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Efficient sequestration of carbon dioxide into calcium carbonate using a novel carbonic anhydrase purified from liver of camel (*Camelus dromedarius*)

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ABSTRACT

Carbonic anhydrase (CA) was successfully used for carbon dioxide (CO_2) sequestration. In fact, sequestration of CO_2 into value-added products, calcium carbonate ($CaCO_3$), requires a stable and efficient CA that should tolerate high concentrations of CO_2 and Ca^{2+}, high pH and high temperature of working process. Herein, for the first time, the present manuscript described the sequestration of CO_2 into $CaCO_3$ using a novel CA which was purified and characterized from liver of camel (*Camelus dromedarius*), an animal that survive extreme desert conditions. The enzyme was a monomer with lower molecular weight (25 kDa), contained Fe as a physiologically-relevant cofactor instead of Zn and showed higher optimum pH (pH 9.0) and temperature (45°C). In addition, the enzyme was active and stable at strongly alkaline pH (pH 9.0) and higher temperature (60°C). IC_{50} values revealed that camel liver CA was inhibited by metal ions in the following order: $Cu^{2+} > Zn^{2+} > Fe^{3+} > Cr^{3+} > Ni^{2+} > Cd^{2+} > Co^{2+} > Al^{3+} > Mg^{2+} > Ca^{2+}$, the enzyme needed high concentrations of Ca^{2+} to reach 50% inhibition. Camel liver CA was effective in accelerating $CaCO_3$ formation in presence of 1, 2, 5, 10 and 20% Ca^{2+}. The formed $CaCO_3$ was characterized by SEM, XRD and FTIR. Interestingly, camel liver CA showed high CO_2 sequestration capacity (966.67 mg $CaCO_3$/mg enzyme) in presence of high concentration of Ca^{2+} (up to 20%). Camel CA represents promising candidate for harsh industrial applications.

1. Introduction

Nowadays, climate change is a major threat to our planet. Emissions of carbon dioxide (CO_2), the most abundant greenhouse gas in the atmosphere, are the major contributor to climate change. Indeed, continuous emissions of CO_2 due to human activities, such as burning of fossil fuels (oil, coal and natural gas) and deforestation, significantly increase CO_2 concentration in the atmosphere [1]. In fact, atmospheric CO_2 concentration in May 2019 and 2020 was found to be 414.65 and 417.07 ppm, respectively [2]. Accordingly, the rapid and continuous increase in the atmospheric CO_2 concentration causes an increase in temperature, sea level, extreme weather and climate events [1]. Therefore, to combat CO_2 emissions, various CO_2 capture and sequestration technologies were reported in recent years, including chemical, physical and biological technologies [3]. Among the biological technologies, carbonic anhydrase (CA, EC 4.2.1.1) was successfully used for CO_2 sequestration [4].

CA is ubiquitous zinc enzyme that plays an important role in life by catalyzing the reversible hydration/dehydration of CO_2 and water (H_2O) into bicarbonate ion (HCO_3^-) and proton (H^+) (Reaction 1) [5]. Biotechnologically, HCO_3^- generated by CA can be mineralized using divalent metal ions to form value-added products, such as calcium carbonate ($CaCO_3 \downarrow$), which can be used in pharmaceuticals, cosmetics, construction materials as well as a nutritional supplement in animal

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foods [6,7]. In fact, in alkaline pH, HCO_3^- generated by CA can dissociate into carbonate ion (CO_3^{2-}) (Reaction 2). Subsequently, in the presence of calcium ion (Ca^{2+}), CO_3^{2-} reacts with Ca^{2+} to form $CaCO_3$ (Reaction 3) [6]. Using CA for sequestration of CO_2 into $CaCO_3$ is the most effective biological technology because it offers a win-win strategy to confront the growing threat of climate change by reducing atmospheric CO_2 concentration and efficiently exploit the value-added products ($CaCO_3$) [4,7]. However, in order to meet CO_2 sequestration requirements, this process requires an efficient CA that should tolerate high concentrations of CO_2 and Ca^{2+}, also, the enzyme should have high activity and stability at high pH and temperature of working process during CO_2 sequestration [8]. In this context, CA should be purified and characterized to investigate its possible application for CO_2 sequestration.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad \text{(Reaction 1)}$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad \text{(Reaction 2)}$$

$$CO_3^{2-} + Ca^{2+} \leftrightarrow CaCO_3 \downarrow \quad \text{(Reaction 3)}$$

In mammals, most purification and characterization studies of CA were performed on erythrocytes [9–12], where the enzyme was first discovered [13]. Furthermore, in erythrocytes, CA plays an important role in the process of transport and excretion of CO_2 [5]. The presence of CA in liver is imperative, due to its crucial roles in physiological and biochemical functions of respiration and CO_2/HCO_3^- buffer system, where, this buffer system regulates acid–base balance in cells which allows countless biochemical reactions to occur [5].

Indeed, CA was extensively purified and characterized from bovine erythrocytes, which makes bovine erythrocytes CA the most commercial CA used in CO_2 sequestration studies [14–17]. However, few studies were reported on the purification and characterization of CA from other organs, such as liver. Consequently, studies regarding the potential use of liver CA for CO_2 sequestration are non-existent. In fact, CA was purified and characterized from liver of sheep [18] and pig [19]. On the other hand, few studies were attempted to use purified CA from mammals for CO_2 sequestration [20–22]. While, recombinant and purified CA from bacteria were widely used for CO_2 sequestration [23–26]. To the best of our knowledge, there has never been a thorough investigation to purify and characterize CA from camel (*Camelus dromedarius*) liver, consequently, there has never been a thorough investigation of the potential use of camel CA for CO_2 sequestration.

Camel is a predominant domestic mammal in desert areas that can efficiently produce and reproduce. Camel represents the main source of meat and milk for human populations in desert areas [27]. Its unique behavioral, anatomical, physiological and biochemical properties make it unique compared to other mammals. Moreover, camel is able to live in its specific ecosystem including a tolerance of high temperature, direct solar radiation and dehydration [28]. With this unique behavior of camel and in light of current climatic changes, camel enzymes, like CA, represent promising candidates for industrial applications and could be exploited in extreme industrial conditions, like CO_2 sequestration technology.

In this paper, in order to test the ability of camel CA to sequester CO_2 into $CaCO_3$, the enzyme was purified and characterized for the first time. Morphological features, structural properties and surface functional groups of the formed $CaCO_3$ with camel liver CA were characterized by SEM, XRD and FTIR, respectively. The results of the present study will provide novel biochemical properties of CA in mammalian species as well as novel promising candidate for CO_2 sequestration technology.

2. Materials and methods

2.1. CA activity assays and protein determination

2.1.1. Hydratase activity assay

Hydratase activity of camel liver CA was assayed as described by Wilbur and Anderson [29]. The assay was performed at 4 °C using CO_2 as a substrate. The enzymatic reaction was initiated by adding 1 ml of ice-cold CO_2-saturated water in a mixture containing 50 µl of enzyme solution and 1 ml of 50 mM Tris-HCl/100 mM Na_2SO_4 (pH 8.0). One unit of CA hydratase activity (U) was defined as the amount of CA required to drop the pH from 8.0 to 7.0. All experiments were performed in triplicate. It is important to note that hydratase activity assay was used only throughout all purification steps.

2.1.2. Esterase activity assay

Esterase activity of camel liver CA was assayed as described by Verpoorte et al., [30] using 4-nitrophenyl acetate (4-NPA) as a substrate. Enzyme solution (50 µl) was incubated with a mixture containing 500 µl of 4 mM 4-NPA and 950 µl of 50 mM Tris-SO_4 (pH 7.4) at room temperature for 3 min. After incubation, the amount of 4-nitrophenolate released from 4-NPA was measured at 348 nm using the molar extinction coefficient of 5400 M^{-1} cm^{-1}. One unit of CA esterase activity (U) was defined as 1 µmol of 4-nitrophenolate released per minute under assay conditions. All experiments were performed in triplicate.

2.1.3. Protonography assay

Hydratase activity of camel liver CA was also assayed electrophoretically following the protonography technique of De Luca et al., [31]. Nondenaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4% stacking gel and 12% separation gel as described by Laemmli [32]. In fact, in order to avoid enzyme denaturation, purified CA from camel liver was not treated with β-Mercaptoethanol (β-ME) and not boiled. After electrophoresis, the gel was incubated for 30 min at 4 °C with 0.1% bromothymol blue prepared in Tris-HCl buffer (100 mM, pH 8.3). Hydratase activity of camel liver CA was detected by immersing the gel in ice-cold CO_2-saturated water. The yellow band appeared on gel corresponds to CA activity.

2.1.4. Protein determination

Protein concentration was determined by measuring absorbance at 595 nm according to Bradford's method [33] using bovine serum albumin as a standard.

2.2. Purification of CA from camel liver

Purification of CA from camel liver was performed using the procedure reported for purification of CA from sheep liver [18]. Fresh camel liver was obtained from the municipal slaughterhouse of Settat–Morocco in accordance with the good veterinary practices. Liver tissues were carefully cut into small pieces, washed several times with cold distilled water to remove all traces of blood and homogenized in 2 volumes of Tris-HCl buffer (50 mM, pH 7.4) using Waring blender. The homogenate was centrifuged at 5000×g for 20 min at 4 °C. After centrifugation, pH of the obtained supernatant was adjusted to pH 8.7 with solid Tris in order to achieve the maximum binding of CA to sulfonamide-containing affinity chromatography as well as to fully preserve the enzyme activity during the loading and washing steps [34]. Then, the adjusted supernatant was applied to p-aminomethylbenzenesulfonamide–agarose affinity column (1.6 × 6 cm) pre-equilibrated with 25 mM Tris–HCl/100 mM Na_2SO_4 (pH 8.7). Affinity column was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7) to remove unbound proteins. Subsequently, CA was eluted with 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6). Acetate anion (CH_3COO^-) and perchlorate anion (ClO_4^-) with lower pH value (pH 5.6) act competitively with sulfonamide. In fact, the binding of these

monovalent anions with CA increases with decreasing pH, while the strength of sulfonamide binding in pH 5.6 becomes weaker [35]. The elution was performed at room temperature and at a flow rate of 60 ml/h. Fractions of 2 ml were collected and those containing CA activity were pooled and dialyzed against four changes of Tris-HCl buffer (25 mM, pH 8.0) in order to remove the monovalent anions and to fully preserve the enzyme activity for further characterization studies. The dialyzed solution was stored at −24 °C until use.

2.3. Characterization of purified CA from camel liver

2.3.1. Determination of molecular weight

Molecular weight of camel liver CA was determined by SDS-PAGE under denaturing and nondenaturing conditions. SDS-PAGE was performed as described by Laemmli [32] using 4% stacking gel and 12% separation gel. On the one hand, camel liver CA was boiled for 5 min in sample buffer containing 5% β-ME. On the other hand, in order to avoid enzyme denaturation, camel liver CA was not boiled and was mixed with loading buffer without β-ME. Following electrophoresis, protein bands in gel were visualized using silver staining method [36]. Molecular weight of camel liver CA was determined using prestained protein molecular weight markers (PageRuler™ Plus Prestained Protein Ladder (10–250 kDa), Thermo-Scientific).

2.3.2. Determination of optimum pH and pH stability

Optimum pH of camel liver CA was determined by assaying esterase activity at different pH values using the following buffers: 50 mM acetate buffer (pH 5.0 and 5.5), 50 mM phosphate buffer (pH 6.0, 6.5, 7.0 and 7.5), 50 mM Tris-HCl buffer (pH 8.0, 8.5, 9.0 and 9.5) and 50 mM glycine-NaOH buffer (pH 10.0 and 10.5). Also, stability of camel liver CA at pH 7.0, 8.0 and 9.0 was determined by assaying esterase activity after incubation for 0, 6, 12, 24, 48, 72 and 96 hours at each desired pH.

2.3.3. Determination of optimum temperature and thermal stability

Optimum temperature of camel liver CA was determined by assaying esterase activity at various temperatures in range from 20 to 120 °C. In addition, thermal stability of camel liver CA at 30, 40, 50 and 60 °C was determined by assaying esterase activity after incubation for 10, 20, 30, 60, 120, 180 and 240 minutes at each desired temperature.

2.3.4. Effect of metal ions on enzyme activity

Since CA is a metalloenzyme, transition metals can easily displace a physiologically-relevant cofactor from the active site of CA [37]. Because of this, it is important to evaluate the inhibition profile of a broad range of metal ions toward camel liver CA. Metal ions used with their tested concentrations were: Al^{3+} (0, 2, 5, 10, 20 and 50 mM), Ca^{2+} (0, 5, 10, 20, 50, 100 and 200 mM), Cd^{2+} (0, 0.5, 1, 2, 5, 10, 20, 50 and 100 mM), Co^{2+} (0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 mM), Cr^{3+} (0, 0.1, 0.2, 0.5, 1, 2 and 5 mM), Cu^{2+} (0, 0.05, 0.1, 0.2, 0.5 and 1 mM), Fe^{3+} (0, 0.05, 0.1, 0.2, 0.5 and 1 mM), Mg^{2+} (0, 5, 10, 20, 50 and 100 mM), Ni^{2+} (0, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 mM) and Zn^{2+} (0, 0.1, 0.2, 0.5, 1, 2 and 5 mM). Prior to esterase activity assay, camel liver CA was pre-incubated with the appropriate concentration of each metal ion for 15 min. IC_{50} and Hill slope of camel liver CA for each metal ion were calculated using GraphPad Prism software version 7.

2.3.5. Effect of anions and chemicals on enzyme activity

Effect of various anions (Br^-, CH_3COO^-, $C_6H_5O_7^{3-}$, ClO_4^-, CN^-, CO_3^{2-}, F^-, HCO_3^-, I^-, N_3^-, NO_3^-, SCN^-, SeO_3^{2-} and SO_4^{2-}) on enzyme activity was investigated in order to evaluate their inhibition profile toward camel liver CA, in particular, anions constitute an important class of CA inhibitors due to their affinity for metal ions in the enzyme active site [38]. In addition, effect of various chemicals, such us reducing agents (β-ME, cysteine (Cys), D,L-dithiothreitol (DTT)), chelating agent (ethylenediaminetetraacetate (EDTA)), serine protease inhibitor (phenylmethylsulfonylfluoride (PMSF)), ionic surfactant (SDS) and non-ionic surfactant (Triton X-100 (TX-100)), on enzyme activity was also investigated in order to understand, in part, the molecular structure of camel liver CA. Esterase activity was assayed in the presence of 1, 2 and 5 mM of each anion and chemical. Prior to esterase activity assay, camel liver CA was preincubated with the appropriate concentration of each anion and chemical for 15 min.

2.3.6. Determination of kinetic parameters

Kinetic parameters of camel liver CA for substrate (4-NPA) and inhibitor (sulfanilamide) were determined. In order to determine kinetic parameters for 4-NPA, esterase activity was assayed using different concentrations of 4-NPA (0, 0.1, 0.2, 0.5, 1, 2, 3 and 4 mM). k_m and V_{max} were then determined by nonlinear regression using GraphPad Prism software version 7. In addition, turnover number (k_{cat}) and catalytic efficiency (k_{cat}/k_m) for 4-NPA were calculated. On the other hand, in order to determine kinetic parameters for sulfanilamide, esterase activity was assayed in the presence of three different concentrations of sulfanilamide (0.2, 2 and 20 μM), where for each sulfanilamide concentration, 4-NPA was used at five different concentrations (0.2, 0.5, 1, 2 and 3 mM). Subsequently, k_i and inhibition type were determined by nonlinear regression using GraphPad Prism software version 7. Moreover, IC_{50} and Hill slope for sulfanilamide were determined by varying sulfanilamide concentrations from 0 to 300 μM and keeping 4-NPA concentration constant at 4 mM.

2.3.7. Determination of metal content

Purified CA from camel liver was analyzed for Zn, Fe, Cd, Cu and Co using quadrupole Inductively Coupled Plasma Mass Spectrometry (Agilent 7800 Quadrupole ICP-MS, Agilent Technologies, USA) standardized with standard solutions of the corresponding metal. Zn, Fe and Cd are the physiologically-relevant cofactors of CA. While, Cu and Co can easily bind to the three-histidine moiety within the CA active site in in vitro experimental conditions [37].

2.4. Sequestration of CO_2 into $CaCO_3$ and characterization studies

2.4.1. Sequestration of CO_2 into $CaCO_3$

Sequestration of CO_2 into $CaCO_3$ was performed in reaction mixtures containing 5 ml of Tris-HCl buffer (50 mM, pH 8.0) pre-bubbled with CO_2 gas for 30 min, 300 μl of purified CA from camel liver (0.12 mg/ml) and 5 ml of 1, 2, 5, 10 and 20% Ca^{2+} prepared in Tris-HCl buffer (200 mM, pH 9.0). In parallel, control reactions were also run without addition of enzyme. The mixtures were incubated at room temperature for 30 min with orbital shaking at 150 rpm to produce $CaCO_3$. After incubation, precipitated $CaCO_3$ was collected by centrifugation and dried at 60 °C to weigh its mass [39].

2.4.2. Characterization of the formed $CaCO_3$

The formed $CaCO_3$ without and with camel liver CA was then characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) and fourier transform infrared spectroscopy (FTIR). Morphological study using SEM was performed in Zeiss Sigma 300 microscope at an accelerating voltage of 15 kV. The samples were placed on a holder and covered with very thin layer of gold (about 3 Å/sec). Structural properties were examined by XRD analysis. The analysis was performed at room temperature under ambient air conditions on PANalytical Empyrean diffractometer using Cu Kα1 radiation (λ =1.54060 Å) at 40 mA, 5 kV and a 2 Theta angle ranging from 5 to 90°. Surface functional groups were identified using FTIR spectroscopy recorded on a Bruker Vertex 70v spectrometer. Spectra were recorded in the range of 400–4000 cm^{-1} with spectral resolution of 4 cm^{-1}.

3. Results and discussion

3.1. Purification and molecular weight determination

CA was purified from camel liver in a single step using p-aminomethylbenzenesulfonamide–agarose affinity chromatography. This single step resulted in pure CA with a specific activity of 111.23 U/mg, a purification fold of 2291.17 and a yield of 45.52% (Table 1). Furthermore, purity of camel liver CA was confirmed by electrophoretic analysis using SDS-PAGE under denaturing and nondenaturing conditions. On the one hand, under denaturing conditions, purified CA from camel liver showed a single protein band (Fig. 1A, lane 2). On the other hand, a single protein band was also observed under nondenaturing conditions (Fig. 1A, lane 4). In addition, protonogram showed a single protein band corresponding to the hydratase activity of purified CA from camel liver (Fig. 1A, lane 5). Accordingly, denatured camel liver CA displayed similar band with nondenatured camel liver CA. Therefore, from SDS-PAGE and protonogram results, it is concluded that camel liver CA is a monomer with a molecular weight of 25 kDa (Fig. 1B). Generally, purified CAs from different sources of mammalian species were reported to have monomeric structures with a molecular weight of 29 kDa in sheep erythrocytes [9], 30 kDa in bovine stomach [40] and cat erythrocytes [11], 31 kDa in bovine erythrocytes [41], 34 kDa in human erythrocytes [42] and 36 kDa in bovine bone [43]. However, in comparison with molecular weight of mammalian CAs, camel liver CA exhibited lower molecular weight. Similar to this observation, in our previous studies, we also found that purified superoxide dismutase (SOD) [44] and glutathione peroxidase (GPx) [45] from camel liver exhibited lower molecular weights compared to those found in mammalian species.

3.2. Determination of optimum pH and pH stability

Esterase activity of camel liver CA was assayed in the pH range of 5.0–10.5 (Fig. 2A). Camel liver CA was active at both acidic and alkaline pH and its high activity was recorded at alkaline pH, where, more than 70% of its activity was retained between pH 8.0 and 9.5. In fact, the enzyme was optimally active at pH 9.0, which is strongly alkaline pH. Interestingly, camel liver CA has a higher optimum pH than that of mammalian species, where, optimum pH of purified CAs from different sources of mammalian species was found to be neutral to moderately alkaline: pH 7.0 in bovine bone [43], pH 7.5 in sheep erythrocytes [9] and bovine stomach [40], pH 8.0 in erythrocytes of human [10] and cat [11], and pH 8.5 in sheep kidney [46].

Effect of pH on camel liver CA stability was investigated by incubating the enzyme at pH 7.0, 8.0 and 9.0 for 0, 6, 12, 24, 48, 72 and 96 hours. As shown in Fig. 2B, camel liver CA retained more than 50% of its activity at pH 7.0, 8.0 and 9.0 after 96 hours. Stable pH of camel liver CA was found to be different depending on incubation time, where, stable pH of 7.0, 8.0 and 9.0 was found after 96, 72 and 6–48 hours, respectively. Similarly, in other purified CAs, different stable pH with different incubation time were reported in literature, for example: pH 8.5/336 hours in sheep kidney [46], pH 8.0/72 hours in fish gills [47], and pH 7.0/1 hour [48] and pH 7.0–8.5/24 hours [49] in bacteria. Obviously, camel liver CA was optimally active and stable at strongly alkaline pH, which are good indications for potential applications of camel CA in the alkaline solvent-based CO_2 absorption/desorption process [4].

3.3. Determination of optimum temperature and thermal stability

Esterase activity of camel liver CA was assayed at various temperatures in range from 20 to 120 °C (Fig. 2C). Enzyme activity increased with increasing temperature and reached maximum at 45 °C, after that, enzyme activity decreased with further increasing temperature and completely lost at 120 °C. Camel liver CA was able to maintain high enzyme activity at high temperatures, where, it retained 85, 72, 70, 51, 43 and 36% of its activity at 50, 60, 70, 80, 90 and 100 °C, respectively. Optimum temperature of camel liver CA was found to be 45 °C, which is higher than that found in mammalian CAs, including human erythrocytes (25 °C [10]); erythrocytes (37 °C [12]), stomach (35 °C [40]) and bone (35 °C [43]) of bovine; erythrocytes (30 °C [9]) and kidney (35 °C [46]) of sheep; and cat erythrocytes (40 °C [11]). Likewise, purified SOD [44], GPx [45,50] and catalase (CAT) [51] from camel organs were found to have an optimum temperature higher than that found in mammalian species. In contrast, purified CA from sheep erythrocytes was found to have an optimum temperature of 70 °C [52].

Thermal stability of camel liver CA was also investigated by incubating the enzyme at 30, 40, 50 and 60 °C for 10, 20, 30, 60, 120, 180 and 240 min (Fig. 2D). Noticeably, the enzyme was very stable up to 60 °C after 240 min of incubation. Camel liver CA preserved more than 85% of its activity at all temperatures and incubation times tested. In human erythrocytes CA, after 80 min of incubation, the enzyme retained 79, 56 and 24% of its activity at 30, 40 and 50 °C, respectively [10]. Furthermore, in bacteria CA, 80% of enzyme activity was retained within the range of 35–45 °C after 60 min of incubation [48]; 25% of enzyme activity was retained at 60 °C after 120 min of incubation [23]; and 100, 89, 86 and 21% of enzyme activity was retained at 35, 40, 45 and 50 °C after 60 min of incubation, respectively [49]. In these reported studies [10,23,48], immobilized CA was also assayed for its thermal stability and showed a better thermal stability than free CA. Thus, immobilization of CA increases thermostability as compared to free CA. For example, immobilized CA from human erythrocytes retained 89, 93 and 35% of its activity at 30, 40 and 50 °C after 80 min of incubation, respectively [10]. In all cases, camel liver CA was found to be very stable at high temperatures and retained its activity for a longer period of time compared to free CA as well as immobilized CA from other sources. We expect that the immobilization of camel liver CA could significantly increase the enzyme stability at elevated temperatures (>60 °C). Overall, higher optimum temperature and thermostability of camel liver CA could be related to the adaptation of camel to live in prolonged and intense exposure to high temperature and direct solar radiation in desert areas. Temperature profile of camel liver CA revealed that this enzyme is a good candidate for CO_2 absorption/desorption process, in particular, stability between temperature range of $40 - 80$ °C is the most important criteria for this process [4].

3.4. Effect of metal ions on enzyme activity

Effect of Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+} and Zn^{2+} on camel liver CA activity was investigated. Esterase activity was assayed in the presence of various concentrations of each metal ion, then, the measured residual activity was plotted against metal ion concentration (Figure S1). All metal ions showed inhibitory effects on camel liver CA activity. IC_{50} and Hill slope of camel liver CA for each metal ion were calculated (Table 2). Among metal ions tested, Cu^{2+},

Table 1
Purification scheme of CA from camel liver by p-aminomethylbenzenesulfonamide–agarose affinity chromatography.

Step	Total proteins mg/ml	Total activity* U/ml	Specific activity U/mg	Purification -fold	Yield %
Homogenate	582.39	28.27	0.05	1.00	100.00
Affinity chromatography	0.12	12.87	111.23	2291.17	45.52

* The enzymatic activity was determined using hydratase activity assay.

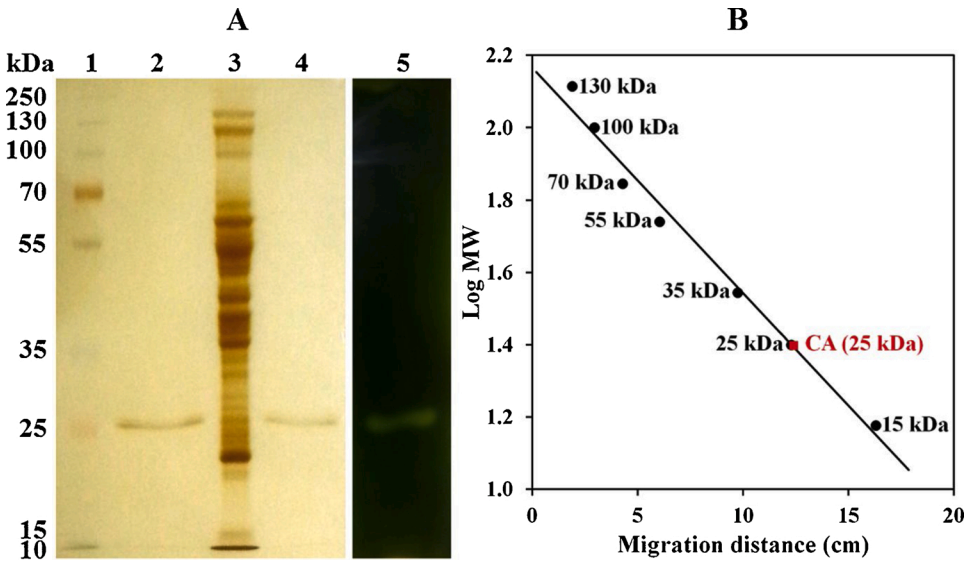

Fig. 1. SDS-PAGE analysis of purified CA from camel liver. (A) Lane 1, molecular weight marker; lane 2, denatured camel liver CA; lane 3, camel liver homogenate; lane 4, nondenatured camel liver CA; lane 5, protonogram showing a band (yellow color) corresponding to the hydratase activity of purified CA from camel liver. (B) Calibration curve for determination of molecular weight of camel liver CA. Red square indicates camel liver CA.

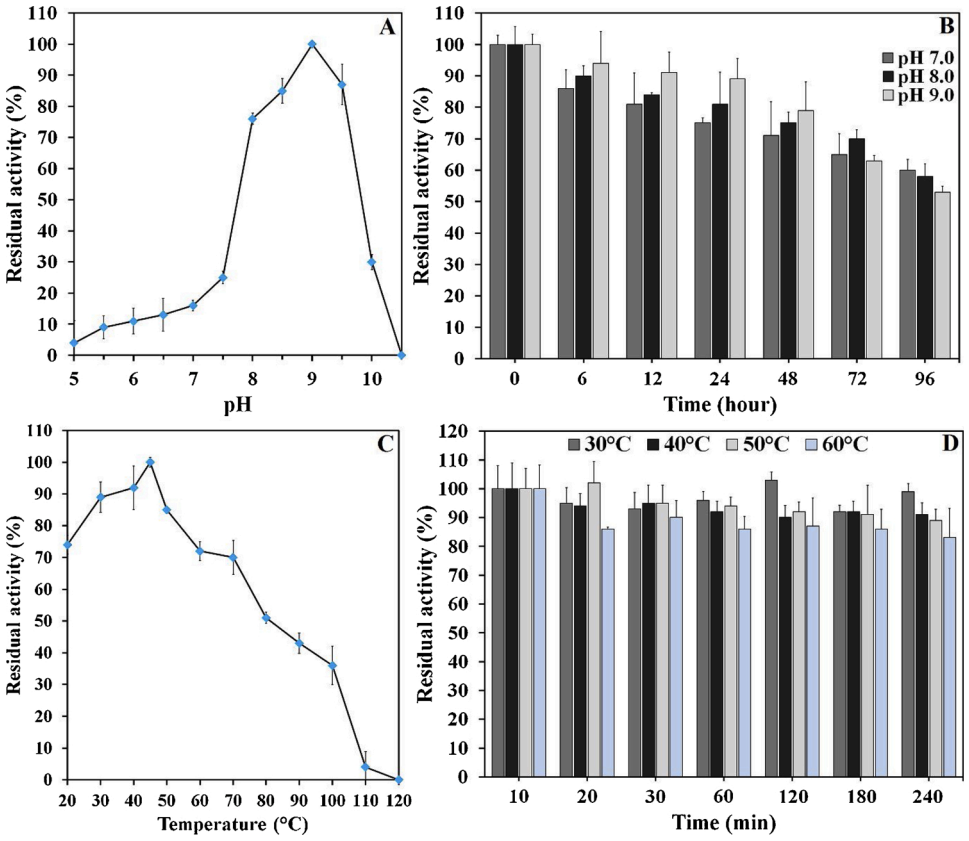

Fig. 2. pH and temperature profile of camel liver CA. (A) optimum pH, (B) pH stability, (C) optimum temperature and (D) temperature stability of camel liver CA were determined as described in "Materials and methods" section. Results were presented as mean ± SD (n = 3).

Fe^{3+} and Zn^{2+} were found to be the most potent inhibitors of camel liver CA with an IC_{50} of 0.18, 0.64 and 0.59 mM, respectively. While, Cd^{2+}, Co^{2+}, Cr^{3+} and Ni^{2+} showed a moderate to relatively strong inhibitory effect with an IC_{50} of 8.19, 10.23, 2.12 and 2.48 mM, respectively. Although, Al^{3+}, Ca^{2+} and Mg^{2+} showed a weak inhibitory effect, where they inhibited enzyme activity at high IC_{50}, 25.19, 78.41 and 45.56 mM, respectively. Overall, inhibition order of metal ions tested on camel liver CA activity was as follow: $Cu^{2+} > Zn^{2+} > Fe^{3+} > Cr^{3+} > Ni^{2+} > Cd^{2+} > Co^{2+} > Al^{3+} > Mg^{2+} > Ca^{2+}$. In the measure of cooperativity in binding process, a Hill slope less than one was found for all metal ions tested, indicating that metal ions-CA interactions showing negative cooperativity.

All living organisms are inevitably exposed to heavy metals from various sources, including camel [53]. In fact, heavy metals are known

Table 2
IC_{50} and Hill slope values of metal ions for camel liver CA.

Metal ions	IC_{50} (mM)*	r^2*	Hill slope*
Al^{3+}	25.19	0.9848	−1.21
Ca^{2+}	78.41	0.9885	−2.56
Cd^{2+}	8.19	0.9878	−0.61
Co^{2+}	10.23	0.9824	−0.65
Cr^{3+}	2.12	0.9893	−1.26
Cu^{2+}	0.18	0.9798	−1.04
Fe^{3+}	0.64	0.9616	−0.77
Mg^{2+}	45.56	0.9944	−2.21
Ni^{2+}	2.48	0.9881	−0.71
Zn^{2+}	0.59	0.9954	−0.56

* Data were obtained from nonlinear regression plots given in Figure S1.

to disrupt catalytic functions of enzymes by decreasing the activities of various enzymes, including CA [9,18,54,55]. In sheep erythrocytes CA, *IC_{50}* of 0.42, 0.63 and 1.22 mM was found for Co^{2+}, Cu^{2+} and Fe^{2+}, respectively [9]. In addition, in sheep liver CA, *IC_{50}* of 0.66, 0.75, 0.00041, 1.44 and 0.058 mM was found for Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, respectively [18]. Also, *IC_{50}* of 0.19, 0.14 and 0.92 mM for Cd^{2+}, Fe^{3+} and Ni^{2+} was found in fish gills, respectively [54]. In fish liver, *IC_{50}* of 0.07, 0.32, 0.07 and 0.39 mM was found for Al^{3+}, Co^{2+}, Cu^{2+} and Zn^{2+}, respectively [55]. In our previous study, we found that camel liver accumulated higher concentrations of Cd^{2+} compared to other studies [53]. Herein, *IC_{50}* of camel liver CA for Cd^{2+} was higher than that found in other CAs. Overall, camel liver CA needed high concentrations of such metal ions to reach 50% inhibition, it seems to has a low sensitivity to metal ions, which could not affect its catalytic functions. Interestingly, Mg^{2+} and Ca^{2+} were found to be the weak inhibitors of camel liver CA, indicating that the enzyme could function optimally for CO_2 sequestration under salt environments [56]. In fact, in the presence of CO_3^{2-} in alkaline media, all together, CA, Mg^{2+} and Ca^{2+} could play a critical role in the sequestration of CO_2 into carbonate of Mg^{2+} and Ca^{2+} [4].

3.5. Effect of anions and chemicals on enzyme activity

Effect of Br^-, CH_3COO^-, $C_6H_5O_7^{3-}$, ClO_4^-, CN^-, CO_3^{2-}, F^-, HCO_3^-, I^-, N_3^-, NO_3^-, SCN^-, SeO_3^{2-} and SO_4^{2-} on esterase activity of camel liver CA was investigated (Table 3). No significant effect on enzyme activity was observed when concentrations of Br^-, $C_6H_5O_7^{3-}$, HCO_3^- and SO_4^{2-} were increased from 1 to 5 mM, where the residual enzyme activity was found to be higher than 100%, indicating that these anions could be considered as weak activators. However, increasing concentrations of CN^-, I^-, NO_3^- and SeO_3^{2-} inhibited enzyme activity, where it reached 32, 15, 42 and 39% at 5 mM, respectively. While, enzyme activity was weakly inhibited with increasing concentrations of CH_3COO^-, CO_3^{2-}, F^-, N_3^- and SCN^-, it retained more than 70% at 5 mM. Anions constitute an important class of CA inhibitors [38]. In bacteria, purified CA from *Bacillus subtilis* showed residual activity of 36 and 126 % in presence of 1 mM of NO_3^- and SO_4^{2-}, respectively [57], while, an *IC_{50}* of 600 and 800 mM was found for purified CA from *Micrococcus lylae* for NO_3^- and SO_4^{2-}, respectively [48]. In our study, camel liver CA was moderately inhibited by NO_3^-, while no significant effect on enzyme activity was observed in the presence of SO_4^{2-}. These observations suggest the possible utility of camel liver CA for CO_2 sequestration from flue gas, where NO_x and SO_x are the main components of flue gas [17]. Purified CA from bovine bone was greatly inhibited by N_3^- and SCN^-, where an *IC_{50}* of 1.15 and 1.53 mM was obtained, respectively [43]. Similarly, bovine stomach CA showed an *IC_{50}* of 0.77 and 0.50 mM for N_3^- and SCN^-, respectively [40]. Herein, camel liver CA retained more than 50% of its activity at 5 mM of N_3^- (73%) and SCN^- (84%). Otherwise, our results were different compared to the *IC_{50}* found in bacteria for Br^- (700 mM), F^- (300 mM), HCO_3^- (600 mM) and I^- (700 mM) [48].

Table 3
Effect of anions on esterase activity of camel liver CA.

Anions	Residual activity (%)		
	1 mM	2 mM	5 mM
Control	100 ± 1.81	100 ± 1.28	100 ± 2.64
Br^-	100 ± 0.70	103 ± 1.07	105 ± 3.50
CH_3COO^-	98 ± 4.36	87 ± 0.45	80 ± 7.86
$C_6H_5O_7^{3-}$	101 ± 3.37	105 ± 3.21	104 ± 3.46
ClO_4^-	75 ± 7.45	69 ± 2.80	51 ± 7.61
CN^-	44 ± 2.47	41 ± 5.43	32 ± 6.05
CO_3^{2-}	99 ± 9.63	95 ± 5.76	94 ± 4.20
F^-	102 ± 1.32	86 ± 0.45	77 ± 4.90
HCO_3^-	106 ± 3.37	103 ± 3.09	101 ± 4.94
I^-	40 ± 0.41	30 ± 5.56	15 ± 6.56
N_3^-	88 ± 3.62	80 ± 1.40	73 ± 3.42
NO_3^-	82 ± 1.77	68 ± 7.61	42 ± 5.64
SCN^-	105 ± 3.70	93 ± 1.40	84 ± 3.13
SeO_3^{2-}	43 ± 1.07	41 ± 0.95	39 ± 4.36
SO_4^{2-}	109 ± 1.32	112 ± 2.88	110 ± 2.76

Esterase activity was also assayed in the presence of 1, 2 and 5 mM of β-ME, Cys, DTT, EDTA, PMSF, SDS and TX-100 (Table 4). Increasing concentrations of β-ME, Cys and DTT resulted in stimulation of enzyme activity, where the residual enzyme activity was found to be higher than 100%. Whereas, enzyme activity decreased with increase in concentrations of EDTA, PMSF, SDS and TX-100. In fact, EDTA and SDS significantly decreased enzyme activity from 71 and 94% at 1 mM to 17 and 3% at 5 mM, respectively, while, PMSF and TX-100 progressively decreased enzyme activity from 115 and 94% at 1 mM to 96 and 70% at 5 mM, respectively. Camel liver CA activity was not affected by the presence of reducing agents, β-ME, Cys and DTT, suggesting that SH-groups were not required for enzyme activity. However, the chelating agent EDTA inhibited camel liver CA, which indicates that a metal ion is essential for enzyme activity. From the effect of a serine protease inhibitor, PMSF slightly inhibited enzyme activity of camel liver CA, indicating that serine residues are not significantly involved in enzyme activity. In addition, camel liver CA activity was strongly inhibited by the ionic surfactant SDS. This could be due to the strong binding of SDS to the monomeric structure of camel liver CA in the positively charged amino acid, consequently, causing conformational changes at the active site of the enzyme. In contrast, camel liver CA activity was weakly inhibited by the nonionic surfactant TX-100, which indicates that TX-100 could prevent the aggregation of the monomeric structure of camel liver CA thereby reducing inhibitory effect loss.

3.6. Determination of kinetic parameters

Kinetic parameters, k_m and V_{max}, of camel liver CA for 4-NPA as a substrate were determined using nonlinear regression plot. As shown in Fig. 3A, camel liver CA displayed a hyperbolic kinetic response to increasing 4-NPA concentrations. Thus, the dependence of the reaction rate catalyzed by camel liver CA on 4-NPA concentrations obeyed Michaelian kinetics. In fact, k_m and V_{max} of camel liver CA were found to be 1.45 mM and 120.50 U/ml, respectively, as well as, k_{cat} and k_{cat}/k_m were calculated to be 47.37 s^{-1} and 32.67 s^{-1} mM^{-1}, respectively. Camel liver CA exhibited higher catalytic efficiency for 4-NPA, as a

Table 4
Effect of chemicals on esterase activity of camel liver CA.

Chemicals	Residual activity (%)		
	1 mM	2 mM	5 mM
Control	100 ± 2.25	100 ± 2.52	100 ± 3.78
β-ME	106 ± 2.22	114 ± 1.07	112 ± 6.67
Cys	112 ± 7.98	119 ± 6.34	122 ± 5.51
DTT	108 ± 4.36	119 ± 8.64	122 ± 5.48
EDTA	71 ± 1.73	52 ± 1.28	17 ± 0.49
PMSF	115 ± 1.19	98 ± 1.81	96 ± 4.61
SDS	94 ± 0.21	47 ± 2.68	3 ± 1.65
TX-100	94 ± 4.36	80 ± 7.86	70 ± 3.29

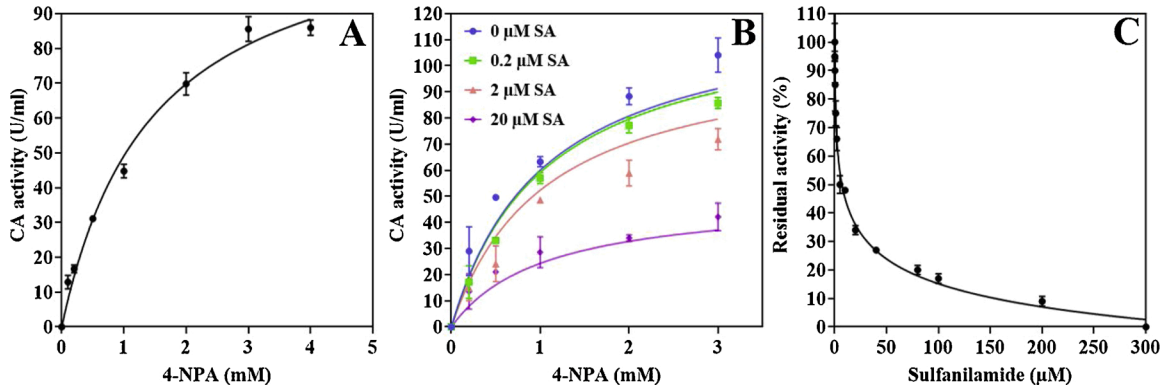

Fig. 3. Kinetic parameters of camel liver CA for substrate (4-NPA) and inhibitor (sulfanilamide (SA)). (A) Nonlinear regression plot to determine k_m and V_{max} for 4-NPA. (B) Nonlinear regression plot to determine k_i and inhibition type for sulfanilamide. (C) Nonlinear regression plot to determine IC_{50} and Hill slope for sulfanilamide. Experimental procedures were detailed under "Materials and methods".

consequence of low k_m value (high affinity for substrate) and high k_{cat} value (rapid turnover number). In comparison with mammalian species, k_m of purified CA for 4-NPA was found to be different as follows: 0.48 mM in human erythrocytes [10], 0.54 mM in bovine stomach [40], 3.60 mM in bovine erythrocytes [12], 7.34 mM in sheep erythrocytes [9], 17.87 mM in sheep kidney [46] and 80 mM in sheep erythrocytes [52]. In addition, k_{cat} and k_{cat}/k_m of purified CA from human erythrocytes for 4-NPA were found to be different as follows: 0.92 s^{-1} and 191.70 s^{-1} mM^{-1} [10].

Likewise, kinetic parameters, k_i, inhibition type, IC_{50} and Hill slope, of camel liver CA for sulfanilamide as a potent inhibitor were determined using nonlinear regression plots. Esterase activity was assayed in the presence of three different concentrations of sulfanilamide (0.2, 2 and 20 µM) while keeping 4-NPA concentrations constant at five different concentrations (0.2, 0.5, 1, 2 and 3 mM). By increasing sulfanilamide concentrations, the reaction rate decreased from 129.60 U/ml (0 µM sulfanilamide) to 47.93 U/ml (20 µM sulfanilamide), while k_m remains unaffected (Fig. 3B). This behavior corresponds to noncompetitive inhibition. In addition, k_i for sulfanilamide was determined to be 13.66 µM. Sulfanilamide is the most potent inhibitor of CA [46]. In contrast to our study, sulfanilamide inhibited purified CA from bone and stomach of bovine uncompetitively with k_i of 7.18 mM [43] and 0.29 µM [40], respectively. Also, different k_i value for sulfanilamide was reported to be 69.31 µM in sheep kidney [46].

Furthermore, esterase activity of camel liver was also assayed by varying sulfanilamide concentrations (0 to 300 µM) and keeping 4-NPA concentration constant at 4 mM. From plot shown in Fig. 3C, IC_{50} for sulfanilamide was determined to be 14.20 µM. The maximum inhibition of camel liver CA was achieved by 300 µM sulfanilamide. In addition, Hill slope for sulfanilamide was determined to be −0.31, indicating that sulfanilamide-CA interactions showing negative cooperativity. IC_{50} of sulfanilamide was found to be 14.20 µM for camel liver CA, which is lower than that found in bone (1.15 mM [43]) and stomach (1.36 mM [40]) of bovine, while it is higher than that found in bacteria (7 µM [49]). Somewhat, camel liver CA showed lower k_i for sulfanilamide, indicating that this enzyme exhibited higher affinity for sulfanilamide. This observation is consistent with the lower IC_{50} found for this inhibitor, where, the enzyme was sensitive towards sulfanilamide and required lower concentration to reach half inhibition.

3.7. Determination of metal content

Determination of metal content in camel liver CA using ICP-MS revealed interesting and unexpected results. The enzyme contained 0.00, 1.00, 0.00, 0.00 and 0.00 µg of Zn, Fe, Cd, Cu and Co per mg of protein, respectively. While, based on its native molecular weight (25 kDa), the enzyme contained 0.44 mol of Fe per mol of protein. In contrast to our study, purified CA from erythrocytes of human [42] and bovine [41] contains 1 mol of Zn per mol of protein. Thus, unlike mammalian CAs, that contain Zn as a physiologically-relevant cofactor, camel liver CA contains very low content of Fe instead of Zn. This finding might be attributed to the unique metabolism [58] and low concentrations of Zn [53] in camel compared to other domestic animals.

3.8. Sequestration of CO_2 into $CaCO_3$ and characterization studies

3.8.1. Sequestration of CO_2 into $CaCO_3$

The ability of camel liver CA to sequester CO_2 into $CaCO_3$ was examined. As shown in Fig. 4A, camel liver CA was successfully enhanced CO_2 sequestration and effective in accelerating $CaCO_3$ formation in presence of 1, 2, 5, 10 and 20% Ca^{2+} after 30 min, where, the amount of precipitated $CaCO_3$ was found to be 2.10, 4.40, 24.10, 29.60 and 34.80 mg, respectively. Notable changes in precipitation patterns of $CaCO_3$ was observed in presence of purified CA from camel liver. In fact, CO_2 sequestration capacity of camel liver CA was 58.33, 122.22, 669.44, 822.22 and 966.67 mg $CaCO_3$/mg enzyme after 30 min at pH 9.0 and room temperature in presence of 1, 2, 5, 10 and 20% Ca^{2+}, respectively. Interestingly, CO_2 sequestration capacity of camel liver CA was much higher than that found for immobilized (56 mg $CaCO_3$/mg enzyme) and free (58 mg $CaCO_3$/mg enzyme) CA from human erythrocytes after 60 min at pH 10.0/25 °C in presence of 1% Ca^{2+} [59], immobilized (227 mg $CaCO_3$/mg enzyme) and free (241 mg $CaCO_3$/mg enzyme) CA from bovine erythrocytes after 10 min at pH 10.0/25 °C in presence of 4% Ca^{2+} [60], immobilized (253 mg $CaCO_3$/mg enzyme) and free (267 mg $CaCO_3$/mg enzyme) CA from *E. coli* MO1 after 20 min at pH 9.5/37 °C in presence of 1% Ca^{2+} [24], immobilized (480 mg $CaCO_3$/mg enzyme) and free (340 mg $CaCO_3$/mg enzyme) CA from *Bacillus subtilis* VSG-4 after 20 min at pH 9.5/37 °C in presence of 1% Ca^{2+} [23], purified CA from *Citrobacter freundii* (230 mg $CaCO_3$/mg enzyme at pH 8.0/room temperature [26]) and *Serratia* sp. ISTD04 (26 mg $CaCO_3$/mg enzyme at pH 10.3/20 °C [25]) in presence of 2% and 25 mM Ca^{2+}, respectively. Our results showed that camel liver CA displayed higher efficiency and capacity in the sequestration of CO_2 into $CaCO_3$ in presence of high concentration of Ca^{2+} (up to 20%).

Sequestration of CO_2 into $CaCO_3$ requires a robust CA functioning at higher pH and temperature conditions. In this context, sequestration efficiency of CO_2 into $CaCO_3$ using bovine erythrocytes CAs and indigenous bacterial strains CA was achieved at pH 9.5/40 °C [61], pH 10.0/30 °C [62] and pH 9.5/35-45 °C [63], respectively. In the present study, sequestration efficiency of CO_2 into $CaCO_3$ using camel liver CA was achieved at pH 9.0 and at room temperature. Biochemically, camel liver CA was active and stable at strongly alkaline pH (pH 9.0) and higher temperature (60 °C). Based on these biochemical properties, camel liver CA can efficiently sequester CO_2 into $CaCO_3$ at higher pH

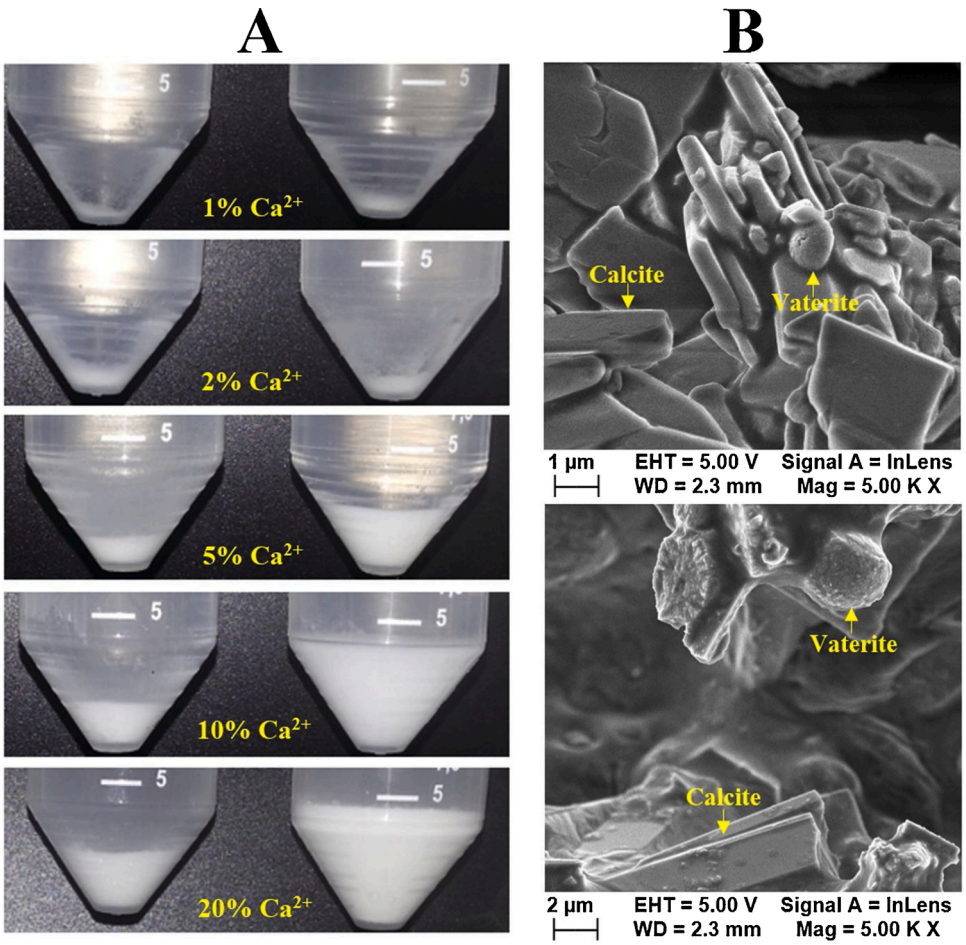

Fig. 4. (A) Precipitation patterns of $CaCO_3$ without (left) and with (right) camel liver CA in presence of 1, 2, 5, 10 and 20% Ca^{2+}. (B) SEM images of the formed $CaCO_3$ without (top) and with (below) camel liver CA in presence of 20% Ca^{2+}.

and temperature conditions.

Overall, as compared to both free and immobilized CA from other sources, camel liver CA showed its ability to efficiently sequester CO_2 into $CaCO_3$ in presence of high concentration of Ca^{2+} at strongly alkaline pH and room temperature. It is well known that the immobilization improves the catalytic activity and the stability of the enzyme [64]. We expect that the immobilization of camel liver CA could significantly increase the enzyme stability under high-salt environments, higher pH and higher temperature. Therefore, camel liver CA represents a promising candidate for CO_2 sequestration under harsh industrial conditions.

3.8.2. *Characterization of the formed $CaCO_3$*

In order to confirm the formation of $CaCO_3$ with camel liver CA, $CaCO_3$ precipitated with 20% Ca^{2+} was characterized by SEM, XRD and FTIR and compared to the $CaCO_3$ formed without enzyme. As shown in SEM images (Fig. 4B), the formed $CaCO_3$ with camel liver CA exhibited rhombohedral and spherical shapes, indicating the presence of calcite (stable phase) and vaterite (unstable phase) polymorphs. Similarly, the formed $CaCO_3$ without enzyme showed the same crystal polymorphs. Furthermore, XRD results provided further evidence (Fig. 5A). XRD patterns demonstrated that the crystal polymorphs of the formed $CaCO_3$ without and with camel liver CA were calcite and vaterite polymorphs.

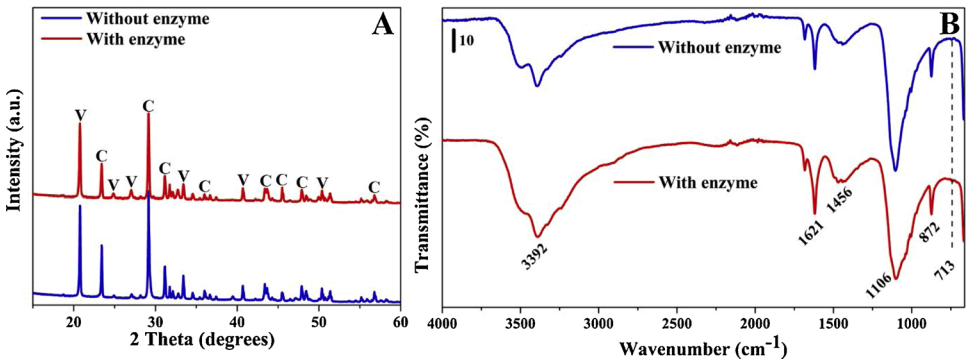

Fig. 5. (A) XRD and (B) FTIR patterns of the formed $CaCO_3$ without (blue line) and with (red line) camel liver CA in presence of 20% Ca^{2+}. 'C' and 'V' indicated XRD patterns for calcite and vaterite, respectively.

Calcite phase diffraction peaks appeared at 2θ = 23°, 29°, 31°, 36°, 44°, 46°, 48° and 57°, where, the characteristic peak of calcite phase was 2θ = 29°. Whereas, vaterite phase diffraction peaks appeared at 2θ = 21°, 24°, 27°, 33°, 41° and 51°. From FTIR spectrum of the formed $CaCO_3$ without and with camel liver CA, the bands were located at 713, 872, 1106, 1456, 1621 and 3392 cm^{-1} (Fig. 5B). In fact, characteristic peaks of $CaCO_3$ were located at 713, 872, 1106 and 1456 cm^{-1}. A weak band at 713 cm^{-1} belonged to the in-plane bending vibration mode of CO_3^{2-}. The peaks at 872 and 1456 cm^{-1} corresponded to CO_3^{2-} out-of-plane deformation vibration mode, which further confirm the formation of $CaCO_3$. Appearance of $CaCO_3$ absorption band at 1621 cm^{-1} was due to C – O stretching vibrations. Whereas, a distinct band at 1106 cm^{-1} and a broad band at 3392 cm^{-1} belonged to symmetric vibration of $CO_3^{2-}$and O–H stretching mode, respectively. Overall, morphological features, structural properties and surface functional groups of the formed $CaCO_3$ with camel liver CA determined by SEM, XRD and FTIR, respectively, were very similar to those described previously for the formed $CaCO_3$ in presence of CA from human erythrocytes [59], *Citrobacter freundii* [26] and *Serratia* sp. ISTD04 [25].

4. Conclusion

For the first time, the present manuscript described the sequestration of CO_2 into $CaCO_3$ using a novel CA which was purified and characterized from liver of an animal that survive extreme desert conditions, camel (*Camelus dromedarius*). Purified CA from camel liver contained Fe as a physiologically-relevant cofactor instead of Zn and showed lower molecular weight, higher optimum pH, higher optimum temperature and lower sensitivity to metal ions. Furthermore, camel liver CA was active and stable at strongly alkaline pH (pH 9.0) and higher temperature (60 °C), also, it needed high concentrations of Ca^{2+} to reach 50% inhibition. These biochemical properties are good indications for potential application of camel CA to sequester CO_2 into $CaCO_3$. Indeed, camel liver CA clearly demonstrated its efficiency and ability to be successfully used for sequestration of CO_2 into $CaCO_3$. Interestingly, camel liver CA showed higher capacity in this process with a value of 966.67 mg $CaCO_3$/mg enzyme in presence of high concentration of Ca^{2+} (up to 20%). Camel CA represents promising candidate for harsh industrial applications. In fact, further studies are strongly needed to improve the stability of camel CA by immobilizing the enzyme and to be used for CO_2 sequestration under harsh industrial conditions.

Author Statement

Abdelbasset Chafik and Safinur Yildirim Çelik purified enzyme and characterized its properties, Kaoutar El Hassani helped with interpretation of data, Ahmet Mavi and Abdelkhalid Essamadi conceived and supervised project, and wrote paper with contributions from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcou.2020.101310.

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