large enzyme concentrations.
The selection of CA forms able to operate under the typical harsh industrial conditions is an essential prerequisite for the design and set up of the biomimetic CO_2 capture process. Indeed, conditions as high temperature, high salts concentration and elevated alkalinity may affect enzyme performance. Usually, the temperature of the absorption unit ranges between 40°C and 60°C while the temperature of the desorption unit is about 100°C but it can be lowered by carrying out the process under vacuum (about 0.3 bar) (Chen et al., 2007; Russo et al., 2013a). Moreover, the presence of pollutant in the flue gases - such as fly ashes, NO_x, SO_2, mercury, and chlorine - may significantly affect the enzyme performance. Many contributions on the performance of recombinant CA species are reported in the literature and the characterizations are expressed in terms of kinetic assessment and long term stability under the typical process conditions.
Lu et al. (2011) characterized a CA form of microbial origin by means of absorption tests in a stirred cell reactor. Tests were carried out by using pure CO_2 as gas phase and 20% wt K_2CO_3 aqueous solutions as liquid phase, at 25, 40 and 50°C, and at CA concentration of 300 mg L^{-1}. Results pointed out that the CA enhanced the CO_2 absorption rate of about 10, 5 and 4 times with respect to tests carried out without promoter, at 25. 40 and 50°C, respectively. The investigated CA was characterized by low affinity towards SO_4^{2-}, NO_3^-, Cl^- ions.
Russo et al. (2013b) characterized the kinetics of the recombinant CA form SspCA, isolated from the thermophilic bacterium *Sulfurihydrogenibium yellowstonens*. The CO_2 absorption tests were carried out into in a stirred cell reactor in 0.5 M Na_2CO_3-0.5M $NaHCO_3$ buffer solution (pH 9.6) at 25°C. k_{cat}/K_M value was $9.16 \cdot 10^6$ $M^{-1}s^{-1}$. SspCA was characterized by a good thermal stability, presenting half-life of 53 and 8 days at pH 8.3 and at 40 and 70°C, respectively. The enzyme was characterized by low affinity for hydrogen sulfide, bicarbonate, and carbonate.
Ye and Lu (2014) characterized an engineered CA form supplied by *Novozymes* in a stirred cell reactor. The CA was characterized into K_2CO_3 20% aqueous solution and the effects of carbonate to bicarbonate (CTB) conversion (0-40%) and temperature (25-50°C) were analyzed. CA was characterized by k_{cat}/K_M of about $1.24 \cdot 10^8$ $M^{-1}s^{-1}$ and it was not affected by CTB conversion and temperature, under operating conditions tested. Moreover, the biocatalyst was characterized by a good stability in presence of SO_4^{2-}, NO_3^-, Cl^- ions.
Zhang and Lu (2015) characterized an engineered CA form provided by *Novozymes*. They carried out batch tests of CO_2 absorption into K_2CO_3 20% in conditions of lean (20% CTB conversion) and rich (55% CTB conversion) solvent at 50°C. They assessed k_{cat}/K_M to be $9.0 \cdot 10^8$ $M^{-1}s^{-1}$, without any influence of CTB conversion. They also developed a theoretical model to simulate the CA performance in a packed-bed column at the scheduled conditions including the assessed kinetic parameters. The same simulation was carried out considering 5M MEA as liquid phase, in condition of lean (40% MEA conversion) and rich (90% MEA conversion) solvent. Results pointed out that the overall rate of CO_2 absorption into 5M MEA solution and into K_2CO_3 20% promoted by 3g L^{-1} CA were about the same. The long term stability of this CA at 40, 50, and 60°C into 20% wt K_2CO_3/$KHCO_3$ (20 and 40% CTB conversion) was reported by Ye and Lu (2014). CA retained: i) 80% of the activity after 6 months at 40°C; ii) 50% of the activity after 2 months and 20% after 4 months at 50°C; iii) 40% of the activity after 1 months and 20% after 2 month at 60°C. No influence of CTB conversion was observed. The results marked the high potentiality of CA as process promoter in K_2CO_3 aqueous solutions and encouraged further investigations for the

replacement of MEA-based solvents with CA-based solvents.
Hu et al. (2017) characterized a CA form of microbial origin by means of absorption tests in a wetted wall column. They used K_2CO_3 30% aqueous solutions as liquid phase at 50°C and different CTB conversions (0-20%). The CA was characterized by k_{cat}/K_M of about $5.3 \cdot 10^8$ $M^{-1}s^{-1}$ and it slightly decreased with the CTB conversion. The decrease may be due to the CA catalysis of the backward reaction of CO_2 hydration that occurs at high bicarbonate concentration and that influences the apparent reaction rate. The CA retained more than 70% of its initial activity after incubation into K_2CO_3 30% at 50°C for 8h.
Gladis et al. (2017) characterized a recombinant CA form (*Novozymes*) through absorption tests in a wetted was column. They used K_2CO_3 aqueous solutions at various concentrations (5-20%) as liquid phases and set the temperature between 25 and 55°C. The results pointed out that the enzyme activity was particularly influenced by the temperature, reaching in all the cases a k_{cat}/K_M of about $5 \cdot 10^3$ $m^3 \cdot kg^{-1} \cdot s^{-1}$ at low solvent concentrations (5-15 wt%). On the other hand, at 20% wt K_2CO_3 a visible increase of the rate constant was observed, passing from $1.2 \cdot 10^4$ $m^3 \cdot kg^{-1} \cdot s^{-1}$ at 25°C to $2.1 \cdot 10^4$ $m^3 \cdot kg^{-1} \cdot s^{-1}$ at 55°C.
In conclusion, all these studies confirm the remarkable potential of some CA forms as biocatalysts, providing a realistic demonstration of the feasibility of the biomimetic CO_2 capture processes.

1.4 Absorption unit configurations

Although the wide variety of several reactor configurations proposed in literature, the design of the absorption unit is still an open issue and a critical challenge. In general, the reactor configuration strongly depends on the use of the dissolved (homogeneous catalysis) or immobilized enzyme (heterogeneous catalysis). The use of CA-based heterogeneous catalysts can provide several advantages with respect to the use of the biocatalyst in dissolved form. In particular:

a) The use of the proper immobilization technique significantly improves the enzyme stability under the industrial process conditions (Garcia-Galan et al., 2011);
b) The immobilization of the CA in the absorption unit allows the confinement of the enzyme in the absorption unit and avoid the flow of the enzyme in the desorption unit characterized by harsh conditions, in particular temperature close to 100°C. Indeed, these conditions may be scarcely consistent with enzyme activity and stability, even though highly thermostable CA forms are available. In addition, immobilization techniques allow the use of high enzyme loadings, e.g. concentrations larger than 300 mg L^{-1} (Lu et al.,2011; Ye and Lu, 2014; Zhang and Lu, 2015);
c) Under the high enzyme and salts concentrations required for the CO_2 absorption (Ye and Lu, 2014), the free CA may form enzyme aggregates and it may reduce the homogeneous biocatalyst efficiency;
d) The use of immobilized CA also provides the possibility of easy recovery and reuse of the enzyme.

The morphology of the solid biocatalyst and the reactor configuration should be carefully designed to maximize the CO_2 absorption rate. It has been highlighted that the immobilized CA must be available at the gas-liquid interface to observe beneficial effects of the enzyme catalysis on the CO2 absorption rate (Iliuta and Larachi, 2012;

Penders-van Elk, 2013; Russo et al., 2013a; Hou et al., 2015; Russo et al., 2016). According to this criterion, several technical solutions based on the use of immobilized CA are reported in literature.

Iliuta and Larachi (2012) reported a theoretical model of a three-phase monolith slurry reactor where HCA II was covalently immobilized on monolith wall. The monolith was a bundles of identical parallel straight channels (honeycomb like) with a cross section diameter of about 3 mm. The solvent was continuously regenerated by fine particles (*slurry*) composed by ionic-exchange resin (Amberlite IRN-150) which remove HCO_3^- ions and avoid CA product inhibition. The reactor was operated continuously with respect to both the liquid and the gas phase in a co-current flow mode: liquid and gas superficial rate was set between 0.05 and 0.1 m/s and 0.05 and 0.2 m/s, respectively. Under the operating conditions set the system, the Taylor flow regime was expected: gas travels as bubbles of diameter close to the size of the monolith channel, alternated by the liquid (slags). According to the ‘‘two films’’ theory, different mass transfer phenomena inside the monolith channel take place: gas-solid, gas-liquid and liquid-solid mass transfer. Simulations showed a remarkable increase of CO_2 absorption rate with respect to that obtained in the absence of CA thanks to the large gas-solid mass transfer and CO_2 conversion catalyzed by CA in the liquid film around gas bubble.

Hou et al. (2015, 2016) developed an absorption unit made by a cylindrical hydrophilic-superhydrophobic membrane. It was obtained by: i) coating a polyvinylidene fluoride membrane (0.45mm pore size) with TiO_2 nanoparticles and ii) by functionalizing the inner wall of the membrane with carboxylated multiwalled carbon nanotubes (8–15 nm outer diameter, length 10-50 mm) and the outer wall with 1H,1H,2H,2H-per fluorododecyltrichlorosilane (FTCS). The inner hydrophilic wall of the membrane was in contact with the liquid phase while the hydrophobic side was oriented toward the gas phase which circumvented the membrane. CA (from bovine erythrocytes) was immobilized on the inner wall by adsorption (Hou et al., 2015) or covalently attached on titania nanoparticles dispersed in the liquid phase (Hou et al., 2016). In both cases, CA catalysis occurred close to the gas-solvent interface, within a gas-liquid membrane contactor. The CA immobilization on titania nanoparticles was characterized by larger benefits in terms of higher immobilization yields and easier biocatalyst recovery with respect to CA adsorbed to the inner wall of the membrane. Moreover, the immobilized enzyme showed similar performances, in terms of the CO_2 hydration rate, with respect to the dissolved CA, indicating that the immobilization process only had very a negligible effect on the activity of CA.

Finally, the use of the *slurry* reactors seems to be one of the optimal strategy to allow an effective enhancement of CO_2 absorption rate. Indeed, the ability of the catalytic fine particles (*slurry*) to be available close to the gas-liquid interface provides beneficial effects on the enhancement of the CO_2 absorption rate (Russo et al., 2013a; Alper et al., 1980a; Ramachandran, 2007; Penders-van Elk et al., 2013). Russo et al. (2016) developed a theoretical model of a staged bubble column for the biomimetic CO_2 capture in potassium carbonate solution as liquid solvent. CO_2 absorption simulation were carried out in the absence of CA and in the presence of fine particles of immobilized CA suspended in the liquid phase (*slurry*). The staged bubble column was described according to the “tanks-in-series” approach and the reversible Michaelis-Menten kinetics was used to model the reaction rate of CO_2 hydration catalyzed by CA. Simulation results showed that the enhancement of the CO_2 absorption rate in presence of CA immobilized on fine particles (50-80 mg/g_{solid} , k_{cat} = 10^6 s^{-1}) was three times larger than the absorption rate into pure alkaline

solvent. This study highlights the crucial dependence of the absorption unit performance on the enzyme kinetics and on the properties of immobilized enzyme particles. Therefore, it remarks the importance of the selection of thermoactive CA forms and of the proper support/immobilization technique, in order to minimize the enzyme activity lowering due to immobilization. Finally, the assessment of intrinsic kinetics of the immobilized CA is an essential tool for the design of the absorption unit.

1.5 Enzyme immobilization techniques: overview and application to carbonic anhydrase

The enzymes as industrial biocatalysts are characterized by high potential in a wide spectrum of applications thanks to their high selectivity, specificity and activity under mild conditions. They provide significant benefits in terms of: reduced processing time, low energy input, cost effectiveness, nontoxic and environmentally friendly features. Singh et al. (2016) reported a detailed review about the current industrial employments of enzymes. The main applications are in pharmaceutical and diagnostics, food, textile, organic synthesis, as well as waste treatments. However, significant drawbacks are strictly related to their biological origin: e.g., under the process conditions they may be characterized by low activity and stability, low solubility, substrate and/or product inhibition. A valid solution to this drawbacks may be the use of enzymes-based solid biocatalyst by means of immobilization techniques (Garcia-Galan et al., 2011; Rodrigues et al., 2013; dos Santos et al., 2015; Bilal et al., 2017). Indeed, the enzyme immobilization offers an effective and remarkable approach to reduce enzyme instability. Moreover, solid biocatalysts may be easily recovered and used in different reactor configurations.

A wide spectrum of immobilization techniques is available, providing a great versatility for the solid biocatalysts preparation, in dependence on enzyme applications and reactor configurations. In general, the immobilization techniques can be divided in three main groups based on the nature of the interaction between the enzyme and other reagents/phases involved in the process (Moehlenbrock and Minteer, 2011):

- *Adsorption*. Physical interactions between proteins and the surface of solid carriers by means of Van der Waals forces, hydrogen bridge bonds and electrostatic interactions. These interactions depend on the properties of the enzyme and of the support, such as electrostatic charge, polarity, pK values. Usually, adsorption is a mild immobilization technique and enzyme leaching may occur (Moehlenbrock and Minteer, 2011);
- *Enzyme entrapment*: Polymerization of a monomer or low molecular weight polymer around the protein (e.g., sol-gel processes) traps enzymes into the polymeric matrix. The porous structure of the polymer must ensure the diffusion of substrates and products and must avoid enzyme leaching. The advantage of this technique is the confinement of the enzyme in a limited zone, preventing a premature denaturation under the process conditions. Drawbacks of this technique include: harsh alkaline or acidic conditions during the sol-gel process that can compromise the enzyme activity, limited porosity of the matrix that limits the diffusion of substrate/products, shrinking of the matrix during the curing phase, relatively long time (hours to days) for the material to undergo polycondensation and cure to the final product (Johnson and Luckarift, 2011);

- *Covalent immobilization techniques*. Two solutions may be found: chemical interactions may occur between the enzyme and a solid surface and cross-linking of enzyme aggregates.

The production of Cross-Linked Enzyme Aggregates (CLEAs) is a carrier-free immobilization technique. It consists in (i) the formation of insoluble supramolecular structures (enzyme aggregates) by treatment with a precipitant agent, and (ii) cross-linking with a bifunctional agent, to stabilize the aggregates by covalent bonds and to avoid the re-dissolution of the enzymatic aggregates in the aqueous medium after removing of the precipitant agent. There is a wide spectrum of precipitant agents, such as organic solvents, polymers (e.g. polyethilenimine) and inorganic salts. The selection of the proper precipitant agent depends on the nature of the enzyme. The aim is the formation of enzymatic aggregates characterized by significant activity, therefore the modification of the native enzyme structure during enzyme aggregation must be prevented. As regards the cross-linking step, glutaraldehyde is the most used bifunctional agent because it is inexpensive and easy to handle. It is able to form covalent bonds with a number of enzymes through nucleophilic attack by the amino group of the lateral lysines to the aldehyde groups of glutaraldehyde. However, glutaraldehyde may be associated to significant activity losses, in particular when a lysine is close to the active site (e.g. nitrilases) (Barbosa et al., 2014). Indeed, the small size of glutaraldehyde allow the molecule to penetrate within the enzyme structure and to react with the internal amino groups close to the active site. Moreover, intramolecular cross-linkings may occur with glutaraldehyde inducing strong distortions of the enzyme structure. In this case, the use of macromolecular cross-linking agents is suggested, e.g. cross-linkers derived by oxidation of polysaccharides to give aldehyde-functionalized macromolecules (Mateo et al., 2004; Zhen et al., 2013). Regarding the enzyme immobilization on solids, the supports may be the inner walls of the bioreactor, packing materials or granular solids. The supports must have some specific requirements, such as low cost, no toxicity, and high long term stability, due to their storage times which may last weeks or months (Garcia-Galan et al., 2011). Among the numerous supports, a particular attention deserve the magnetic granular beads. Many contributions have been reported in the literature about the immobilization of enzymes on magnetic granular beads (Tural et al., 2015; Cao et al., 2016). Indeed, the use of magnetic supports may facilitate the recovery of biocatalysts from reaction mixtures by the application of a magnetic field.
The selection of the immobilization technique is an important prerequisite to ensure a high quality of the solid biocatalyst. Moreover, the immobilization parameters must be carefully selected. Conditions such as temperature, ionic strength, and pH may promote denaturation, especially under prolonged exposure. In many cases, the immobilization of enzymes may cause the decrease of their activity. However, it provides a great stability improvement under the various process conditions (Rodrigues et al., 2013). In general, the enzyme activity decrease may be due to possible conformational alterations of the enzyme, active center blockage and substrate diffusional hindering. The control of the enzyme orientation - by selecting proper support and tuning of the immobilization conditions (e.g. pH and temperature) - plays a key role on the final activity of the immobilized enzyme (Torres et al., 2003; Hernandez and Fernandez-Lafuente, 2011). Enzyme deactivation may also be due to multipoint attachment and remarkable physical interactions between the enzyme and supports. A common technique to prevent the conformational alterations is the use of a long and flexible spacer arm between the enzyme and the support. However, this

technique does not contribute to the stabilization of the enzyme (Garcia-Galan et al., 2011).
The mechanical resistance and the physical properties of the biocatalyst are crucial issues to be addressed in the immobilization strategy: these features must be consistent with the selected reactor configuration (Garcia-Galan et al., 2011). Criteria for the selection of the solid support include the mechanical properties. Supports to be used for biocatalyst utilization in internal mechanical stirring reactors include flexible polymers, such as agarose, cellulose, etc. Supports to be used for biocatalyst utilization in fixed bed reactors include rigid structures (inorganic supports like porous glass, silicates, etc.) and the size should be compatible with the pressure drop typical of packed columns. CLEAs may have tunable mechanical resistance depending on the cross-linking extent. However, CLEAs are considered too soft and their application may be limited in some reactor configurations (e.g. stirred or pneumatically mixed reactors or liquid solid fluidized beds). The entrapment of the immobilized enzyme in polymeric matrixes may provide a good mechanical resistance (Bentagor et al., 2005; Wiemann et al., 2011).

Innovative and promising biocatalysts can be developed by co-immobilization of two or more enzymes on the same carrier or by means of CLEA technique (combi-CLEA). These biocatalysts - known as combi-catalysts – are often characterized by properties higher with respect to the use of a mixture of individually immobilized enzymes. Takelar et al. (2013) proposed a combi-CLEA of glucoamylase and pullulanase to catalyze the starch hydrolysis reaction. Their results showed that 100% starch conversion was achieved by the combi-CLEA after 120 min of hydrolysis reaction, whereas 60% and 40% starch conversion was achieved by the separate CLEAs mixture and dissolved enzyme mixture, respectively. The higher performances of the co-immobilized enzymes systems with respect to single enzymes may be due to the proximity of the different enzymes in the combi-catalyst. Indeed, the effect called substrate channeling may be proposed: the product of an enzyme is directly transferred to the other co-localized enzyme where it can act in turn as substrate or co-reagent. The reduced diffusional distances increases the speed of the reaction and leads to enhanced performance of the cascade reactions compared to their soluble or individual immobilized form. However, active sites should be properly oriented to take the advantage of the co-immobilization. The substrate channeling effect is schematically described in Fig 1.3 (Ji et al., 2016).

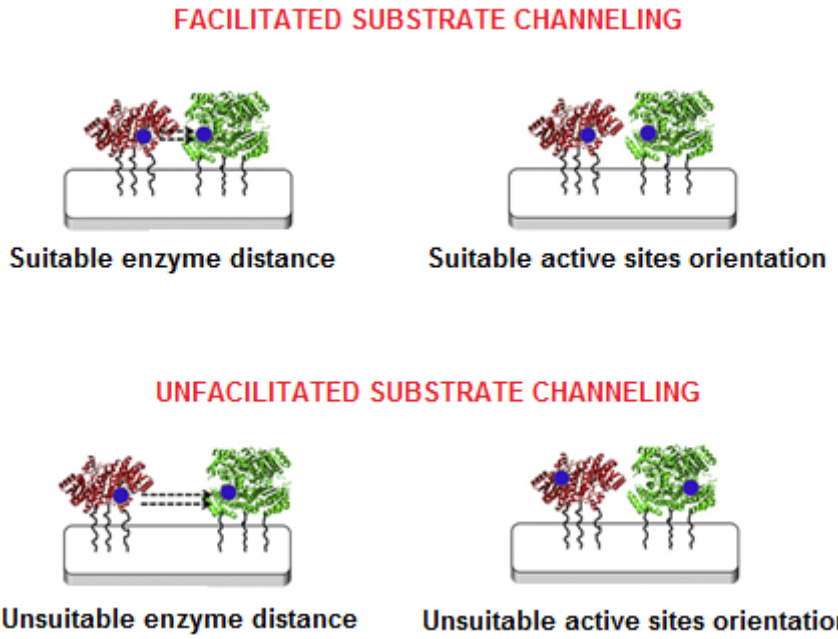

Fig. 1.3. The main parameters influencing the substrate channeling: enzymes distance and active sites orientation (Ji et al., 2016).

Combi-catalysts have been widely applied to redox and hydrolysis reactions (Ji et al., 2016). In general, the product (or the by-product) of the first cascade reaction is the substrate for the enzyme that catalyzes the second reaction, and so forth. The synthesis of mandelic acid from benzaldehyde is an example of these applications: the hydrocyanation catalyzed by hydroxynitrile lyase produces mandelonitrile that is subject to by nitrilase-mediated hydrolysis (van Rantwijk and Stolz, 2015). In some cases, the (by-)product of the first reaction is not the substrate of the second reaction but it can be used to modify the substrate of the second reaction. An example of this kind of application is the use of oxidases to produce hydrogen peroxide utilized in the second step by lipases to produce peracids (Aouf et al., 2014). Finally, cascade reactions may be involved to convert by-products that may interfere the main reaction path. An example of this kind of application is the catalase to convert hydrogen peroxide in the presence of reactions catalyzed by oxidases (Godjevargova et al., 2004). Cascade reaction application is also use for the conversion of CO_2 to methanol by three step dehydrogenases: formate dehydrogenase, formaldehyde dehydrogenase, and alcohol dehydrogenase. In particular, formate dehydrogenase catalyzes the CO_2 conversion to formate, then formaldehyde dehydrogenase catalyses the reduction of formate to formaldehyde, and alcohol dehydrogenase catalyzes the reduction of formaldehyde to methanol. This reaction chain requires 3 moles of reduced nicotinamide adenine dinucleotide (NADH) per mole of produced methanol. Therefore, the glutamate dehydrogenase can also be co-immobilized together with the other enzymes for continuous regeneration of the cofactor (Ji et al., 2015). Although the remarkable benefits, the co-immobilization of enzymes presents several drawbacks. The main drawback is that the combi-catalyst must be wasted as soon as the less stable enzyme is inactivate even though the other enzymes are still active. Moreover, the use of the same immobilization technique and operational conditions may not be an optimal solution for all the involved enzymes.

Many contributions are available in the literature regarding the CA immobilization on

solid supports. Various examples are reported hereinafter.
Ozdemir et al. (2009) immobilized the bovine carbonic anhydrase (bCA) by covalent attachment within a polyurethane foam matrix. The activity was measured by using the paranitrophenyl acetate (p-NPA), in Tris buffer (50 mM, pH 7.5) and 10% acetonitrile (esterease activity). The immobilized CA was characterized by activity close to that of the free enzyme. Moreover, it presented an excellent stability after incubation in Tris buffer (50 mM, pH 7.5) at 4°C for 45°C even though free bCA lost all its activity under the same conditions. Immobilized bCA preserved all its activity after 7 cycles.
Vin*oba et al. (2012)* immobilized the human carbonic anhydrase I (HCAI) on gold nanoparticles assembled over amine/thiol-functionalized mesoporous silica matrix (SBA-15). The activity was measured by carrying out the hydration reaction for 5 min in 0.5 M Tris-HCl buffer (pH 6.4) at 25°C. Then, the enzyme was recovered and the resulting solution was treated with $CaCl_2$ aqueous solution in 0.5 M Tris base (pH 10.). The precipitated $CaCO_3$ was recovered and CO_2 hydration rate was indirectly assessed by processing the $CaCO_3$ measured mass. Results showed that free and immobilized HCAI were characterized by the same results in terms of biocatalytic CO_2 hydration. The immobilized HCAI was characterized by unaltered activity after incubation inTris-HCl buffer (50 mM, pH 6.4) for 20 days at 25°C,even though the dissolved catalyst preserved just the 70% of its initial activity under the same conditions.
Yadav et al. (2012) immobilized bCA by entrapment in sodium alginate beads. The activity was measured according to a titrimetric assay by measuring the pH decay from 8.3 to 6.3 in 12 mM Tris Buffer at 4°C. The hydration reaction tests followed by $CaCO_2$ conversion were carried out according to a procedure similar to that reported by Vinoba et al. (2012).The immobilized CA showed a higher activity retention at 40°C in Tris Buffer (20 mM, pH 7) with respect to free enzyme. The immobilized CA presented an excellent stability after incubation in Tris buffer (20 mM, pH 7), preserving the 80 and 60% of the initial activity at 4°C for 25°C, respectively, even though the free bCA preserved just the 60% of its initial activity at both temperature. The immobilized enzyme preserved 50% of the initial activity after 10 reuse cycles. CO_2 hydration/$CaCO_3$ conversion tests results reported a similar amount of $CaCO_3$ product in both cases of free and immobilized enzymes. This result highlighted that the activity of the immobilized bCA did not change after the immobilization procedure.
Wanjari et al. (2011) immobilized a CA form extracted from *B. Pumilus* on cross-linked chitosan based beads by physical adsorption. Physical interactions were based on Van der Waals attractions, hydrogen bonding and ionic interaction between the positively charged surface of the material and negative charge on enzymes. The activity was measured according to several procedures (titrimetic method, esterase activity measurements, CO_2 hydration/$CaCO_3$ conversion tests). In all the cases, the activity of the immobilized enzymes was lower than its free counterpart: the reduction may be due to a possible change in the enzyme conformation and to the accessibility reduction of the active site to the substrate. Immobilized enzyme were more stable than the free enzymes: half-life was 216 and 456 hours after incubation into phosphate buffer (100 mM, pH 7) at -20 and 25°C and it was 192 and 408 hours for the free enzymes.
Zhu et al. (2017) immobilized the Bovine carbonic anhydrase (bCA) by entrapment in alginate polymers and formation of covalent bindings via glutaraldehyde cross-linking. The activity was measured according to titrimetic method. CO_2 capture tests were carried out by using a vertical reactor. Distilled water was used as liquid phase

and a gas mixture of N_2 and CO_2 (14%) was used as gas phase. pH measurement up to a constant value indicated the end of the reaction. The CO_2 volume fraction of the gas phase was continuously measured by an infrared CO_2 analyzer to indirectly assess the CO_2 absorption rate. The immobilized CA was characterized by activity of about 56.3% with respect to the free enzymes. The immobilized CA preserved the 40% of its initial activity after I hour incubation at 60°C, while the dissolved enzyme activity was almost null. The immobilized enzyme was characterized by a resistance to the SO_4^{2-}, NO_3^-, and Cl^- impurities (90% of retained activity) higher than the free enzymes that lost 51% of the initial activity after the incubation in buffer solution (pH 9) at 30°C. The immobilized CA showed a good reusability and it could be used for more than six cycles with nearly 61% preservation of the initial activity. To conclude, the immobilized bCA showed a remarkable enhancement of the CO_2 absorption rate in the adopted vertical reactor.

Remarkable studies on CA immobilization were presented by Zhang et al. (2013) and by Reardon et al. (2014). These works focused on the characterizations of solid CA-based biocatalysts under conditions close to those adopted in the biomimetic CO_2 capture process. Zhang et al. (2013) analyzed the kinetics of both the dissolved bCA and the bCA-covalently immobilized on non-porous silica nanoparticles. The characterization was carried out in 0.1 M $KHCO_3/K_2CO_3$ buffer solution (pH 10.5) within a wide temperature interval (4-50°C). Carbon dioxide absorption tests were carried out in a batch reactor at lab scale. Results showed that the immobilized bCA showed, at 40 and 50°C, the same k_{cat}/K_M value of about $1.3 \cdot 10^7 M^{-1}s^{-1}$. Moreover, it was less active than the dissolved enzyme at 40°C but the immobilized bCA was three times more active than the free enzyme at 50°C. These results suggested that the possible deactivation of the dissolved enzyme is hindered at temperature higher than 40° by the immobilization that provides improved stability to the immobilized bCA. The enzymes in the immobilized form are characterized by high long term stability: it preserved 70% of its initial activity after 30 days even in solution of SO_4^{2-}, NO_3^-, and Cl^- ions. Reardon et al. (2014) carried out absorption tests in a pilot-scale packed column installed at the National Carbon Capture Center (Wilsonville, AL) using 20% potassium carbonate solution as liquid phase at 40°C. CA was immobilized in organosilicate matrix at the walls of the structured packing. Tests were carried out for 3460 hours feeding the unit with coal flue gas stream. Results pointed out that the biocatalyst presented an exceptional stability and provided about 80% CO_2 capture.

From these contributions, it is evident that the use of CA as biocatalyst offers a great opportunity for the promotion of the CO_2 absorption rate. This encourages further investigations upon the development of solid CA-based biocatalysts for the implementation of a biomimetic CO_2 capture process.

2. AIM OF THE THESIS

The aim of the thesis was the development of solid CA-based biocatalysts to be used in a three-phase slurry CO_2 absorption unit. The present contribution is part of a wide research activity aimed to the development of a biomimetic process for CO_2 capture and utilization in microalgal cultivations. An innovative technique aimed to the production of combi-catalysts was also investigated as alternative potential strategy to exploit enzymatic systems in multi-phase systems. The PhD research activity was organized according to three lines:

- Performances of recombinant carbonic anhydrase in carbonate solutions as biocatalyst for biomimetic CO_2 absorption;
- Development and characterization of CA-based solid biocatalysts for biomimetic CO_2 absorption;
- Development of innovative protocols for enzymes co-immobilization.

The activities regarding the first two lines were carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale (DICMAPI) of the Università degli Studi di Napoli *Federico II*. The activity regarding the third line was carried out at the Instituto de Catálisis y Petrolquímica (ICP) of the Consejo Superior de Investigaciones Cíentificas (CSIC) in Cantoblanco (Madrid, Spain), under the supervision of Dr. Roberto Fernández-Lafuente from October 2015 to April 2016.

Performances of recombinant carbonic anhydrase in carbonate solutions as biocatalyst for biomimetic CO_2 absorption

The selection of the thermostable recombinant CA form is the first fundamental step for the implementation of a biomimetic CO_2 absorption process. Indeed, the enzyme must be active and stable at the typical industrial conditions: high temperature (313-333 K), large salts concentration (> 10 %), large alkalinity (pH > 10), presence of pollutants (fly ashes, NO_x, SO_2, etc.). The kinetic characterization of the selected CA by means of CO_2 absorption tests in carbonate solutions under the typical conditions of an absorption unit was carried out. The enzyme behavior at the high biocatalyst concentrations suggested in the literature (≥ 0.3 kg m^{-3}) was investigated to assess the potential CO_2 absorption rate enhancement reachable in an industrial absorption unit.

Development and characterization of CA-based solid biocatalysts for biomimetic CO_2 absorption

The development of the solid biocatalysts - characterized by morphology consistent with the potential absorption reactor configuration- was carried out. The immobilization technique was carefully selected as a function of the typology (attachment on a granular solid/packing materials or carrier-free technique). The solid biocatalyst was characterized under the typical process conditions in terms of catalytic performance and potential activity losses. Indeed, post-immobilization conformational modifications and/or resistance to mass transfer may reduce the activity of the enzyme.

Development of innovative protocols for enzymes co-immobilization

Innovative methodologies for enzymes co-immobilization were investigated as a versatile tool for further development of CO_2 capture and utilization processes based

on enzymatic conversions. The study was aimed at addressing drawbacks commonly associated with the co-immobilization procedures: the immobilization of two or more enzymes on the same support (or through combi-CLEA technique) and under the same operating conditions that can not be optimal for all immobilized enzymes.

3. PERFORMANCES OF RECOMBINANT CARBONIC ANHYDRASE IN CARBONATE SOLUTIONS AS BIOCATALYST FOR BIOMIMETIC CO_2 ABSORPTION

The present contribution reports about the methodology adopted for the kinetic assessment of a recombinant form of carbonic anhydrase (CA) selected as potential biocatalyst for biomimetic CO_2 absorption processes. According to Chapter 2, the main aim of this investigation was the feasibility assessment of the use of the recombinant CA form at conditions close to those adopted in CO_2 absorption processes. Moreover guidelines for rigorous CA kinetic assessment have been retrieved from data analysis. Several absorption tests were performed in a stirred cell apparatus by using carbonate solutions (pH>10) at different liquid solvent compositions, different temperature and carbonate to bicarbonate (CTB) conversion in the presence of the enzyme and in the only solvent. Firstly, a qualitative study on the CA behavior was carried out, basing on the experimental results in terms of effect of enzyme concentration on CO_2 absorption rate. Then, the assessment of the first order kinetic constant k_{cat}/K_M of the CA-catalyzed CO_2 hydration reaction was carried out applying theoretical analysis on the experimental data. Furthermore, investigations were carried out to assess long term stability and CO_2 absorption rate at large CA concentrations (≥ 0.3 kg m^{-3}) into potassium carbonate solvent since such enzyme concentrations are representative of those assumed in the literature for industrial applications.

3.1.1 Materials and methods

Materials. The biocatalyst was a liquid crude extract containing carbonic anhydrase at the concentration of about 60% of the total proteins content. The protein mixture was kindly supplied for research purposes by *Novozymes*. All the other chemicals were supplied by Sigma Aldrich®: potassium carbonate and bicarbonate (99% wt), sodium carbonate and bicarbonate (99% wt), phosphate buffer saline, bovine serum albumin (BSA) as lyophilized powder (≥ 96% wt), Bradford reagent (Coomassie brilliant blue). Gaseous carbon dioxide was purchased at technical grade.

Analytics. Total protein concentration in the crude protein mixture was assessed according to the Bradford assay (Bradford, 1976), CA concentration was calculated as 60% of the measured total protein content according to the manufacturer information. On the basis of the assessed CA concentration in the stock protein mixture, the liquid phases for the absorption tests were prepared by diluting the stock mixture into the carbonate solutions up to the desired biocatalyst dosage. Regarding the solutions with large CA concentrations (> 0.3 kg m^{-3}), the formation of possible protein aggregates was detected through spectroscopic analysis, by measuring the total protein concentration at 280 and the solutions optical density at 600 nm. In particular, the optical density at 600 nm was periodically registered till a constant value was reached. At that time the achievement of protein precipitation equilibrium was fixed. The total protein concentration was assessed both immediately after stock mixture addition to carbonate solution (t=0) and after the proteins precipitation reached the equilibrium. The difference between the total initial protein content the residual dissolved protein content after precipitation gave the assessment of precipitated proteins.

Experimental apparatus and procedures. The whole experimental apparatus, already used in previous work (Russo et al., 2013b), is sketched in Fig. 3.1. The main unit is a jacketed stirred cell (Applikon Biotechnology®) having 0.013 m^2 internal diameter and 0.17 m height. The vessel, made of Pyrex®, is equipped with two impellers in order to ensure an uniform mixing in the liquid and gas phases, and with a flanged steel cap connected to the gas and liquid feeding units. The stirred cell jacket is connected with a thermostatic bath (Julabo F33) to keep the reactor temperature at the set value. A differential pressure transducer (DPT) (Druck, PMP4165) was housed on the steel cap to measure the pressure of the gas phase within the reactor. The pressure signal was acquired at 2 Hz and processed with a LabView 7.1® code in order to display and store the pressure vs time data in real time.

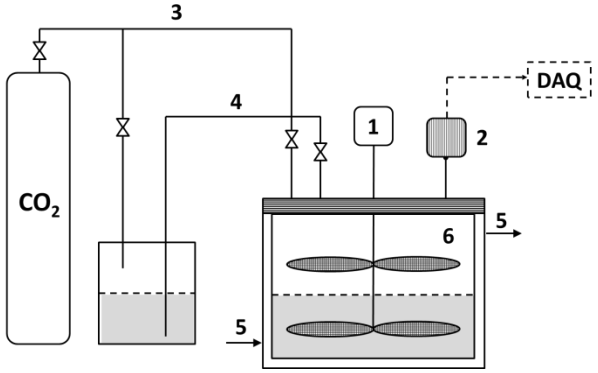

Fig. 3.1: Experimental apparatus for CO_2 absorption tests (1) stirrer motor, (2) differential pressure transducer, (3) CO_2 feeding line, (4) liquid phase pneumatic transfer, (5) thermostatic bath water line, (6) stirred cell.

Absorption tests were carried out batchwise with respect to both gas and liquid phase. The stirring rate was fixed at 100 rpm while the temperature of the thermostatic bath was tuned in order to obtain a gas and liquid phase constant temperature at the desired values (298 or 313 K). With reference of Fig. 3.1, the steps of the complete experimental procedure, aimed to the kinetic characterization of the enzyme are listed hereafter:

- The reactor vessel (6) was firstly flushed for about 20 minutes with CO_2, fixing the stirring rate at 200 rpm, at an overpressure of about 70 mbar;
- The liquid was pneumatically transferred from the tank to the reactor vessel through the loading port (4) up to a final volume of about $5.0x10^{-4}$ m^3;
- The reactor head space was pressurized with CO_2 up to a overpressure of about 140 mbar and pressure decay data acquisition was started;
- Finally, CA solution/suspension at the desired concentration was injected through the loading port (4) and again the pressure decay data acquisition was started.

Monitoring the pressure decay in the time, and assuming the ideal behavior of the gas phase, it was possible to assess the CO_2 absorbed amount in the time through pressure measurements.

3.1.2 Assessment of CO_2 absorption rate

The following assumptions were adopted in order to assess CO_2 absorption rate from experimental data: (i) the system was isothermal, (ii) the CO_2 behaved as an ideal

gas, (iii) the gas-side mass transfer resistance was negligible because pure CO_2 was adopted as gas phase, (iv) gas-liquid interfacial area corresponded to the reactor cross sectional area. Under these assumptions, the molar rate of CO_2 absorption per unit of gas-liquid interfacial area (R_{CO2}) was calculated by working out the acquired pressure decay rate according to the Eq. 3.1:

$$R_{CO2} = -\frac{dp_{CO2}}{dt} * \frac{V_G}{RTA_{GL}} \quad (3.1)$$

where p_{CO2} is the CO_2 partial pressure, V_G is the volume of the gas phase, T is the temperature fixed during the absorption test, R is the ideal gas constant , A_{GL} is the gas-liquid interfacial area.

The list of the adopted conditions during the CO_2 absorption tests, in terms of enzyme concentrations, temperature, solvent composition, CTB conversion level (α) and pH is reported in Tab. 3.1.

Tab. 3.1: Adopted operating conditions in CO_2 absorption tests.

Liquid phase	CA (kg m^{-3})	Temperature (K)	α (-)	pH
Na_2CO_3/$NaHCO_3$ 0.5 M	0-1.5·10^{-2}	298	-	9.6
	0-5.0·10^{-3}	313	-	9.6
10% wt K_2CO_3 10%	0-1.5·10^{-2}	298	0	12.3
	0-1.5·10^{-2}		0.2	10.8
	0-1.5·10^{-2}		0.4	10.4
20%wt K_2CO_3	0-1.8·10^{-2} 0.3-0.6	298	0	12.3
	0-1.8·10^{-2} 0.3-0.6	313		12.1
30%wt K_2CO_3	0-1.8·10^{-2} 0.3-0.6	298	0	12.8

3.1.3 Assessment of long term stability of CA

The long-term stability of CA was assessed through CO_2 absorption rate measurements. CA sample (1.2·10^{-2} kg m^{-3}) was incubated into 20% wt K_2CO_3 at 313 K for 26 days. The CO_2 absorption rate measurements were performed with the freshly prepared CA solution and with the same solution after the incubation period at 313 K.

3.1.4 Assessment of CA kinetics

In this section the procedure adopted to work out experimental data according to chemical absorption theory (Danckwerts, 1970) is reported. The procedure is aimed at the assessment of kinetic constant for pseudo-first order reaction occurring during CO_2 absorption process.

The general rate equation for first order reaction between the dissolving gaseous specie A and the reactant B in the liquid in agitated gas-liquid systems is given by Eq. 3.2 (Levenspiel, 1998):

$$r = \frac{1}{\left(\frac{1}{k_G} + \frac{H}{Ek_G} + \frac{H}{\varepsilon_L k_1 C_B}\right)} p_A \quad (3.2)$$

where p_A and C_B are the partial pressure and the concentration in the bulk of the gas and liquid phases for specie A and B, respectively, E is the enhancement to mass transfer by the reaction occurring in the stagnant liquid film close to the gas-liquid interface, ε_L is the liquid hold-up, k_L and k_G are the liquid and gas side physical mass transfer coefficient, respectively. As already mentioned in the Chapter 1, in the absence of any catalyst, at pH > 8 the contribution of the hydroxylation reaction is dominant to the overall CO_2 conversion rate. Under certain conditions, the kinetics of hydroxylation reaction may be described by the pseudo-first order model since the OH^- concentration in the buffer solution can be assumed almost constant at the gas-liquid interface during CO_2 absorption. In particular, this condition can be verified by the Danckwerts criterion (Eq. 3.3):

$$C^* \left(\frac{1}{[HCO_3^-]} + \frac{2}{[CO_3^{2-}]}\right)\left(\sqrt{1 + \frac{D_{CO_2} k_i}{k_L^2}} - 1\right) \ll 1 \qquad (3.3)$$

where k_L is the liquid side physical mass transfer coefficient, D_{CO2} the CO_2 molecular diffusivity in the K_2CO_3 solutions, C* the CO_2 concentration at the gas-liquid interface calculated according to the Henry's law (Eq. 3.4), k_i the overall first order kinetic constant defined according to Eq.s 3.5 and 3.6 for absorption in the only carbonate solution or in the carbonate solution supplemented with CA, respectively:

$$C^* = \frac{p_{CO2}}{H} \qquad (3.4)$$

$$k_0 = k_{H2O} + k_{OH}[OH^-] \qquad (3.5)$$

$$k_1 = k_0 + k_E[CA] \qquad (3.6)$$

Under these conditions, the overall rate equation provides the average absorption rate R_{CO2} expressed by Eq. 3.7 (Danckwerts and Sharma, 1966):

$$R_{CO2} = k_L \sqrt{1 + \frac{k_i D_{CO2}}{k_L^2}} * (C^* - C_{CO2}) \qquad (3.7)$$

where k_{H2O} and k_{OH} are the CO_2 hydration and hydroxylation kinetic constants, respectively and k_E is the kinetic constant relative to the linear approximation of Michaelis-Menten model for the CA-catalyzed hydration reaction defined according Eq. 3.8.

$$r_{CA} = \frac{k_{cat}}{k_M}[CA](C_{CO2} - C_{eq}) = k_E[CA](C_{CO2} - C_{eq}) \qquad (3.8)$$

The case of fast reaction (Danckwerts, 1970; Levenspiel, 1998) is verified whenever the Hatta modulus (Eq. 3.9) is larger than 2. In that case, by definition of fast reaction, the CO_2 conversion mainly occur within the liquid layer close the gas liquid-interface, so that the concentration of the carbon dioxide in the liquid bulk, C_{CO2}, approaches the CO_2 equilibrium concentration (C_{eq}). Since in alkaline carbonate solutions C_{eq} is negligible with respect to C* (Danckwerts & Sharma, 1966), the CO_2 absorption rates per unit of interfacial area with and without the enzyme can be assessed according to Eq. 3.10 and 3.11:

$$Ha = \sqrt{\frac{k_i D_{CO2}}{k_L^2}} \quad (3.9)$$

$$R_{CO2}^0 = C^* \sqrt{k_L^2 + k_0 D_{CO2}} \quad (3.10)$$

$$R_{CO2} = C^* \sqrt{k_L^2 + k_1 D_{CO2}} \quad (3.11)$$

The square of the ratio between Eq. 3.11 and 3.10 combined with Eq. 3.5 and 3.6 yields to Eq. 3.12:

$$\left(\frac{R_{CO2}}{R_{CO2}^0}\right)^2 = 1 + \frac{k_E}{k_0 + {k_L^2}/{D_{CO2}}} [CA] \quad (3.12)$$

According to Eq. 3.12, k_E can be assessed by linear regression of $\left(\frac{R_{CO2}}{R_{CO2}^0}\right)^2$ vs [CA] data sets following to the procedure detailed in the physicochemical parameters section (end of this chapter).

3.1.5 Assessment of liquid-phase mass transfer coefficient

The value of k_L in the Na_2CO_3/$NaHCO_3$ 0.5 M at 298 K was fixed according to Russo et al. (2013b) since the experimental test were accomplished with the same reactor and stirring rate. The value of k_L into potassium solutions was assessed adjusting the value assessed in sodium solutions by the relation provided by Hikita and Ishikawa (1969) (Eq. 3.13) according to the procedure detailed in the physicochemical parameters section:

$$Sh = 0.322\, Re^{0.7} Sc^{0.33} \quad (3.13)$$

3.2 Results and Discussion

As mentioned in the section 3.1.1, the total proteins concentration contained in the stock crude mixture was assessed through the Bradford assay and it resulted 320 kg m^{-3}. Because about 60% of total protein was carbonic anhydrase, the resultant enzyme concentration in the stock crude mixture was 192 kg m^{-3}.

3.2.1 Assessment of performances of CA as homogeneous catalyst and its long term stability

In order to carry out a qualitative analysis on the enzyme activity, the absorption tests were carried out at the operating conditions listed in Tab. 3.1. Fig. 3.2 and 3.3 show the results in terms of R_{CO2} vs CA concentration for K_2CO_3 solutions at low CA concentrations [0-1.5·10^{-2} kg m^{-3}].

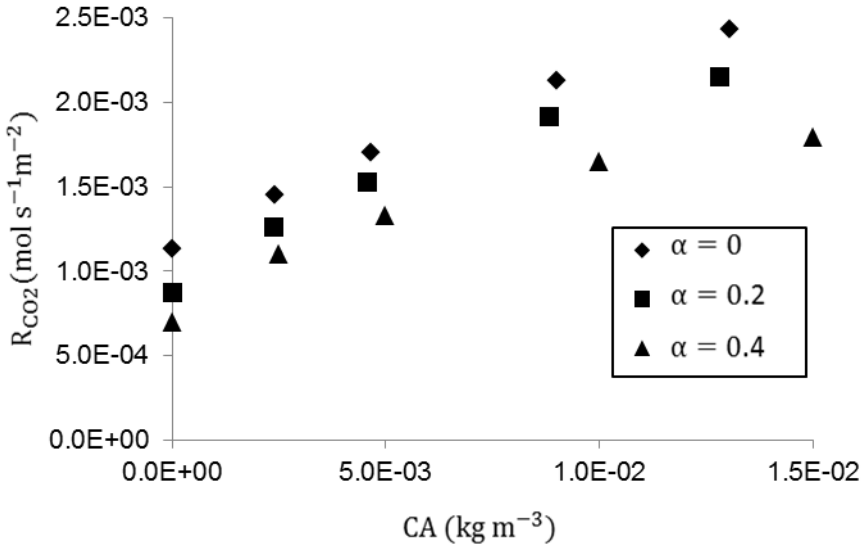

Fig. 3.2: R_{CO2} vs. CA concentration data obtained during absorption tests carried out with CA in 10%wt K_2CO_3 with α = 0, α = 0.2 and α = 0.4 at 298 K.

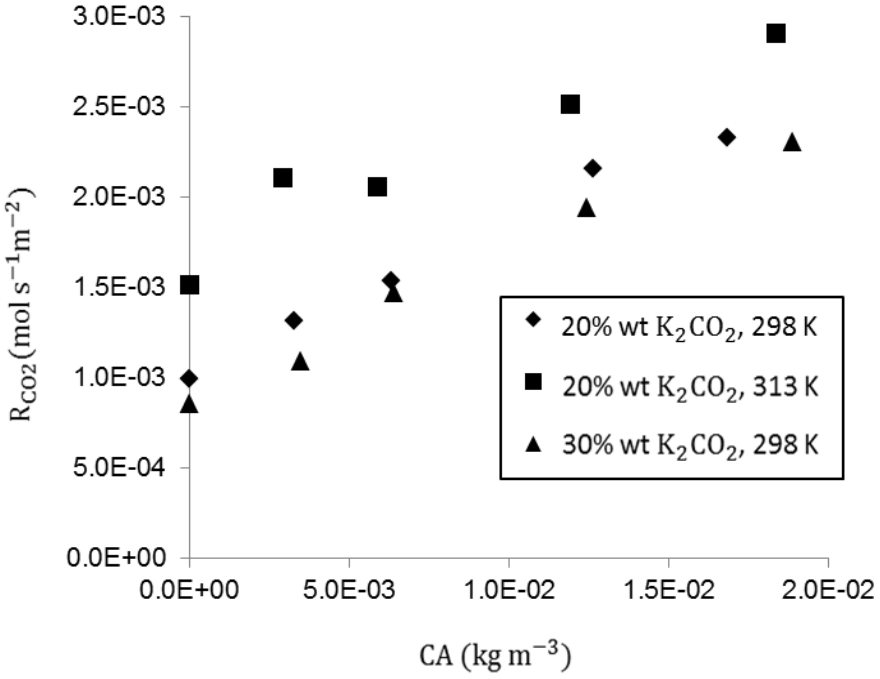

Fig. 3.3: R_{CO2} vs. CA concentration data obtained during absorption tests carried out with CA in 20%wt K_2CO_3 at 298 K, 20%wt K_2CO_3 at 313 K, 30%wt K_2CO_3 at 298 K.

Results show that at increasing CA concentration the CO_2 absorption rate increased. This result is valid in all the investigated conditions, in other words CA is active in presence of K_2CO_3 concentration ranging between 10 and 30% wt with different CTB conversions and at temperature ranging between 298 and 313 K. The overall data of R_{CO2} did not allow to quantitatively assess the contribution of CA or its extent with respect to the mass transfer rate and to the CO_2 hydroxylation rate. In order to assess the contribution of CA catalysis on the overall CO_2 absorption rate, data

analysis was extended to the calculation of the ratio between CO_2 absorption rate in the presence of the enzyme and in the only alkaline solvent, namely $\mathbf{R_{CO2}/R^0_{CO2}}$. In Fig 3.4 and 3.5 the data sets R_{CO2}/R^0_{CO2} vs [CA] are reported for the absorption tests in potassium carbonate solutions.

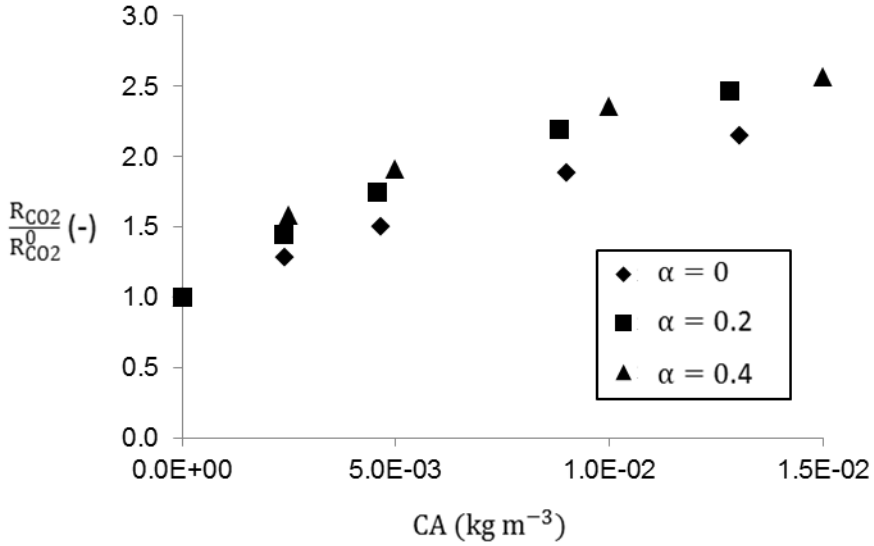

Fig. 3.4: $\mathbf{R_{CO2}/R^0_{CO2}}$ vs. CA concentration data obtained during absorption tests carried out with CA in 10%wt K_2CO_3 with α = 0, α = 0.2 and α = 0.4 at 298 K.

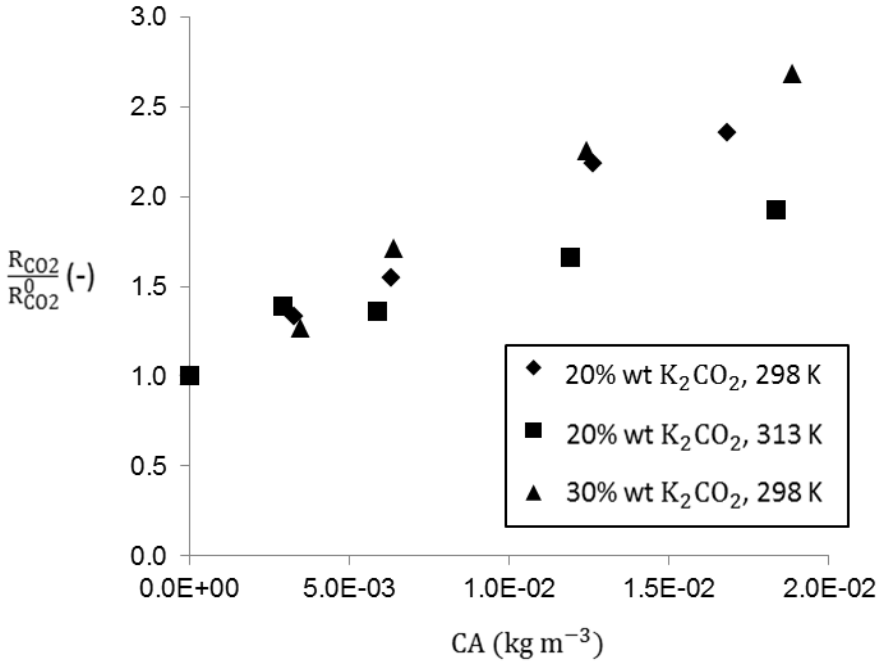

Fig. 3.5: $\mathbf{R_{CO2}/R^0_{CO2}}$ vs. CA concentration data obtained during absorption tests carried out with CA in 20%wt K_2CO_3 at 298 K, 20%wt K_2CO_3 at 313 K, 30%wt K_2CO_3 at 298 K.

Ratios reported in Fig 3.4 and 3.5 represent the enhancement of the CO_2 absorption rate with respect to the absorption rate in the only aqueous solvent due to the

contribution of CA at different initial operating conditions. The enhancement ranged between 1.5 and 2.5 for the investigated enzyme concentrations and this range of values is almost constant when solvent composition and temperature are changed within the investigated values. These results can be explained by the wide complexity of the process that embodies CO_2 dissolution in the liquid electrolyte solution, CO_2 convective transfer across the liquid and its chemical conversion into bicarbonate by hydroxylation reaction and CA catalyzed hydration. Both mass transfer rate and CO_2 hydroxylation rate depends on temperature and solvent composition and the overall absorption rate is non-linear function of parameters describing such phenomena according to the general expression reported in section 3.1.4.
The same analysis has been applied to data regarding tests at large CA concentrations (0.3-0.6 kg m^{-3}). Results are reported in Tab. 3.2. CA concentrations between 0.3 and 0.6 kg m^{-3} have been proposed in several theoretical and experimental studies on biomimetic CO_2 capture promoted by CA (Lu et al., 2011; Ye and Lu, 2014b). The formation of protein aggregates through precipitation was verified through spectroscopic measurements of the total proteins concentration and solutions optical density as described in section 3.1.2. Due to the high CA concentrations, it is worth to note that solutions containing 0.3 and 0.6 kg m^{-3} of CA are characterized by 0.5 and 1 kg m^{-3} of total dissolved proteins. The ratio R_{CO2}/R^0_{CO2} was assessed in order to characterize CA performances at large enzyme concentration and in the presence of precipitated protein aggregates. Tab. 3.2 reports the values of R_{CO2}/R^0_{CO2} ratios obtained at the above mentioned concentrations and the protein precipitation yields assessed through spectroscopic measures. In all the cases, when the crude extract was added to the K_2CO_3 solutions, liquid turbidity increased quite rapidly. The optical density at 600 nm was 0.1 and 0.22 for 0.3 and 0.6 kg m^{-3} of CA, respectively, and the value remained almost unaltered after 30 minutes.

Tab. 3.2 CO_2 Absorption rate enhancement R_{CO2}/R^0_{CO2} and enzyme precipitation yields for K_2CO_3 solutions supplemented with CA.

Run	CA concentration (kg m^{-3})	K_2CO_3 concentration (% wt)	Temperature (K)	R_{CO2}/R^0_{CO2}	Precipitation yield (%)
A1	0.3	20	298	5.2 ± 0.1	0
A2			313	4.9 ± 0.4	0
A3		30	298	5.2 ± 0.6	0
B1	0.6	20	298	5.6 ± 0.1	40 ± 2
B2			313	6.4 ± 0.2	39 ± 3
B3		30	298	8.3 ± 0.8	48 ± 3
B4	0.36	20	298	5.4 ± 0.1	-
B5	0.37		313	5.0 ± 0.6	-
B6	0.31	30	298	5.2 ± 0.6	-

No protein precipitation was detected by spectroscopic protein concentration measures for solution at 0.3 kg m^{-3} of CA (run A1-3), this could be due to the different sensitivity of the optical density measures (indicating slight increase at 600 nm) and the UV measures providing total dissolved protein concentration in the liquid phase. A remarkable precipitation was observed in solutions having 0.6 kg m^{-3} CA (run B1-

3). In particular, about 40% decrease in total protein concentration was observed at 20% wt K_2CO_3 and this value did not change increasing temperature from 298 to 313 K (run B1-2). A slight increase in protein precipitation, up to about 48%, was observed into 30%wt K_2CO_3 (run B3). At 0.3 kg m^{-3} of CA, no remarkable effects of temperature and salt concentration were observed according to data in Tab. 3.2 (run A1-3) in agreement with results reported in Fig 3.4 and 3.5. On the contrary, at 0.6 kg m^{-3} CA, R_{CO2}/R^0_{CO2} increased both with increasing temperature (B2) and K_2CO_3 concentration (B3). The results obtained for CO_2 absorption in 20% K_2CO_3 solution at 298 K with 0.3 and 0.6 kg m^{-3} CA (run A1 and B1) are consistent with the assessed total protein concentration because similar enhancement factor R_{CO2}/R^0_{CO2} has been obtained from solvent having similar CA concentrations. As a matter of fact, after 40% protein precipitation at 0.6 kg m^{-3} CA (B1) the resultant liquid phase had CA concentration close to 0.3 kg m^{-3} (A1). In order to elucidate the effect of precipitated enzyme on the CO_2 absorption rate, further tests (B4-6) were carried out with K_2CO_3 solutions having CA concentrations equal to those assessed in the liquid phase at the equilibrium with precipitated proteins. Results have been compared with those obtained at 0.6 kg m^{-3} CA (B1-3). CO_2 absorption rate enhancement R_{CO2}/R^0_{CO2} obtained in 20% K_2CO_3 at 298 K and CA concentration close to solubility limit but lacking in precipitated enzyme (B4) is similar to that obtained under the same conditions of ionic strength and temperature in the presence of saturated solution with precipitated enzyme (B1). As expected due to similar dissolved CA concentrations both run B5 and B6 showed similar CO_2 absorption rate enhancement. These values were lower than those observed for the saturated solution in the presence of precipitated CA aggregates (B2 and B3). This suggests that some not null contribution to CO_2 absorption rate enhancement may be provided by the precipitated CA aggregates that can be assumed to be made by active enzyme. With this figures, even at large CA concentration (almost equal to saturation condition), if no precipitated aggregates are present the behavior of the enhancement is similar to those observed at small concentration, whenever protein aggregates are not negligible a further increase in the enhancement was observed. This suggest a possible activity of protein aggregates.
Finally, concerning the long term stability assessment of CA in 20%wt K_2CO_3 solution at 313 K (Section 3.1.3), CO_2 absorption rate resulted $2.3 \cdot 10^7$ e $1.9 \cdot 10^7$ mol·s^{-1} for freshly prepared solution and for solution stored at 313 K for 26 days, respectively. Thus it can be conclude that the enzyme retained about 83% of its initial activity after the incubation at the abovementioned conditions.
Following section reports results on further data analysis that was performed to quantify the effect of CA on the enhancement ratio R_{CO2}/R^0_{CO2} according to the theory described in section 3.1.4 in terms of the parameter k_{cat}/K_M.

3.2.2 Assessment of CA kinetics

As reported in the section 3.1.5, the values of k_L at each operating condition were calculated as reported in the physicochemical parameters section. The results are reported in Tab. 3.3.

Tab. 3.3: Values of liquid side mass transfer coefficient k_L (see physicochemical parameters section).

Liquid phase	Temperature (K)	k_L (m s^{-1})
Na_2CO_3/$NaHCO_3$ 0.5 M*	298	$5.3 \cdot 10^{-5}$
	313	$6.7 \cdot 10^{-5}$
K_2CO_3 10%	298	$5.5 \cdot 10^{-5}$
K_2CO_3 20%	298	$4.7 \cdot 10^{-5}$
	313	$4.6 \cdot 10^{-5}$
K_2CO_3 30%	298	$3.1 \cdot 10^{-5}$

* k_L value calculated from Russo et al. (2013b)

According to the theory reported in section 3.1.4., Eq. 3.12 was adopted to perform linear regression of data sets $(R_{CO2}/R^0_{CO2})^2$ vs. CA concentration. Fig. 3.5, 3.6 and 3.7 report the results of linear regressions on the entire experimental data obtained at the conditions listed in Tab. 3.1. Fig.s 3.5-3.7 show satisfactory agreement between the theoretical ratio in Eq. 3.12 and the experimental data as a function of CA concentration. The kinetic constant k_{cat}/K_M was calculated from the slope of the linear curve resulting from each linear regression, results are reported in Tab. 3.4.

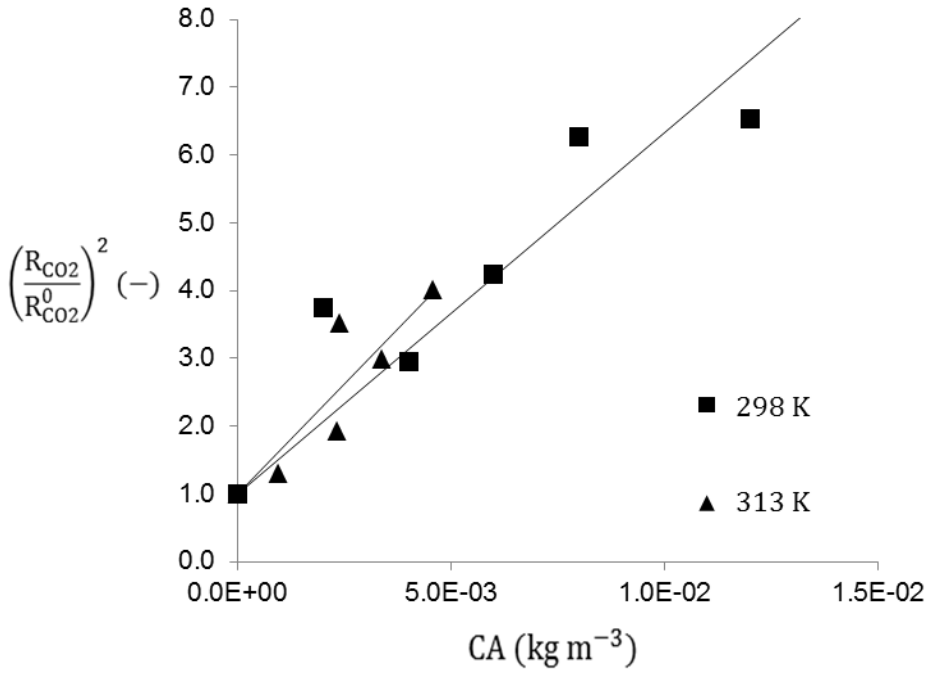

Fig. 3.5: Linear regression of $(R_{CO2}/R^0_{CO2})^2$ vs CA concentration data sets according to Eq. 3.12. CO_2 absorption tests were carried out in Na_2CO_3 /$NaHCO_3$ 0.5 M at 298 and 313 K.

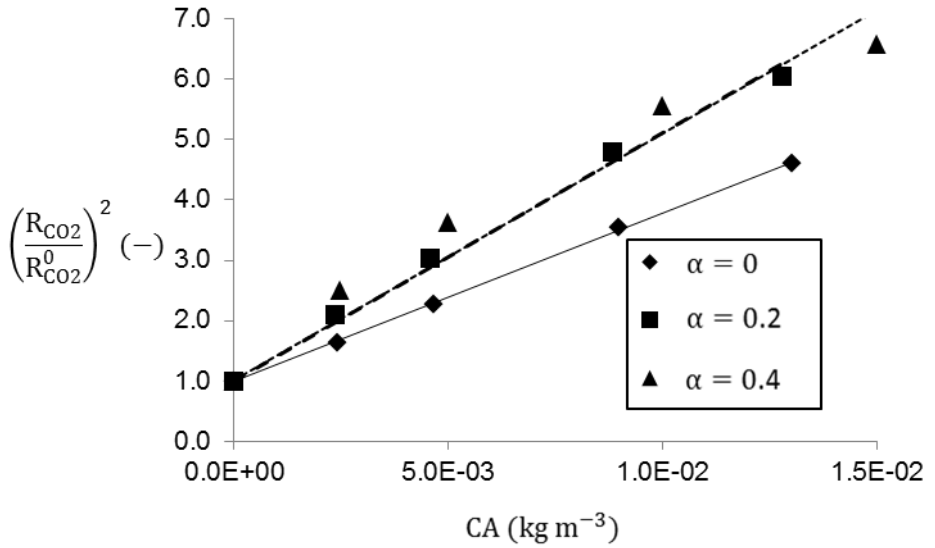

Fig. 3.6: Linear regression of $(\mathbf{R_{CO2}/R^0_{CO2}})^2$ vs. CA concentration data sets according to Eq. 3.12. CO_2 absorption tests were carried out in 10%wt K_2CO_3 at 298 K and α = 0, α = 0.2 and α = 0.4.

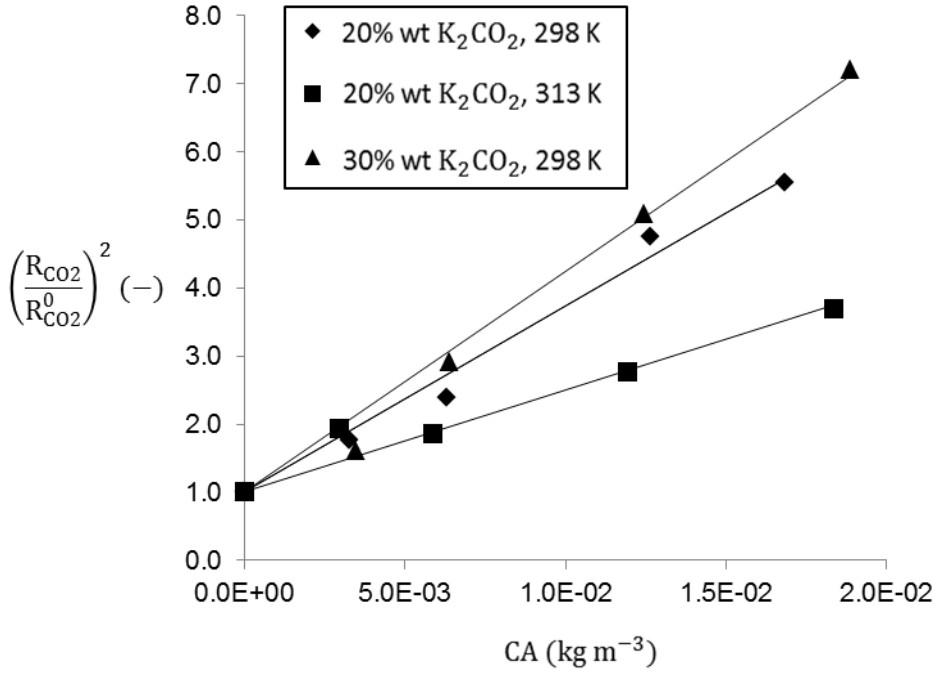

Fig. 3.7: Linear regression of $(\mathbf{R_{CO2}/R^0_{CO2}})^2$ vs. CA concentration data sets according to Eq. 3.12. CO_2 absorption tests were carried out in 20%wt K_2CO_3 at 298 and 313 K and in 30% wt K_2CO_3 at 298 K.

Tab. 3.4: Values of the slopes, Danckwerts criterion (Eq. 3.3), Hatta numbers and kinetic constant k_{cat}/K_M at the adopted conditions.

Solvent composition	α (-)	T (K)	Slope (Eq. 3.12) ($m^3\,kg^{-1}$)	R^2 (-)	LHS term (Eq. 3.3) (-)	Ha (-)	k_{cat}/K_M ($m^3 \cdot kg^{-1} \cdot s^{-1}$)
Na_2CO_3	-	298	$5.3 \cdot 10^2$	0.88	0.02	0.7	$1.4 \cdot 10^3$
$NaHCO_3$ 0.5M	-	313	$6.5 \cdot 10^2$	0.81	0.09	2.2	$8.1 \cdot 10^3$
10%wt K_2CO_3	0	298	$2.8 \cdot 10^2$	0.99	14.6	5.5	$1.5 \cdot 10^4$
	0.2		$4.1 \cdot 10^2$	0.99	0.19	2.1	$3.3 \cdot 10^3$
	0.4		$4.1 \cdot 10^2$	0.94	0.08	1.6	$1.9 \cdot 10^3$
20%wt K_2CO_3	0	298	$2.7 \cdot 10^2$	0.99	4.6	8.5	$3.3 \cdot 10^4$
		313	$1.5 \cdot 10^2$	0.94	5.4	19.9	$7.2 \cdot 10^4$
30%wt K_2CO_3	0	298	$3.2 \cdot 10^2$	0.99	11.6	22.8	$1.8 \cdot 10^5$

Results in Tab 3.4 shows that the condition for fast reaction (Ha>2) is verified for almost every set of operating conditions except for the 10%wt K_2CO_3 solution having the largest CTB conversion degree and into Na_2CO_3 $NaHCO_3$ 0.5 M at 298 K. This result is due to the effect of both k_{OH} and OH^- concentration, in particular OH^- is almost constant at increasing carbonate concentration but it decreases with increasing CTB conversion. Moreover, k_{OH} is dependent on both ionic strength and CTB conversion as reported in the physicochemical parameters section, in particular it increases with the ionic strength and decreases with the CTB conversion. Concerning Na_2CO_3 /$NaHCO_3$ solution, the condition for fast reaction is not verified in the absence of CA at 298 K due to the low values of both k_{OH} and OH^- concentration. The criterion on the depletion of OH^- ions close to the gas-liquid interface (Eq 3.3) is verified in sodium carbonate/bicarbonate buffer and in potassium carbonate solutions having not null CTB conversion. This results depend on the effect of increasing of HCO_3^- concentrations (larger in sodium buffer and in partially carbonated potassium solution) that can balance the effect of large C* caused by the use of pure CO_2 at almost atmospheric pressure as gas phase during the experiments.
It reasonable to assume Ha>>2 for all the test at not null CA concentration, since the contribution of enzyme catalysis leads to further increase in CO_2 conversion rate. The changes in solvent speciation occurring after any test in presence of CA are not so large to provide relevant decrease in the LHS term of eq. 3.3.
In conclusion, satisfactory regressions of data in reported Fig. 3.5, 3.6 and 3.7 suggest that the values of k_{cat}/K_M should be reliable because the contribution of CA made the overall CO_2 conversion rate fast with respect to the physical mass transfer even though the Danckwerts criterion is satisfied only in three cases.
On the basis of the assumption reported in the section dedicated to the physicochemical parameters, for the assessment of CO_2 solubility, CO_2 diffusivity, kinetic constant of CO_2 hydroxylation and equilibrium constant for reversible ionization reactions of carbonic acid and bicarbonate, the values of k_{cat}/K_M reported in Tab 3.4 increased at increasing temperature, mainly due to the effect of k_{OH} which increased of almost one order of magnitude, while all the other parameters were scarcely influenced by the temperature change. Moreover, the k_{cat}/K_M values are influenced by CTB conversion when it is increased from 0 to 20%. Indeed, although the slopes values increased with the CTB conversion, however the $k_{OH}[OH^-]$ product significantly decreased passing from α = 0 to α = 0.2, with the final effect to obtain a kinetic constant value at null CTB conversion of one order magnitude higher with respect to the case where α = 0.2. Finally, the increasing of k_{cat}/K_M at the K_2CO_3 concentration raising, passing from 10% to 30% (α = 0) at 298 K, is mainly due to

synergic effect the k_{OH} increasing and diffusivity decreasing with the ionic strength, with the final effect to raise the kinetic constant value of about one magnitude order.
For further validation of the presented data analysis comparison of this results with relevant data reported in the literature has been detailed in the next section

3.3 Discussion and conclusions

The crude protein mixture containing 60%wt CA was characterized through CO_2 absorption rate assessment in Na_2CO_3/$NaHCO_3$ 0.5 M and 10% to 30%wt K_2CO_3 solutions at various CTB conversions. The tests were performed at 298 and 313 K. The results showed that the rough data on CO_2 absorption at increasing CA concentration can give qualitative information on enzyme activity. Even though data demonstrate the activity of the enzyme at large salt concentration and at 313 K, further data analysis was required in order to obtain quantitative characterization of CA performances in terms of kinetic parameters. Accordingly, the study was extended to data analysis on the basis of theoretical models describing reactions between dissolving gaseous species and reactant in the liquid solvent, namely the chemical absorption process.
The entire set of data collected at different solvent composition and temperature was analyzed and the kinetic constant k_{cat}/K_M was assessed assuming the overall reaction as fast pseudo-first order. The resulting values of k_{cat}/K_M were compared with the kinetic constants reported in literature for other CA forms (Tab. 3.5). It is possible to observe that k_{cat}/K_M value of the investigated CA form, obtained into Na_2CO_3/$NaHCO_3$ 0.5 M at 298 K, is one of magnitude order higher than the others CA forms characterized under the same conditions (bovine CA and SspCA from *Sulfurihydrogenibium yellowstonense*) Moreover, it is possible to observe that the k_{cat}/K_M averaged value obtained by Ye and Lu (2014b) was about $4.1 \cdot 10^3$ m^3 kg^{-1} s^{-1} and was obtained under conditions of about 20% K_2CO_3 (α = 0-0.4) at 298-323K. Moreover, all the calculated k_{cat}/K_M values resulted to be not dependent on temperature and CTB conversion. This k_{cat}/K_M averaged value was similar to those obtained in this study in 10% wt K_2CO_3 (α = 0.2-0.4) at 298 K, while it was one and two order of magnitude lower than the values obtained into 10 and 20% wt K_2CO_3 at 298 and 313 K However, it should be taken into account that, in this study, the Danckwerts' criterion (Eq. 3.3) was not satisfied in all the cases having null CTB conversions (Tab. 3.4). The experimental difficulty to carry out the CO_2 absorption tests under controlled conditions in order to satisfy the Danckwerts criterion at α = 0 was also remarked by Ye and Lu (2014b). In their work, they carried out the experiments at CO_2 total pressures lower than 100 mbar, in order to keep as small as possible the C* term (Eq. 3.3). As the Danckwerts criterion could not be satisfied in all the cases, the reliability of the k_{cat}/K_M values obtained in this work at conditions of α = 0 may be prejudged. However, it has to be regarded that the real composition of the solvents in the industrial applications is far from the condition of null CTB conversion. For example, as reported by Zhang and Lu (2015), the conditions corresponding to the top and the bottom of a packed-bed column are 20% and 55% of CTB conversion. Therefore, the knowledge of the kinetic behavior of the enzyme at conditions α≠0, instead of at null CTB conversion, becomes a more fundamental tool for the design of an absorption unit. In general, the increasing of the CTB conversion leads to conditions more favorable to the enzyme stability, as it decreases the solvent pH, while the ionic strength remains unaltered. In the present contribution, the results from qualitative study of the enzyme kinetic performances under the conditions of null CTB conversion, defined as the worst pH conditions for

the enzyme activity/stability, confirmed the ability of this enzyme to be active under such harsh conditions.

Tab. 3.5: Comparison between kinetic constant for different CA forms.

CA source	pH	Solvent	T (K)	k_E , ($m^3 \cdot kg^{-1} \cdot s^{-1}$)	Reference
Bovine CA	9.6	$NaHCO_3/Na_2CO_3$ 0.5M	298	$9.0 \cdot 10^2$	Alper and Deckwer, 1980
Bovine CA	10.5	$KHCO_3/K_2CO_3$ 0.1M	313	$5.7 \cdot 10^3$	Zhang et al., 2013
SspCA from *Sulfurihydrogenibium yellowstonense*	9.6	$NaHCO_3/Na_2CO_3$ 0.5M	298	$3.5 \cdot 10^2$	Russo et al., 2013
α-CA1 (*Novozymes*)	10-11	K_2CO_3 1.8 M (20% wt) α = 0-0.4	298-323	$4.1 \cdot 10^3$	Ye and Lu., 2014b
α -CA2 (*Novozymes*)	11	K_2CO_3 1.8 M (20% wt) α = 0.2	323	$3.0 \cdot 10^3$	Zhang and Lu., 2015
Crude protein mixture with 60% CA (*Novozymes*)	9.6	$NaHCO_3/Na_2CO_3$ 0.5M	298-313	$1.4\text{-}8.1 \cdot 10^3$	This study
Crude protein mixture with 60% CA (*Novozymes*)	12.3-10.3	K_2CO_3 0.9 M (10% wt) α = 0-0.4	298	$1.5 \cdot 10^4$ $1.9 \cdot 10^3$	This study
Crude protein mixture with 60% CA (*Novozymes*)	12.3-12.1	K_2CO_3 1.8 (20%)	298-313	$3.3\text{-}7.2 \cdot 10^4$	This study
Crude protein mixture with 60% CA (*Novozymes*)	12.7	K_2CO_3 2.8 M (30% wt)	298	$1.8 \cdot 10^5$	This study

Regarding CA characterization at high biocatalyst concentration, the R_{CO2}/R^0_{CO2} values were in accordance with the results reported by Lu et al. (2011) and Ye and Lu (2014b) when absorption tests were carried out into 20% K_2CO_3 at 40°C in presence of CA 300 mg L^{-1}: in all the cases, absorption rates ratios of about 5 were obtained. Moreover, by increasing the CA concentration up to 0.6 kg m^{-3}, Lu et al. (2011) reported that R_{CO2}/R^0_{CO2} value was about 7, similar values were obtained in this work. However, Lu et al. (2011) reported absorption rates ratio up to 9, when absorption tests were carried out into 20% K_2CO_3 at 25°C in presence of CA 0.3 kg m^{-3}. An attempt was made in the present study to assess the contribution of enzyme aggregates that may form in the presence of largely salty solvent at CA concentration larger than 0.3 kg m^{-3}. The results suggested that the presence of not negligible amount of enzyme aggregates (about 40% of initial protein amount) may give further contribution to absorption rate enhancement. Quantitative characterization of such contribution may be the object of future investigations as well as the characterization of the composition in the liquid phase at the equilibrium with the aggregates since the initial 60%wt CA of the stock protein mixture may change once the equilibrium after protein precipitation is approached.

The CA showed good long-term stability in 20%wt K_2CO_3 at 40°C, this result further encourages the use of such protein mixture as biocatalyst for biomimetic CO_2 absorption process.

In conclusion the protein mixture having CA activity has been successfully characterized to some extent in terms of its performances as biocatalyst for biomimetic CO_2 capture processes. The experimental study and the theoretical analysis allowed to highlight the criticalities related to the selection of proper operating conditions that should be representative of CO_2 capture process and that at the same time that satisfy criteria for rigorous kinetics assessment (Eq. 3.3 and 3.9) according to the case of fast first order reaction. It is worth to note that, in general, the process of CO_2 chemical absorption catalyzed by enzymes might be described by detailed models based on mass balances that take into account physical processes and any kind of chemical reactions occurring in the system (see as an example Russo et al. 2016). Such models can be use as both predictive tools and for experimental data analysis, the latter approach may allow the assessment of enzyme kinetic parameters in any condition provided that reliable chemical and physical data are available for any other processes (mainly CO_2 solubility and diffusivity in non-ideal electrolyte solutions, chemical equilibrium constants and kinetics of hydroxylation reaction).

The results reported in this section make the adopted CA form a potential candidate as CO_2 absorption promoter in alkaline carbonate solutions and encourage further investigation on the development of biocatalysts based on immobilized CA. the latter task has been accomplished according to the procedures reported in the next chapter.

Nomenclature

T	Temperature (K)
R	Ideal gas constant (Pa m^3 mol^{-1} K^{-1})
V_G	Gas volume (m^3)
V_L	Liquid volume (m^3)
A_{GL}	Gas-liquid interface (m^2)
a	Gas-liquid interface per unit volume of liquid (m^{-1})
H	Henry constant (mol m^{-3} Pa^{-1})

ρ Liquid density (kg m^{-3})
μ Liquid density (Pa s^{-1})
I Ionic strength (mol m^{-3})
α CTB conversion grade [0-1] (-)
p_{CO2} CO_2 pressure (Pa)
C* Dissolved CO_2 concentration at gas–liquid interface (mol m^{-3})
C_{CO2} Dissolved CO_2 concentration in liquid bulk (mol m^{-3})
$C_{CO2,eq}$ Dissolved CO_2 concentration at chemical equilibrium (mol m^{-3})
D_{CO2} CO_2 diffusivity in the liquid (m^2 s^{-1})
Ha Hatta modulus (-)
Sc Schmitd number (-)
K_1 Carbonic acid ionization constant (mol m^{-3})
K_2 Bicarbonate ionization constant (mol L^{-3})
K_w Water ionization constant (mol^2 m^{-6})
k_E First order kinetic coefficient for enzyme catalyzed CO_2 hydration (m^3 kg^{-1} s^{-1})
k_0 Overall kinetic constant for CO_2 conversion in carbonate solution (s^{-1})
k_1 Overall kinetic constant for CO_2 conversion in carbonate solution supplemented with CA (s^{-1})
k_{cat} Turnover number (s^{-1})
k_L Liquid-phase mass transfer coefficient (m s^{-1})
k_M Michaelis-Menten constant (mol m^{-3})
k_{OH} CO_2 hydroxylation kinetic constant (m^3 $kmol^{-1}$ s^{-1})
k_{H2O} CO_2 hydration kinetic constant (s^{-1})
E_a Activation energy (kJ mol^{-1})
r_{CA} CO_2 hydration kinetic rate catalyzed by CA (mol s^{-1} m^{-2})
R_{CO2} Molar rate of CO_2 absorption per unit of gas-liquid interfacial area (mol s^{-1} m^{-3})

Physicochemical parameters

The selection of the physicochemical parameters used to work out the experimental data is detailed hereinafter.

Ionization constants. Stoichiometric ionization constants for carbonic acid (K_1) and bicarbonate (K_2) into Na_2CO_3/$NaHCO_3$ 0.5 M and K_2CO_3 solutions were calculated as a function of temperature, according to other contributions about the kinetic assessment of other CA. The analytic expressions of K_1 and K_2 are given by Eq. A and B, respectively (Danckwertz and Sharma, 1966):

$$\mathrm{Log}(K_1) = -\frac{3404.7}{T} + 14.843 - 0.03279T \quad \text{(A)}$$

$$\mathrm{Log}(K_2) = -\frac{2902.4}{T} + 6.498 - 0.0238T \quad \text{(B)}$$

The ionic product of water was calculated according to Eq. C (Tsonopoulos et al., 1976):

$$-\mathrm{Log}\left(\frac{K_W}{\rho_W}\right) = \frac{5839.5}{T} + 22.4773\log[T] - 61.2063 \quad \text{(C)}$$

Kinetic constants. Kinetic constants for CO_2 hydration reaction k_{H2O}, into Na_2CO_3/$NaHCO_3$ 0.5 M and K_2CO_3 solutions, were calculated as only function of temperature according to Eq. D (Danckwerts and Sharma, 1966):

$$\mathrm{Log}(k_{H2O}) = 329.85 - 110.541\log[T] - \frac{17265.4}{T} \quad \text{(D)}$$

CO_2 hydroxylation constants k_{OH} into Na_2CO_3/$NaHCO_3$ 0.5 M were calculated as functions of temperature through Eq. E:

$$\mathrm{Log}(k_{OH}) = 13.65 - \frac{2895}{T} \quad \text{(E)}$$

k_{OH} values into K_2CO_3 solutions were calculated as function of the temperature, CTB conversion (α) and of ionic strength I through the Eq.s F-J (Ye and Lu, 2014a). These relations can be applied in a temperature range of 25-80°C and up to the total ionic strengths up to 12 kmol m^{-3} (unless specified otherwise):

$$\ln(k_{OH}) = \ln(A) - \frac{E_a}{RT} \quad \text{(F)}$$

$$E_a = 171.1\alpha^2 - 128.74\alpha + 47.03 \quad \text{(G)}$$

$$\ln(A) = 0.24I + 26.4 \qquad (\alpha = 0) \quad \text{(H)}$$

$$\ln(A) = 0.5I + 19.79 \qquad (\alpha = 0.2) \quad \text{(I)}$$

$$\ln(A) = 0.64I + 17.83 \qquad (\alpha = 0.4,\ I \leq 8.8 \text{ kmol } m^{-3}) \quad \text{(J)}$$

where I is the ionic strength calculated as:

$$I = \frac{1}{2}\sum_{i=1}^{N} C_i z_i^2 \quad \text{(K)}$$

C_i are the ionic species concentration and z_i the corresponding ion charge.

Carbon dioxide solubility. Henry's law constants for CO_2 solubility in pure water (H_0) and in the electrolyte solution (H) as function of ionic strength at atmospheric pressure were calculated for all the adopted solutions according to the Eq.s L-N (Danckwerts and Sharma 1966):

$$\log[H_0] = \frac{1140}{T} - 5.3 \quad \text{(L)}$$

$$\log\left[\frac{H}{H_0}\right] = -K_s I \quad \text{(M)}$$

$$K_s = i_+ + i_- + i_g \quad \text{(N)}$$

where coefficients i_+, i_- and i_g correspond to the contributions of cations, anions and gaseous species, respectively. The values of coefficients i_+, i_- and i_g are reported in Table A:

Table A: i_+, i_- and i_g constant values (Danckwerts and Sharma, 1966)

i-specie	i (m^3 mol^{-1})
Na^+	94
CO_3^{2-}	21
HCO_3^-	21
OH^-	61
CO_2	17

Diffusivity of carbon dioxide. Diffusivity of carbon dioxide into Na_2CO_3/$NaHCO_3$ 0.5 M was calculated as function of temperature and ionic strength through the Eq. O:

$$\frac{D_{CO2}}{D^0_{CO2}} = 1 - (C_1[CO_3^{2-}] + C_2[HCO_3^-] + C_3[OH^-]) \quad (O)$$

Values of constants C_1, C_2 and C_3 are 0.261, 0.14 ,0.129 kmol m^{-3} respectively, while D^0_{CO2} is the CO_2 diffusivity in water and was $1.92 \cdot 10^{-9}$ and $2.7 \cdot 10^{-9}$ m^2 s^{-1} at 298 K and 313 K, respectively (Versteeg et al., 1987). The values reported by Ye and Lu (2014a) for diffusivity of CO_2 in the K_2CO_3 solutions were adopted. In particular, diffusivity of carbon dioxide into 10%wt K_2CO_3 solutions at the various α values was assumed to be the same as that of the K_2CO_3 solution at the equivalent concentration (α= 0) and was $1.61 \cdot 10^{-9}$ m^2 s^{-1}. According to Ye and Lu (2014a). Diffusivity of carbon dioxide into 20%wt K_2CO_3 at 298 and 313 K and into 20%wt K_2CO_3 at 298 K resulted $1.36 \cdot 10^{-9}$, $1.74 \cdot 10^{-9}$ and $1.92 \cdot 10^{-10}$ m^2 s^{-1}, respectively

Composition of aqueous solvent. The behavior of the bulk liquid was assumed to be ideal, that is the chemical equilibrium for all the dissociation reaction was assumed (Eq.s P-R) as well as the electroneutrality (Eq. S). Accordingly, the chemical composition of the carbonate solutions in terms of [CO_2], [HCO_3^-], [CO_3^{2-}], [OH^-], was calculated solving the equations P-S and fixing the value of hydrogen ions concentration [H^+] according to the initial value of pH measured during CO_2 absorption tests.

$$K_1 = \frac{[H^+][HCO_3^-]}{[CO_2]} \quad (P)$$

$$K_2 = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]} \quad (Q)$$

$$K_w = [H^+][OH^-] \quad (R)$$

$$[HCO_3^-] + 2[CO_3^{2-}] + [OH^-] = [H^+] + [K^+] \quad (S)$$

Finally, diffusivity of carbon dioxide D_{CO2}, density ρ and viscosity μ of the K_2CO_3 solutions (Ye et Lu, 2014a) and ρ and μ values for Na_2CO_3/$NaHCO_3$ (Perry, Chemical Engineering Handbook, 8th Edition, 2007) at the various experimental conditions are reported in tables A2 and A3. For the 10 %wt K_2CO_3 solutions, ρ and μ of the were assumed to be the same of the 10% wt K_2CO_3 solution (α= 0). For the Na_2CO_3/$NaHCO_3$, ρ and μ of the were assumed to be the same of the 10% wt Na_2CO_3 solution.

Table B: CO_2 diffusivity D_{CO2}, density ρ and viscosity μ for 10, 20 and 30%wt K_2CO_3 solutions at 298 and 313 K (Ye et Lu, 2014a)

K_2CO_3 concentration (%wt)	Temperature (K)	ρ (kg/m^{-3})	μ (Pa·s)	D_{CO2} (m^2·s^{-1})
10	298	$1.09 \cdot 10^{3}$	$1.13 \cdot 10^{-3}$	$1.61 \cdot 10^{-9}$
20	298	$1.19 \cdot 10^{3}$	$1.36 \cdot 10^{-3}$	$1.36 \cdot 10^{-9}$
	313	$1.18 \cdot 10^{3}$	$1.12 \cdot 10^{-3}$	$1.74 \cdot 10^{-9}$
30	313	$1.30 \cdot 10^{3}$	$2.20 \cdot 10^{-3}$	$9.20 \cdot 10^{-10}$

Table C: Density *ρ* and viscosity *μ* for Na_2CO_3/$NaHCO_3$ at 298 and 313 K (Perry, 2007)

Temperature (K)	*ρ* (kg·m^{-3})	*μ* (Pa·s)
298	$1.11 \cdot 10^{3}$	$1.13 \cdot 10^{-3}$
313	$1.08 \cdot 10^{2}$	$8.3 \cdot 10^{-4}$

Assessment of the liquid mass transfer coefficient. According to Russo et al. (2013b) the value of k_L in Na_2CO_3/$NaHCO_3$ 0.5 M at 298 K (Na,298K) was $5.3 \cdot 10^{-5}$ m s^{-1}. The value of k_L at the other conditions ($k_{L,i}$) were assessed by the relation derived from the ratio between the Hikita and Ishikawa correlation (Eq. 3.13) at the condition i and at Na_2CO_3/$NaHCO_3$ 0.5 M at 298 K, according to the procedure adopted by Ye and Lu (2014a). The expression is given by Eq. T:

$$\frac{k_{L,i}}{kL_{,Na,298}} = \left(\frac{\rho_i}{\rho_{Na,298}}\right)^{0.7} \left(\frac{\mu_i}{\mu_{Na,298}}\right)^{-0.7} \left(\frac{D_i}{D_{Na,298}}\right) \left(\frac{Sc_i}{Sc_{Na,298}}\right)^{0.33} \qquad \text{(T)}$$

4. DEVELOPMENT AND CHARACTERIZATION OF CA-BASED SOLID BIOCATALYST FOR BIOMIMETIC CO_2 ABSORPTION

In this chapter, the development and characterization of solid CA-based biocatalysts for CO_2 absorption processes are reported. Two immobilization techniques have been selected: covalent attachment on non porous nano-particles and cross-linking of enzymes aggregates. The thermostable CA form characterized in the Chapter 3, and the bovine carbonic anhydrase (bCA) were used as enzyme models.

4.1 PERFORMANCES OF CARBONIC ANHYDRASE IMMOBILIZED ON MAGNETITE NANOPARTICLES AS BIOCATALYST FOR BIOMIMETIC CO_2 CAPTURE

The study reported in this subsection has been carried out in collaboration with Dott. Clemente Capasso and Prof. Mosè Rossi of the Istituto di Bioscienze e Biorisorse (IBBR) of the National Research Council. The study regards the development and characterization of a solid CA-based biocatalyst to be used in CO_2 slurry absorbers. In particular, the thermostable CA form (characterized as homogeneous catalyst, as reported in Chapter 3) was immobilized by covalent bonding on paramagnetic Fe_3O_4 nanoparticles via carbodiimide activation. The performances of the solid biocatalyst were characterized according to the protocol used for the characterization of dissolved CA (Sections 3.1.1-2-4). In particular, CO_2 absorption tests were carried out in a stirred cell reactor using carbonate solutions (pH>10) at different liquid solvent compositions, different temperatures, and carbonate to bicarbonate (CTB) conversion. The Danckwerts theory for gas-liquid absorption with reaction was applied to calculate the intrinsic first order kinetic constant k_{cat}/K_M of the immobilized CA relative to the CA-catalyzed CO_2 hydration reaction.

4.1.1 Materials and methods

The enzyme source was the liquid crude extract containing 60% of the recombinant CA (characterization reported in the Chapter 3). All the other chemicals were supplied by Sigma Aldrich®: sodium carbonate and bicarbonate (99% wt), potassium and bicarbonate (99% wt). Technical grade gaseous CO_2 was used for the absorption tests.

4.1.2 Preparation of the CA-based solid biocatalyst

The synthesis of carbodiimide activated magnetite nano-particles (NPs) and immobilization of CA on the resulting solids was carried out by Clemente Capasso and Mosè Rossi and their co-workers at the Istituto di Bioscienze e Biorisorse, according to the procedure reported by Huang et al. (2003). Few details on the immobilization procedure are: Fe_3O_4 magnetic nanoparticles were synthetized by co-precipitation of Fe^{2+} and Fe^{3+} ions in ammonia solutions followed by treatment under hydrothermal conditions. Then CA was immobilized by covalent bonding on the magnetite NPs via carbodiimide activation.

4.1.3 Assessment of CO_2 absorption rate promoted by CA immobilized on magnetite NPs

The CO_2 absorption tests were carried out to assess the contribution of the solid biocatalyst (CA on magnetite NPs) on CO_2 absorption rate in carbonate aqueous solutions. The experimental apparatus and the procedure of the absorption tests

were developed for the characterization of dissolved CA as reported in the sections 3.1.1 and 3.1.2, respectively. The conditions adopted in CO_2 absorption tests with CA immobilized on magnetite NPs are listed in Tab. 4.1. Low biocatalyst concentrations were used, the maximum concentration was $1.5 \cdot 10^{-2}$ kg of immobilized CA per m^3 of liquid volume. The biocatalyst concentration was varied by dosing the solid holdup according to the data on CA loading per unit mass of NPs measured at IBBR.

Tab. 4.1: Operating conditions in CO_2 absorption tests with CA immobilized on magnetite NPs.

Liquid phase composition	Temperature (K)	Carbonate to bicarbonate conversion α (-)	pH
$Na_2CO_3/NaHCO_3$ 0.5 M	298	-	9.6
	313	-	9.6
10%wt K_2CO_3	298	0	12.3
		0.2	10.8
		0.4	10.4
20%wt K_2CO_3	298	0	12.3

4.1.4 Kinetics of CA immobilized on magnetite NPs

As reported by Alper et al. (1980), the kinetic characterization of the immobilized CA on fine particles - in terms of the kinetic constant k_{cat}/K_M relative to the CA-catalyzed CO_2 hydration - can be carried out according to the theoretical analysis adopted for the homogeneous biocatalyst (dissolved CA). To this aim, the kinetic assessment of the biocatalyst made by CA immobilized on magnetite NPs was carried out according to the Danckwerts theory described in the section 3.1.4.
The following assumptions were made to support the adopted theoretical approach:

- The size of solid biocatalyst fine particles is sufficiently small to penetrate within the liquid boundary layer (gas-liquid structure according to the “two films theory”). According to the “two films theory” the order of magnitude of the liquid boundary thickness, δ, is defined by Eq. 4.1:

$$\delta = \frac{D_{CO2}}{k_L} \qquad (4.1)$$

  As the size of the biocatalyst particles is smaller than δ, the enzyme may be considered active close to the gas-liquid interface. Under these conditions, the enhancement of the overall rate of CO_2 absorption depends on enzyme activity and solid holdup.

- The slurry phase is described according to the pseudo-homogeneous approach (Ramachandran, 2007). Biocatalyst particle size is sufficiently small to assume negligible liquid-solid mass transfer resistance. Under this condition, the concentration of reacting species at the solid surface is set equal to that in the liquid phase.

Taking into account the reported assumption and according to the theory described in section 3.1.4, Eq. 3.12 is equivalent to Eq. 4.2:

$$\left(\frac{R_{CO2}}{R^0_{CO2}}\right)^2 = 1 + \frac{k_E}{k_0 + {k_L^2}/{D_{CO2}}}[CA]_{imm} \qquad (4.2)$$

where $[CA]_{imm}$ is the concentration of immobilized enzyme per unit volume of liquid.

4.1.5 Results

The samples of CA immobilized on magnetite NPs prepared at IBBR had an enzyme loading of 18.5 mg/g of solids. The immobilization yield was about 64.8%.

The presence of NPs can influence the values of mass transfer coefficient k_L and of gas-liquid interfacial area *a* (Fan, 1989; Ramachandran, 2007; Alper et al., 1980), in dependence of their structure and composition (Alper et al., 1980). For example, if the NPs present highly porous structure and high adsorbing property (e.g. activated carbon), they may move right into gas-liquid boundary, adsorb the gas molecules and then go back into the depths of liquid phase to desorb the gas. In other words, they may act as *carriers* of the gaseous specie. Therefore, the transfer of the gas molecules from the gas-liquid boundary to the liquid phase bulk may result more facilitated with respect to the absence of the solid, providing a remarkable enhancement of k_L. To verify the relevance of these effects, preliminary CO_2 absorption tests were carried out in Na_2CO_3/$NaHCO_3$ 0.5 M buffer solution with the inert solids dispersed at condition close to those set during the absorption tests. The temperature was set at 298 K. The NPs concentration was the maximum solid loading set for the CO_2 absorption tests with immobilized CA (0.5 kg m^{-3}), constituting the 0.1% by weight. The results in terms of pressure decay data occurring during CO_2 absorption were compared with those obtained during CO_2 absorption in the homogeneous liquid solvent (Fig. 4.1).

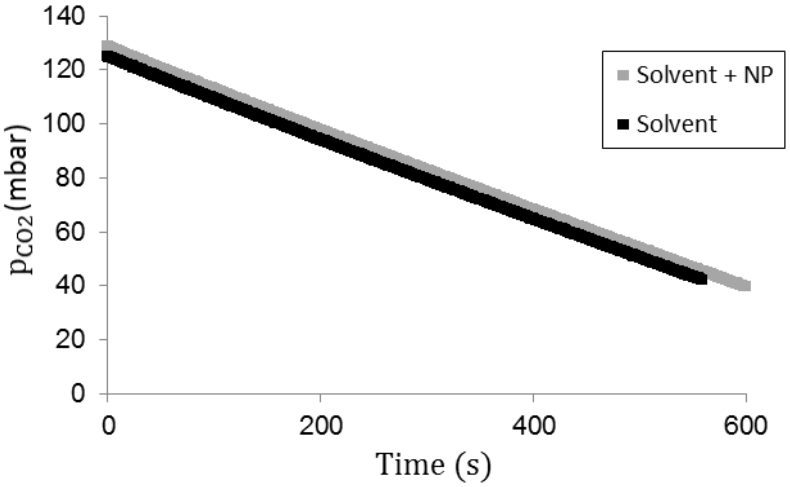

Fig. 4.1: P_{CO2} vs. time data measured during absorption tests carried out in Na_2CO_3/$NaHCO_3$ 0.5 at 298 K with and without dispersed magnetite NPs (solid concentration 0.5 kg m^{-3}).

Observing Fig. 4.1, P_{CO2} decay showed in both cases linear trend, having the same slope (about 0.15 mbar s^{-1}). Since, under the same conditions, possible modifications

of this slope value are indicative of possible variations of k_La due to the presence of the solid, this result confirmed that the NPs did not influence the k_La value even at the maximum solid concentration adopted for assessment of immobilized enzyme performances. Due to the very low NPs concentration (~ 0.1%$_{WT}$), the gas-liquid interface per unit volume of liquid was assumed not to be influenced by the presence of the solid. Accordingly, the k_L value used for data analysis was the one assessed for CO_2 absorption tests in the homogeneous liquid solvent (Tab. 3.3).
Therefore in this case, in analogy to the chemical absorption in presence of the homogeneous catalyst, the liquid side mass transfer in presence of the solid biocatalyst in form of fine particles may be exclusively defined as a combination of CO_2 diffusion and chemical reaction. In particular, the CA-catalyzed hydration reaction leads to a sharper CO_2 concentration gradient, enhancing the diffusion and, therefore, the mass transfer through the liquid layer.
Fig. 4.2 and 4.3 show the results in terms of R_{CO2}/R^0_{CO2} vs immobilized CA concentration collected after CO_2 absorption tests in K_2CO_3 solutions and Na_2CO_3/$NaHCO_3$ 0.5 M, respectively (immobilized CA concentrations: 0-1.5·10^{-2} kg m^{-3}).

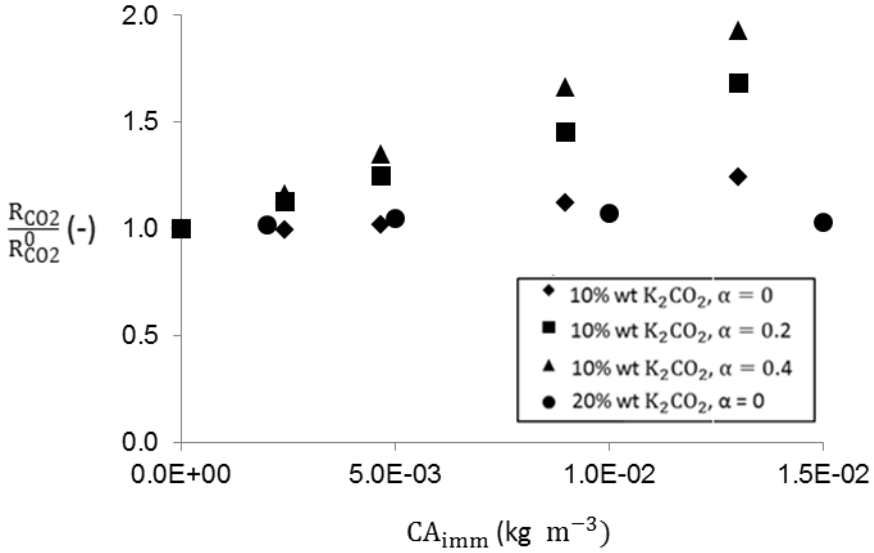

Fig. 4.2: R_{CO2}/R^0_{CO2} vs. [CA]$_{imm}$ data sets calculated during from CO_2 absorption tests in 20% wt K2CO3 with α = 0 and 10%wt K2CO3 with α = 0, α = 0.2 and α = 0.4 at 298 K.

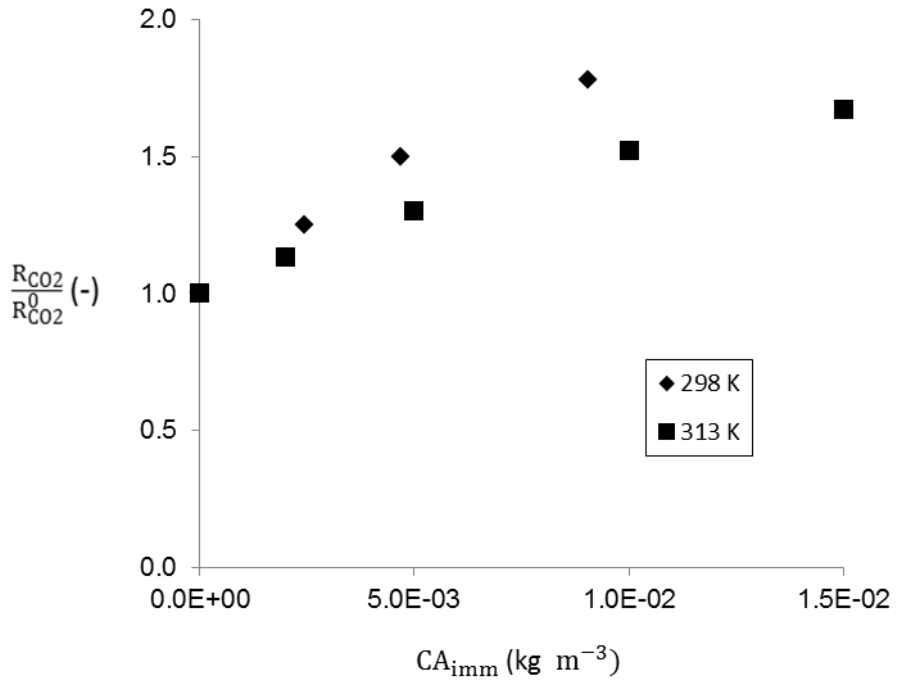

Fig. 4.3: R_{CO2}/R^0_{CO2} vs. $[CA]_{imm}$ data calculated from CO_2 absorption tests in Na_2CO_3/$NaHCO_3$ 0.5 at 298 and 313 K.

The analysis of Fig.s 4.2-3 points out that the contribution of immobilized CA in terms of R_{CO2}/R^0_{CO2} >1 is evident in all the data sets except in that related to CO_2 absorption in 20%wt K_2CO_3. For CO_2 absorption in 20%wt K_2CO_3 the ratio R_{CO2}/R^0_{CO2} is very close to the unit for the entire set of immobilized CA concentration investigated (Fig. 4.2).
The R_{CO2}/R^0_{CO2} assessed for the absorption tests carried out in 10% wt K_2CO_3 with α=0 was smaller than that assessed under the same conditions at α>0 (Fig. 4.2).
The contribution of CA immobilized on magnetite NPs on the overall CO_2 absorption rate was assessed in terms of the parameter k_{cat}/K_M according to the hypothesis described in the sections 3.1.4 and 4.1.4. The Fig. 4.4 and Fig. 4.5 report the plot of the linear regressions of data $(R_{CO2}/R^0_{CO2})^2$ vs. immobilized CA concentration carried out according to the Eq. 4.2. Fig. 4.4 refers to data measured during the CO_2 absorption tests in Na_2CO_3/$NaHCO_3$ 0.5 M buffer solution at 298 K and 313 K. The Fig. 4.5 refers to data measured during the CO_2 absorption tests in 10%wt K_2CO_3 solutions (0<α<0.4).

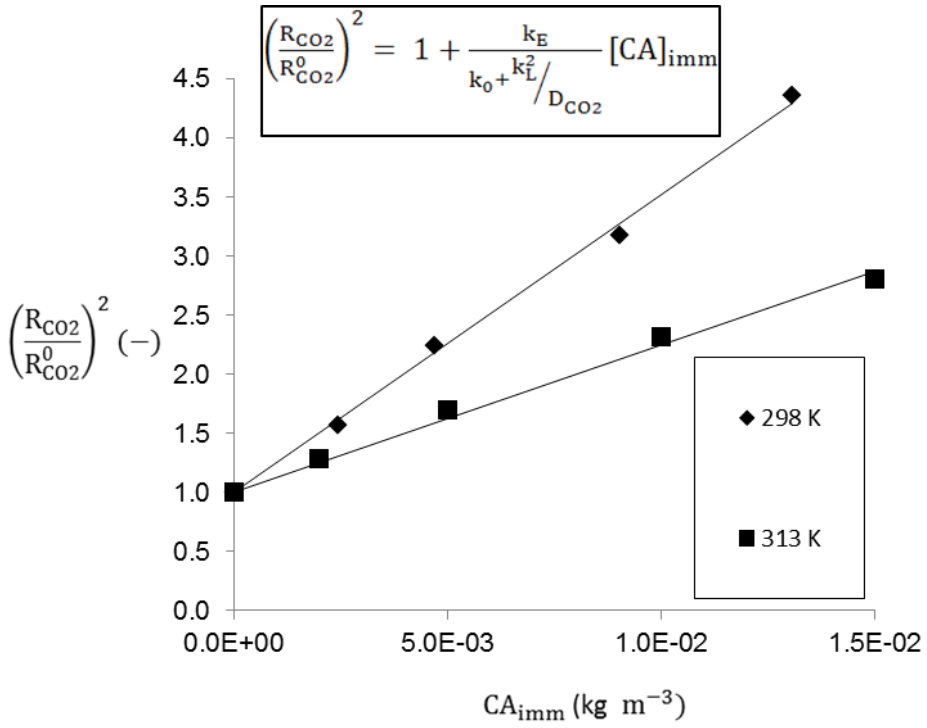

Fig. 4.4: $(R_{CO2}/R^0_{CO2})^2$ vs $[CA]_{imm}$ data sets and linear regression according to Eq. 4.2. CO_2 absorption tests in Na_2CO_3/$NaHCO_3$ 0.5 M buffer solution at 298 K and 313 K.

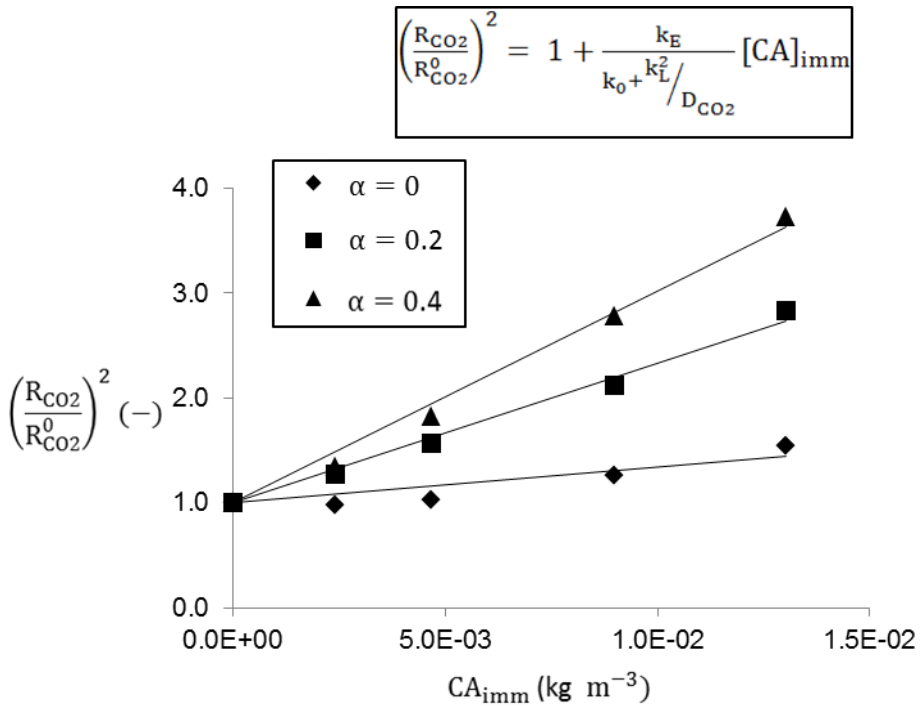

Fig. 4.5: $(R_{CO2}/R^0_{CO2})^2$ vs $[CA]_{imm}$ data set and linear regression according to Eq. 4.2. CO_2 absorption tests in 10%wt K_2CO_3 with α = 0, α = 0.2 and α = 0.4 at 298 K.

Results point out that all the experimental data sets were satisfactorily regressed by the linear function in Eq. 4.2. The slope of the linear curves is reported in tables 4.2 and 4.3 for the tests carried out in Na_2CO_3/$NaHCO_3$ 0.5M and in 10%wt K_2CO_3,

respectively. According to Eq. 4.2, the slope is proportional to the kinetic parameter k_{cat}/K_M and it is inversely proportional to the term that include the effect of physical mass transfer rate and CO_2 hydroxylation rate on the overall absorption rate. The k_{cat}/K_M values obtained under the investigated experimental conditions by working out the values of the slopes are reported in Tab. 4.2 and 4.3.

Tab. 4.2: Kinetic constant k_{cat}/K_M assessed for CA immobilized on magnetite NPs in Na_2CO_3 /$NaHCO_3$ at 298 and 313 K.

Slope of the curves in Fig. 4.4 ($m^3\,kg^{-1}$)	Mean square displacement R^2 (-)	Temperature (K)	k_{cat}/K_M ($m^3 \cdot kg^{-1} \cdot s^{-1}$)
$1.2 \cdot 10^2$	0.99	298	$6.6 \cdot 10^2$
$2.2 \cdot 10^2$	0.99	313	$1.6 \cdot 10^3$

Tab. 4.3. Kinetic constant k_{cat}/K_M assessed for CA immobilized on magnetite NPs in 10% wt K_2CO_3 at α = 0, 0.2, 0.4

Slope of the curves in Fig. 4.5 ($m^3\,kg^{-1}$)	Mean square displacement R^2 (-)	Carbonate to bicarbonate conversion, α (-)	k_{cat}/K_M ($m^3 \cdot kg^{-1} \cdot s^{-1}$)
$3.4 \cdot 10^1$	0.84	0	$1.9 \cdot 10^3$
$1.3 \cdot 10^2$	0.99	0.2	$1.2 \cdot 10^3$
$2.0 \cdot 10^2$	0.99	0.4	$9.3 \cdot 10^2$

Data reported in the Tab. 3.4 are now analyzed considering the Ha values relative to the conditions set for the characterization of CA immobilized on NP too. It is possible to observe that the condition for fast reaction (Ha>2) in absence of CA is verified for almost all set of operating conditions except for the 10%wt K_2CO_3 solution having α=0.4 and into Na_2CO_3 $NaHCO_3$ 0.5 M at 298 K. Moreover, the Danckwerts' criterion (Eq. 3.3) was verified in all the operating conditions, except that in the potassium carbonate solution with null CTB conversion. It is possible to assume Ha> 2 in all the tests carried out in the presence of immobilized CA. Moreover, the LHS of Eq. 3.3 is still smaller that unit after any absorption test with immobilized CA (exception for absorption in 10%wt K_2CO_3 at α = 0). Therefore, the assessed values of k_{cat}/K_M are considered reliable according to the theory.

The analysis of data in Tab 4.2 is carried out taking into account the increase of the contribution of CO_2 hydroxylation rate as temperature increased (larger Ha and larger denominator assessed for the slope in Eq. 4.2). The increase in slope must be due to larger kinetic parameter k_{cat}/K_M for immobilized enzyme catalysis. Similarly, the kinetic parameters k_{cat}/K_M assessed for immobilized CA catalysis in potassium carbonate solution decreased as increasing CTB conversion degree. Indeed, Ha and the denominator of the ratio representing the slope of the linear curves in Eq. 4.2 decreased at increasing CTB conversion ($k_{OH}[OH^-]$ presents a value of about 55 s^{-1} when α= 0 which decreases to 8.1 s^{-1} when α= 0.2 and 4.6 when s^{-1} when α= 0.4). In other words, at fixed $[CA]_{imm}$, lower rate of catalyzed CO_2 hydration is required to enhance CO_2 absorption rate up to $R_{CO2}/R^0_{CO2} > 1$ because the absorption rate in the alkaline solvent decreased as CTB conversion increased.

4.1.6 Discussion

The results reported in the previous section support the assumption listed in section 4.1.4. According to the Eq. 4.1 and to the values of D_{CO2} and k_L related to the investigated conditions (Tab. 3.3 and physicochemical parameters section in Chapter

3), the liquid layer thickness, δ, should be about 30 μm. The NPs averaged diameter synthesized according to the procedure proposed by Huang et al. (2003) should be about 13 nm and it should not increase after the enzyme immobilization: the binding process did not significantly promotes NPs aggregation and change in size of CA-NPs. Therefore, in all the cases the biocatalyst particles size can be considered sufficiently small to penetrate the liquid boundary layer at gas-liquid interface allowing CO_2 absorption rate enhancement by immobilized CA catalysis. The calculated k_{cat}/K_M values were compared to those obtained for dissolved CA and are listed in tables 4.4 and 4.5.

Tab. 4.4: Kinetic constant k_{cat}/K_M assessed for dissolved CA and CA immobilized on magnetite NPs in Na_2CO_3 /$NaHCO_3$ 0.5M.

T (K)	**k_{cat}/K_M dissolved CA ($m^3 \cdot kg^{-1} \cdot s^{-1}$)**	**k_{cat}/K_M CA immobilized on NPs ($m^3 \cdot kg^{-1} \cdot s^{-1}$)**
298	$1.4 \cdot 10^3$	$6.6 \cdot 10^2$
313	$8.1 \cdot 10^3$	$1.6 \cdot 10^3$

Tab. 4.5: Kinetic constant k_{cat}/K_M assessed for dissolved CA and CA immobilized on magnetite 10%wt K_2CO_3 at 298 K

α (-)	k_{cat}/K_M dissolved CA ($m^3 \cdot kg^{-1} \cdot s^{-1}$)	k_{cat}/K_M CA immobilized on NPs ($m^3 \cdot kg^{-1} \cdot s^{-1}$)
0	$1.5 \cdot 10^4$	$1.9 \cdot 10^3$
0.2	$3.3 \cdot 10^3$	$1.2 \cdot 10^3$
0.4	$1.9 \cdot 10^3$	$9.3 \cdot 10^2$

At fixed solvent composition and temperature, results in Tab.s 4.4-5 suggest that CA immobilized on magnetite NPs is characterized by k_{cat}/K_M values smaller than that assessed for dissolved CA.
The values of k_{cat}/K_M obtained in this study were compared with those obtained in an analogous study reported by Zhang et al. (2013) for bovine carbonic anhydrase. In particular, they assessed the CA kinetic into $KHCO_3/K_2CO_3$ 0.1 M buffer solution (pH 10.5) at various temperatures through absorption tests in batch reactor at lab scale. The study concerned both the dissolved enzyme and the enzyme immobilized on non-porous silica nanoparticles. They found a k_{cat}/K_M averaged value of about $5.3 \cdot 10^3$ m^3 kg^{-1} s^{-1} for the immobilized enzyme whose order of magnitude is consistent with those obtained in the present study even though did not depend on temperature in the range 313-323 K. Again, the k_{cat}/K_M value for the immobilized enzyme was slightly lower than the one related to free enzyme at 313K. On the contrary, they found a k_{cat}/K_M value which was 3.5 times larger than k_{cat}/K_M of dissolved enzyme at 323 K. The Authors suggested that the covalent attachment of the enzyme to the silica nanoparticles might have reduced the conformational flexibility of the enzyme molecules allowing to keep constant enzyme activity in the presence of small temperature increase.

4.1.7 Conclusions

The recombinant carbonic anhydrase obtained as concentrated protein mixture was immobilized through covalent attachment on paramagnetic magnetite NPs via carbodiimide activation in order to develop a biocatalyst for biomimetic CO_2 capture processes. The Danckwerts theory was applied to assess the kinetic parameter k_{cat}/K_M for the immobilized CA. The results were compared to those obtained for dissolved CA and reported in Chapter 3. Absorption tests were carried out at condition close to those of industrial application, in particular into carbonate aqueous solutions at different composition (pH > 10) and temperatures (298 and 313 K). Results showed that, although the immobilized CA had activity smaller than that of dissolved CA, k_{cat}/K_M were in agreement with those reported for other CA forms immobilized on NPs.

4.2 MAGNETIC CROSS-LINKED ENZYMES AGGREGATES (CLEAs) OF BOVINE CARBONIC ANHYDRASE AS PROMOTER OF BIOMIMETIC CO_2 CAPTURE

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Abstract

CO_2 absorption into aqueous solutions promoted by the enzyme carbonic anhydrase (CA) is a potential post-combustion CO_2 capture process. The enzyme CA (EC 4.2.1.1) catalyzes the CO_2 hydration and it is characterized by large *turnover numbers*. The present contribution is aimed at the development of CA-based solid biocatalysts using the technique of magnetic Cross-Linked Enzyme Aggregates (CLEAs) on bovine carbonic anhydrase (bCA) as model enzyme and magnetic nanoparticles (NPs). CLEAs were produced by glutaraldehyde cross-linking of precipitated bCA/NPs aggregates. Magnetic NPs were mixed with the CA solution before the enzyme aggregation in order to promote their inclusion in the bCA CLEAs. The optimum conditions of the immobilization procedure were assessed in terms of type of precipitating agent, concentration of glutaraldehyde and cross linking operating conditions, as well as concentration of magnetic NPs. The optimization was carried out with respect to the maximum immobilization yield and to the CLEAs activity. The maximum immobilization yield was about 90% and the maximum activity (1268 WAU/mg_{immbCA}) was measured for CLEAs prepared with 100 mM glutaraldehyde at 4°C, after 16 h crosslinking and 0.5 g_{NPs}/g_{bCA}. Morphological analysis of CLEAs prepared under different conditions was carried out through optical microscopy. Results pointed out that vigorous stirring during precipitation step provided reduction of aggregates size that was slightly modified by Magnetic field separation. Moreover, possible explanation of detrimental effect of too large NPs concentration on CLEAs activity was supported by the observed CLEAs structure

Keywords: carbonic anhydrase, CO_2 capture, CLEAs

Highlights

- Bovine carbonic anhydrase CLEAs were prepared with 90% immobilization yield
- Optimal conditions for CA precipitation and cross-linking were selected
- bCA CLEA were active during CO_2 absorption rate in carbonate buffer pH 9.6
- CLEA forming branched porous structure showed largest activity
- Increasing NPs to enzyme ratio branching and porosity of CLEAs clusters decreased

4.2.1 Introduction

Carbon capture and storage (CCS) processes can be regarded as powerful strategies to mitigate CO_2 emissions from stationary sources, e.g. power plants fired

with fossil fuels [1]. The CCS processes include post-combustion CCS technologies based on CO_2 capture from flue gases after removal of fly ashes, NO_x and SO_x [1]. According to the recent literature, CO_2 adsorption into aqueous solutions of alkanolamines followed by desorption of pure CO_2 gas stream and solvent regeneration is one of the most advanced process of post-combustion CCS technologies [2]. However, amines-based processes are characterized by environmental and economical drawbacks related to amine degradation, equipment corrosion, and high energy consumption [3]. An environmental friendly alternative to absorption into amine solutions is the CO_2 absorption process in potassium carbonate solutions [4]. However, the poor absorption rate provided by such solvents has driven researchers to investigate the enhancing effects of inorganic and organic promoters [4], the enzyme carbonic anhydrase (CA, E.C. 4.2.1.1) included. The CA has been proposed as an environmental friendly promoter for CO_2 absorption because it is an ubiquitous enzyme with remarkable catalytic activity against CO_2 hydration reaction [5-7]. Indeed, the CA catalyzes the CO_2 hydration reaction at *turnover number* ranging between 10^4 and 10^6 s^{-1} depending on the class of CA [8, 9].

A prerequisite of using CA in CCS absorption processes is that the CA forms are active and stable under the typical harsh industrial operating conditions [6]. Indeed, the absorption unit temperature ranges between 40 and 60 °C, the desorption unit temperature is close to 100 °C, salt concentration ranges between 20% and 30% wt, and pH is about 10 [6]. A potential solution to increase the enzyme stability is the covalent-immobilization of enzymes by attachment on solid supports [10] or by formation of cross-linked aggregates [11]. Moreover, the CA-based solid biocatalysts should be easily recovered from the liquid solvent and reused [11].

In order to exploit immobilized CA as CO_2 absorption rate promoter the biocatalyst should be active as close as possible to the gas-liquid interface. Russo et al. [6] reviewed the several systems proposed in the literature and pointed out the role of the morphology of the solid biocatalyst and the reactor configuration in the maximization of the performance of biomimetic units. The most recent contributions are hereinafter presented. Reardon et al. [12] carried out absorption tests in a pilot-scale packed column using potassium carbonate 20% wt solution as liquid phase. CA was immobilized in an organosilicate matrix at the walls of the structured packing. Results pointed out that the biocatalyst was characterized by an exceptional stability and provided about 80% CO_2 capture efficiency. Hou et al. [13] developed an innovative system made by a hydrophilic–superhydrophobic biocatalytic membrane. The liquid phase flowed along the inner hydrophilic side of the membrane and the gas phase along the hydrophobic side of the membrane. CA was immobilized on the inner side by adsorption [13] or covalently attached on titania nanoparticles and dispersed in the liquid phase [14]. In both cases, CA catalysis occurs close the gas–solvent interface: within the gas-liquid-membrane region. The use of the slurry biocatalyst proposes an optimal strategy for an effective enhancement of CO_2 absorption rate [6, 15, 16]. The contribution of the catalytic fine particles to absorption rate enhancement is due to their presence close to the gas–liquid interface [6, 16, 17]. Zhang et al. [18] reported a contribution about bovine CA immobilized on non-porous silica nanoparticles and characterized at conditions close to those applied in CO_2 absorption processes (carbonate buffer pH 10.5, 20-50°C). The immobilized CA provided absorption rate about those provided by the dissolved enzyme. Moreover, the biocatalyst was more active than the free enzyme at high temperature. Watson et al. [19] used whole cell biocatalysts made by *Escherichia coli* displaying CA at the

cell wall. In this way, an efficient solid biocatalyst was provided without enzyme purification and the immobilization procedure, although this may have the disadvantage of lack of enzyme improvement via a proper immobilization. Another effective CA immobilization technique was proposed by Shanbhag et al. [20]: the CA was provided with a self-assembling peptide. The non-covalent interaction between the fusion proteins provided nanometric (50-200 nm) enzyme aggregates at pH values close to 6.7.

The covalent carrier-free immobilization techniques include the Cross-Linked Enzyme Aggregates (CLEAs). CLEAs are very attractive due to the stability of the solid biocatalysts, the simplicity of the procedure, the large yield of immobilization [11, 21]. The method includes two main steps: (i) precipitation of enzyme in solution by means of precipitating agents (salts, water miscible organic solvents, or nonionic polymers); (ii) cross-linking of enzyme molecules precipitated in the aggregates to make the structure insoluble after the removal of the precipitating agent. Optimization of the CLEAs preparation should concern two main issues: selection of the best precipitating agent and selection of the cross-linking agent/conditions. Enzyme precipitation should occur as rapid as possible with respect to enzyme denaturation reaction in the presence of concentrated solutions of the precipitating agent: the enzyme molecules must hold their active structure in the aggregates [22]. The cross-linking is typically carried out with glutaraldehyde: it is the most used bifunctional agent because it is economic and it forms covalent bonds with the enzyme by nucleophilic attack of the amino group of the lateral lysines to the aldehyde groups [23]. The glutaraldehyde treatment may also cause chemical modifications of the enzyme molecules. The chemical modification acts on the amino groups and gives the amino/glutaraldehyde and amino/glutaraldehyde/glutaraldehyde moieties [23]. The amino/glutaraldehyde moiety has good activity towards other amino/glutaraldehyde groups while amino/glutaraldehyde/glutaraldehyde group is less prone to give cross-linking reactions. To obtain satisfactory cross-linking, it is necessary to use moderate glutaraldehyde concentration (e.g. 0.1-1% v/v, pH 7): the amino group will be activated by only one glutaraldehyde molecule [23]. Uncontrolled chemical modifications occurring when glutaraldehyde reacts with enzyme molecules may give both positive effects on the enzyme stability as well as negative effects. Cases of significant inactivation have been reported in the literature and regard enzymes with lysine residues close to the active site (e.g. nitrilases): glutaraldehyde [24, 25], thanks to its small size, can penetrate within the enzyme structure and react with the internal amino groups. Glutaraldehyde may be substituted by a macromolecular cross-linking agent, e.g. polysaccharides functionalized with polyaldehyde [24, 25], to avoid this undesired effect. In other cases, the low amount of Lys residues makes to have a proper crosslinking difficult, this may be solved using a feeder protein [26, 27], an aminated polymer [28] or aminating the enzyme [29] to facilitate the crosslinking. Furthermore, cross-linking gives rigidity to the enzyme structure: this phenomenon increases the enzyme stability but it may also lead to a reduction of the enzyme activity. Therefore, cross-linking conditions should be carefully selected in order to obtain the best results in terms of immobilization yield and preserved activity. Temperature and reaction time should also be carefully selected in order to avoid an extensive cross-linking, since long reaction times and relatively high temperature may promote high cross-linking degrees which may influence the resultant enzyme activity [11, 30]. Entrapment in more rigid materials [31] or co-immobilization with magnetic particles [32-36] may help to solve this problem. The scenario reported in the literature suggests that CA immobilization for

CO_2 capture should be carefully designed in order to fulfil several requirements including: stability of the interaction among enzyme molecules, carrier and/or cross-linker at CO_2 capture conditions (large pH and ionic strength, T>40°), stability of biocatalyst morphology, easy handling and recovery in the case of dispersed solids [6]. CLEAs may offer a safe way to covalently immobilize CA preserving the enzyme activity. However, the CLEAs stability and the CLEAs morphology ask for further investigation because both features strongly affect mass transfer phenomena, then the overall performance of the heterogeneous biocatalyst.
The present study reports the development of magnetic CLEAs using bovine CA (bCA) as model enzyme, glutaraldehyde as cross-linker and paramagnetic amino-functionalized nanoparticles (NPs). The investigated operating conditions were the type of precipitating agent, the cross-linker concentration, cross-linking time and temperature, and the NPs to bCA mass ratio. The performance of the CLEAs was characterized in terms of immobilization yields and CLEAs activity. Performances of CLEAs were assessed through CO_2 absorption tests in a lab scale batch reactor. The effect of the stirring modality used during CLEAs preparation on the morphology and on the biocatalyst performances was also investigated in terms of CLEAs particle size and CO_2 absorption rate.

4.2.2 Materials and methods

Carbonic anhydrase from bovine erythrocytes (lyophilized powder, purity≥ 75 % wt) was supplied by Sigma Aldrich® as well as other chemicals: glutaraldehyde grade I (25%vol), tris(hydroxymethyl)aminomethane (≥99.9 % wt), sulfuric acid (95 – 98 %), phosphate buffer saline (PBS), ammonium sulfate (≥99%wt), sodium bicarbonate (99%), sodium carbonate (99%). Paramagnetic nanoparticles (NPs) fluidMAG-Amine® (average diameter 100nm) had an external aminosilane matrix and a magnetite core and were supplied as aqueous suspension by Chemicell GmbH.

4.2.2.1 Preparation of bCA CLEAs

The procedure to prepare CLEAs was developed according to the protocol reported in the literature [22] and was adapted to the use of bCA and paramagnetic NPs. Three main steps were scheduled: adsorption of bCA on NPs, enzyme precipitation and cross-linking. The protocol was optimized with respect to the immobilization yield and specific activity by tuning: type of precipitating agent, concentration of the cross-linker, concentration of NPs, cross-linking time and temperature. The CLEAs preparation included the following steps:

a) NPs suspension (pre-set amount of NPs) was mixed with 1 mL of 10 g/L bCA dissolved in 10 mM PBS (pH 7.4);

b) The NPs-enzyme suspension was incubated under gentle mixing on rotating shaker (Stuart SB3) at 25°C for 30 min;

c) The suspension was added dropwise to 9 mL of 100 mM PBS containing the precipitating agent and the pH was adjusted to 7.4. The fraction of initial suspension and of PBS solution were selected to have final concentration of bCA and of the precipitant agent at about 1 g/L and 80%$_W$, respectively;

d) The new suspension was incubated for pre-set time and temperature under gentle mixing;

e) The glutaraldehyde 25%$_{vol}$ solution was mixed with the suspension of bCA-aggregate-NPs up to the pre-set concentration;

f) The mixture was incubated for the cross-linking step under gentle mixing in rotating shaker at the pre-set time and temperature;

g) The produced CLEAs were recovered by means of magnets;

h) The recovered CLEAs were re-suspended and washed 3 times with PBS 10 mM (pH 7.4). The CLEAs samples were stored in PBS at 4°C.

The immobilization yield was assessed on the CLEAs produced according to the reported procedure a) through d) steps. The specific activity was assessed on the CLEAs produced according to the reported procedure a) through f) steps and a) through h) steps. Finally, the produced bCA CLEAs were observed at optical microscopy, using the optical microscope OlympusBX41.

4.2.2.2 Selection of the precipitating agent

Ammonium sulfate, propanol, and acetone were used as precipitating agents. The selection criterion was the minimization of the activity loss of CA after precipitation in the presence of the selected agent and re-dissolution in the original buffer [25, 37, 38].
Tests were carried out according to the reported procedure a) through d) steps and setting: the mass of NPs (5 mg of NPs in 200 µL suspension); the incubation time and temperature of the suspension of glutaraldehyde and bCA-aggregate-NPs - step d) - at 30 minutes and 25°C, respectively. The enzyme aggregates-NPs suspension (1 mL) at the end of the step d) was recovered by centrifugation (10000 rpm for 5 min). Then, the recovered enzyme aggregates were dissolved by resuspension in 1 mL of 10 mM PBS (pH 7.4).
The protein concentration in the supernatant recovered after the centrifugation was measured. The mass of precipitated bCA was calculated as the difference between the initial mass of bCA (10 mg) and the recovered mass in the supernatant. The precipitation yield was defined as the ratio between the mass of precipitated bCA and the initial mass of bCA.

4.2.2.3 Optimization of the bCA aggregate cross-linking

Tests were carried out according to the reported procedure a) through h) steps. The operating conditions were: the NPs to bCA mass ratio – step a) - changed between 0.5 and 2 g/g; the incubation time and temperature of the suspension of glutaraldehyde and bCA-aggregate-NPs - step d) - at 30 minutes and 25°C, respectively, and 1 hour at 4°C; the glutaraldehyde concentration – step e) - ranged between 50 and 200 mM as suggested in the literature[22, 37]. Cross linking time and temperatures were fixed so that the reaction lasted 3 hours at 25°C and 16 or 22 hours at 4°C.
The activity of CLEAs (A_{CLEA}) was calculated according to Schoevaart et al. [22]. The procedure includes: the assay of the CLEAs after the cross-linking step (A_{TOT}) (recovered after step f); the assay of the bCA activity of the liquid recovered after precipitation and crosslinking (A_{LIQ}). The activity of CLEAs can be expressed in accordance to the Eq. 4.3:

$$A_{CLEA} = A_{TOT} - A_{LIQ} \quad (4.3)$$

Some tests were aimed to assess bCA leaching from CLEAs. The assessment required the assay of the CLEAs activity after the washing cycles (A_{WASH}) and the assay of the bCA activity in the washing liquids fractions ($A_{released}$).
Some tests were carried out to investigate the effects of type of mixing applied during precipitation on CLEAs morphology. The tests were carried out at the optimal selected operating conditions except for the mixing system adopted in the step f). An overhead stirrer equipped with a 2-bladed propeller (stirring rate set at 850 rpm) was used for the suspension gentle mixing. The CLEAs produced according to the two procedure/systems were compared in terms of absorption rate enhancement and morphology.

4.2.2.4 Analytics and activity assay

The protein concentration in the liquid phase (e.g: the supernatant recovered after the suspension centrifugation) was assessed through optical absorbance measurements at 280 nm in order to assess the immobilization yield, as reported in the section 4.2.2.1.
The activity of the free bCA and of the bCA-CLEAs was characterized according to the Wilbur-Anderson assay [39]. The assay is a titrimetric method proposed to assess the activity of dissolved CA. In the present work it was adapted for the assessment of the bCA-CLEAs activity according to Russo et al. [40]. The bCA activity was measured as the rate of hydration reaction of carbon dioxide dissolved in 2 mL of a saturated aqueous solution added to 3 mL of 20 mM tris(hydroxymethyl)aminomethane sulfate buffer (TRIS sulfate) at 0°C. In particular, the time elapsed during pH decay from 8.3 to 6.3 was measured. CLEAs concentration was properly tuned so that the measured time at least 20s and no longer than 100s. The activity A was expressed as Wilbur-Anderson units per unit volume of CLEAs suspension (WAU/mL) according to Eq. 4.4:

$$A = \frac{t_{blank} - t_{CA}}{t_{CA}} \frac{df}{v_{CA}} \quad (4.4)$$

where t_{blank} and t_{CA} are hydration times measured during tests carried out in absence and presence of CLEAs, respectively, d_f is the dilution factor of the CLEAs sample, and v_{CA} the added volume of the CLEAs suspension (0.05 mL). Assays were repeated at least three times. The pH values were measured with a pH-meter (Hanna HI 3222).

4.2.2.5 CO_2 absorption tests

The CO_2 absorption tests were carried out to assess the contribution on CO_2 absorption rate of the washed bCA-CLEAs in alkaline aqueous solutions according to the method adopted by Russo et al. [41] for the absorption rate enhancements by dissolved CA. A system modified with respect to Russo et al. [41] was developed to operate the absorption unit with CLEAs. The apparatus is sketched in Fig. 4.6. It was equipped with a stirred cell made by a jacketed polymethyl-methacrylate vessel (5.3 cm ID, 10 cm height). The cell was equipped with two impellers to evenly mix the liquid and gas phases. The head flange was equipped with ports for the gas feeding, the liquid feeding, the hydraulic connection to a differential pressure transducer (DPT) (Druck, PMP4165).

The CO_2 absorption tests were carried out with technical grade CO_2 and 0.5 M Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.6) at 25°C. The apparatus was operated batchwise with respect to both gas and liquid phases: dissolved bCA or bCA-CLEAs were used as catalysts.
The vessel was flushed with gaseous CO_2 for 15 min before each test: the impellers rotating speed was set at 200 rpm and CO_2 relative pressure in the vessel was set at about 70-100 mbar. 50 mL of buffer solution containing the enzyme were injected with a syringe in the vessel through the liquid loading port and the reactor head space was pressurized with CO_2 up to a relative pressure of about 140 mbar. The vessel pressure decay was recorded since the injection of the liquid phase. Gaseous pressure decay data were collected, the DPT signal was acquired at 2 Hz and processed with a LabView 7.1® code enabling to display on screen the CO_2 pressure value. During the absorption tests, the stirring rate was set at 100 rpm to keep flat the gas-liquid interface.
Data from each test were analyzed according to the following procedure. Assuming that: (i) the closed system was isothermal, (ii) the CO_2 gas behaves like an ideal gas, (iii) the gas-side mass transfer resistance was negligible because pure CO_2 had been adopted as gas phase and water evaporation was sufficiently slow, (iv) gas-liquid interfacial area corresponded to the reactor cross sectional area, it was possible to calculate the molar rate of CO_2 absorption per unit of gas-liquid interfacial area (R_{CO2}) through the Eq. 4.5:

$$R_{CO2} = -\frac{dp_{CO2}}{dt} * \frac{V_g}{RTA_{gl}} \qquad (4.5)$$

The enhancement factor E was defined according to Eq. 4.6 as the ratio between the CO_2 absorption rate in presence of the enzyme (R^{CA}_{CO2}) and the CO_2 absorption rate in the buffer solution (R^{0}_{CO2}).

$$E = \frac{R^{bCA}_{CO2}}{R^{0}_{CO2}} \qquad (4.6)$$

with R^{bCA}_{CO2} and R^{0}_{CO2} the CO_2 absorption rates in presence and absence of the bCA CLEAs/free bCA, respectively.

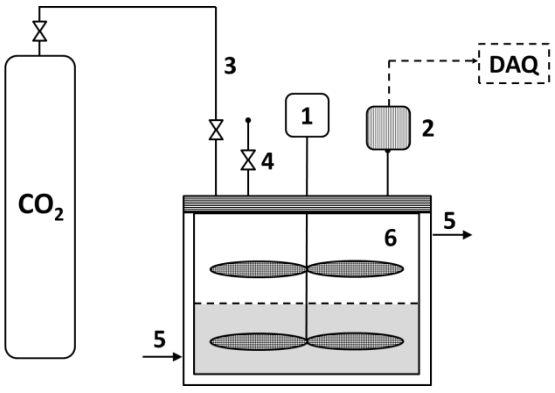

Fig. 4.6: Sketch of the experimental apparatus used for CO_2 absorption tests. (1) Stirrer motor, (2) Differential pressure transducer, (3) CO_2 feeding line, (4) liquid injection port, (5) thermostatic bath water line, (6) Stirred cell. DAQ) data acquisition unit.

4.2.3 Results

4.2.3.1 Optimization of the precipitation step

Three chemical species were investigated as bCA precipitant agents: ammonium sulfate, acetone and propanol. After the precipitation step the enzyme activity of the re-dissolved bCA aggregates was assayed by means of the titrimetric method. The irreversible formation of protein solid aggregates was observed whenever the organic solvents were used. Dissolution of the aggregates in PBS or in distilled water was not observed when enzyme aggregates were formed in acetone or propanol solutions. The complete dissolution of the aggregates in aqueous buffer solution was observed when ammonium sulfate was used. The activity assessment of the re-dissolved bCA enzyme aggregates was about 94±17% of the initial bCA activity. A precipitation yield of about 90±2% was measured. Therefore, $(NH_4)_2SO_4$ 80% aqueous solution was selected as precipitation solvent for the preparation of the bCA CLEAs samples. The reported results are quite expected. As reported by Shoevaart et al. [22], if the enzyme solution is supplemented to a very large volumes of highly concentrated precipitant agent solutions (the used condition), good results can be obtained in terms of precipitation yield and activity preservation of the enzyme aggregates. The latter result can be explained considering the different time scale of enzyme aggregates formation and enzyme denaturation: if the enzyme solution is added slowly to the solvent containing the precipitating agent the formation of the enzyme aggregates can occur faster that the enzyme molecules denaturation.

4.2.3.2 Optimization of the cross-linking step

The cross-linking step was optimized with respect to the glutaraldehyde concentrations between 50 and 200 mM. Other operating conditions were selected assuming that a complete cross-linking can be obtained after 3 hours at 25°C, then both time and temperature were tuned by increasing cross-linking time whenever the reaction was carried out at lower temperature [22]. NPs to bCA mass ratio of 0.5 g/g was selected.

It is worth to note that: i) the activity assay carried out according to the titrimetric method allows to compare CLEAs prepared under different conditions in order to optimize the protocol; ii) the activity assay cannot be used to compare free bCA with bCA-CLEAs because the activity resulting from the assay applied to the CLEAs refers to a heterogeneous reaction between solid biocatalyst and CO_2 in solution. Indeed, the overall rate does not depend only on the enzyme intrinsic kinetics but also on mass transfer phenomena (interphase mass transfer and intra-particle diffusion).

Results on CLEAs activity are reported in Tab. 4.6. Concerning the effect of glutaraldehyde concentration, data show that, even a satisfactory A_{CLEA} was observed, a remarkable $A_{released}$ was measured for samples obtained with cross-linking at 50 mM glutaraldehyde apart from cross-linking time and temperature. Increasing glutaraldehyde concentration up to 100mM, $A_{released}$ was almost null, this suggested a satisfactory cross-linking degree. A_{CLEA} at 100 mM glutaraldehyde was few WA units for samples prepared at 25°C after 3h cross-linking. On the contrary, 980 and 1033 WAU/mg for samples cross-linked at 4°C after 16 and 22 h, respectively. A further increase in glutaraldehyde concentration at 200 mM did not affect the CLEAs activity. According to the reported results, 100 mM glutaraldehyde, 4°C and 16 h were selected as optimal conditions for cross-linking. Moreover, the CLEAs activity did not remarkably change after CLEAs washing (A_{WASH} was about A_{CLEA}) for samples characterized by satisfactory cross-linking degree ($A_{released} \approx 0$).

Tab. 4.7 reports the results of CLEAs activity prepared at the optimal selected operating conditions as regard precipitant agent, temperature, time and at cross-linking agent concentration, and changing the NPs to bCA ratio between 1 and 2 g/g.

Tab. 4.6: CA activity assayed for CLEAs. Precipitating agent: ammonium sulfate 80%. NPs to bCA ratio: 0.5 g/g.

Cross-linking conditions			CLEAs activity		
Glutaraldehyde concentration (mM)	**Time (h)**	**Temperature (°C)**	**A_{CLEA} (WAU/ $mg_{imm.bCA}$)**	**$A_{released}$ (WAU/ $mg_{imm.bCA}$)**	**A_{WASH} (WAU/$mg_{imm.bCA}$)**
50	3	25	87± 25	41 ± 11	N.A.
100			4.2 ± 0.7	0	3.7 ± 0.5
50	16	4	2540± 234	1030 ± 205	N.A.
100			1268 ± 209	0	980 ± 13
200			1300 ± 223	0	1033 ± 187
50	22		2371± 511	906 ± 185	N.A.
100			1056 ± 172	0	842 ± 16
200			1033 ± 187	0	932 ± 15

Free bCA activity: 3622 ± 384 WAU/mg

Tab. 4.7: CA activity assayed for CLEAs prepared at different NPs to bCA ratios. Precipitating agent: ammonium sulfate 80%. Glutaraldehyde concentration: 100 mM. Cross-linking time: 16h. Temperature: 4°C.

Cross-linking conditions				CLEAs activity	
Glutaraldehyde concentration (mM)	**Time (h)**	**Temperature (°C)**	**NPs/bCA (g/g)**	**A_{CLEA} (WAU/ $mg_{imm.bCA}$)**	**A_{WASH} (WAU/ $mg_{imm.bCA}$)**
100	16	4	0.5	1268 ± 209	980 ± 13
			1	5.6 ± 1.0	2.0 ± 0.7
			2	4.3± 0.5	1.3 ± 0.3

Free bCA activity: 3622 ± 384 WAU/mg

The analysis of data in Tab. 4.7 points out that the lower the NPs to bCA ratio the higher the bCA-CLEAs activity. In particular the activity of bCA-CLEAs was few WA units at NPs to bCA ratio larger than 1 g/g. A possible explanation of this result was given by the observation of CLEAs morphology as described in section 4.2.3.4. Concerning the effect of cross-linking temperature (Tab. 4.6), both the desired (inter-molecular) and undesired (intra-molecular) cross-linking reactions [23] occur at larger rate at 25 than at 4°C. Because undesired reactions promotes enzyme deactivation, it is expected that A_{CLEA} can decrease at increasing cross-linking temperature according to data in Tab.4.6.

4.2.3.4 Morphological analysis

The morphological analysis of CLEAs by means of optical microscopy observations was carried out. Figures 4.7 and 4.8 report pictures of washed bCA-CLEAs prepared under optimal operating conditions except for the ratio NPs to bCA. bCA-CLEAs were prepared setting the NPs to bCA ratio at the optimal value (0.5 g/g) (Fig 4.7) and at the larger (1 and 2 g/g) (Fig. 4.8). Dark regions are dense/thick CLEAs: dark intensity increased with the thickness of the CLEAs. The analysis also regarded the effects of

the mixing conditions during precipitation on CLEAs morphology. Two mixing conditions were investigated: mild (rotary shaker) and vigorous (blade stirrer) stirring.
Figure 4.7 shows pictures of bCA-CLEAs sample prepared under mild shaking conditions and NPs to bCA ratio 0.5 g/g. The analysis of the pictures pointed out that under the operating conditions selected the bCA-CLEAs formed high branched clusters characterized by size close to 1 mm (Fig. 4.7A). The thickness of the clusters was high in the inner region and small in the outer region (Fig.4.7B). Moreover, a fractal-branched structure is observed that should enhance the enzymatic efficiency of the CLEAs. Fig. 4.8A and B reports pictures of bCA-CLEAs prepared at NPs to bCA ratio of 1 g/g and 2 g/g, respectively. The main difference among bCA-CLEAs prepared setting the NPs to bCA ratio at 0.5 g/g and those prepared at high NPs to bCA ratio is the round-shape of clusters can be observed. The branched structure observed in the Fig. 4.7 is not observed in Fig. 4.8 even though few debris (Fig 4.8A) and thin porous clusters (Fig 4.8B-D) can be observed.
The observed morphology is in agreement with the effect of NPs to bCA ratio on the activity of CLEAs reported in Tab 4.7. Indeed, as the NPs to bCA ratio increases the size of CLEAs clusters did not remarkably change and the branched-fractal structures disappears: the specific active surface of the CLEAs clusters decreases with the NPs to bCA ratio.
Fig. 4.9 reports pictures of bCA CLEAs prepared setting the ratio NPs to bCA at the optimal value (0.5 g/g) and under vigorous stirring. Fig.s 4.9A and 4.9B are pictures of CLEAs observed at the end of the preparation procedure and Fig.s 4.9C-F are pictures of CLEAs treated with several washings and recoveries by MF assisted solids separation (steps g-h, section 4.2.2.1). The analysis of the Fig.s 4.9A and 4.9B points out that the CLEAs structure under vigorous stirring is characterized by loose clusters of size smaller than 1 mm. These features are partially modified by MF assisted separation of dispersed CLEAs during the washing step. The comparison of Fig.s 4.9A and 4.9B with Fig.s 4.9C trough 4.9F points out an increase of the average size and of the density of clusters (more compact structures).
The analysis of activity data and of the CLEAs morphology highlights that there is room to control CLEAs performances. Indeed, cluster size/structure may be controlled by selecting proper ratio NPs to bCA, type of mixing during enzyme precipitation step and recovery procedure. As regards the MF assisted CLEAs recovery procedure, applied during CLEAs washing, it asks for further investigation to keep the cluster size and porosity as close as possible to the initial values even though, according to data in Tab.4.6, it seems to slightly damage CLEAs structure because A_{WASH} was about A_{CLEA}.

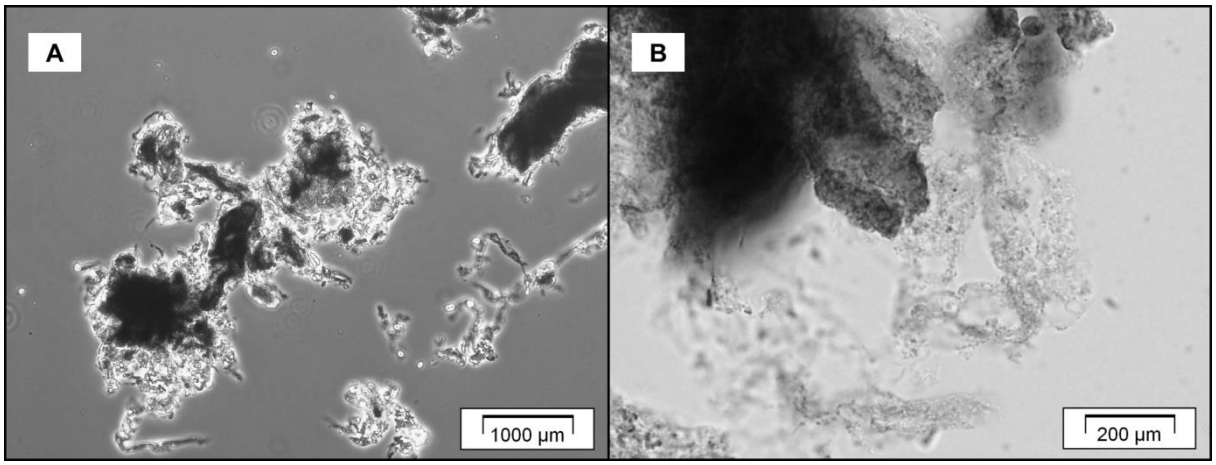

Fig. 4.7: Optical microscopy pictures of bCA-CLEAs samples. Operating conditions set during the CLEAs preparation: 100 mM glutaraldehyde, 16h cross-linking time, 4°C, 0.5 g/g NPs to bCA ratio, mild shaking.

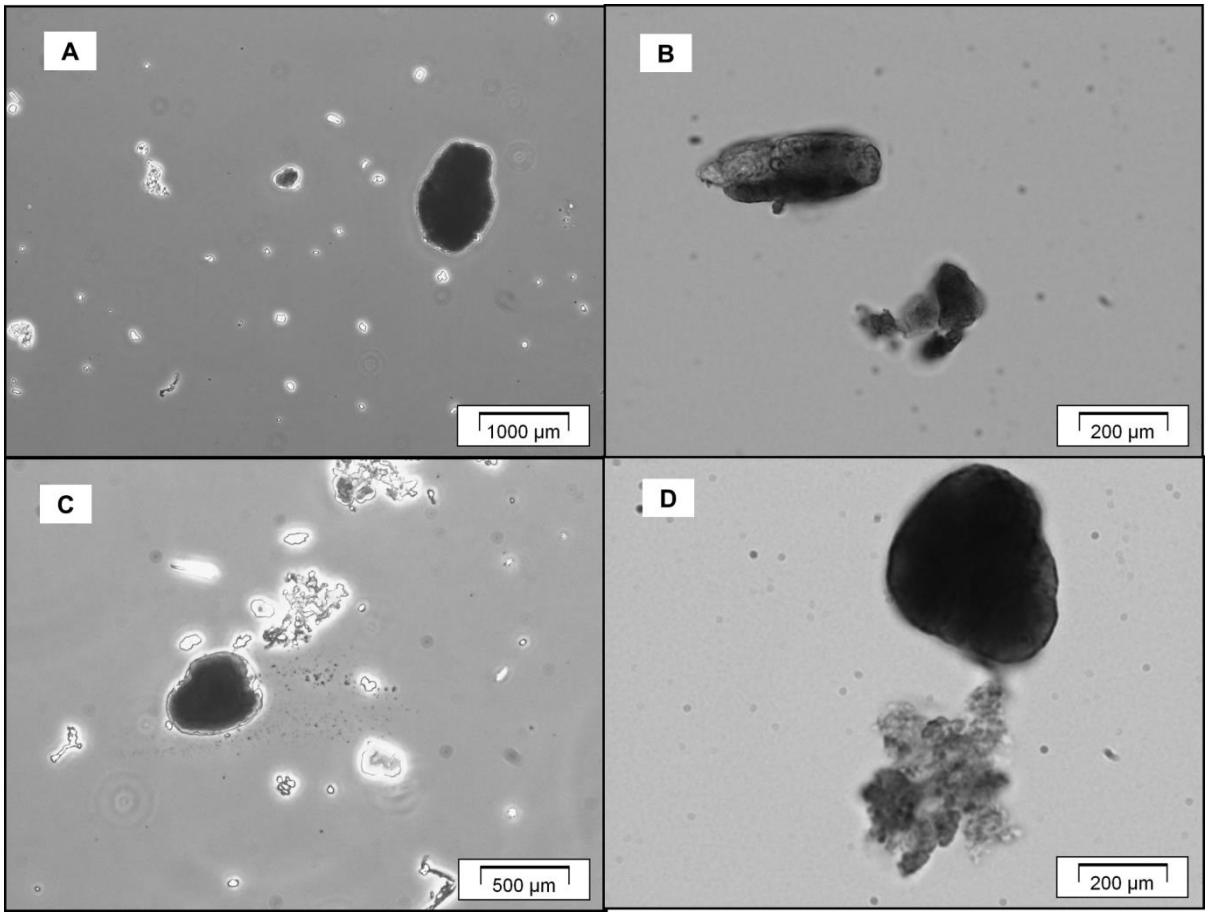

Fig. 4.8: Optical microscopy pictures of bCA-CLEAs samples. Operating conditions set during the CLEAs preparation: 100 mM glutaraldehyde, 16h cross-linking time, 4°C, mild shaking. 0 NPs to bCA ratio: 1 g/g (A, B) and 2 g/g (C, D).

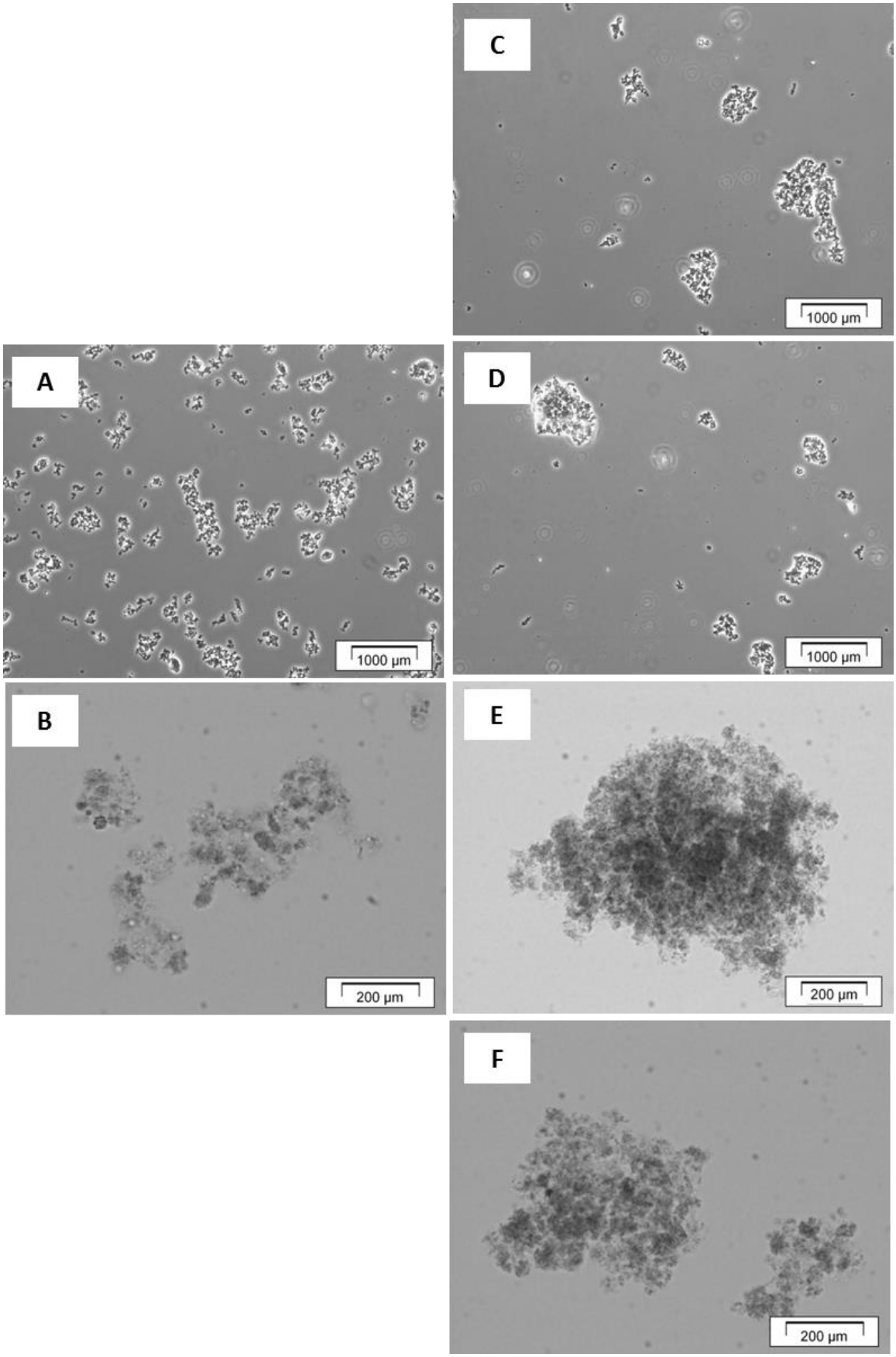

Fig. 4.9: Optical microscopy pictures of bCA-CLEAs samples. Operating conditions set during the CLEAs preparation: 100 mM glutaraldehyde, 16h cross-linking time,

4°C, vigorous stirring, 0.5 g/g NPs to bCA ratio. A and B) CLEAs just prepared; C through F) washed CLEAs and MF assisted recovery.

4.2.3.5 CO_2 absorption tests

Further investigation was accomplished to assess the effect of stirring rate applied during CLEAs preparation on the performance of CLEAs as promoters of CO_2 absorption rate. CO_2 absorption tests were carried out in the apparatus shown in Fig. 4.6 in the presence of bCA-CLEAs suspended in Na_2CO_3/$NaHCO_3$ 0.5 M alkaline solution pH 9.6. CLEAs were prepared under optimal operating conditions: 100 mM glutaraldehyde, 16h cross-linking time, 4°C, vigorous stirring, 0.5 g/g NPs to bCA ratio. bCA-CLEAs prepared under mild (rotary shaker) and vigorous (blade stirrer) stirring were compared. CO_2 absorption tests were also carried out in presence of free bCA.

Fig. 4.10 reports the enhancement factor E (Eq. 4.6) assessed during the CO_2 absorption tests as a function of the bCA-CLEAs concentrations (cross-linker mass was neglected). The bCA-CLEAs characterized by about 980 WAU/mg activity were used. The *E* measured during tests carried out with free20 mg/L bCA is also reported.

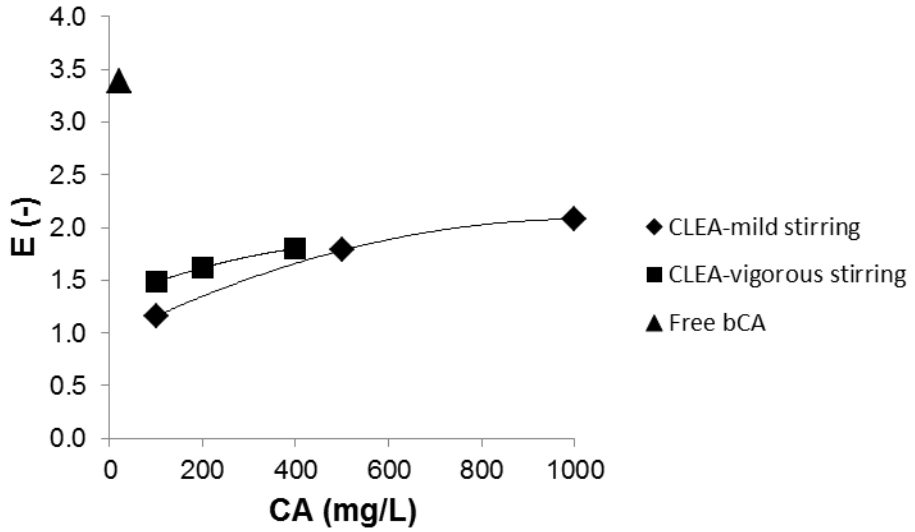

Fig. 4.10: CO_2 absorption rate enhancement factor (*E*) as a function of bCA-CLEAs concentration. The *E* for free dissolved bCA (20 mg/L) is also reported. Operating conditions: 0.5 M Na_2CO_3/0.5 M $NaHCO_3$, buffer solution pH 9.6. bCA-CLEAs were prepared under optimal conditions and with two types of mixing: rotary shaker 30 rpm (mild stirring), blade stirrer 850rpm (vigorous stirring)

The contribution to CO_2 absorption by bCA-CLEAs is evident in Fig. 4.10: E>1 at concentration larger than 100 mg_{CLEA}/L. The comparison among E data related to CLEAs prepared with different type of mixing points out that the vigorous mixing provided higher performance. The good performance was in agreement with the microscopy observation: very fine aggregates and fractal-like behaviour enhanced the catalytic effects of bCA.

As expected, the enhancement factor of free bCA (homogeneous catalysis of CO_2 hydration reaction) is larger than the enhancement factor of bCA-CLEAs (heterogeneous catalysis). Indeed, the E at 1000 mg_{CLEA}/L – heterogeneous biocatalyst - was about 2 and the E at 20 mg/L of free bCA - homogeneous biocatalyst - is about 2.9. The performance of free bCA was definitively high because

enzyme molecules were evenly distributed in the bulk and close to the gas-liquid interface. Altogether, the performance of CLEAs is affected by possible partial inactivation of bCA during cross-linking and also by the scarce efficiency of such a solid biocatalyst. The latter issue can be related to several phenomena including the distribution of the CLEAs particles close to the gas-liquid interface, the interphase mass transfer rate between the liquid and the CLEAs particles, the CO_2 diffusion into the CLEAs particles.

4.2.4 Conclusions

The CLEAs technique was applied to develop (bovine) carbonic anhydrase (bCA) based biocatalyst for CO_2 capture process. Magnetic nanoparticles (NPs) were successfully included in the CLEAs for their recovery during the preparation and after the industrial application. Ammonium sulfate was selected as a promising precipitant agent because enzyme activity was largely preserved (about 90%). Careful design of cross-linking steps allowed to keep as high as possible the activity of bCA-CLEAs. The beneficial effect of decreasing the temperature on glutaraldehyde-bCA interactions was also highlighted. The CLEAs structure was modeled by tuning the NPs to enzyme ratio and to the modality/intensity of mixing during the precipitation step. Minimum NPs concentration and maximum stirring intensity minimize CLEAs cluster size and maximize the fractal behavior. The developed CLEAs were characterized by promising enhancement factor as regard the CO_2 absorption in lab scale stirred cell reactor.

The reported results suggest that the proposed biocatalyst can be successfully proposed for the application in slurry absorber for CO_2 capture. In particular, the tuning of stirring rate during enzyme aggregates formation, the possible use of additives, and the design of magnetic field assisted solid recovery may allow the minimization of CLEAs cluster size and may make their morphology mechanically stable according to the guidelines reported in the literature for the development of biocatalyst for slurry CO_2 absorber [6, 16].

Acknowledgement

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5. DEVELOPMENT OF AN INNOVATIVE PROTOCOL FOR ENZYMES CO-IMMOBILIZATION

The present section reports the research activity aimed at the development of innovative protocols for enzymes co-immobilization. The aim of the research was to address critical issues associated to conventional co-immobilization procedures. In particular, the critical issues under investigation were the immobilization of two or more enzymes on the same support (or according to combi-CLEA technique) and the immobilization under the same operating conditions. The second issue is very critical because the optimal operating conditions to immobilize enzymes change - even drastically - with the enzyme. The research activity was carried out at the Instituto de Catálisis y Petrolquímica (ICP) - Consejo Superior de Investigaciones Cíentificas (CSIC) in Cantoblanco (Madrid, Spain) - under the supervision of the Prof. Roberto Fernández-Lafuente. In the present section, the abstracts of each paper concerning this activity are reported. The full manuscripts are reported in the Appendix.

5.1 Stabilization of Candida antarctica Lipase B (CALB) Immobilized on Octyl Agarose by Treatment with Polyethyleneimine (PEI)

Molecules 21 (2016) 751-760

Peirce S.[1,2], Tacias-Pascacio V.G.[1,3], Russo M.E.[4], Marzocchella A.[2], Virgen-Ortíz J.J.[1]. Fernández-Lafuente R.[1]

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The present contribution reports the immobilization procedure of CALB via interfacial activation on octyl agarose (OC) and subsequent coating with polyethyleneimine (PEI). and characterization of the resultant biocatalyst

Abstract

Lipase B from *Candida antarctica* (CALB) was immobilized on octyl agarose (OC) and physically modified with polyethyleneimine (PEI) in order to confer a strong ion exchange character to the enzyme and thus enable the immobilization of other enzymes on its surface. The enzyme activity was fully maintained during the coating and the thermal stability was marginally improved. The enzyme release from the support by incubation in the non-ionic detergent Triton X-100 was more difficult after the PEI-coating, suggesting that some intermolecular physical crosslinking had occurred, making this desorption more difficult. Thermal stability was marginally improved, but the stability of the OCCALB-PEI was significantly better than that of OCCALB during inactivation in mixtures of aqueous buffer and organic co-solvents. SDS-PAGE analysis of the inactivated biocatalyst showed the OCCALB released some enzyme to the medium during inactivation, and this was partially prevented by coating with PEI. This effect was obtained without preventing the possibility of reuse of the support by incubation in 2% ionic detergents. That way, this modified CALB not

only has a strong anion exchange nature, while maintaining the activity, but it also shows improved stability under diverse reaction conditions without affecting the reversibility of the immobilization.

5.2 Reuse of anion exchangers as supports for enzyme immobilization: Reinforcement of the enzyme-support multiinteraction after enzyme inactivation

Process Biochemistry 51 (2016) 1391-1396

Virgen-Ortíz J.J.[1], Peirce S.[1,2], Tacias-Pascacio V.G.[1,3], Cortes-Corberan V.[1], Marzocchella A.[2],. Russo M.E.[4], Fernandez-Lafuente R.[1]

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The present contribution reports a preliminary study upon the feasibility of support recovery after the β-gal inactivation. Agarose beads coated with PEI were used as support, in order to emulate the external part of the whole OCCALB-PEI biocatalyst. This study was carried out to analyze the difficulties of the support recovery which can be occur after the enzyme inactivation. Indeed, the denatured enzyme may produce partially unfolded structures which lead a more intense ionic exchange, allowing a no easy reuse of the anion exchanger. This phenomenon is successfully described in Fig. 5.1 (Graphical abstract).

Abstract
Galactosidase from *Aspergillus oryze* (β-gal) has been immobilized on agarose beads coated with polyethyleneimine. The fresh enzyme was released from the support using 500 mM NaCl at pH 7. After thermal inactivation or inactivation in the presence of organic solvents, the active enzyme still could be easily released from the support using similar conditions. However, SDS-PAGE of the enzyme contained in the support after enzyme desorption showed that enzyme molecules remained in the support (inactivated enzyme molecules). This effect was stronger on enzyme preparations inactivated an organic medium. Now the conditions should be greatly strengthen to permit the full enzyme desorption: only after incubation in 2 M sodium phosphate at pH 2 and 50°C full release of the enzyme molecules was achieved. This could be repeated several cycles with any difference neither in the immobilization performance nor on the SDS-PAGE analysis. Therefore, the reversibility of the immobilization is a real fact, but recovery of a support fully free of protein molecules is not an easy objective after enzyme inactivation, because the inactivated enzymes seemed unfold increasing in a great way the interaction with the support, driving to a very strong enzyme-support multi-interactions which make more difficult its desorption.

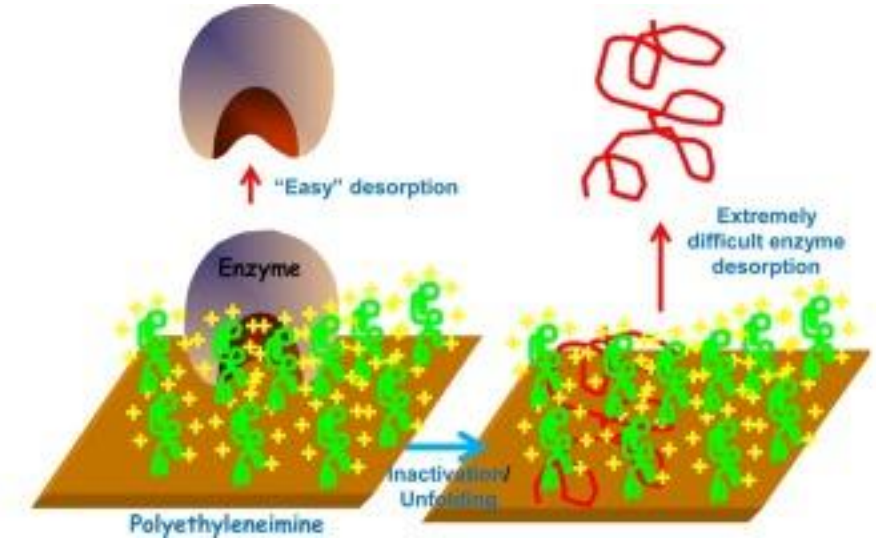

Fig. 5.1: Graphical abstract relative to the contribution of Virgen-Ortíz et al.(Process Biochemistry, 2016).

5.3 Development of simple protocols to solve the problems of enzyme coimmobilization. Application to co-immobilize a lipase and a β-galactosidase

RSC Advances 6 (2016) 61707-61715

Peirce S.[1,2], Virgen-Ortíz J.J.[1], , Tacias-Pascacio V.G.[1,3], Rueda N.[1,4], Bartolomeo-Cabrero R.[1], Fernández-Lopez L.[1], Cortes-Corberan V.[1], Russo M.E.[5], Marzocchella A.[2], Fernández-Lafuente R.[1]

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In this contribution, the protocol for the preparation of the CALB/β-gal combi-catalyst has been reported. Characterizations of the resultant catalyst have been performed, mainly basing on the expedients aimed to the support recovery after β-gal inactivation, focalizing also on the CALB residual activity after the β-gal loading, inactivation and desorption cycles. A schematic description of this system is reported in Fig. 5.2.

Abstract
This paper shows the co-immobilization of β-galactosidase from *Aspergillus oryzae* (β-gal) and lipase B from *Candida Antarctica* (CALB). The combi-biocatalyst was designed in a way that permits an optimal immobilization of CALB on octyl-agarose (OC) and the reuse of this enzyme after β-gal (an enzyme with lower stability and altogether not very stabilized by multipoint covalent attachment) inactivation, both of them serious problems in enzyme co-immobilization. With this aim, OCCALB was

coated with polyethylenimine (PEI) (this treatment did not affect the enzyme activity and even improved enzyme stability, mainly in organic medium). Then, β-gal was immobilized by ion exchange on the PEI coated support. We found that PEI can become weakly adsorbed on an OC support, but the adsorption of PEI to CALB was quite strong. The immobilized β-gal can be desorbed by incubation in 300 mM NaCl. Fresh β-gal could be adsorbed afterwards, and this could be repeated for several cycles, but the amount of PEI showed a small decrease that made re-incubation of the OCCALB–PEI composite in PEI preferable in order to retain the amount of polymer. CALB activity remained unaltered under all these treatments. The combi-catalyst was submitted to inactivation at 60°C and pH 7, conditions where β-gal was rapidly inactivated while CALB maintained its activity unaltered. All β-gal activity could be removed by incubation in 300 mM NaCl, however, SDS analysis showed that part of the enzyme β-gal molecules remained immobilized on the OCCALB–PEI composite, as the inactivated enzyme may become more strongly adsorbed on the ion exchanger. Full release of the β-gal after inactivation was achieved using 1 M NaCl and 40°C, conditions where CALB remained fully stable. This way, the proposed protocol permitted the reuse of the most stable enzyme after inactivation of the least stable one. It is compatible with any immobilization protocol of the first enzyme that does not involve ion exchange as only reason for enzyme immobilization.

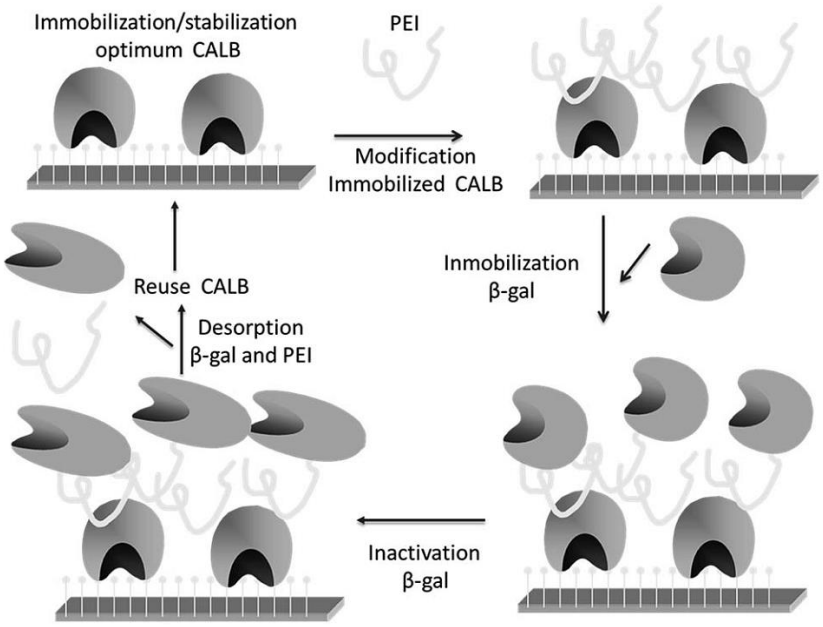

Fig. 5.2. Schematic representation of the model of the multi enzymes based biocatalyst proposed in this research activity (Peirce et al., .RSC Adv, 2016)

5.4 Ion exchange of β-galactosidase: The effect of the immobilization pH on enzyme stability

Process Biochemistry 51 (2016) 875-880

de Alburquerque T.L.[1,2], Peirce S.[1,3], Rueda N.[4], Marzocchella A.[3], Gonçalves L.R.B.[2], Ponte Rocha M.V.[2], Fernández-Lafuente R.[1]

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This contribution is not directly connected with the topic of the combi-catalysts protocols development. In this study, the effects of the immobilization conditions (pH and ionic strength) have been analyzed upon the performances of β-gal, used as enzyme model, immobilized on an aminated support (MANAE) via ionic exchange. The final biocatalyst performances were interpreted by regarding the possible enzyme orientation and thus the different enzyme areas involved in the immobilization, which strictly depend on the adopted operational conditions.

Abstract
β-Galactosidase from *Aspergillus oryzae* has been immobilized at pH 5, 7 and 9 on an aminated support using 5 mM buffer. The immobilization was total in 30 min, maintaining 75-80% of activity. These preparations were inactivated at different pH values and in presence of 50% ethanol. The stability of the enzyme immobilized at pH 9 was much lower than that of the enzyme immobilized at pH 5 under all studied conditions but the differences decreased as the ionic strength of the inactivation solution increased. The likeliest explanation to these different stabilities depending on the immobilization pH was that the enzyme presented a different orientation on the support. The enzyme immobilized at pH 5 was more stable than the free enzyme at pHs 5 and 9 (by a 2 or a 6 fold factor respectively), while at pH 7 the free enzyme was clearly more stable than the immobilized enzyme.

CONCLUSIONS

The study carried out during the present Ph.D program aimed at the development of solid CA-based biocatalysts for their use in a three-phase slurry CO_2 absorption unit. The main tasks were articulated according to the following paths:

a. Study of the kinetic performances of a CA recombinant form
b. Development and characterization of solid CA-based biocatalysts

Moreover, a further research activity was focused on the development of innovative protocols for preparation of combi-catalysts finalized at solving some critical issues associated to the conventional co-immobilization procedures. The task relative to the first research activity was carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale (DICMAPI) of the Università degli Studi di Napoli Federico II, while the activity regarding the development of innovative combi-catalysts typologies was carried out at the Instituto de Catálisis y Petrolquímica (ICP) of the Consejo Superior de Investigaciones Cíentificas (CSIC) in Cantoblanco (Madrid, Spain), under the supervision of Dr. Roberto Fernández-Lafuente.

Study of the kinetic performances of a CA recombinant form

The crude protein mixture containing 60% wt of recombinant CA form was characterized through CO_2 absorption rate assessment in a stirred cell reactor using as liquid phases Na_2CO_3/$NaHCO_3$ 0.5 M and 10% to 30%wt K_2CO_3 solutions at various carbonate to bicarbonate conversion degrees. The tests were performed at 298 and 313 K. The results showed that the rough data on CO_2 absorption at increasing CA concentration provided qualitative information of enzyme contribute on the overall CO_2 absorption rate. Then, the entire set of data collected at different solvent compositions and temperatures was analyzed and the kinetic constant k_{cat}/K_M was assessed, assuming the overall reaction as fast pseudo-first order and applying the Danckwerts' theory for gas-liquid absorption with reaction. The resulting values of k_{cat}/K_M obtained in Na_2CO_3/$NaHCO_3$ 0.5M at 298K resulted to be higher than those obtained with bCA (Alper and Deckwer, 1980) and SspCA from *Sulfurihydrogenibium yellowstonense* (Russo et al., 2013b). Moreover, k_{cat}/K_M values obtained in this study in 10% wt K_2CO_3 (CTB conversions 20 and 40%) at 298K were comparable to those reported by Ye and Lu (2014b) and Zhang and Lu (2015) for other recombinant CA forms, obtained using highly concentrated potassium carbonate solutions (20%wt) in a wide range of temperatures.

The validity of the performed kinetic assessment is supported by both the verification of criteria allowing the adoption of fast pseudo-first order model for description of CO_2 absorption process in the presence of free CA (even not in the whole set of operating conditions) and by the operating conditions adopted during the experimental investigation (potassium carbonate solvents at concentration and CTB conversion degree consistent with industrial application of CO2 capture process).

The assessment of CA performances at high biocatalyst concentration (≥ 0.3 kg m^{-3}) was carried out into highly concentrated K_2CO_3 solutions (20-30% wt) at 298 and 313 K according to conditions reported in literature for experimental and theoretical models of industrial CO_2 capture units (Lu et al., 2011: Ye and Lu, 2014; Zhang and Lu, 2015). The resultant enhancement factors values, with respect to the absorption rate in the only aqueous solvent, were close to 5 in all the cases at 0.3 kg m^{-3} CA and the formation of possible enzyme aggregates was not detected. An attempt was

made to assess the contribution of enzyme aggregates that may be formed in the presence of largely salty solvent at CA concentration larger than 0.3 kg m^{-3}. The results suggested that the presence of not negligible amount of enzyme aggregates (about 40% of initial protein amount of about 0.6 kg m^{-3}) may give further contribution to absorption rate enhancement. Finally, CA showed a good long term stability, retaining about 83% of its initial activity after the incubation into 20% wt K_2CO_3 at 40°C. The satisfying results reported in this section defined the adopted CA form as potential candidate as CO_2 absorption promoter in alkaline carbonate solutions

Development and characterization of solid CA-based biocatalysts

The thermostable CA form, characterized as homogeneous catalyst, was immobilized through covalent bonding on paramagnetic Fe_3O_4 nanoparticles (NPs) via carbodiimide activation in order to develop a CA-based solid biocatalyst to be used in CO_2 slurry absorbers. The performances of the solid biocatalyst were characterized according to the protocol used for the characterization of dissolved CA. CO_2 absorption tests were carried out in a stirred cell reactor, using carbonate solutions (pH>10) at different solvent compositions, different temperatures and carbonate to bicarbonate (CTB) conversions. Finally, the Danckwerts' theory for gas-liquid absorption with reaction was applied to calculate the first order kinetic constant k_{cat}/K_M of the immobilized CA relative to the CA-catalyzed CO_2 hydration reaction. The assessed enhancement factor values revealed that the CA contribution to the overall CO_2 absorption rate increased with the CTB conversion due to the decreasing of the CO_2 absorption rate in the alkaline solvent. Results showed that, although the immobilized CA had activity smaller than that of dissolved CA, k_{cat}/K_M were in agreement with those reported for other CA forms immobilized on nano-particles (e.g. bCA immobilized on non-porous silica NPs – Zhang et al., 2013)and were about 10^3 $m^3 \cdot kg^{-1} \cdot s^{-1}$.

The CLEAs technique was applied to develop bovine carbonic anhydrase (bCA) based biocatalyst for biomimetic CO_2 capture process. Magnetic NPs were successfully included in the CLEAs for their recovery during the preparation and for possible use of magnetic field assisted solids separation during the industrial application. The optimum conditions of the immobilization procedure were assessed in terms of type of precipitating agent, concentration of glutaraldehyde and cross linking operating conditions, as well as concentration of magnetic NPs. The optimization was carried out with respect to the maximum immobilization yield and to the CLEAs activity. Ammonium sulfate was selected as the best precipitant agent because the enzyme activity was largely preserved (about 90%). CLEAs activity was maximized by performing cross-linking at low temperature a for long time (4°C and 16 h). Such beneficial effect of decreasing temperature resulted an effective strategy to drive glutaraldehyde-bCA interactions towards satisfactory cross-linking degree with minimum enzyme deactivation by undesired reactions. Different CLEAs structure were observed when NPs to enzyme ratio and to the modality/intensity of mixing were changed during the precipitation step. Minimization of NPs concentration and maximization of stirring intensity minimize CLEA cluster size and maximize cluster branching. The maximum immobilization yield was about 90% and the maximum activity (1268 WAU/mg_{CLEA}) was measured for CLEAs prepared with 100 mM glutaraldehyde at 4°C, after 16 h crosslinking and 0.5 g_{NPs}/g_{bCA}. The developed CLEAs showed not negligible enhancement of CO_2 absorption rate in lab scale stirred cell reactor. The reported results suggest that the developed biocatalyst can be successfully proposed for the application in slurry absorber for CO_2 capture.

In both cases, satisfactory results were obtained, confirming the feasibility of the production of highly performing solid CA-based biocatalysts for CO_2 biomimetic absorption. As mentioned above, immobilized CA on NPs showed a satisfactory activity under conditions close to those of industrial interest, which was only slightly lower than that observed in the free enzyme counterpart. This revealed the great potentiality of this immobilization technique, as the enzyme activity was not significantly affected. On the contrary, bCA CLEAs presented lower activity than their free counterparts, maybe due to a partial deactivation during the immobilization phase or loss of specific activity. However, the not negligible activity and the observed high immobilization yields can positively approve this immobilization technique as candidate for the production of CA-based solid biocatalyst in slurry absorbers.

Development of innovative protocols for enzymes co-immobilization

The innovative co-immobilization methodology developed in this research activity was finalized to overcome some drawbacks associated to the conventional co-immobilization procedures, such as the immobilization of two or more enzymes on the same support (or through combi-CLEAs technique) and under the same operating conditions, which seldom may constitute the optimal conditions. Lipase B from *Candida antarctica* (CALB) and β-galactosidase from *Aspergillus oryzae* (β-gal) were used as enzymes models. The combi-biocatalyst was designed in a way that permitted an optimal immobilization of CALB on octyl-agarose (OC) and the reuse of this enzyme after β -gal (the enzyme with lower stability) inactivation. The coating of OC-CALB with polyethylenimine (PEI) did not affect the CALB activity, on the contrary it improved the enzyme stability, mainly in organic medium. The high stability of OC-CALB coated with PEI was also confirmed during the cycles of absorption, inactivation and desorption of β-gal, since its activity ever remained unaltered. Among the still open issues relative to this protocol, there are the stronger adsorption of the inactivated β-gal with respect to the active enzyme, which required drastic conditions for its desorption, e.g. high temperature and/or ionic strengths. Such conditions did not alter CALB activity but made compulsory the re-incubation of the whole combi-catalyst in PEI after each desorption/adsorption cycle of β-gal, due to the release of the polymer from OC-CALB. It is not a problem at laboratory scale, but may be an inconvenient at industrial level and strategies to avoid this necessity should be explored. In conclusion, the proposed protocol permits the reuse of the most stable enzyme after inactivation of the less stable one. It is applicable to a wide range of enzymes. Moreover, it is consistent with any protocol for the immobilization of the more stable enzyme, except the immobilization through the ionic exchange, which cannot be used in order to avoid the enzyme leaching during the desorption operations.

Future perspectives

The study carried out during the PhD activity was addressed to the development of CA-based biocatalysts for biomimetic CO_2 capture. The main tasks were focused on the analysis of recombinant CA forms and the development of protocols for the preparation of solid biocatalysts. The use of various immobilization techniques was not exclusively confined to the case of CA, as the further research activity was addressed to the development of an innovative protocols aimed to the preparation of combi-catalysts and applicable to a wide variety of enzymes.

As mentioned in Chapter 1, CO_2 valorization is an attractive alternative to the CO_2 storage. Indeed, the use of CO_2 as feedstock for the production of commodity chemicals (including fuels) is a promising way to retrieve carbon sources alternative to the fossil ones. For example, the synthesis of formic acid, formaldehyde and methanol via CO_2 hydrogenation through enzymatic catalysis has been recently proposed by several researchers. Even still far from to be competitive with traditional synthesis pathways this enzymatically catalyzed route to CO_2 utilization seems to be a promising alternative to the traditional catalysis, electrochemical and photocatalytic reduction thanks to the higher specificity as well as to the milder reaction conditions required. Moreover, some authors reported that the presence of CA enables a faster enzymatic conversion of CO_2 to other chemicals, such as the conversion of CO_2 to methanol via formate, aldehyde, and alcohol dehydrogenase catalysis (Addo et al., 2011) or the conversion of CO_2 to formic acid via formate dehydrogenase catalysis and using NADH as cofactor (Wang et al., 2015). Indeed, the catalysis of CA leads to the rapid CO_2 conversion into bicarbonate ions being more soluble in water than CO_2. Therefore, CA can contribute to the improvement of the production rates.

These evidences reveal a possible new route to CO_2 conversion in added value chemicals, moreover there are numerous examples in the literature (Kuwabata et al., 1994; Obert and Dave, 1999; Addo et al., 2011; Wang er al., 2015) reporting on the use of co-immobilized enzymes for the biotrasformation of CO_2 in various chemicals.

In this framework, future perspectives of this work may be addressed to the development of innovative combi-biocatalyst for CO_2 capture and utilization processes based on enzyme cascade reactions. The techniques developed during this PhD research activity offer a reliable tools for the achievement of these aims.

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APPENDIX

Information regarding the scientific contributions, research activities in external institutes and attended seminaries/courses are listed hereinafter. Finally, the full manuscripts of published papers are reported.

Permanence at research structures in Europe:

Research activity under the supervision of Dr. Roberto Fernández-Lafuente at the Instituto of Catálisis y Petrolquímica (ICP) - Consejo Superior de Investigaciones Cíentificas (CSIC) – in Cantoblanco (Madrid, Spain). October 27 2015-April 29 2016.

DISSEMINATION AND CONTRIBUTIONS

Conferences

- Peirce S., Russo M. E., De Luca V., Capasso C., Rossi M., Olivieri G., Salatino P., Marzocchella A., "Immobilization of carbonic anhydrase for biomimetic CO_2 capture in slurry absorber", oral presentation. 16th European Congress on Biotecnology (ECB16), 13-16 July 2014, Edimburgh.
- Peirce S., Russo M. E., De Luca V., Capasso C., Rossi M., Olivieri G., Salatino P., Marzocchella A., "Immobilization of Carbonic Anhydrase for Biomimetic CO_2 Capture in a Slurry Absorber as Cross-Linked Enzyme Aggregates (CLEA)", oral presentation. 12th International Conference on Chemical & Process Engineering (ICheaP12), 19-22 May 2015, Milan.
- Peirce S., Russo M.E., Cortese L., Olivieri G., Salatino P., Marzocchella A., "Immobilization of carbonic anhydrase for biomimetic CO_2 capture: carrier free vs supported biocatalyst", poster presentation. 10th European Congress of Chemical Engineering + 3th European Congress of Applied Biotechnology + 5th European Process Intensification Conference (ECCE10 + ECAB3 + EPIC5), 27th September-1th October 2015, Nice.
- Peirce S., Russo M. E., De Luca V., Capasso C., Rossi M., Olivieri G., Salatino P., Marzocchella A., "Kinetic assessment of thermostable carbonic anhydrase for biomimetic CO_2 capture in carbonate solutions: effect of enzyme precipitation", oral presentation. 10th European Congress of Chemical Engineering + 3th European Congress of Applied Biotechnology + 5th European Process Intensification Conference (ECCE10 + ECAB3 + EPIC5), 27th September-1th October 2015, Nice.
- de Albuquerque T.L., Peirce S., Rueda N., Marzocchella A., Gonçalves L.R.B., Valderez Ponte Rocha M., Fernandez-Lafuente R., "Importance of the immobilization pH upon the stability of the immobilized enzyme", poster presentation. 11th International Conference on Protein Stabilization (ProtStab2016), 9-11 May 2016, Istanbul.
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Papers

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- Prof. Marino G., "Coniugare la Biotecnologia. Passato remoto e… futuro prossimo", March 2014;
- Dr. Temur H., "Quality characteristics of bioenergy sources, solid bioenergy `products, liquid bioenergy product, gaseous bioenergy products,possible technical uses, anaerobic digestion: system description and components, planning an anaerobic digestion project realization, commissioning and start-up, operation and maintenance, economics", May 2014;
- Dr. Temur H., "Introduction: The challenge, universal energy carrier, potential market, boundary conditions. Biomass: energy from the sun: photosyntesys, carbon dioxide's key role in climate change, carbon cycle, type of biomass, utilization of biomass", May 2014;
- Dr. Arzutug M.E., "Molecular diffusion in liquids. Diffusion controlles mass transfer in solid-liquid systems and governing equations. Convective mass transfer on a flat plate". May 2014;
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Stabilization of *Candida antarctica* Lipase B (CALB) Immobilized on Octyl Agarose by Treatment with Polyethyleneimine (PEI)

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Abstract: Lipase B from *Candida antarctica* (CALB) was immobilized on octyl agarose (OC) and physically modified with polyethyleneimine (PEI) in order to confer a strong ion exchange character to the enzyme and thus enable the immobilization of other enzymes on its surface. The enzyme activity was fully maintained during the coating and the thermal stability was marginally improved. The enzyme release from the support by incubation in the non-ionic detergent Triton X-100 was more difficult after the PEI-coating, suggesting that some intermolecular physical crosslinking had occurred, making this desorption more difficult. Thermal stability was marginally improved, but the stability of the OCCALB-PEI was significantly better than that of OCCALB during inactivation in mixtures of aqueous buffer and organic cosolvents. SDS-PAGE analysis of the inactivated biocatalyst showed the OCCALB released some enzyme to the medium during inactivation, and this was partially prevented by coating with PEI. This effect was obtained without preventing the possibility of reuse of the support by incubation in 2% ionic detergents. That way, this modified CALB not only has a strong anion exchange nature, while maintaining the activity, but it also shows improved stability under diverse reaction conditions without affecting the reversibility of the immobilization.

Keywords: reversible immobilization; interfacial adsorption; PEI modification; enzyme stabilization; enzyme physical intermolecular crosslinking

1. Introduction

Lipases are the most used enzymes in biocatalysis due to their good activity, specificity, selectivity and robustness in a variety of reaction media [1–3]. They have a peculiar catalytic mechanism based in deep conformational changes called interfacial activation that permits them to act in the hydrolysis of insoluble drops of oils [4,5], and this causes them to have a tendency to become adsorbed on any hydrophobic surface [6–9]. In order to use them as industrial biocatalysts, immobilization is required in many instances in order to facilitate their reuse [10–13]. This step should be used to improve other enzyme features. Usually the focus is on enzyme stability, but other enzyme properties may be also improved, such as purity, activity, selectivity or specificity [14–18].

An ideal industrial enzyme immobilization method must allow one to improve all enzyme features in a very simple way [15]. In this sense, immobilization of lipases on hydrophobic supports [6,7] may be noted as an useful tool to improve lipase properties: this immobilization strategy permits the specific lipase immobilization (purification) [6], involves the surroundings of the active center of the lipase and, in most cases, the open form of the lipase (hyperactivation and stabilization) [19,20], may be used to tune enzyme properties [21], and is very simple to perform and reversible, enabling support reuse [6,7]. Unfortunately, it has a drawback: the enzyme may be released to the medium when exposed to high temperatures or high concentrations of organic cosolvents during operation [22].

To solve this problem, some authors have chemically crosslinked the lipases using aldehyde dextran [23,24] or glutaraldehyde [25]. The intermolecular crosslinking causes the enzymes to be only desorbed when all the enzymes in the aggregate simultaneously break their interaction with the support. The system works, but converts a reversible method into an irreversible one, which makes both enzyme and support disposable after enzyme inactivation.

Another explored solution has been the use of heterofunctional supports having acyl groups (to permit lipase immobilization via interfacial activation) and some moieties able to form covalent attachments [22,26–32]. This treatment prevents enzyme desorption and improves enzyme stability, even enabling the use of the enzyme under conditions where a support with only acyl groups cannot maintain the enzyme immobilized. The method also permits a partial reactivation of some enzymes [33], but again transforms a reversible method in an irreversible one. In a further step, heterofunctional acyl-ionic supports have been designed [34,35]. However, the results were quite complex, although enzyme release was reduced and the reversibility of the immobilization was maintained, the immobilized lipase was destabilized in some cases [34,35].

Polyethylenimine has been used in biocatalyst design with different objectives: generation of hydrophilic environments to reduce the concentration of solvents molecules or oxygen, stabilization of multimeric enzymes, to keep the activated form of lipases, to prevent oxidation, *etc.* [36–46]. The open structure of the coating does not produce steric hindrance to the entry of small substrates, and it may just produce some partition of hydrophobic substrates from the enzyme environment. In some instances the enzyme activity is even increased after coating [47]. As the interaction is reversible, the use of ionic detergents, guanidine, *etc.* may permit the release of the enzyme and the PEI, permitting the reuse of the support. However, to date, it has been never analyzed if the positive effects of the enzyme coating after enzyme immobilization may be, at least in part, due to the intermolecular physical crosslinking between different immobilized enzyme molecules, avoiding that way the enzyme desorption from the support.

As model enzyme we have utilized the most widely used lipase, lipase B from *Candida antarctica* (CALB), a robust enzyme with many applications [48,49]. It has a very small lid that does not fully seclude the active center from the medium [49], but has been successfully immobilized on a handful of hydrophobic supports [22,25]. Moreover, the PEI coating of the immobilized CALB has permitted to design an immobilized CALB form with good properties to be used as "immobilization support" to co-immobilize other enzymes on the already immobilized lipase via anion exchange. The results of this protocol to co-immobilize lipase and other enzymes have been recently published but the effects of the modification on the enzyme properties have not been properly analyzed [50]. Furthermore, there is no knowledge whether this treatment does or does not produce some enzyme crosslinking. Thus, the objective of this paper was to characterize the optimal PEL-OCCALB preparation useful for enzyme co-immobilization. To reach this goal, a large excess of PEI was used trying to prevent that each PEI molecule may cover a large area of enzyme molecule, because a large polymeric bed is intended. This protocol should produce a large shell of ion polymer coating the enzyme surface, and may maximize the partition effects of this coating in the presence of hydrophobic molecules, like organic solvents. Moreover, we have paid special attention to the possibility of achieve some physical intermolecular crosslinking that could explain, at least partially, some of the positive effect.

2. Results and Discussion

2.1. Immobilization of CALB on Octyl-Agarose

Figure 1 shows that CALB is rapidly (30 min) and fully immobilized on OC with low effect on enzyme activity. The lack of a real lid that isolates the enzyme active center [51] caused the adsorption on octyl agarose to fail to produce the usual increase on enzyme activity found with other lipases [6].

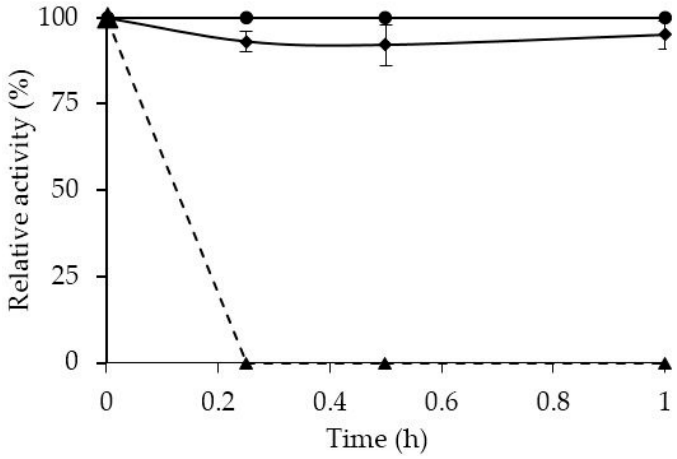

Figure 1. Immobilization courses of CALB at pH 7 on octyl agarose at low ionic strength. Rhombi, solid line: suspension; triangles, dashed line: supernatant; circles, solid line: reference.

2.2. Effects of PEI Modification on Enzyme Activity/Stability

The coating of the enzyme with PEI did not produce any effect on enzyme activity. This result was positive as the presence of the shell covering the enzyme surface could produce some problems to the entry of the substrate. However the open structure of the polymer has not effect on enzyme activity, as it has been found with many other enzymes other than CALB [37,39,45].

Figure 2 shows that while immobilization on OC produced a significant stabilization, the PEI treatment of the immobilized enzyme has a marginal effect on enzyme stability, with small increases on enzyme stability at the three studied pH values. It should be considered that the PEI may be introducing intra- and inter-molecular crosslinkings, but the weak nature of the bonds and the flexible nature of the polymer did not produce a significant change in enzyme stability [52,53].

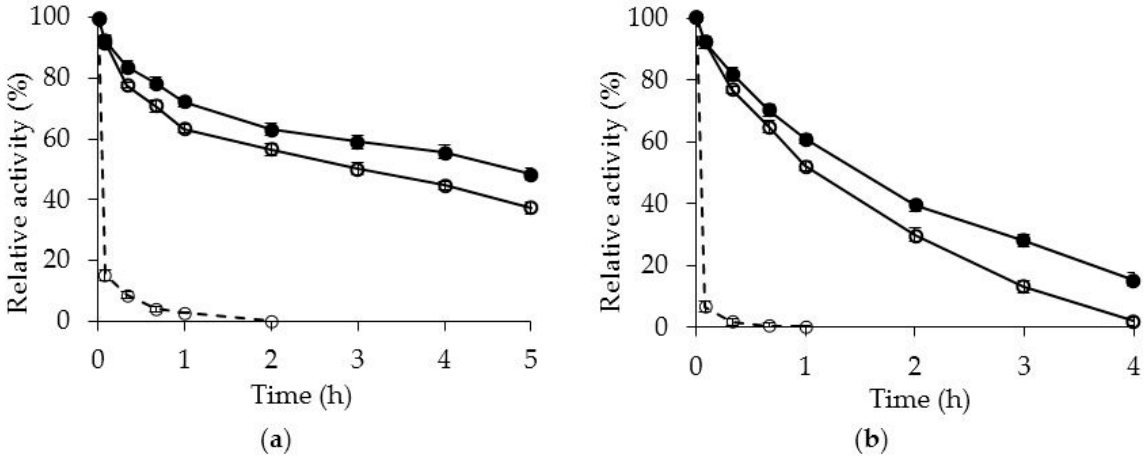

Figure 2. *Cont.*

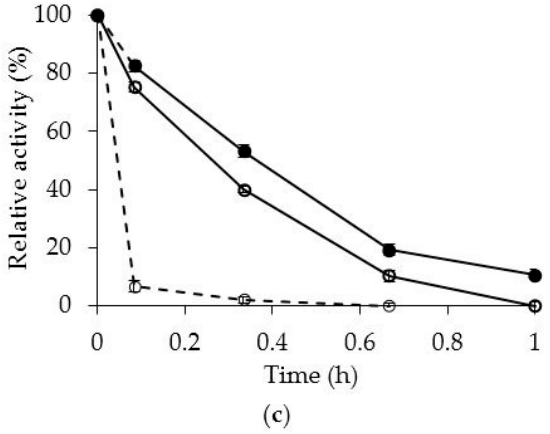

Figure 2. Thermal inactivation courses of the OCCALB and OCCALB-PEI biocatalysts. Panel (**a**): 80 °C and pH 5; panel (**b**): 70 °C and pH 7; panel (**c**): 60 °C and pH 9. Open circles, solid line: OCCALB; closed circles, solid line: OCCALB PEI; dashed line: free CALB.

The results are much better than when using amino-octyl supports [35] to improve the enzyme immobilization, because that support produced enzyme destabilization at pH 5 and 7 and only some stabilization was observed at pH 9. Using glutamic-octyl [34] the stability decreased under acid media value while it increased more clearly than using PEI at neutral pH value. Glutaraldehyde crosslinking gave better results in terms of stabilization [25], because the small size of the crosslinking reagent permitted a higher enzyme rigidification, the combination of inter and intramolecular crosslinking produced a significant stabilization. However, this treatment avoided the enzyme desorption and therefore, transformed this reversible immobilization into an irreversible one, precisely want we wanted to prevent.

Figure 3 shows that this physical modification of the enzyme with PEI has a more significant effect on the stability of the enzyme in the presence of different organic cosolvents. Although OCCALB is already quite stable in this medium, the coating with PEI significantly improved the stability, mainly in the presence of hydrophobic dioxane.

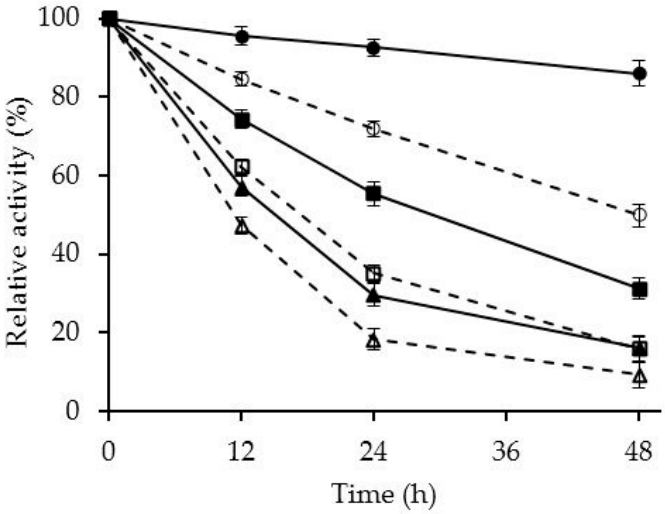

Figure 3. Effect on enzyme activity of the incubation of the immobilized CALB in the presence of different organic solvents. Enzyme preparations were incubated in mixtures of organic solvents/100 mM Tris-HCl pH 7 at 40 °C. Solid line: OCCALB-PEI biocatalyst; dashed line: OCCALB biocatalyst; circles: 80% 1,4-dioxane; squares: 45% acetonitrile; triangles: 70% ethanol.

This stabilization produced by PEI coating in organic solvents has been usually associated to the partition of the hydrophobic organic solvents, but the possibility of enzyme intermolecular physical crosslinking has not been analyzed. The use of amino-octyl [35] almost did not alter the enzyme stability in the presence of organic cosolvents, while glutamic-octyl [34] permitted stabilizations of even 10 folds in these media, higher than those described here. Glutaraldehyde, as in thermal inactivations, provided a higher stabilization (more than 10-fold) than the coating with PEI [25].

2.3. Effect of PEI Treatment in the Desorption of OCCALB by Incubation in non Ionic Detergents

OCCALB and OCCALB-PEI were incubated in growing concentrations of Triton X-100 (Figure 4). The PEI treatment increased the amount of detergent required to desorb CALB from the octyl support. For example using 0.5% almost all activity was released from OCCALB and only a 33% was released from OCCALC-PEI. This higher difficulty of CALB release after PEI treatment suggested that some CALB molecules could be physically crosslinked and that could hinder enzyme desorption. However, finally most enzyme molecules could be desorbed, suggesting that the physical crosslinking using this protocol is not maximized.

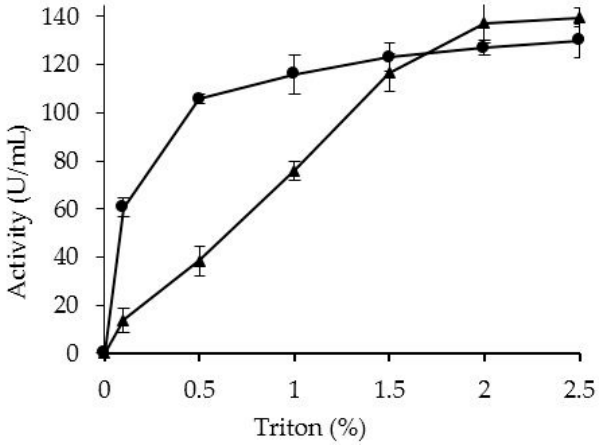

Figure 4. Desorption of the CALB from the OCCALB (circles) and OCCALB-PEI (triangles) derivatives with Triton X-100.

2.4. Effect of PEI Modification in the Release of CALB Molecules to the Medium during Inactivation

It has been described that lipase molecules are desorbed to the medium from OC supports during solvent and thermal inactivations [22]. To check this, the biocatalysts submitted to inactivation were recovered and the amount of protein still attached to the support was analyzed by SDS-PAGE. Figure 5 shows that the OCCALB desorbs part of the enzyme (the intensity of the protein band is lower in the inactivated biocatalysts following densitometry analyses of the gels) during thermal inactivation, mainly at pH 9. The treatment with PEI reduced this decrease in the CALB band (around 50% more protein could be observed). Figure 6 shows that most of the enzyme remained immobilized when incubated in dioxane for 48 h, while ethanol and acetonitrile was able to desorb higher amounts of enzyme. PEI reduced this enzyme release caused by the solvent, mainly using acetonitrile (threefold more protein remained in the biocatalysts after PEI treatment).

2.5. Reuse of the Support

The washing of the OCCALB-PEI with 2% CTAB or SDS at 40 °C permitted to fully eliminate the CALB adsorbed on the support, even after enzyme inactivation (it has been reported that the inactivated enzyme may become strongly adsorbed on the support) [54]. The support, after washing with water, could be reused for five cycles while maintaining the immobilization rate, enzyme stability and activity.

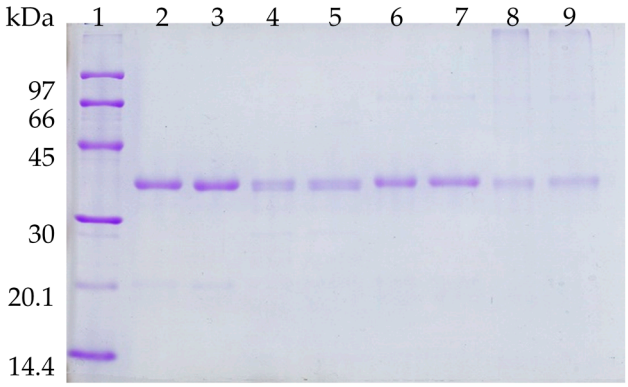

Figure 5. SDS-PAGE analysis of OCCALB and OCCALB-PEI derivatives under different treatments. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: OCCALB; lane 3: OCCALB-PEI; lane 4: OCCALB and lane 5: OCCALB-PEI incubated at pH 5 and 80 °C. Lane 6: OCCALB and lane 7: OCCALB-PEI incubated at pH 7 and 70 °C. Lane 8: OCCALB and lane 9: OCCALB-PEI incubated at pH 9 and 60 °C.

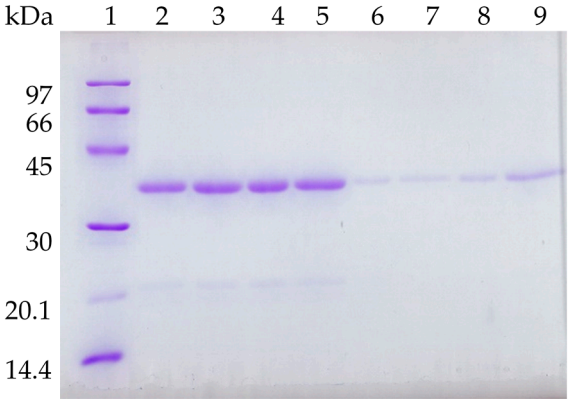

Figure 6. SDS-PAGE analysis of OCCALB and OCCALB-PEI derivatives with organic solvents. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: OCCALB; lane 3: OCCALB-PEI; lane 4: OCCALB and lane 5: OCCALB-PEI incubated in 90% dioxane; lane 6: OCCALB and lane 7: OCCALB-PEI incubated in 70% ethanol; lane 8: OCCALB and lane 9: OCCALB-PEI incubated in 45% acetonitrile.

3. Materials and Methods

3.1. Materials

Solution of lipase B from *Candida antarctica* (CALB) (6.9 mg of protein/mL) was a kind gift from Novozymes (Alcobendas, Spain). Polyethylenimine (PEI) (MW 25,000), Triton X-100 and *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Sigma-Aldrich (St. Louis, MI, USA). Octyl Sepharose CL-4B beads was from GE Healthcare Bio-Sciences (Uppsala, Sweden). Electrophoresis purity reagents were obtained from Bio-Rad (Hercules, CA, USA). All other reagents were of analytical grade. Protein concentration was estimated by the Bradford dye binding method at 595 nm [55] using bovine serum albumin as a standard.

3.2. Standard Measure of Enzyme Activity

The enzyme activity assay was performed by measuring the increase in the absorbance at 348 nm (isobestic point) produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate at pH 7 and 25 °C (ε under these conditions is 5150 $M^{-1} \cdot cm^{-1}$).

A spectrophotometer with a thermostated cell and with continuous magnetic was used. To initiate the reaction, 50–100 μL of lipase solution or suspension were added to 2.5 mL of substrate solution. One international unit of *p*NPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPB min^{-1} (U) under the conditions described above.

3.3. Immobilization of CALB on Octyl (OC) Supports

The standard immobilization was performed using 2 mg of lipase per g of wet support (20 units/g). CALB solution was diluted in the corresponding volume of 5 mM sodium phosphate at pH 7. Then, OC support was added [6]. The activity of both supernatant and suspension was followed using *p*NPB assay. After immobilization the suspension was filtered and the immobilized biocatalyst enzyme was exhaustively washed with distilled water.

3.4. Modification of OCCALB with PEI

A 50 mL solution of 10% PEI (*w*/*v*) was prepared in 5 mM sodium phosphate and the pH was adjusted at pH 7. Then, 5 g of OCCALB was suspended and submitted to gentle stirring for 2 h. Afterwards, the modified enzyme was washed with an excess of distilled water to eliminate the free PEI. The biocatalyst was stored at 4 °C in wet conditions.

3.5. Thermal Inactivation of OCCALB and OCCALB-PEI Preparations

Immobilized biocatalyst (1 g) was suspended in 25 mM sodium acetate (5 mL) at pH 5, sodium phosphate at pH 7 or sodium bicarbonate buffer at pH 9 at different temperatures. Periodically, samples were withdrawn and the activity was measured using *p*NPB.

3.6. Inactivation of OCCALB and OCCALB-PEI in the Presence of Organic Co-Solvents

The biocatalysts were incubated in mixtures of ethanol, acetonitrile or 1,4-dioxane/100 mM Tris-HCl buffer (pH 7) at different temperatures. Periodically, samples were withdrawn and the activity was measured using *p*NPB as described above.

3.7. Desorption of the CALB from the Supports

Samples of 1 g of the immobilized biocatalysts were suspended in 10 mL of 5 mM sodium phosphate buffer at pH 7. Then, Triton X-100 was progressively added to a final concentration of 2.5% (*v*/*v*). Intervals of 30 min were allowed before taking a sample of the supernatant to determine the released enzyme and performing a new detergent addition [22]. Finally, 100% of the enzyme contained in the support was released from the support.

3.8. SDS-PAGE Experiments

SDS-polyacrylamide gel electrophoresis experiments were performed according to Laemmli [56] using a Miniprotean tetra-cell (Bio-Rad), 14% running gel in a separation zone of 9 cm × 6 cm, and a concentration zone of 5% polyacrylamide. One hundred milligrams of the immobilized enzyme samples was re-suspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 8 min and a 10 μL aliquot of the supernatant was used in the experiments. This treatment released all enzyme just interfacially activated on the support [6]. Gels were stained with Coomassie brilliant blue. A low molecular weight calibration kit for SDS electrophoresis (GE Healthcare) was used as a molecular weight marker (14.4–97 kDa).

3.9. Reuse of the Support

These experiments were performed in syringes with a silica plate in the outlet to prevent loss of support. 1 g of support was loaded with CALB, treated with PEI, and incubated in 2% CTAB or SDS at different temperatures (two washings of three volumes of detergent solution for 5 min). Then, the

support was washed 10 times with 10 mL of distilled water to eliminate the detergent (protocols not optimized).

4. Conclusions

The coating of octyl-CALB with PEI following the protocol successfully applied to enzyme co-immobilization has no negative effects on enzyme activity and has positive effects on enzyme stability, mainly in mixtures of organic cosolvents/aqueous buffers, even if this protocol was not optimized for this purpose. The physical coating of the enzyme molecules with PEI reduces enzyme release from the support, both in incubation in nonionic detergents or after enzyme inactivation (thermal or organic solvent) suggesting that some intermolecular physical crosslinking may be relevant for the stabilization results. However, it has been previously described that this modification may also reduce the concentration of solvent in the enzyme environment (e.g., the highest stabilization is in the presence of dioxane, where OCCALB did not release almost enzyme molecules to the medium), therefore the stabilization may be caused by several factors, but the physical intermolecular crosslinking can also play some role. This strategy for enzyme stabilization did not avoid the possibility of desorbing the enzyme and reusing the support after enzyme inactivation because all is reversible: enzyme adsorption on this support is via hydrophobic interaction; PEI crosslinking is via ion exchange. It should be considered that this "physical" enzyme intermolecular crosslinking has been observed using a protocol designed to get a further enzyme adsorption on the PEI coated CALB and not to stabilize the enzyme. Thus, the further optimization of the PEI enzyme coating may produce a better PEI intermolecular physical crosslinking (e.g., chemical succinylation of the enzyme surface, PEI size and concentration). This preliminary results suggest that the strategy may be of general usefulness to prevent any enzyme desorption from supports where the immobilization is not irreversible. Compared to the use of ion-octyl supports, the results on enzyme thermostability were clearly better using this protocol of PEI coating, the results in stability in organic cosolvents/aqueous medium was under that obtained using glutamic, but considering that the optimization was performed to get a good conimmobilization of a second enzyme, the results are quite promising. A proper coating with ionic polymers of CALB immobilized on octyl supports (therefore with the enzyme molecules packed together) should permit to further improve the results.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this paper:

CALB	*Candida antarctica* lipase B
OC	octyl-Sepharose
OCCALB	CALB immobilized on OC
PEI	polyethyleneimine
*p*npb	*p*-nitrophenyl butyrate

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Reuse of anion exchangers as supports for enzyme immobilization: Reinforcement of the enzyme-support multiinteraction after enzyme inactivation

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ABSTRACT

β-Galactosidase from *Aspergillus oryze* has been immobilized on agarose beads coated with polyethyleneimine. The fresh enzyme was released from the support using 500 mM NaCl at pH 7. After thermal inactivation or inactivation in the presence of organic solvents, the active enzyme still could be easily released from the support using similar conditions. However, SDS-PAGE analysis of the enzyme contained in the support after enzyme desorption showed that enzyme molecules remained in the support (inactivated enzyme molecules). This effect was stronger on enzyme preparations inactivated in an organic medium. Now the conditions should be greatly strengthen to permit the full enzyme desorption: only after incubation in 2 M sodium phosphate at pH 2 and 50 °C full release of the enzyme molecules was achieved. This could be repeated several cycles with any difference neither in the immobilization performance nor on the SDS-PAGE analysis. Therefore, the reversibility of the immobilization is a real fact, but recovery of a support fully free of protein molecules is not an easy objective after enzyme inactivation, because the inactivated enzymes seemed to unfold increasing in a great way the interaction with the support, driving to a very strong enzyme-support multi-interaction that difficulty its desorption.

1. Introduction

Enzyme immobilization is a usual requirement in the design of an enzyme biocatalyst in order to solve the issue concerning the solubility of enzymes in aqueous medium and simplify their reuse [1–5]. From this necessity, many researchers have tried to utilize the immobilization step as a tool to improve many other enzyme limitations, like stability, activity, selectivity or specificity, [6–13] even coupling this technique to enzyme purification [14].

Although the immobilization may produce a very positive impact on enzyme performance, it has a certain cost derived from the process and the support. To reduce costs, physical and reversible enzyme immobilization has been proposed as a suitable alternative [10,15,16]. The protocols of these immobilizations tend to be very simple, and the main advantage is that they can permit the recovery of the support after enzyme inactivation [10]. Among them, ion exchange is perhaps the most popular [17–20].

One of the main problems of these physical immobilization techniques is the possibility that the enzyme may be released during operation [10]. This is prevented if a strong adsorption is achieved, and polymeric beds (polyethylenimine (PEI), [21] dextran sulfate (DS) [22]) have been reported to be a good solution for this goal. These beads permit a multipoint adsorption involving many areas of the protein surface (it is a tridimensional net, not a surface [23]) and may be adapted to the protein, without producing undesired conformational changes.

This protocol has moderate to low enzyme structure rigidification effects [10]. Moreover, the final surface may not be fully inert, and that is not the desired situation when immobilizing an enzyme

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because uncontrolled enzyme/support interactions are never welcome [24]. Therefore this is mainly indicated when the enzyme is not very stabilized by multipoint covalent attachment or when the enzyme has good enough stability (e.g., thermophilic enzymes) [25]. However, enzyme operational stability may be improved. For example, they allow the prevention of intermolecular phenomena like aggregation, interaction with hydrophobic interfaces, autolysis [10]. These stabilizing effects are inherent to any enzyme immobilization inside porous supports. Moreover, some stabilization in the presence of organic solvents is also expected; because an artificial ionic enzyme environment may permit some partition of hydrophobic compounds, mainly using polymeric ionic beds [26,27]. Multimeric enzymes, especially dimeric enzymes, may also be involved in the immobilization of some enzyme subunits, reducing subunit dissociation and producing some stabilization effects [11].

Immobilization on these supports is a multipoint process; the enzyme is only incorporated to the support when several ion bridges are formed [28–31]. The more ionic bonds that are formed, the stronger the immobilization results. This permit that supports having the same number of anion and cation groups may efficiently adsorb many proteins, even some that are not adsorbed on equivalent monofunctional supports [32].

However, one point to be considered is that the enzyme will become inactivated during operation, producing (partially) unfolded structures that may permit a more intense ionic exchange and that can produce problems in the enzyme desorption after enzyme inactivation. It has been reported that the presence of ion polymers avoid the possibility of enzyme reactivation after enzyme inactivation, and this is explained by the promotion of new and incorrect enzyme/polymer interactions [33]. That may cause some problems for the support reuse, problems that are not found using these ion exchangers for protein purification. Usually, the conditions utilized for releasing the active enzyme are similar to the ones used to release the protein from the inactivated biocatalyst, and this may be incorrect if the inactivated enzymes are much more strongly adsorbed than the active enzyme.

In this paper, we show the recovery and reuse of PEI-agarose after enzyme thermal and solvent inactivation, using the β-galactosidase from *Aspergillus oryze* (β-gal) as model enzyme, a monomeric and glycosylated enzyme with an isoelectric point of 4.5/4.6 that is active at acidic pH value [34]. The enzyme has been employed in many reactions, like hydrolysis of lactose [35–42] and other alkylgalactosides [43–47].

There are reports on β-gal immobilization on anion exchangers under a wide range of conditions, giving as result biocatalysts with different features [48] while multipoint covalent attachment did not produce a significant stabilization (around 10 folds) [49]. In this paper, β-gal has been immobilized on PEI-agarose, and inactivated for several cycles, analyzing the recovery of the support.

2. Materials and methods

2.1. Materials

β-Galactosidase from *Aspergillus oryzae* (20 units oNPG/mg of protein), *o*-nitrophenyl-β-galactopyranoside (oNPG), polyethylenimine (PEI) (MW 25,000), glycidol, sodium periodate, sodium borohydride, sodium chloride and 1,4-dioxane were purchased from Sigma-Aldrich (St. Louis, USA). Agarose with 4% of cross-linking BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Electrophoresis purity reagents were obtained from Bio-Rad (Hercules, USA). Protein assay reagent was from Thermo scientific (Rockford, IL). All other reagents were of analytical grade. Protein concentration was estimated by the Bradford dye binding method at 595 nm [50] using bovine serum albumin as a standard.

2.2. Standard measure of enzyme activity

The activity of the soluble β-galactosidase and enzyme suspension was determined by measuring the increase in absorbance at 380 nm produced by the release of *o*-nitrophenol produced by the hydrolysis of 10 mM *o*-nitrophenyl-β-galactopyranoside in 100 mM sodium acetate buffer at pH 4.5 and 25 °C (ε was 10493 M^{-1} cm^{-1} under these conditions) [48]. The assay was carried out using a spectrophotometer equipped with a thermostatized cell and with continuous magnetic stirring. To start the reaction, a maximum of 100 μL (0.2 units) of the enzyme solution or suspension were added to 2.5 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmoL of oNPG per minute under the conditions described previously.

2.3. Preparation of supports

The preparation of glyoxyl supports, activated with 25 μmol of aldehyde groups per g of wet support, was carried out by directly oxidizing the diols of the support using sodium periodate (an equimolecular ratio was used in the reaction) following the standard protocol as previously described [51]. The suspensions containing the supports and the sodium periodate were gently stirred for 3 h at 25 °C, and then the supports were filtered and washed with distilled water.

The support polyethyleneimine-agarose was prepared with the amination of glyoxyl-agarose with primary amino groups of PEI. A 50 mL of 10% PEI (w/v) was prepared in 5 mM sodium phosphate and the pH was adjusted at 7. Then, 5 g of glyoxyl-agarose was suspended and submitted to gentle stirring for 2 h at 25 °C. Finally, the support was washed with an excess of distilled water, in a glass funnel, to eliminate the free PEI. The biocatalyst was stored at 4 °C under wet conditions.

2.4. Immobilization of β-galactosidase

The standard immobilization was performed using 20 oNPG units of free β-galactosidase activity per g of wet support (1 mg of enzyme per gram of support). This low loading was used to prevent diffusion limitations that could make more complex the understanding of the results on molecular enzyme properties. In some instances, the amount of enzyme was increased (e.g. to perform SDS-PAGE analysis). The commercial sample of the enzyme was dissolved in the corresponding volume of sodium phosphate at pH 7. Then, the support was added under mild stirring. Aliquots from the suspension and supernatant were withdrawn at an interval of 15 min to determine enzyme activity. After immobilization, the derivatives were extensively washed with distilled water.

2.5. Study of the stability of the β-galactosidase biocatalyst

2.5.1. Thermal inactivation

5 g of immobilized β-gal was suspended in 50 mL of 50 mM of sodium acetate at pH 5, sodium phosphate at pH 7 or sodium bicarbonate buffer at pH 9 at different temperatures. Periodically, samples were withdrawn and the residual activity of the biocatalyst was measured as described above. The remaining activity was calculated as the ratio between the activity at a given time and the activity at zero time of incubation. After thermal inactivation, the suspension was filtered by vacuum and the solid was washed

several times with the buffer of incubation and used to desorption studies and SDS-PAGE analysis.

2.5.2. Inactivation of β-galactosidase biocatalyst in the presence of organic co-solvents

The biocatalyst was incubated in mixtures of ethanol, acetonitrile or 1,4-dioxane/100 mM Tris–HCl pH 7 at 35 °C at different concentrations to have the enzyme inactivated in reasonable time. At different times, samples were withdrawn under stirring to obtain a homogeneous biocatalyst suspension, and the residual activity of the biocatalysts was measured as described above. Inactivated samples used in desorption studies were filtered and washed with plenty of incubation mixture.

2.6. Desorption of β-galactosidase from glyoxyl agarose-PEI

Samples of 1 g of the immobilized β-gal derivative were suspended in 10 mL of 5 mM sodium phosphate buffer at pH 7 and incubated in growing concentrations (0–1 M) of NaCl. The desorption course was monitored by measuring the activity of both supernatant and suspension using *o*-NPG assay as described above. Intervals of 30 min were allowed before taking a sample to determine the released enzyme and performing a new salt addition.

In order to study desorption of inactivated β-gal, samples of 1 g of the inactivated derivative were suspended in 10 mL of the desorption solution and incubated for 60 min at the desired temperature (25, 40 or 50 °C). The support was then filtered, washed thoroughly with desorption solution and analyzed by SDS-PAGE. The solutions used for the desorption tests of inactivated β-gal were 0.5–1 M sodium chloride (pH 7), and 0.5–2 M sodium phosphate at pH 2.

2.7. SDS-PAGE analysis

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [52] using a Miniprotean tetra-cell (Bio-Rad), 14% running gel in a separation zone of 9 cm × 6 cm, and a concentration zone of 5% polyacrylamide. To analyze the amount of proteins adsorbed on PEI-glyoxyl agarose supports, a sample of 100 mg of the support was re-suspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 8 min and a 10 μL aliquot of the supernatant was used in the experiments. Gels were stained with Coomassie brilliant blue. A low molecular weight calibration kit for SDS electrophoresis (GE Healthcare) was used as a molecular weight marker (14.4–97 kDa).

3. Results and discussion

3.1. Immobilization/desorption of the β-gal on PEI-agarose

Fig. 1 shows the inactivation courses of the β-gal on PEI-agarose at pH 5, 7 and 9. The enzyme activity slightly decreased after immobilization on this support. Fig. 2 shows the desorption of the immobilized enzyme when using increasing concentrations of NaCl. The enzyme is fully released from the support when incubated at 500 mM NaCl. The full desorption of the enzyme was confirmed by SDS-PAGE (results not shown). These desorption/adsorption cycles could be repeated by 5 consecutive cycles (results not shown) without any change on the support performance: immobilization rates and enzyme activity remained very similar.

3.2. Enzyme inactivation the β-gal immobilized on PEI-agarose

Fig. 3 shows the thermal inactivation of β-gal immobilized on PEI-agarose at pH 7 and inactivated at pH 5, 7 and 9. The enzyme stability is slightly lower than that of the free enzyme, while using

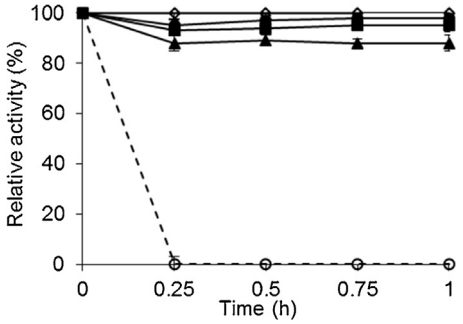

Fig. 1. Immobilization courses of β-galactosidase from *Aspergillus oryzae* on PEI-glyoxyl agarose supports at 5 mM of buffer concentration and pH of 5 (*squares*), 7 (*close circles*) and 9 (*triangles*). *Open circles*: supernatant of the immobilization suspension. *Open rhombus*: free enzyme under identical conditions. Other specifications are described in Section 2.

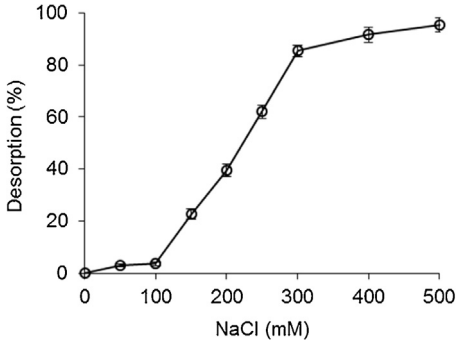

Fig. 2. Desorption of β-galactosidase immobilized on PEI-glyoxyl agarose support using different concentrations of NaCl. Experiments have been performed as described in Section 2.

a support activated with ethylendiamine at pH 5 and 9 the immobilized enzyme is slightly more stable than the free enzyme [48]. This suggested that the enzyme/support interactions were more intense using this polymeric support (apparently with negative effects for enzyme stability), as it may be expected from the better geometric congruence and amount of active groups [23]. The inactivation of β-gal immobilized on PEI-agarose in an organic medium is shown in Fig. 4. Inactivation is quire rapid in all the organic cosolvents assayed.

3.3. Re-use of the support after β-gal immobilized on PEI-agarose inactivation

First, the partially inactivated (under different conditions) enzyme preparations were incubated under the same conditions where the non-inactivated enzyme was fully released. All enzyme activity could be desorbed from the support using 500 mM NaCl, suggesting that results could be similar to those obtained using the immobilized enzyme not exposed to inactivation conditions. Immobilization of fresh enzyme was performed (using 1 mg of enzyme per g of support) for 5 consecutive cycles and no changes in the immobilization rate or recovered activity were detected.

However, Fig. 5 shows that all inactivated preparations (thermal or solvent inactivated β-gal) retained a certain amount of adsorbed enzyme molecules after the washing in 500 mM NaCl, while the non-inactivated immobilized preparation released all enzyme molecules. As all enzyme activity had been released, these protein bands should correspond to inactivated enzyme molecules. This suggested that the inactivated enzyme is strongly adsorbed to the support, as the unfolded structure may have a higher geometrical congruence with the support. The amount of unreleased enzyme

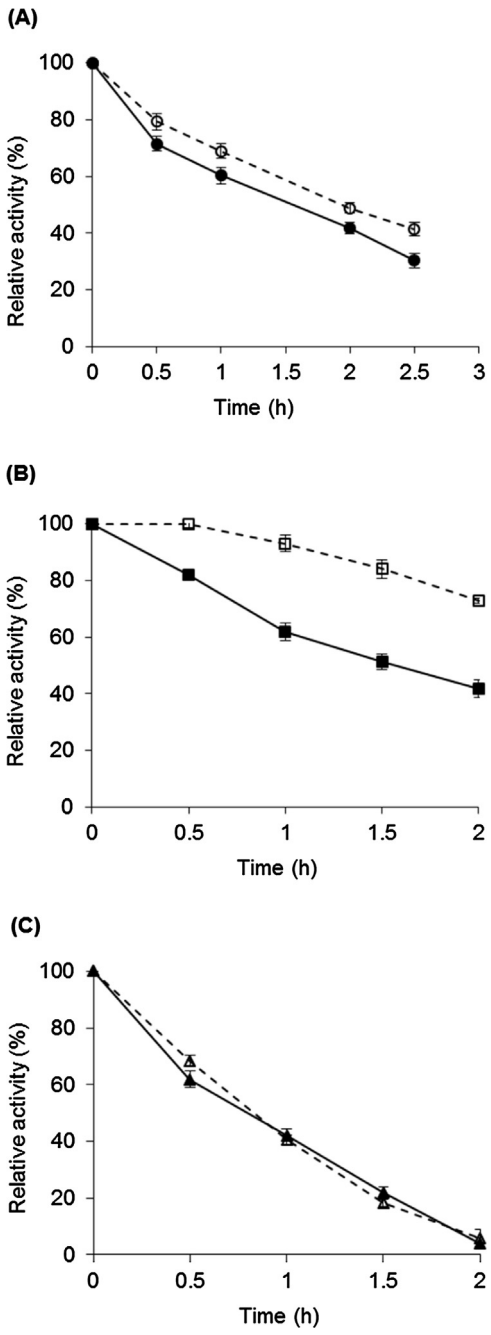

Fig. 3. Thermal stability of the β-galactosidase immobilized on PEI-glyoxyl agarose supports. (A) pH 5 and 40 °C; (B) pH 7 and 52 °C; (C) pH 9 and 35 °C. Experiments were performed as described in Section 2. Free enzyme: *dashed* line; PEI-glyoxyl agarose β-gal derivative: *solid* line.

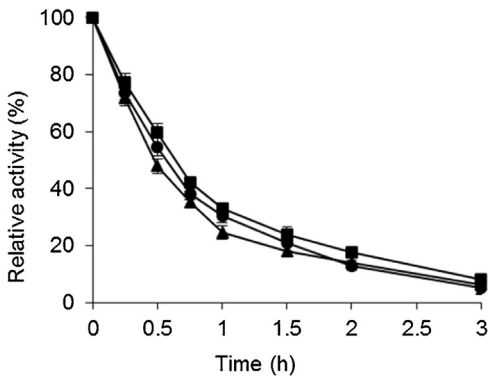

Fig. 4. Effect of different organic cosolvents on the activity of PEI-glyoxyl agarose β-gal derivative. The buffer was 100 mM Tris–HCl pH 7 at 35 °C. Triangles: 45% (V/V) acetonitrile; squares: 80% (V/V) 1, 4 dioxane,; circles: 70% (V/V) ethanol. Experimental details are described in Section 2.

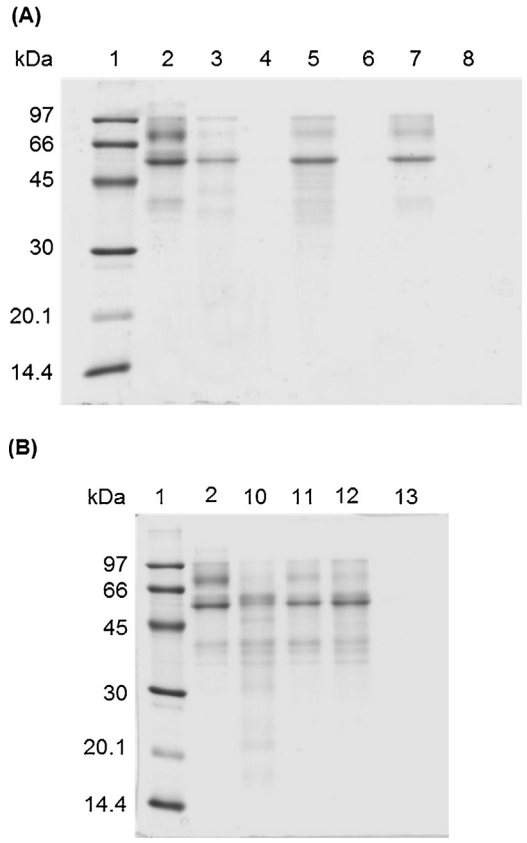

Fig. 5. SDS-PAGE analysis of PEI-glyoxyl agarose support after desorption of β-galactosidase with 500 mM NaCl. The immobilized enzymes were submitted to the processes described in Section 2. Panels A and B show the enzyme that remains bound to the support after desorption. Lane 1: molecular weight marker. Lane 2: commercial β-galactosidase immobilized PEI-glyoxyl agarose before desorption. Lanes 3, 5 and 7: desorption after thermal inactivation at pH 5, 7 and 9, respectively. Lanes 4, 6 and 8: desorption of no inactivated β-galactosidase at pH 5, 7 and 9, respectively. Lanes 10, 11 and 12: desorption after inactivation at 35 °C with 90% dioxane, 45% acetonitrile and 70% ethanol, respectively. Lane 13: desorption of no inactivated β-galactosidase derivative.

was higher when the enzyme had been inactivated in the presence of organic solvents than in thermal inactivations.

To improve the enzyme release, the β-gal inactivated preparations were incubated at growing concentrations of NaCl (the highest concentration was 1 M) and at increasing T (the highest T was 50 °C). Fig. 6 shows that even under the most drastic conditions, some protein still remained adsorbed on the support. This showed the magnitude of the problem; it is not just a certain increase in the adsorption strength, the dramatic increase of the adsorption strength of some of the inactivated enzyme molecules could question the reversibility of the immobilization process, while the active enzyme is relatively easily desorbed.

To improve the inactivated enzyme molecules desorption, the pH was decreased to 2 (trying to neutralize the anionic groups of the enzyme) and 500 mM sodium phosphate was used. Moreover, the temperature was increased from 25 to 50 °C. Fig. 7A and B shows that even under these conditions many of the inactivated enzyme molecules remained immobilized on the support.

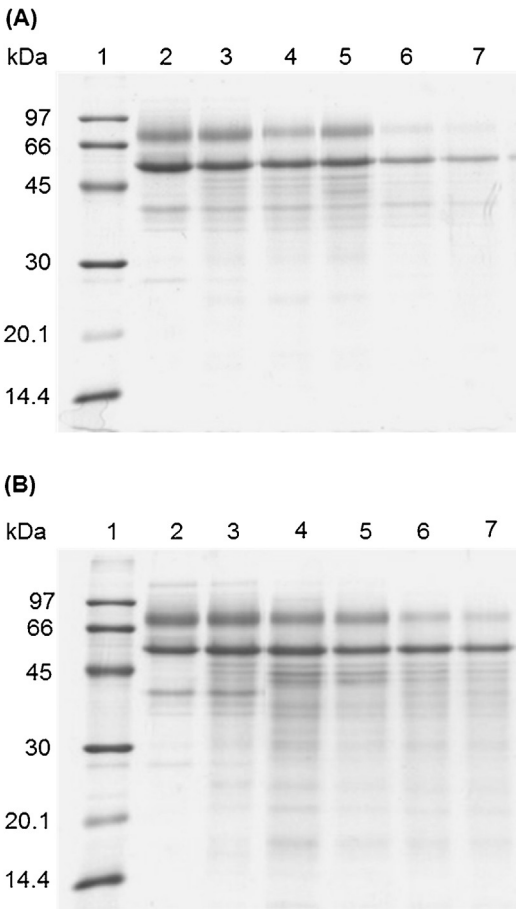

Fig. 6. SDS-PAGE analysis of PEI-glyoxyl agarose support after desorption of β-galactosidase with different concentrations of NaCl at different temperatures. (A) Desorption after thermal inactivation at 52 °C and pH 7. (B) Desorption after inactivation with organic cosolvent at 35 °C. The immobilized enzymes were submitted to the processes described in Section 2. Gels show the enzyme that remains bound to the support after the desorption. Lane 1: molecular weight marker. Lane 2: commercial β-galactosidase immobilized on PEI-glyoxyl agarose before desorption. Lane 3: inactivated β-galactosidase derivative. Lane 4: desorption of proteins with 0.5 M NaCl (25 °C); lane 5: with 1 M NaCl (25 °C); lane 6: with 1 M NaCl (40 °C); lane 7: with 1 M NaCl (50 °C).

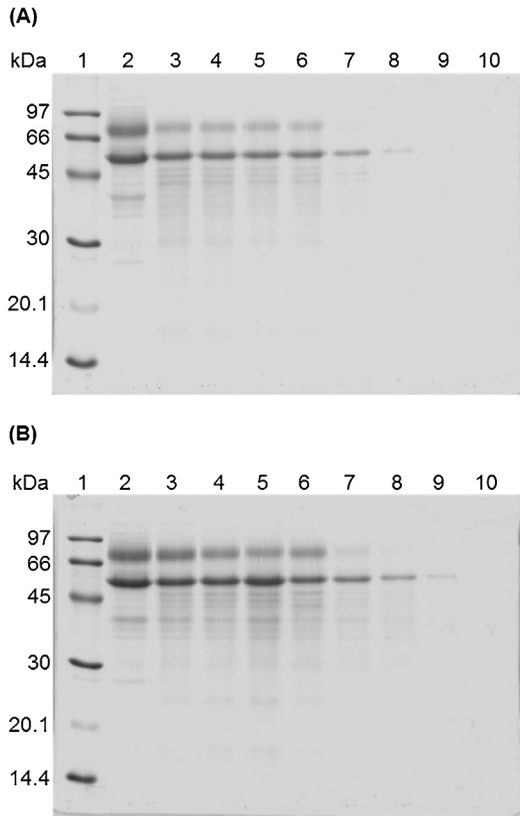

Fig. 7. SDS-PAGE analysis of PEI-glyoxyl agarose support after desorption of inactivated β-galactosidase with different concentrations of sodium phosphate at pH 2 and different temperatures. (A) Desorption after thermal inactivation at 52 °C and pH 7. (B) Desorption after inactivation with organic cosolvent at 35 °C. The immobilized enzymes were submitted to the processes described in Section 2. Gels show the enzyme that remains bound to the support after desorption. Lane 1: molecular weight marker. Lane 2: commercial β-galactosidase immobilized on PEI-glyoxyl agarose before desorption. Lane 3: inactivated β-galactosidase derivative. Lane 4: desorption of proteins with 0.5 M sodium phosphate (25 °C); lane 5: with 1 M sodium phosphate (25 °C); lane 6: with 1 M sodium phosphate (40 °C); lane 7: with 1 M sodium phosphate (50 °C); lane 8: with 2 M sodium phosphate (25 °C); lane 9: with 2 M sodium phosphate (40 °C); lane 10: with 2 M sodium phosphate (50 °C).

To eliminate the thermally inactivated enzyme, it was necessary to use pH 2, 2 M of sodium phosphate and 40 °C (Fig. 7). However, even under the very drastic conditions still some protein could be detected when the enzyme was inactivated in the presence of organic medium, clean support was only obtained after incubation at 50 °C

The process was repeated by 5 cycles, and the results remained consistent: no enzyme remained attached to the support after the desorption treatment.

4. Conclusions

The reversibility of enzyme immobilization should not be considered a granted process just because the active enzyme may be released from the support under moderate conditions. After enzyme inactivation, the enzyme may become partially unfolded and establish a very high number of interactions with the support, producing serious difficulties to release all enzyme molecules from the support, even though the enzyme molecules are still desorbed. In the case of β-gal immobilized in PEI-agarose, before inactivation the enzyme could be released from the support just by incubation at pH 5 in 500 mM NaCl. After inactivation, a significant percentage of enzyme molecules remained adsorbed even in 1 M of NaCl at 50 °C at pH 5. The desorption of the enzyme was obtained using pH 2, 2 M of sodium phosphate and 50 °C. This treatment permitted a real support reuse.

The very strong adsorption of the inactivated enzyme molecules inactivated in the presence of organic solvents suggest that this medium favors enzyme unfolding, penetration in the polymeric bead and reinforces enzyme-support interaction.

Acknowledgments

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Development of simple protocols to solve the problems of enzyme coimmobilization. Application to coimmobilize a lipase and a β-galactosidase†

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This paper shows the coimmobilization of β-galactosidase from *Aspergillus oryzae* (β-gal) and lipase B from *Candida antarctica* (CALB). The combi-biocatalyst was designed in a way that permits an optimal immobilization of CALB on octyl-agarose (OC) and the reuse of this enzyme after β-gal (an enzyme with lower stability and altogether not very stabilized by multipoint covalent attachment) inactivation, both of them serious problems in enzyme co-immobilization. With this aim, OC-CALB was coated with polyethylenimine (PEI) (this treatment did not affect the enzyme activity and even improved enzyme stability, mainly in organic medium). Then, β-gal was immobilized by ion exchange on the PEI coated support. We found that PEI can become weakly adsorbed on an OC support, but the adsorption of PEI to CALB was quite strong. The immobilized β-gal can be desorbed by incubation in 300 mM NaCl. Fresh β-gal could be adsorbed afterwards, and this could be repeated for several cycles, but the amount of PEI showed a small decrease that made reincubation of the OC-CALB–PEI composite in PEI preferable in order to retain the amount of polymer. CALB activity remained unaltered under all these treatments. The combi-catalyst was submitted to inactivation at 60 °C and pH 7, conditions where β-gal was rapidly inactivated while CALB maintained its activity unaltered. All β-gal activity could be removed by incubation in 300 mM NaCl, however, SDS analysis showed that part of the enzyme β-gal molecules remained immobilized on the OC-CALC–PEI composite, as the inactivated enzyme may become more strongly adsorbed on the ion exchanger. Full release of the β-gal after inactivation was achieved using 1 M NaCl and 40 °C, conditions where CALB remained fully stable. This way, the proposed protocol permitted the reuse of the most stable enzyme after inactivation of the least stable one. It is compatible with any immobilization protocol of the first enzyme that does not involve ion exchange as only reason for enzyme immobilization.

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1. Introduction

Enzymes are powerful tools in green organic chemistry due to their high activity under environmentally mild conditions coupled to a high selectivity and specificity.[1] Among the many uses of enzymes as biocatalysts, the so-called cascade or domino reactions have a relevant role because they allow very complex reactions (*e.g.*, copying metabolism) to be carried out.[2] These reactions mean that the product (or side product) of the first reaction is the substrate of the second one and the product of this one is the substrate of the third one and this continues in a successive manner. One of the first and most remarkable examples of multiple reactions catalyzed by several enzymes to obtain one highly added value product was reported by Wong in the production of sialyl Lewis X.[3] However, most examples of cascade reactions are more modest. For example, the relatively simple sequential hydrolysis of an oil, a protein or a polysaccharide may be considered a cascade reaction, even though the order of the modifications is not fully determined in all cases and may depend on the enzyme mixture used (*e.g.*, carboxypeptidase A should be used always after chymotrypsin in a selective hydrolytic process of proteins, but trypsin and chymotrypsin may act in a more free order).[4] In other cases, the

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objective is to perform several modifications with a strict order, like in the transformation of benzaldehyde into mandelic acid by sequential HCN addition and hydrolysis catalyzed by oxynitrilase and nitrilase.[5] In other cases, the second enzyme function is to regenerate a cofactor used by the main enzyme (NAD(P)H or $NAD(P)^+$,[6] or ATP or a phosphorylated compound[7]). In some instances, the side product of one enzyme is used to perform a modification of the target substrate by the other enzyme, like using oxidases that produce hydrogen peroxide that is utilized by lipases to produce peracids,[8] or by peroxidases or laccases to oxidize the desired compound.[9] Some examples involve the use of a cascade reaction just to destroy one side product with a second enzyme that may affect the main product or the main enzyme (*e.g.*, to destroy hydrogen peroxide by catalase in reactions catalyzed by oxidases).[10] All these reactions are just some examples of the huge variety of cascade reactions, keeping in mind that the casuistic is very broad. Moreover, cascade reactions may involve the same or different enzymes. For example, in some cases the cofactor recycling using dehydrogenases may be achieved using the same enzyme and two different substrates[11] and in many instances full hydrolysis of oils or production of biodiesel are performed using just one lipase. However, a more general case is that each reaction is catalyzed by a different enzyme, as this has some advantages.[2]

Immobilization is a requirement for most industrial uses to facilitate the recovery of the enzymes and their reuse provided that they are stable enough.[11] However, nowadays the objective of immobilization must be far more than a simple enzyme reuse; the improvement of many enzyme features (stability, but also activity, selectivity or specificity) may be accomplished by a proper immobilization, transforming this step in a powerful instrument in the biocatalyst design.[12]

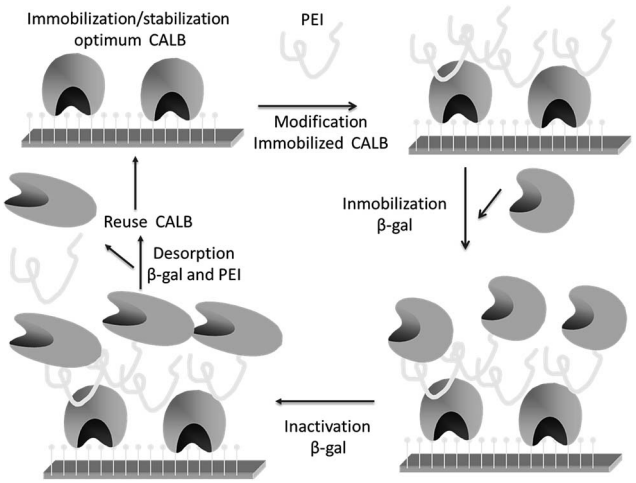
From an industrial point of view, cascade reactions are better performed in one pot.[2] In fact, in some instances such as in the regeneration of cofactors, there are no alternatives to the one pot configuration. This makes finding conditions where all involved enzymes are active and stable compulsory, and this may produce additional difficulties in the design of the process[2] and enhance the interest of having as improved a biocatalyst as possible (*e.g.*, *via* immobilization).[12] Moreover, the enzymes co-immobilized on a same particle are usually preferred, because that way the second enzyme may act on a higher concentration of their substrate from the beginning of the reaction time.[2] This avoids the lag-time usually observed in these reactions, permitting the second enzyme to act from the beginning and may shorten the full reaction course depending on the kinetic properties of the enzymes and the concentration of substrate.[2] In other instances, like when the product of the first enzyme is unstable (production of alpha-keto acids using D-aminoacid oxidases and catalase,[13] or mandelic acid from benzaldehyde[5]) or if this product is able to render the first enzyme inactive (oxidases and catalases),[14] the coimmobilization is fully required.

However, coimmobilization of enzymes has several problems which are usually overlooked.[15] The first one is that when the least stable enzyme is inactivated, both enzymes need to be discarded. The second one refers to the necessity of immobilizing all enzymes on the same support, and usually using the same protocol, that may not be optimal for both enzymes. Recently, a brilliant solution has been reported: the use of heterofunctional supports, where one enzyme is immobilized on one kind of support group and the second enzyme is immobilized on the other kind of group.[16] However, this nice strategy has some problems yet. Both groups will be under the enzyme surface of both enzymes, and that may produce some problems in the intensity of the desired enzyme–support interactions and the existence of some undesired ones, and this may reduce the final stabilization for both enzymes achieved *via* immobilization.[17]

Our group is trying to advance on the solution of these problems concerning coimmobilization. In this first approach, we have focused on a situation where one of the enzymes may be just marginally stabilized *via* multipoint immobilization and it is less stable that the other enzyme. The strategy is simple: an optimal immobilization protocol may be applied for the more stable enzyme, and this enzyme is later coated with an ionic polymer. This treatment with ionic polymers generally does not alter the enzyme activity and has been even used to stabilize the enzymes *versus* diverse inactivating causes (subunit dissociation, oxygen, solvents, *etc.*)[18] or even to improve enzyme properties.[19] Then, the labile and hard to stabilize enzyme may be immobilized *via* ion exchange on the already immobilized one. If the first enzyme remains active and immobilized at high ionic strength, after the labile enzyme inactivation, this enzyme may be desorbed while the support immobilized one is reused. That way, it is possible to have an optimal biocatalyst for the most stable enzyme that can be reused many times to immobilize the labile enzyme, and some cycles of inactivation, desorption and reloading of the second enzyme may be accomplished reusing the most stable enzyme. This is not a fully general situation, but many enzymes couples may fulfill these requirements.

For example, in this proof of concept paper we have employed two very widely used enzymes. The lipase B from *Candida antarctica* is among the most used ones in biocatalysis,[20] it is very stable and may be further stabilized *via* immobilization. For example, CALB has been greatly stabilized by immobilization on octyl-agarose supports *via* interfacial activation on the hydrophobic surface of the support. The final stability thus achieved by even gives a higher stabilization than the same biocatalyst prepared *via* multipoint covalent attachment.[21] This immobilization is reversible[22] and may be useful to study the molar relation of both enzymes *via* SDS-PAGE. Therefore, we have selected this immobilization strategy.

The CALB modification with PEI produced a further enzyme stabilization, mainly in organic medium, without affecting the enzyme activity.[23] As a second model enzyme to get the combi-biocatalyst *via* this new strategy, we have selected the β-galactosidase from *Aspergillus oryzae*, an enzyme employed in many reactions and which has a high transglycosylation activity.[24] This enzyme is quite stable, but a maximum of 12 folds using epoxy-amino supports (best results reported for this enzyme) can be stabilized,[25] and immobilization *via* ion exchange gave good results.[26] This has been explained by its high glycosylation (this reduces the exposition of the protein structure of the enzyme) and the low stability at alkaline pH value (reducing the



Scheme 1

possibilities of forcing the enzyme–support reactions)[27] making their covalent immobilization not recommendable as that way support and enzyme should be discarded.[15] Thus, this enzyme may be valid for the proposed strategy: it is difficult to stabilize the enzyme *via* multipoint covalent attachment in a support but the immobilization *via* ion exchange produced reasonable good results in terms of activity and stability. Both enzymes might be used to produce galactose modified in the position 1 with 1,2-diacetin *via* a glycosidic bond, using triacetin and lactose as substrates. 1,2-Diacetin is produced by CALB in hydrolysis of triacetin, but it is unstable tending to isomerize.[28] Moreover, in a kinetically controlled process like the proposed, the concentration of the nucleophile may be a key point to reach good yields,[29] therefore coimmobilization could have a double justification in this reaction.

In this paper, we just studied if both enzymes may be coimmobilized on the same particle but using different immobilization strategies, permitting an individual support surface optimization for each of them, and we have analyzed the activity/stability features of the biocatalyst compared to that of the individual ones. Finally, we have checked the actual possibility of reusing the immobilized CALB after the β-gal inactivation, a main problem in the standard design of coimmobilized biocatalysts. Scheme 1 resumes the strategy and objectives.

2. Materials and methods

2.1. Materials

Solution of lipase B from *C. antarctica* (CALB) (6.9 mg of protein per mL) was a kind gift from Novozymes (Spain). β-Galactosidase from *Aspergillus oryzae* (β-gal) (20 units oNPG per mg of protein), *o*-nitrophenyl-β-galactopyranoside (ONPG), polyethylenimine (PEI) (MW 25 000), dextran sulfate (DS) (9–20 000 MW), Triton X100, cetyltrimethylammonium bromide (CTAB), sodium dodecylsulfate (SDS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), diethyl *p*-nitrophenylphosphate (D-*p*NPP) and *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Sigma-Aldrich (St. Louis, USA). Octyl Sepharose CL-4B beads and 4% CL agarose beads were from GE Healthcare. PEI and DS supports were prepared as previously described.[26a,30] Electrophoresis reagents were obtained from Bio-Rad (Hercules, USA). All other reagents were of analytical grade. Protein concentration was estimated by the Bradford dye binding method[31] at 595 nm using bovine serum albumin as a standard.

2.2. Standard determination of enzyme activity

2.2.1. β-Galactosidase. This assay was performed by measuring the increase in absorbance at 380 nm produced by the release of *o*-nitrophenol in the hydrolysis of 10 mM ONPG in 25 mM sodium acetate buffer at pH 5 and 25 °C (ε was 10 493 M^{-1} cm^{-1} under these conditions),[26b] using a spectrophotometer with a thermostatized cell and with continuous magnetic stirring. To start the reaction, 100 μL of the enzyme solution or suspension were added to 2.5 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG per minute under the conditions described previously.

2.2.2. Lipase. This assay was performed by measuring the increase in absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate buffer at pH 7.0 and 25 °C (ε under these conditions is 5150 M^{-1} cm^{-1}). 50–100 μL of lipase solution or suspension were added to 2.5 mL of substrate solution to start the reaction. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-NPB per minute under the conditions described.

2.3. Immobilization of CALB on octyl (OC) supports

The standard immobilization was performed using 10 units of lipase per g of wet support. In some instances, like those to perform SDS-PAGEs or to determine maximum loading of the enzymes, the amount of offered CALB was increased up to 80 mg g^{-1} of support. CALB solution was diluted in the corresponding volume of 5 mM sodium phosphate buffer at pH 7 at 25 °C. Then, OC support was added to reach the desired loading.[22] The activity of both supernatant and suspension was followed using *p*-NPB assay. After immobilization the suspension was filtered and the immobilized biocatalyst enzyme was exhaustively washed with distilled water.

2.4. Modification of OCCALB with PEI

A 50 mL solution of 10% PEI (w/v) was prepared and the pH was adjusted at pH 7. Then, 5 g of OCCALB was suspended and submitted to gentle stirring for 2 h. Afterwards, the modified enzyme was washed with an excess of distilled water to eliminate the free PEI.[23] The enzyme activity was maintained unaltered and the stability improved, mainly in the presence of organic solvents.[23]

2.5. Immobilization of β-galactosidase *via* ion exchange

The standard immobilization was performed using 20 ONPG units of free beta-galactosidase activity per g of wet support (1

mg of enzyme per gram of support), although in some cases maximum enzyme loading was utilized (4 mg). The support could be PEI, DS or OCCALB–PEI. This low loading was used to prevent diffusional limitations that could make the understanding of the results on molecular enzyme properties more complex. In some instances, the amount of enzyme was increased (*e.g.* to determine maximum loading of the support, or to perform SDS-PAGE analysis). The commercial sample of the enzymes was dissolved in the corresponding volume of sodium acetate at pH 5, sodium phosphate at pH 7 or sodium bicarbonate buffer at pH 9 at 25 °C, and then the support was added to reach the desired enzyme loading.

2.6. Thermal stability of the enzyme preparations

Immobilized or coimmobilized enzymes were incubated at different pH values (5, 7 and 9) and different ionic strengths (25 or 500 mM of the buffers indicated in the above section). Periodically, samples were withdrawn and the enzyme activity was measured using oNPG and *p*-NPB, depending on the enzyme analyzed. Half-lives were calculated from the observed inactivation courses.

2.7. Desorption of β-galactosidase from OCCALB–PEI

The coimmobilized derivatives were suspended in 5 mM sodium phosphate and incubated in growing concentrations of NaCl at pH 7 and the activities of both supernatant and suspension were followed using *o*-NPG and *p*-NPB.

2.8. Primary amino titration of the different preparations using TNBS

0.5 g of the enzyme preparation were suspended in 5 mL of 100 mM sodium phosphate at pH 8, and then 0.5 mL of TNBS commercial solution were added.[32] After 30 minutes of gentle stirring, the colored support was exhaustively washed with sodium phosphate at pH 8. Finally, 200 mg of the treated support were suspended in 5 mL of sodium phosphate at pH 8 in a cuvette (1 cm) and submitted to continuous stirring. Spectrum acquisition was performed from 350 to 600 nm of the different supports compared to the non TNBS-treated supports, and the wavelength that permitted an absorption of 425 nm was selected for the comparisons.

2.9. SDS-PAGE experiments

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli[33] using a Miniprotean tetra-cell (Bio-Rad), 14% running gel in a separation zone of 9 cm × 6 cm, and a concentration zone of 5% polyacrylamide. One hundred milligrams of the immobilized enzyme samples was resuspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 8 min and a 10 μL aliquot of the supernatant was used in the experiments. This treatment released all enzyme which was just interfacially activated on the support.[34] Gels were stained with Coomassie brilliant blue. A low molecular weight calibration kit for SDS electrophoresis (GE Healthcare) was used as a molecular weight marker (14.4–97 kDa).

3. Results and discussion

3.1. Immobilization of CALB on octyl support

The immobilization course of CALB on octyl support is shown in Fig. 1Sa.† In less than 30 minutes, using a ratio of 1 g of support and 10 mL of enzyme suspension, CALB was immobilized and the activity remained almost unaltered. Immobilization yield is over 95% and the activity is maintained at 100%. This result agreed with previous reports in literature using this support and enzyme.[34] Although the immobilization involves the open form of the lipase and stabilizes it,[35] the CALB lid is so small that the enzyme did not experiment a real activation after immobilization.[36] Fig. 1Sb† shows that the immobilized CALB is far more stable than the free enzyme, maintaining 70% of activity when the free enzyme retained less than 10% of the initial activity. This stabilization of lipases immobilized on octyl supports has been explained by the high stability of the adsorbed open form of the lipases when compared to lipases in the standard conformational equilibrium.[37] The coating with PEI under the conditions used in this paper has been described to present no effect on enzyme activity (activity remained at 100%) and improved stability (mainly in organic solvents).[23] Therefore, we have decided to use this biocatalyst as a method to prepare he coimmobilized biocatalyst. Thus, the OCCALB–PEI seems a very adequate system to be used as "support" to immobilize other enzymes.

3.2. Immobilization of β-gal on PEI and DS supports at different pHs values

Fig. 2S† shows the immobilization of the β-gal on supports activated with DS or PEI at pH 5 and 7. While using PEI the enzyme immobilization is complete after only 20 minutes at pH 5 and 7, the enzyme is only partially immobilized on DS at pH 5 and negligible at pH 7. Therefore, PEI was selected for all further studies. Immobilization yield was 100% and activity recovery over 90%. The stability of this enzyme preparations is shown in Fig. 3S,† showing that the immobilization has a marginal effect on the stability of this enzyme, similar to that found using standard ion exchangers.[26b] Although the enzyme could be immobilized at pH 9 on PEI coated supports, this pH offered a lower stability of the enzyme:[26b] for this reason we discarded the immobilization under this pH condition.

3.3. Immobilization of β-gal on octyl-CALB–PEI

Fig. 1 shows the immobilization of (0.5 mg, 10 U g^{-1}) β-gal on the composite OC-CALB (2 mg g^{-1})–PEI. Immobilization proceeds very rapidly at both pH values (5 and 7) and the activity of the enzyme remained unaltered. The stability and activity of the CALB of this composite was identical to that of the lipase immobilized on octyl and coated with PEI (results not shown) and the β-gal stability also was identical to that of the enzyme immobilized on the support coated with PEI (results not shown). The difference in stabilities of CALB and β-gal enzymes

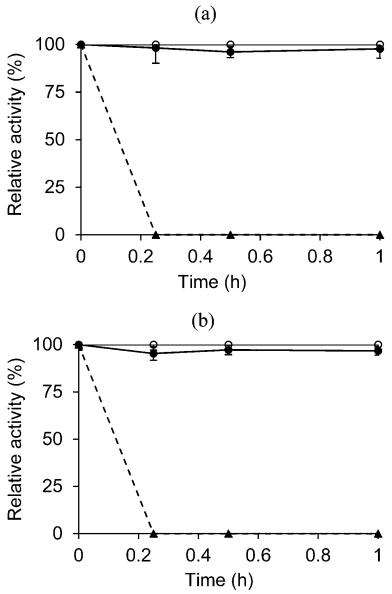


Fig. 1 Immobilization courses of β-galactosidase using 1 mg of enzyme (20 U) at pH 5 (panel a) and 7 (panel b) on octyl-CALB–PEI. Experiments were performed as described in Section 2. Close circles: suspension; triangle, dashed line: supernatant; open circles: reference.

was very significant, being the CALB much more stable than the β-gal.

3.4. Desorption of β-gal immobilized on octyl-CALB–PEI

We performed cycles of adsorption/desorption of the β-gal on the PEI–lipase composite. That way, OCCALB could be reused after β-gal inactivation. Fig. 2 shows that all β-gal activity could be released to the medium using 300 mM of NaCl at pH 7, without affecting the CALB activity that remained fully immobilized and active, and this operation could be repeated several cycles. After enzyme desorption, new β-gal could be immobilized on the OCCALB–PEI. While in the first cycles 100% of the β-gal was immobilized, it was found that after 6 cycles, the amount of β-gal immobilized decreased to 60%. This result suggested that the PEI could be released from the OCCALB at

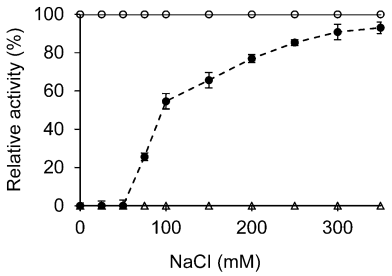


Fig. 2 Relative activity profiles of the supernatants, with respect to the initial value, during the desorption tests of β-galactosidase and CALB immobilized on octyl-CALB–PEI, at different NaCl concentrations. The β-galactosidase activity was 20 U g^{-1}. Experiments were performed as described in Section 2. Triangles, dashed line: CALB activity; close circles: β-galactosidase; open circles: reference.

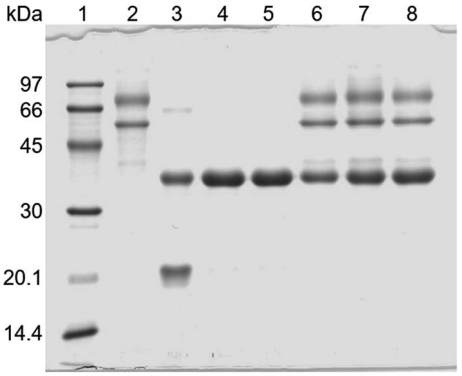


Fig. 3 SDS-PAGE analysis of different biocatalyst preparation and free enzymes used in this study. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: commercial free β-galactosidase. Lane 3: commercial free CALB. Lane 4: octyl-CALB (CALB 5 mg g^{-1} of support). Lane 5: octyl-CALB–PEI (CALB 5 mg g^{-1} of support). Lane 6: β-galactosidase on octyl-CALB–PEI (CALB 2 mg g^{-1} of support). Lane 7: β-galactosidase on octyl-CALB–PEI (CALB 4 mg g^{-1} of support). Lane 8: β-galactosidase on octyl-CALB–PEI (CALB 5 mg g^{-1} of support).

300 mM of NaCl, reducing the amount of PEI and that way decreasing the amount of immobilized enzyme. Therefore, we decided to prepare biocatalysts with maximum loading of β-gal at different CALB amounts to analyze in a more precise manner the intensity of the problem. Surprisingly, we found that we could immobilize a maximum of 4 mg of β-gal per g of OCCALB independently of the amount of the CALB on the support (results not shown). Fig. 3 shows the SDS-PAGE analysis of these preparations, showing that although the amount of CALB increased, the maximum amount of β-gal remained constant. β-gal presented two bands, one at 60 kDa and the other at 72 kDa, both have been previously described.[38] This could be caused by the closing of the pores of the agarose with the β-gal and the PEI, thus we did not reach the maximum values of loading with the β-gal, or maybe because β-gal can be immobilized on the support surface and not only in the CALB. Fig. 4 shows that while β-gal did not immobilize on OC support, it immobilized very rapidly on OC–PEI. This occurred although agarose is supposed to be an inert matrix, and suggests that some sulfate from agarose remains or that the chemical treatment of the agarose to introduce the octyl groups has produced some oxidations in the agarose hydroxyl groups. PEI is a poly-cation that requires a very low amount of anion groups in the support to establish multiple ionic bridges.

To confirm that PEI was adsorbed on OC, TNBS assay was utilized. Table 1 offers the results, which confirmed that PEI could be adsorbed on OC agarose beads. The incubation of this composite in 300 mM NaCl released almost completely the PEI. As a comparison, OC–PEI and OC-CALB (maximum loading)–PEI were used, and this showed that PEI was only marginally desorbed from the support having maximum CALB loading when incubated in 300 mM NaCl, while a significant percentage of the PEI was released when using OC–PEI preparations (Table 1). Thus, PEI was more strongly attached to CALB than to the OC support.

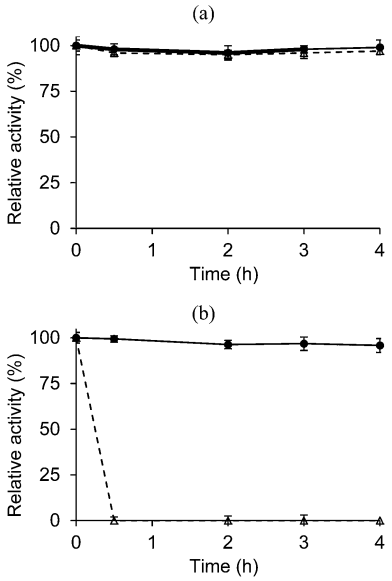


Fig. 4 Immobilization courses of β-galactosidase on octyl (panel a) and octyl–PEI (panel b) supports at pH 7. Experiments were performed as described in Section 2. Circles: suspension; triangles: supernatant.

Table 1 Adsorption of polyethyleneimine on octyl–PEI and octyl-CALB–PEI composites before and after treatment with sodium chloride. The PEI content was determined by the TNBS assay and is expressed in absorbance units at 425 nm

Condition	Octyl–PEI	Octyl-CALB–PEI
Without treatment	0.49 ± 0.07	0.84 ± 0.06
After added 300 mM NaCl	0.15 ± 0.03	0.81 ± 0.05

The stability of the β-gal (0.5 mg to prevent diffusion problems) was rechecked using OCCALB–PEI with maximum CALB loading and the results in terms of activity recovery and stability were identical to the ones previously presented in this paper.

The fact that the commercial OC support could be coated with PEI may become an unexpected advantage, as we can immobilize (while keeping the activity and stability of both enzymes) the desired amounts of both enzymes, *e.g.* an excess of β-gal regarding the CALB. If the support cannot be modified with PEI, to have an excess of β-gal (or other second enzyme, this paper is just a proof of concept using a model bienzymatic system) could be a complex problem, and this may be a requirement on the design of some reactions.

3.5. Inactivation, desorption/reimmobilization of β-gal immobilized on octyl-CALB–PEI

Next, the combi-biocatalyst prepared using 0.5 mg of β-gal and 2 mg of CALB was incubated at 60 °C and pH 7 (Fig. 5). Under these conditions, β-gal activity decreased rapidly while the activity of CALB remained unaltered. When the activity of the β-gal was lower than 40%, the combi-biocatalyst was incubated in 300 mM NaCl to release all β-gal and fresh enzyme was

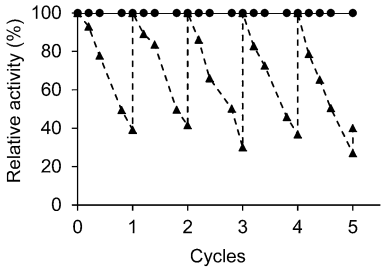


Fig. 5 Cycles of β-galactosidase thermal inactivation–desorption–ionic binding from octyl-CALB–PEI composite. Experiments were performed as described in Section 2. Circles: lipase activity, rhombus: galactosidase activity.

immobilized. This protocol was repeated for 5 cycles: the activity of CALB was unaltered after the last desorption/adsorption experiment, while the amount of immobilized β-gal decreased only after the sixth cycle, very likely due to the loss of PEI. To check if this problem also existed using the PEI adsorbed on the CALB, we used a support with maximal loading of CALB and just 0.5 mg of β-gal. In this case, we can immobilize 100% of the β-gal for 6 cycles. Using the maximum loading of β-gal (in this case the preparations were submitted to the same inactivation conditions but the activity was not followed, due to the diffusion problems), results could be repeated for 6 cycles. However, when the amount of PEI was determined in the OCCALB–PEI biocatalysts after each cycle by TNBS titration (Table 2), a decrease in the amount of PEI attached to the support was appreciated. Apparently this PEI loss was not enough to prevent β-gal adsorption, but it was significant. To prevent this, the OCCALB preparations were incubated in a solution of 10% PEI after each desorption step of β-gal. This permitted to maintain the amount of PEI on the composite for 6 cycles (results not shown). In case that another enzyme was used and that this was able to immobilize on PEI stronger than CALB the reloading of PEI should be a requirement after each enzyme desorption step because all PEI would be released from the OCCALB.

It has been recently shown that the desorption of inactivated enzyme immobilized on PEI support may be more difficult that the desorption of the active enzyme.[39] Fig. 2 shows that 300 mM NaCl was enough to release all β-gal following β-gal activity. Fig. 6 shows the SDS-PAGE analysis of the combi-catalysts. While the non-inactivated enzyme showed no enzyme on the support after desorption using 300 mM NaCl, the inactivated preparations after desorption under those conditions showed both bands of the β-gal, the smaller one with a relative higher

Table 2 Residual polyethyleneimine on octyl-CALB–PEI after various cycles of union–thermal inactivation–detachment of β-galactosidase

Cycle	Residual PEI (%)
2	82 ± 3
4	72 ± 2
6	55 ± 1

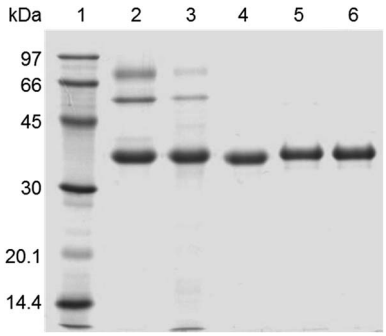


Fig. 6 SDS-PAGE analysis of β-galactosidase desorption from octyl-CALB–PEI composite. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: octyl-CALB–PEI–β-gal. Lane 3: desorption of β-gal with 0.3 M NaCl after thermal inactivation. Lane 4: desorption of β-gal with 0.3 M NaCl without previous thermal inactivation. Lanes 5 and 6: desorption of β-gal with 1 M NaCl with- and without previous thermal inactivation, respectively.

intensity. As the objective was to release all β-gal molecules, the desorption of the β-gal was assayed at different salts concentrations before and after β-gal inactivation using maximum loading of both enzymes (including a step of PEI incubation between cycles). Fig. 6 shows that using 1 M NaCl, all β-gal molecules were desorbed from the support (and also almost all PEI). The activity of CALB remained unaltered even under these conditions, but the incubation of the OCCALB preparation with PEI was fully necessary. The established protocol was β-gal immobilization, inactivation, desorption, PEI incubation, and a new β-gal immobilization. And after 6 cycles, OCCALB exhibited more than 90% of the initial activity.

4. Conclusions

The protocol proposed in this paper overcomes some of the problems associated to coimmobilization of two enzymes: it is possible to optimize the immobilization of one of them, and it is possible to reuse this enzyme after the inactivation of less stable enzyme. The requirement for this strategy is that the immobilization of the first enzyme is not only based on ion exchange (otherwise we can desorb the enzyme when desorbing the other enzyme). The example used in this paper is interfacial activation on hydrophobic support, a method reported as very adequate for lipase immobilization. The strategy is mainly useful if one of the enzymes is not stabilized *via* multipoint covalent attachment, and it is the least stable enzyme among those involved in the combi-biocatalyst. The coating with PEI (but other ionic polymers may be used) produced even some positive effects on CALB stability,[23] and it has been used for stabilizing many other enzymes, with low to null effect on activity due to the random coil structure. The strategy permitted to reuse CALB after several cycles of β-gal inactivation. However, the enzyme inactivation produces a stronger adsorption of the inactivated enzyme on the PEI and makes it harder to regain a CALB–PEI composite free of inactivated enzyme molecules. This is possible to achieve using higher salt concentration and temperatures.[39] These conditions did not affect CALB activity, but make re-incubation of the OCCALB–PEI composite with PEI in each desorption/adsorption cycle compulsory. This re-incubation in PEI is not a problem at laboratory scale, but may be an inconvenient at industrial level and strategies to avoid this necessity should be explored.

The proposed strategy has fulfilled the initial objectives and may be extrapolated to many other enzyme couples involved in cascade reactions. However, to prepare a real combi-biocatalyst, an adequate relation between the catalytic activity of CALB and β-gal will be required to maximize the product conversion. The optimization of the reaction and preparation of the specific biocatalyst is under way in our laboratory.

Acknowledgements

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Short communication

Ion exchange of β-galactosidase: The effect of the immobilization pH on enzyme stability

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ABSTRACT

β-Galactosidase from *Aspergillus oryzae* has been immobilized at pH 5, 7 and 9 on an aminated support using 5 mM buffer. The immobilization was total in 30 min, maintaining 75–80% of activity. These preparations were inactivated at different pH values and in the presence of 50% ethanol. The stability of the enzyme immobilized at pH 9 was much lower than that of the enzyme immobilized at pH 5 under all studied conditions but the differences decreased as the ionic strength of the inactivation solution increased. The likeliest explanation to these different stabilities depending on the immobilization pH was that the enzyme presented a different orientation on the support. The enzyme immobilized at pH 5 was more stable than the free enzyme at pHs 5 and 9 (by a 2 or a 6 fold factor respectively), while at pH 7 the free enzyme was clearly more stable than the immobilized enzyme.

1. Introduction

Enzyme immobilization is a pre-requisite for many uses of the enzymes as industrial biocatalysts, because the primary objective of the immobilization is to facilitate enzyme reuse [1]. Many researchers have focused their research on how immobilization may improve other enzyme properties, like stability, activity or selectivity [2–9]. There are many immobilization strategies and the adsorption of proteins on supports is one of the preferred protocols, due to the simplicity and reversibility of the techniques [10]. However, adsorption of the enzyme on a support is not always advantageous; the support (that needs to be highly activated to be used in immobilization) will be never fully inert and can continue establishing new enzyme-support interactions during storage or operation, leading to enzyme inactivation [11]. Thus, although enzyme adsorption is not really a "mild" immobilization strategy, it is a very simple one. Moreover, a real rigidification of the enzyme may not be possible due to the very weak ionic bridges [10]. Stabilization may be expected in some instances, e.g., multimeric proteins may become stabilized if some subunits are involved in the adsorption [12] or the enzymes may be stabilized by some partition phenomena, or by generating favorable nanoenvironments [13].

Protein ion exchange on ion exchangers supports is a tool utilized for both, purification [14–16] and immobilization of proteins [17–21]. As an immobilization tool, the immobilization on an ion exchanger is very simple, it consists on the mixture of the support and the protein under conditions where protein adsorption is possible [10]. Immobilization of a protein via ion exchange is a multipoint process; the enzyme is fixed to the support only when a high enough number of ionic bridges to compensate the ionic strength of the media is established between the protein and the support [22–25]. This way, a protein may become adsorbed on supports having the same amount of cationic and anionic groups (that is, a mixed ion exchanger), as the really relevant point is the possibility of establishing an ionic net of interactions between enzyme and support, in fact some proteins may become adsorbed on mixed cationic/anionic exchangers while they cannot

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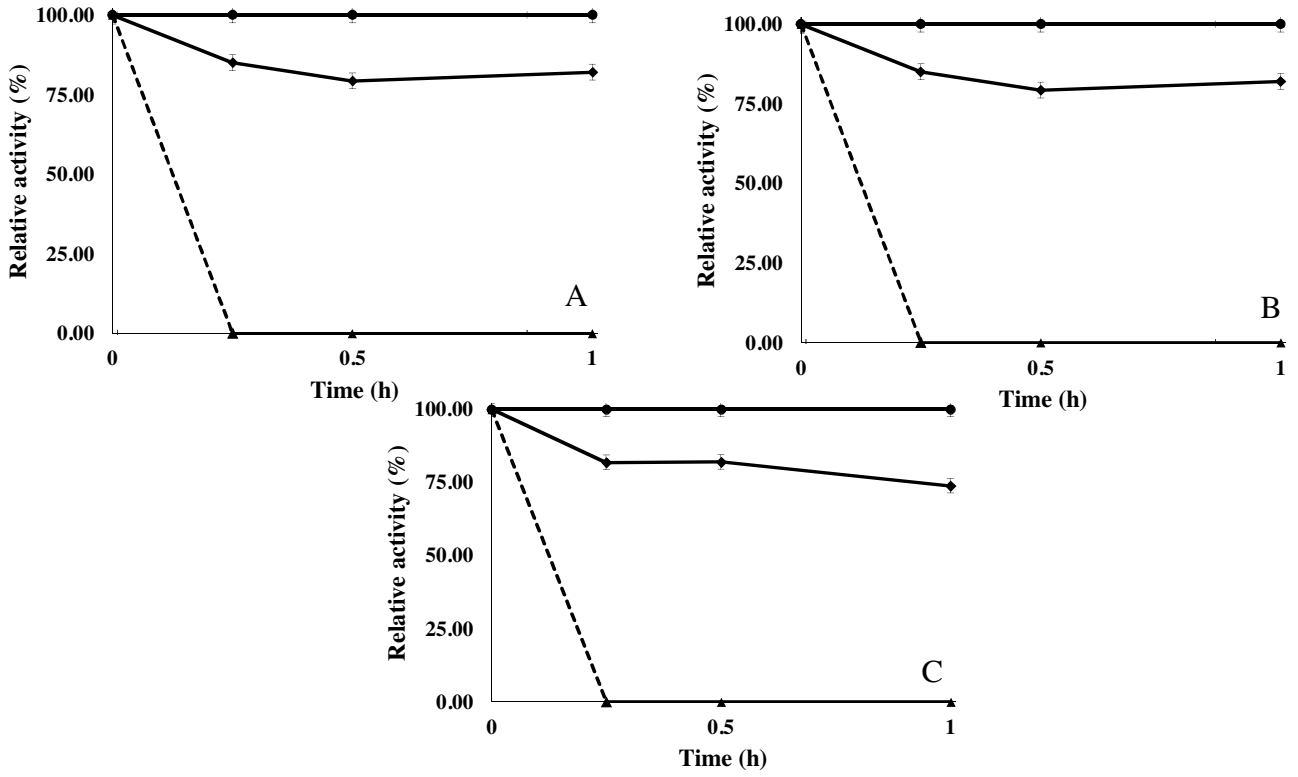

Fig. 1. Immobilization courses of β-galactosidase from *Aspergillus oryzae* on MANAE supports at 5 mM of buffer and pH 5 (A), 7 (B) and 9 (C). Other details are described in Methods section. Rhombus: activity of the immobilization suspension; Triangles: supernatant of the immobilization suspension; Circles: free enzyme under identical conditions.

be adsorbed on any of the respective monofunctional supports [26]. That mechanism of ion exchange adsorption of proteins has been used to develop purification systems for large proteins via specific adsorption on lowly activated supports of the target protein [27,28] and even to shift association/dissociation equilibria of proteins towards the association state [29]. The coupling of these tailor made ion exchangers to some chemical reactive group has permitted to develop heterofunctional supports that can be used for the one step immobilization and purification of large multimeric proteins [30,31].

Moreover, it has been shown that the ionic strength during immobilization may favor the orientation of the enzyme that permits to maximize the number of enzyme-support ionic bridges: the higher the ionic strength, the higher the number of enzyme support interactions required to immobilize the enzyme [32]. That means that the ion exchange may be a more complex and versatile immobilization tool that supposed, and depending on the support activation and experimental conditions the area of the protein involved in the immobilization may be different, as it is necessary that an intense enough multi interaction process may occur.

One of the experimental conditions that can alter the balance between cationic and anionic groups on a protein surface is the pH. It is well known and accepted that this variable determines if an enzyme is adsorbed or not during anion exchange [22–25]. However, to the best of our knowledge, it has been never studied if the immobilization on an ion exchanger support may involve different areas depending on the immobilization pH value and how this may affect the final enzyme properties, e.g, stability, activity or selecitvity.

In this paper, we have analyzed the effect of the immobilization conditions (mainly pH and ionic strength) on the final performance of the β-galactosidase from *Aspergillus oryzae*, a monomeric and glycosylated enzyme with a isoelectric point of 4.5/4.6 active mainly at acidic pH value [33] and utilized in the production of galactosyl polysaccharides [34–38]. The enzyme has been immobilized by many authors using very different methodologies, including some adsorption ones [39–44]. Now, we have immobilized this interesting enzyme on an aminated support (MANAE), having a primary (pK 6.9) and a secondary (pK 10.2) amino group [45] at different pH value and analyzed the effect of the pH on the final biocatalysts performance, looking for changes cause by the implication of different enzyme areas on its immobilization.

2. Materials and methods

All experiments were performed at least by triplicate and the values are given as the mean and the standard deviation.

2.1. Materials

β-Galactosidase from *A. oryzae* (20 Units oNPG/mg of protein), *o*-nitrophenyl-β-galactopyranoside (ONPG) and *o*-nitrophenol (ONPG) were purchased from Sigma–Aldrich (St. Louis, USA). 6% CL agarose beads was from GE Healthcare. MANAE supports were prepared as previously described [45]. All other reagents were of analytical grade.

2.2. Standard determination of enzyme activity

This assay was performed by measuring the increase in absorbance at 380 nm produced by the release of *o*-nitrophenol in the hydrolysis of 10 mM ONPG in 100 mM sodium acetate at pH 4.5 and 25◦C (the calculated ε was 10493 $M^{-1}cm^{-1}$ under these conditions). To start the reaction, 100 μL of the enzyme solution or suspension were added to 2.5 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes

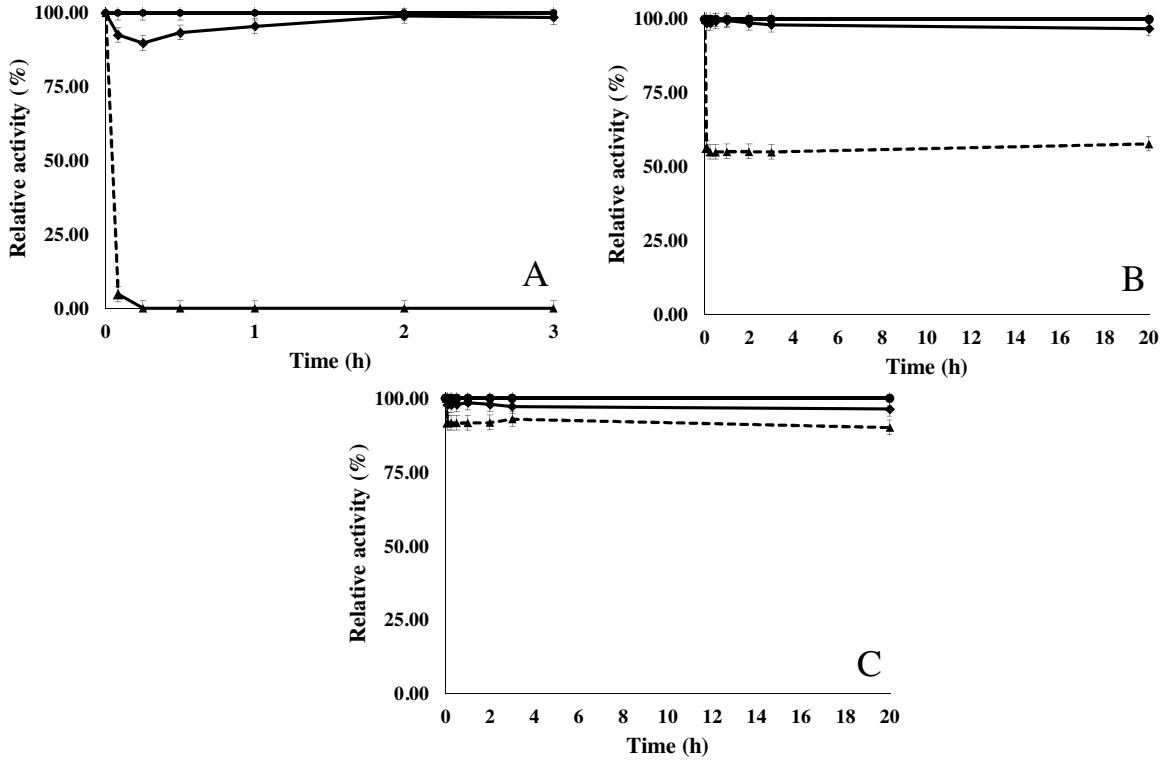

Fig. 2. Immobilization courses of β-galactosidase from *Aspergillus oryzae* on MANAE supports at 25 mM of buffer and pH 5 (A), 7 (B) and 9 (C). Other details are described in Methods section. Rhombus: activity of the immobilization suspension; Triangles: supernatant of the immobilization suspension; Circles: free enzyme under identical conditions.

1 μmoL of ONPG per minute under the conditions described previously. Protein concentration was determined using Bradford's method [46].

2.3. Immobilization of β-galactosidase on MANAE-agarose

The immobilization was carried out employing 20 oNPG units of free beta-galactosidase activity per g of wet support (1 mg of enzyme per gram of support). Maximum loading should be over 20 mg/g of support. This low loading was used to prevent diffusion limitations that could distort the results. The commercial samples of the enzymes were dissolved in the corresponding volume of 5 or 50 mM sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 9.

2.4. Thermal stability of the enzyme preparations

Enzyme was incubated at different pH values (4.5, 7.0 and 9.0) and different ionic strengths (5 and 25 mM of the buffers indicated in the above section). Periodically, samples were withdrawn and the enzyme activity was measured using oNPG. Half-lives were calculated from the observed inactivation courses.

2.5. Ethanol inactivation of different enzyme preparations

1 g of immobilized enzyme was suspended in 10 mL of 50% ethanol in 5 mM or 25 mM sodium acetate at pH 5.0 at 30 °C. Periodically, samples were withdrawn and the enzyme activity was measured using oNPG. Half-lives were calculated from the observed inactivation courses.

3. Results and discussion

3.1. Enzyme immobilization in MANAE supports ***under different conditions***

First, we tried to immobilize the enzyme at pHs 5, 7 and 9 in MANAE agarose using 5 mM of buffer. To control the pH, the support needs to be washed with a large volume of the immobilization buffer, otherwise the buffering properties of the support make the immobilization pH very similar between runs, because the concentration of amino groups in the support may be very high compared to the concentration of the buffer [3]. After this precaution, Fig. 1 shows that the enzyme may be immobilized on MANAE agarose at the 3 pH values, in only a few minutes, and maintaining over 80% of activity at all studied pH values.

Using a buffer concentration of 25 mM, the immobilization yield of the enzyme on MANAE support decreased, except at pH 5 (Fig. 2). This effect is expected considering the competition of the ions of the buffer [22–25] and the enzyme for the support and these conditions did not seem to be recommended for immobilization of this enzyme. At pH 5, the charge of the support is maximum [45] and immobilization proceeds rapidly, as the isoelectric point of the enzyme is 4.5/4.6.

However, the enzyme was not released when immobilized under a concentration of buffer of 5 mM and incubated at 25 mM of the corresponding buffer at all the studied pH values. Therefore, after the enzyme-support multi-ion exchange, the ionic strength that may prevent the immobilization is not enough to produce enzyme desorption from the MANAE support. All enzymes could be released to the medium using 150 mM of buffer, and the support could be reused in several adsorption/desorption cycles without any detrimental effect on its performance.

Table 1
Half-lives the different biocatalysts under different conditions (in minutes). Inactivations were performed in 5, 25 or 50 mM of the different buffers. The conditions were sodium acetate at pH 4.5 and 55 °C; sodium phosphate at pH 7 and 50 °C, or sodium carbonate at pH 9 and 36 °C. The inactivations in 50% ethanol were performed at pH 5 in 5 or 25 mM sodium acetate at 50 °C.

Biocatalyst	Inactivation Experimental conditions										
	5 mM buffer			25 mM buffer			50 mM buffer			% Ethanol pH 5 (5 mM)	50% Ethanol pH 5 (25 mM)
pH	4.5	7.0	9.0	4.5	7.0	9.0	4.5	7.0	9.0		
MANAE-Betagalactosidase (immobilized at pH 5)	270 ± 9	900 ± 54	500 ± 70	240 ± 11.0	20 ± 3	90 ± 30	420 ± 23	240 ± 22	30 ± 5	260 ± 13.0	220 ± 40
MANAE-Betagalactosidase (immobilized at pH 7)	330 ± 42	120 ± 6.0	350 ± 40	180 ± 8.3	18 ± 2	50 ± 14	4100 ± 12	215 ± 31	28 ± 4	100 ± 16	210 ± 25
MANAE-Betagalactosidase (immobilized at pH 9)	10 ± 1.4	3 ± 0.4	20 ± 2	12 ± 0.8	5 ± 0.7	22 ± 3	420 ± 17	35 ± 9	10 ± 1.2	15 ± 2	240 ± 30
Free enzyme	130 ± 9	1500 ± 110	40 ± 8	20 ± 9	150 ± 20	30 ± 6	300 ± 40	230 ± 20	28 ± 5	–	–

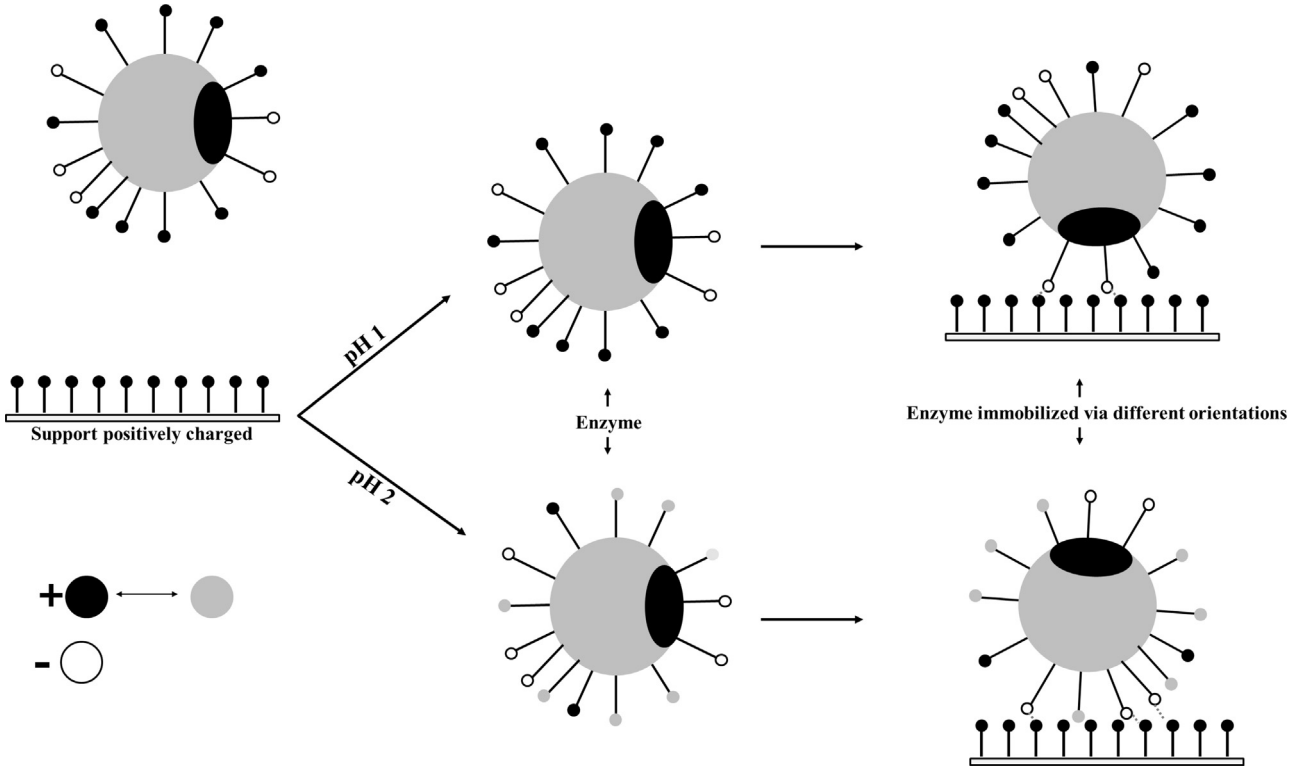

Fig. 3. Scheme of the hypothesis of effect of immobilization pH on enzyme orientation regarding the support surface.

3.2. Thermal stability of the different MANAE-enzyme preparations under different conditions

Table 1 shows the stability at pH 4.5 and different sodium acetate concentrations of the enzymes immobilized at different pH values on MANAE agarose beads. Using 5 mM of buffer (properly equilibrated and checking the inactivation pH to ensure that it was the desired one), one fact is remarkable. The enzyme immobilized in MANAE support at pH 9 is by far the least stable preparation, even less stable than the free enzyme. However, the immobilized preparations produced at pH 5 or 7, were slightly more stable than the free enzyme. Using 25 mM of inactivating buffer, the stability of the enzyme immobilized at pH 9 remained the least stable enzyme-MANAE preparation, while the enzymes immobilized at pH 5 or 7 were clearly more stable than the free enzyme. No enzyme could be found in the supernatant during enzyme inactivation under these conditions for any of the 3 preparations. At 50 mM of buffer, the stability of all enzyme-MANAE preparations at pH 5 became similar, but we could detect most of the activity of the immobilized enzyme in the supernatant.

Considering the very different stability of the enzyme immobilized in MANAE support at pH 9 with that of the enzyme immobilized at the other pH values, the only likely explanation is that the enzyme is immobilized on the support via different areas at pH 9 and the other two pH values. Otherwise, the stability should be similar because the support and the enzyme are the same, and the inactivation conditions are identical. This could be because at different pH values, the ionic balance of different areas of the protein may be diverse, permitting enzyme immobilization via different areas of the protein. We have tried to represent this idea in Fig. 3.

Ionic adsorption is not expected to have great positive effects on enzyme rigidity, but may improve enzyme operational stability [10,11] under certain conditions, e.g., if the enzyme tends to precipitate, because the immobilized enzyme cannot precipitate. The weak one point ionic interaction [22–25] and the fact that there are many ionic groups under the protein surface that can fix incorrect enzyme structures [11] are the main reasons that make to expect a

poor stabilization of a monomeric enzyme after ionic exchange. In the case of this enzyme, the area involved in the immobilization at pH 9 seemed to suffer negative interactions with the support, while these effects were lower if the immobilization was performed at pH 5 or 7. At 5 mM, the stabilization found for the enzyme immobilized at pH 5 or 7 was negligible, while using 25 mM a 2–3 stabilization factor could be found. This could be due to a lower interaction between the enzyme and the support. Using 50 mM of buffer and 55 °C the enzyme become desorbed and stabilities are similar to that of the free enzyme (just the time required to desorb the enzyme may explain the short differences in enzyme stability).

Although the enzyme is mainly used at acidic pH values, from an academic point of view the stability at other pH may have interest. At pH 7.0, all the immobilized enzyme preparations are less stable than the free enzyme using 5 mM of buffer (Table 1), as expected from the physical "activity" of the activated support [11]. In this case, the enzyme immobilized at pH 9 remains the least stable preparation, while that immobilized on MANAE agarose at pH 5 is significantly more stable than the enzyme immobilized at pH 7 in MANAE support. Using 25 mM of buffer, that weakened the enzyme-support interactions [22–25], differences between the immobilized enzymes in MANAE support are shorter, but still the enzyme immobilized at pH 9 is significantly less stable. At 50 mM of buffer, the stability of the enzymes immobilized in MANAE support at pH 5 and 7 are similar to that of the free enzyme, while the enzyme immobilized at pH 9 is still less stable. Although the enzyme is desorbed in these conditions after some time, the low stability of the enzyme immobilized at pH 9 make that the enzyme is inactivated before it is desorbed.

The inactivations at pH 9.0 show a new situation (Table 1). The enzyme immobilized in MANAE support at pH 9 is the least stable, even less than the free enzyme, while the enzyme immobilized at pH 5 is more stable than the enzyme immobilized at pH 7, and both are more stable than the free enzyme. In this case the differences of the stabilities of the different MANEA-enzyme decreased when the buffer concentration increase, but the stability is not like the free enzyme in any case, although at 50 mM some enzyme could be found in the supernatant. Explanations should be similar to those previously described.

Thus, the enzyme immobilized in MANAE support at pH 5 seems to be the most stable preparation, and that immobilized in MANAE support at pH 9 seem to be clearly the least stable under all the inactivations pH studied. This confirmed the hypothesis that the most likely explanation is that presented in Fig. 3.

If the enzyme immobilization rate is higher than the rate of diffusion of the enzyme in the pores of the support, the enzyme molecules will be packed together, if the enzyme immobilization rate is low, the distance between enzyme molecules increases. The data on this paper on immobilization rate agree with the data of other papers where it has been shown a closing packing of the enzyme molecules [47,48]. The ionic strength controls the immobilization rate, therefore may control the distance between enzyme molecules. We have compared the stabilities using 5 and 25 mM of buffer in the immobilization buffer and we have not found significant differences (results not shown) suggesting that enzyme packing is not relevant for the observed results The use of higher enzyme loading produced strong diffusion limitations and the measurement of the enzyme activity and stability was not reliable. Using 5 mg/g of MANAE support, less than 25% of activity is expressed; the apparent stability of all preparations increase but still the preparation immobilized at pH 9 is the least stable one.

3.3 Stability of different enzyme-MANAE preparations in the presence of ethanol

Table 1 shows the results obtained in the inactivation of the 3 MANAE support immobilized enzyme preparations in the presence of ethanol, at two buffer concentrations. The visible instant aggregation of the free enzyme have caused that we do not include the results using the non-immobilized enzyme. It is evident again that the stability of the enzyme immobilized at pH 9 is lower than that of the other two preparations using low buffer concentration, but this is not the case using 25 mM of buffer. The results confirm those obtained in thermal inactivations: the enzyme immobilized at pH 9 suffers some negative interactions with the support, which are decreased when the ionic strength is increased.

4. Conclusion

In the specific case of the enzyme used in this paper, the immobilization on MANAE support permit to maintain very high level of enzyme activity at the 3 immobilization pHs used (75–85%). The enzyme remained immobilized under conditions where enzyme adsorption did not occur, suggesting that enzyme immobilization and enzyme desorption are not fully similar processes. Enzyme immobilization requires the establishment of several enzyme-support linkages, very difficult by the increment of the ionic strength. However, enzyme desorption requires the simultaneous breaking of the ionic bridges formed, very likely more than those just required for the enzyme immobilization because the closeness of enzyme and support may facilitate the enzyme-support interaction.

The enzyme immobilized in MANAE support at pH 5 is far more stable than the enzyme immobilized at pH 9, while the comparison with the free enzyme is only favorable when the free enzyme suffered some intermolecular processes, avoided after immobilization. In inactivations at pH 9, the apparent stabilization of the enzyme immobilized at pH 5 in MANAE support is of almost a 6 fold factor, while the "destabilization" of the enzyme immobilized at pH 9 is 2–3 folds, compared to the free enzyme. The increase in the ionic strength weaken both, positive and negative effects, as this decreased the support/enzyme possibility of establishing new interactions and also may break some of the already formed ionic bridges.

However, the most relevant conclusion of this communication is a more general one. The enzyme immobilization on ion exchangers may be more versatile that supposed. If the protein has specially enriched areas in anion groups, the change in the immobilization pH during ion exchange may alter the immobilized enzyme functional properties, and the simplest explanation for this is that the enzyme orientation regarding the support may be altered by using different pH value (Fig. 3). This has been probed for the first time just with this galactosidase in this paper and requires further work to validate the general application of this strategy. That opens new opportunities to the tuning of enzyme properties via immobilization [3] using ionic exchange as the first step of the immobilization [29–32], in covalent immobilization where the first step is an ionic exchange (e.g., glutaraldehyde [49,50] or epoxy-amino supports [31]). These results may have also impact in the chromatography or proteins.

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