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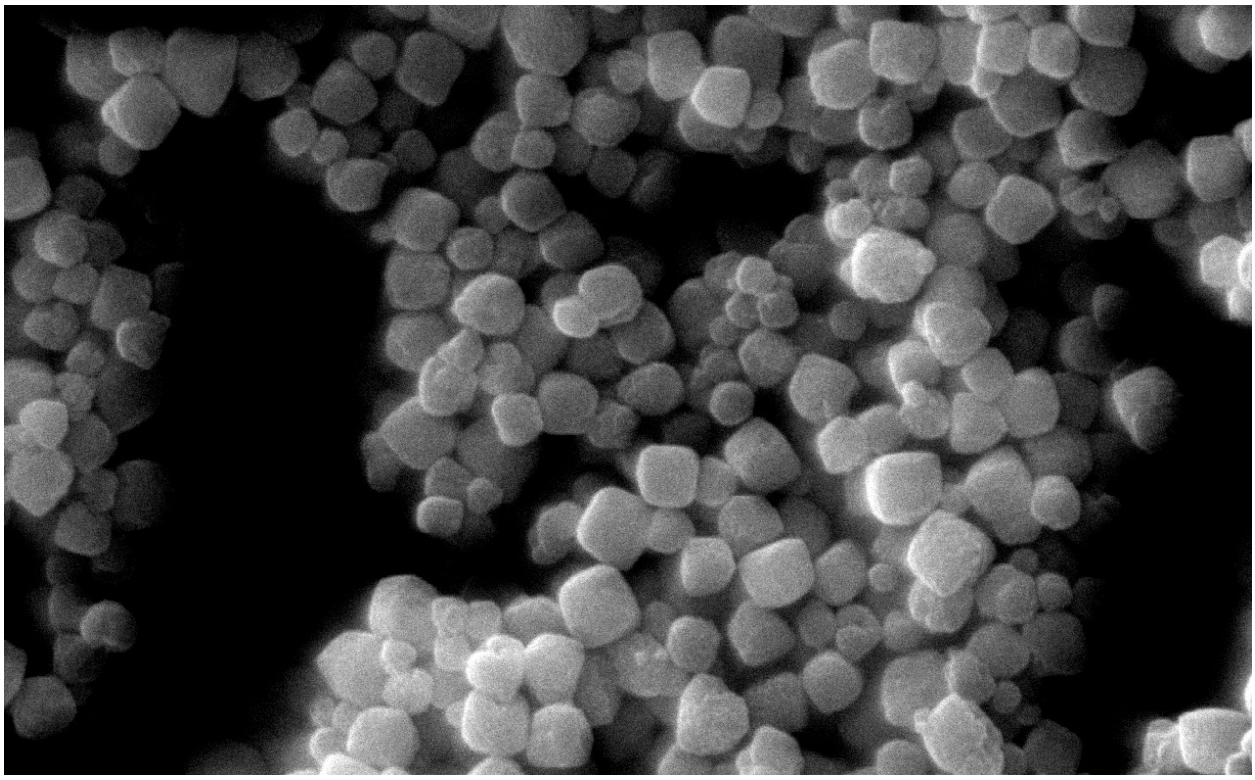

Encapsulation of carbonic anhydrase in metal-organic frameworks to facilitate CO_2 capture

Master's thesis in Innovative and Sustainable Chemical Engineering

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DEPARTMENT OF LIFE SCIENCES

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Cover: Micrograph of SazCA encapsulated in ZIF-8.

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Abstract

Reducing carbon emissions is crucial to stop global warming. The current methods to capture and store carbon dioxide are expensive and energy demanding. Therefore, a new technique using the enzyme carbonic anhydrase was evaluated in this project. Five protocols were tested to encapsulate SazCA, which is a carbonic anhydrase from the thermophilic bacterium *Sulfurihydrogenibium azorense*, in metal-organic frameworks (MOFs) called zeolitic imidazolate framework-8 (ZIF-8). Encapsulating enzymes in metal-organic frameworks has previously been shown to increase their reusability and thermostability.

The enzyme was first produced through heterologous expression from *Escherichia coli* and purified with affinity chromatography. The MOF was then synthesized together with SazCA. It was confirmed through X-ray diffraction that two of the protocols were successful in producing pure SazCA@ZIF-8. The structure was then identified with scanning electron microscopy, which showed that the protocol with a ratio of 70:1 of the precursors 2-methylimidazole and zinc nitrate hexahydrate had particles with the most desirable cubical shape and were also mostly uniform in size. However, evaluation of the enzymatic activity with Wilbur-Anderson units assay after the encapsulation showed that it only reached about 2-5 % of the activity in free SazCA. This was shown to most likely partly be because the free zinc ions were inhibiting the enzyme. However, it was also shown that the activity improved with 122.41 % by introducing sonication, which increases the diffusion rate. Finally the thermostability was tested and compared to that of free SazCA at the temperatures 75, 85 and 95 °C for 1 and 4 hours. At 85 °C it was observed that the thermostability in the encapsulated SazCA was higher than in the free SazCA. After 1 h at this temperature the relative activity compared to the free SazCA was 11.85 ± 0.72 %, compared to 1.97 ± 0.30 %, which it was initially.

In conclusion, even though the SazCA encapsulated in ZIF-8 lost most of its original activity, important design principles were identified, which could benefit future research within the field.

Keywords: metal-organic framework (MOF), zeolitic imidazolate framework-8 (ZIF-8), carbonic anhydrase (CA), SazCA, enzyme encapsulation, carbon capture and storage (CCS), carbon dioxide (CO_2).

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Ellen Sjöstrand, Gothenburg, January 2026

List of Acronyms

2-MIM	2-Methylimidazole
BCA	Bovine Carbonic Anhydrase
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase
EDTA	Ethylenediaminetetraacetic Acid
LB	Lennox Broth
MOF	Metal-Organic Framework
MQ-water	Milli-Q Water
pNPA	p-Nitrophenyl Acetate
SazCA	CA from *Sulfurihydrogenibium azorense*
SEM	Scanning Electron Microscopy
TEA	Triethylamine
XRD	X-Ray Diffraction
ZIF-8	Zeolitic Imidazolate Framework-8
ZIF-L	Leaf-shaped Zeolitic Imidazolate Framework

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1
Introduction

Global warming is becoming an increasing problem with temperatures expecting to rise with 1.5 °C above pre-industrial levels between 2030 and 2052 [1]. The consequences of global warming are already noticeable in the form of increased droughts and floods, sea level rise and loss of biodiversity. Since carbon dioxide (CO_2) is one of the major greenhouse gases contributing to global warming, it is essential to reduce CO_2 emissions. However, current methods are still very expensive and energy demanding [2]. An emerging low-cost and eco-friendly technique to remove CO_2, from especially industrial emissions, is through the use of an enzyme called carbonic anhydrase (CA). It utilizes the natural process of biomineralization, in which living organisms form inorganic minerals, which for example can be used to build tissues such as bones and teeth in vertebrates [3]. Compared to traditionally researched carbon dioxide sequestration techniques, such as geological and deep ocean sequestration, using CA has multiple advantages [2]. By using this technique stable carbonate minerals can be produced from CO_2, which are permanent and safe, making them suitable for long-term storage. Additionally, they also have applications in construction materials.

1.1 Background

A proposed design for a process using CA to capture and store CO_2 can be seen in Figure 1.1. In this design a scrubber column would first be used to dissolve the carbon dioxide, coming from a waste gas stream, in water. The scrubber column would also contain the CA encapsulated in a metal-organic framework (MOF), which would increase its reusability and thermostability. There the CA would catalyze the conversion of CO_2 to bicarbonate ion (HCO_3^-). The produced HCO_3^- would then be transported to a precipitation chamber, where it would be mixed with sea water and spontaneously turn into carbonate ions (CO_3^{2-}). The sea water contains calcium ions (Ca^{2+}), which would make it possible for calcite ($CaCO_3$) to precipitate. The produced $CaCO_3$ can then either be stored as a permanent carbon sink or used for applications in the construction industry, such as cement. The enzyme and the mentioned reactions will be explained further in Theory 2.1.

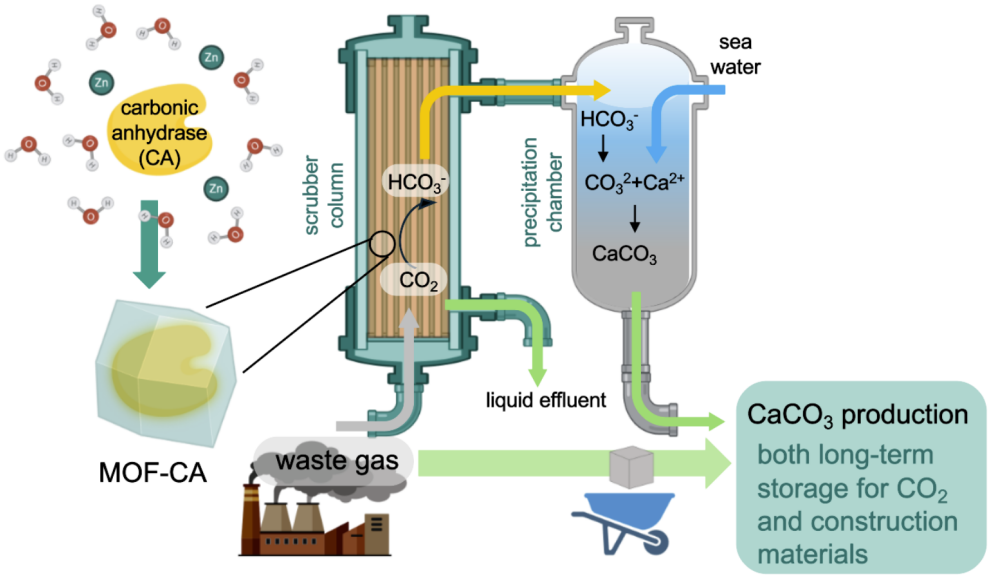

Figure 1.1: Design of a process to capture and convert CO_2 to $CaCO_3$. Created with BioRender.com.

1.2 Aim and Objectives

The aim of the project is to encapsulate CA from the thermophilic bacterium *Sulfurihydrogenibium azorense*, called SazCA, in the MOF zeolitic imidazolate framework-8 (ZIF-8), while maintaining the enzyme's catalytic activity and improving its thermostability. This will be achieved through first heterologous expression and purification of SazCA from *Escherichia coli* (*E. coli*) by affinity chromatography. ZIF-8 will then be synthesized together with the purified SazCA. The catalytic activity and thermostability of SazCA after integration in ZIF-8 will also be tested. Finally the structure will be identified through X-ray diffraction (XRD) and scanning electron microscopy (SEM).

The following questions will be investigated in this master's thesis.

- What is the optimal composition of ZIF-8 precursors and synthesis conditions to encapsulate SazCA in ZIF-8?
- How much of the SazCA's catalytic activity is maintained after encapsulation in ZIF-8?
- How is the thermostability of SazCA affected by encapsulation in ZIF-8?
- What is the structure of ZIF-8 with encapsulated SazCA?

1.3 Delimitations

The project is limited to a proof of concept, rather than direct implementation for real industry use. This means that only one native type of CA (SazCA) will be tested. The variation of MOF materials tested will also be limited to only include ZIF-8, since it has suitable properties for this application and has already been shown to work with biomacromolecules, including another type of CA. The experiments will also only be performed in a small lab scale, without any real waste gas or a scrubber column. Only the part concerning the MOF-CA complex in the proposed process design in Figure 1.1 will be evaluated. The further conversion of HCO_3^- to $CaCO_3$ in the precipitation chamber will also not be addressed. Other than that no economic analysis will be performed.

2
Theory

2.1 Carbonic anhydrase

CA is an enzyme found in both prokaryotes and eukaryotes [2]. It is a metalloenzyme with a zinc metal ion in its active site. It is important in processes such as photosynthesis, pH homeostasis and electrolyte secretion.

2.1.1 Biomineralization

CA catalyzes the hydration of carbon dioxide at very high rates, as can be seen in Equation 2.1 [2].

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \tag{2.1}$$

During the biomineralization of CO_2, gaseous carbon dioxide is first dissolved in water, see Equation 2.2.

$$CO_2(g) \longleftrightarrow CO_2(aq) \tag{2.2}$$

In Equation 2.3 it can be seen that the dissolved carbon dioxide then forms carbonic acid (H_2CO_3) with water, which is dissociated into HCO_3^- and CO_3^{2-}, as seen Equation 2.4 and 2.5.

$$CO_2(aq) + H_2O \longleftrightarrow H_2CO_3 \tag{2.3}$$

$$H_2CO_3 \longleftrightarrow H^+ + HCO_3^- \tag{2.4}$$

$$HCO_3^- + OH^- \longleftrightarrow CO_3^{2-} + H_2O \tag{2.5}$$

The carbonate ions can then precipitate as a mineral carbonate, such as $CaCO_3$, when there is Ca^{2+} in the solution, see Equation 2.6.

$$CO_3^{2-} + Ca^{2+} \longrightarrow CaCO_3 \tag{2.6}$$

2.1.2 CA for industrial carbon capture

There are multiple aspects to consider when using CA for CO_2 capture and storage from industry. The enzymes could potentially be used freely in the solution or in whole cells, removing the need for enzyme purification [2]. However, for industrial-scale applications immobilization of CA would be more beneficial, since it would

make it possible to reuse the enzymes, improve their stability and enable easy separation from products.

The CAs can also be engineered for increased stability, such as halotolerance, by adding mutations [4]. Already natural existing CAs with desired characteristics, such as high thermostability, can be used as a starting point for conducting structure-based rational design or directed evolution. Additionally, immobilization techniques can be used to stabilize CA. One of the tested immobilization techniques include covalent bonding by chemical crosslinking. However, it can cause a loss in flexibility of the enzyme conformation, decreasing its activity. Another option is to use mesoporous materials because of their high specific surface area and variable pore size etc. Alternatively, materials known as MOFs can be used, which offer mild synthesis conditions and is the focus of this project.

An engineered enzyme with the desired properties in an immobilized form would presumably result in the best candidate for industrial applications. However, this project will only focus on the immobilization.

2.1.3 CA from *Sulfurihydrogenibium azorense*

In this project a CA from the thermophilic bacterium *Sulfurihydrogenibium azorense*, called SazCA, will be used. It is an α-CA and was during its time of characterization the fastest CA enzyme discovered with a $k_{\text{cat}} = 4.40 \times 10^6$ s^{-1} and $k_{\text{cat}}/K_M = 3.5 \times 10^8$ $\text{M}^{-1}\,\text{s}^{-1}$ [5]. It was also shown to exhibit a high thermostability, since it did not lose any catalytic activity even after hours of incubation at temperatures of $90 - 100\,^{\circ}\text{C}$. These properties make it suitable for industrial applications of CO_2 capture.

One study [6] investigated how sensitive free SazCA is to different metal ions, which is something that could affect its compatibility with MOFs. They found that 1.0 mM of Zn^{2+} severely inhibited the enzyme activity, with only 4.6 % activity remaining. The activity was even affected at 0.5 mM, but not as much, still keeping 75 % of its activity. All other metal ions tested also resulted in a decrease in the activity at a concentration of 1 mM, except for K^+ and Fe^{3+}, where the activity almost remained entirely (99.99 %) and even improved (131.41 %), respectively.

2.2 Metal-organic frameworks

MOFs have previously been shown to be able to encapsulate biomacromolecules, such as proteins, DNA and enzymes, while retaining their bioactivity [7]. MOFs are hybrid materials, made up of both organic and inorganic components and are chemically as well as thermally stable. They are especially appropriate to use with biomacromolecules like enzymes because of their large pore volume and open structure, facilitating their interaction with substrates in the surrounding environment.

The MOF, which will be used in this work, is called ZIF-8 and its structure can be seen in Figure 2.1. ZIF-8 is created by coordination between Zn^{2+} and 2-methylimidazole (2-MIM) [7]. The pore aperture diameter and pore diameter of ZIF-8 have been determined to be 3.4 Å and 11.6 Å, respectively [8]. The advantage with using ZIF-8 for encapsulating proteins is that it can be synthesized in a water-based solution and at room temperature. A lot of other MOFs require harsh synthesis conditions with solvents and high temperatures that could damage the protein.

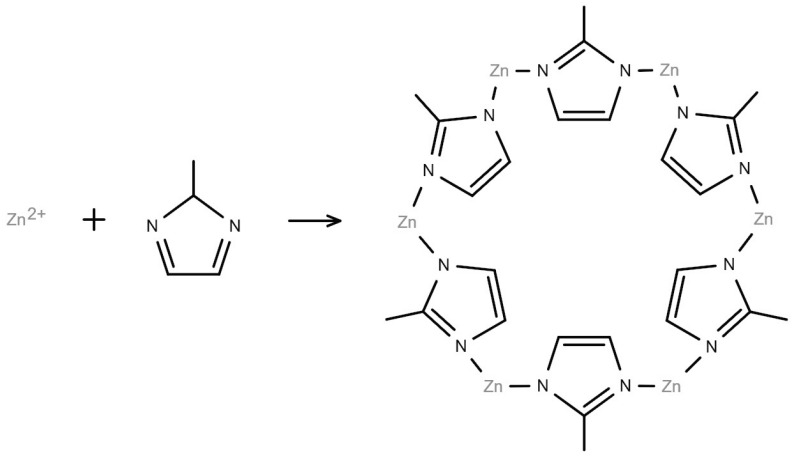

Figure 2.1: Synthesis of ZIF-8. Picture illustrated mostly in FreeChemDraw.com.

2.2.1 MOF-enzymes

There are mainly three different classes of MOF-enzymes [9]. Firstly, there are surface bound, called enzyme-on-MOF, in which either covalent bonds or noncovalent interactions are used to attach an enzyme to the surface of some MOF. Secondly, there are pore infiltration, called enzyme@MOF, in which the enzyme is placed within the pores of already synthesized MOF. Thirdly, there is the class which will be used in this project, encapsulation, called enzyme@MOF, which means that the enzyme is enclosed by the MOF crystals.

2.2.1.1 Enzyme encapsulation

Encapsulation of enzymes can be accomplished by synthesizing the MOF with the enzyme present. The enzyme then acts as the nucleus when the MOF is growing and gets encapsulated through a heterogeneous nucleation mechanism [9]. The pore size, where the enzyme is encapsulated, has also shown to affect how well the enzyme is protected and its activity. The size of the protein does not affect the size of the pore aperture in the MOF with this method. However, there are limitations when it comes to the size of the substrate that would need access to the enzyme. Also, because the entire synthesis needs to take place under biocompatible conditions, such as room temperature, this limits the types of MOFs that can be used. Whereas, during the synthesis of the other classes of MOF-enzymes mentioned, the steps involving MOF synthesis and the addition of the enzyme are separated, which allows for a

wider range of MOF materials. A big advantage with encapsulated enzymes is that they usually perform better than surface bound during recycling, with less leaching occurring during washing, etc. The decision to in this project encapsulate SazCA in MOF was taken for its increased protection from the environment, compared to binding the SazCA to the surface. Specifically, synthesizing the MOF together with the enzyme was chosen, because the SazCA being somewhat larger than 40 Å in all of its dimensions in its common dimer form [10] makes it too big to be able to enter the pores after the synthesis of ZIF-8, preventing the use of pore infiltration.

The specific encapsulation method, which is mainly investigated in this master's thesis, is called biomimetic mineralization. In this method the biomacromolecule itself promotes the growth of the MOF in water, in comparison to using additional additives and/or organic solvents [9]. The formation of the MOF-CA complex is facilitated by the biomacromolecules natural ability for biomineralization, which is, as previously mentioned, a self-assembly process occurring in a lot of living organisms to produce molecular structures providing support [7]. The MOF material ZIF-8 has been shown to work for this application and is suitable because of its good chemical and thermal stability as well as its high surface area.

2.2.1.2 Previous CA@ZIF experiments

Other research groups have already tested a few different methods and materials to synthesize CA@ZIFs, which have mostly been successful.

One group tested a protocol to encapsulated CA from bovine erythrocytes in ZIF-8 [11]. They mixed a 200 mL zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6\,H_2O$) water solution with the same volume of a 2-MIM and CA water solution, while using a ratio of 1 to 4. The mix was then vortexed for 10 s and aged in room temperature for 15 h. They found that the enzyme only retained about half of its activity in ZIF-8 compared to its free form. However, after measuring the activity after 1 h of incubation at 60 °C, it was shown that the CA@ZIF-8 retained about 90 % of its original activity, compared to free CA, which only retained about 8 %.

Another study, also using bovine CA (BCA), made the encapsulation by mixing 5 mL of the CA solution (in Tris buffer) with 2-MIM and zinc perchlorate hexahydrate ($Zn(ClO_4)_2 \cdot 6\,H_2O$) solutions in water [12]. Afterwards, they added 0.3 mL triethylamine (TEA). They then stirred the solution for 1 h and finally centrifuged it at 6 000 rpm for 20 min and washed the precipitate 3x with Tris buffer and water. The encapsulated BCA showed improved thermostability, as well as an increased storage stability and reusability. After 1 h of incubation at 55 °C the activity decreased with 45 % for the free BCA, while only decreasing with 14 % for the BCA@ZIF-8. The study also evaluated the effect of different MOF precursors on the activity of BCA. They found that at 110 mM $Zn(NO_3)_2$ the activity was reduced to only 6 % of its initial value. However, when 40 mM of $Zn(ClO_4)_2$ was tested, the activity remained at 80 %. The conclusion was drawn that it was the nitrate (NO_3^-) and not Zn^{2+} that caused the loss in activity. Using 650 mM 2-MIM the activity was shown to increase with 174 % and with 240 mM TEA 90 % of the activity remained.

Recently a group encapsulated the thermohpilic bacterium *Persephonella marina* carbonic anhydrase (PmCA) in a leaf-shaped zeolitic imidazolate framework (ZIF-L), which was made from 2-MIM and $Zn(NO_3)_2 \cdot 6\,H_2O$ with a molar ratio of 8:1 [13]. They found that using low protein concentrations of just 0.25 mg/mL, compared to 2 mg/mL lead to a much higher entrapment efficiency. They also concluded that the PmCA was mainly located inside the ZIF-L instead of at the surface. However, they also found that the enzymatic activity decreased in the PmCA@ZIF-L compared to the free CA, which they suggested might be because of diffusion/mass-transport limitations introduced by the MOF structure. If comparing the CO_2 hydration rate per μg at around 0.33 μg free PmCA to 0.25 mg/mL PmCA@ZIF-L at 0.4 μg PmCA, only about 32 % of the activity in the free PmCA can be estimated to remain in the encapsulated PmCA.

These studies indicate that encapsulating SazCA in ZIF-8 or ZIF-L should be possible and lead to an increase in thermostability, even if the initial activity might decrease compared to the free CA.

3
Materials and Methods

3.1 Protein production and purification

The *E. coli* strain BL21 was used to produce the enzyme SazCA. The strain contained the pET15b vector, in which the sequence for the SazCA protein had been added. The DNA sequence of SazCA had been codon optimized and connected to a 6xHis-tag for simpler purification, but other than that the protein was native. New SazCA was continuously produced during the project, since the the enzyme degraded after some time.

E. coli was first cultivated in Lennox broth (LB) media and the production of SazCA was induced by addition of isopropyl β-d-1-thiogalactopyranoside (IPTG). The produced SazCA protein was then purified using centrifugation, cell lysis, 6xHis-tag affinity chromatography with imidazole elution and finally desalting/buffer exchange with a PD10 desalting column. Most of the buffer was usually exchanged to a 2-MIM solution to be used during ZIF-8 synthesis. A smaller portion was stored in a buffer solution of 25 mM Tris-HCl (pH 8.0) or a solution called sol. A (solution A) to be used for testing the activity of free SazCA. The solution A contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 10 % (V/V) glycerol.

Gel electrophoresis was then conducted with the Mini-PROTEAN TGX Precast Gel to estimate the molecular weight of the protein in the sample. From this it was possible, with a high probability, to confirm if SazCA was present in the sample and its purity, since it is known that the SazCA protein should weigh about 26 kDa [5]. The purity of the sample was estimated with the help of Bio-Rad's Image Lab Software.

The protein concentration was then measured using the Bradford protein assay, following the standard protocol, with bovine serum albumin (BSA) as the protein standard. Up to 20 μL of the protein sample or standard were pipetted into cuvettes and mixed with 980 μL Bradford reagent. A spectrophotometer was then used to measure the absorbance and, based on the standard curve, the concentration of the sample solution could then be calculated. The standard curve was made by plotting the measured absorbance at 595 nm against the known BSA concentration (in μg/mL). The unknown concentration of protein in the SazCA samples could afterwards be calculated with Equation 3.1, where a is the slope, b is the intercept, A is the measured absorbance of the unknown sample and f is the fraction of the

added 20 μL containing the protein sample. This is because the SazCA sample sometimes had to be diluted for it to be within the acceptable absorbance range.

$$\text{Protein concentration } (\mu\text{g/mL}) = \left(\frac{A-b}{a}\right) \cdot \frac{1}{f} \tag{3.1}$$

3.2 Synthesis of ZIF-8

3.2.1 Pure ZIF-8

Some pure ZIF-8, without any protein, was made to use as a reference. Based on a protocol used in a previous study [14] one solution of 50 mL 120 mM zinc nitrate hexahydrate was made with 25 mL methanol and 25 mL ethanol. A second solution of 50 mL was made with 480 mM 2-MIM, which was also dissolved in 25 mL methanol and 25 mL ethanol. These solutions were then mixed through magnetic stirring during 1 hour. Afterwards the solution was centrifuged at 7 000 rpm for 10 minutes and the supernatant was removed. It was then washed 3x with ethanol and lastly dried overnight in 80 °C.

3.2.2 SazCA@ZIF-8

To start with four already established protocols (protocol 1-4), previously used to encapsulate protein in ZIF-8 or to synthesize pure ZIF-8, were used with some modifications to encapsulate the protein BSA in ZIF-8, for comparison. These protocols, along with protocol 5 were then also used with SazCA, with variations in volume and concentration of SazCA. The chemicals, concentrations and volumes used in protocols 1-5 can be found in Table 3.1. The final ratio between 2-MIM and zinc ions were 4:1, 70:1, 16:1, 60:1 and 3:1 for protocol 1-5, respectively.

Table 3.1: Protocols tested to synthesize ZIF-8 with encapsulated protein.

Protocol	Chemical	Concentration (mM)	Volume (mL)
1 [7]	2-Methylimidazole	160	20
	Zinc acetate dihydrate	40	20
2 [15]	2-Methylimidazole	2800	2
	Zinc nitrate hexahydrate	40	2
3[#]	2-Methylimidazole	792	20
	Zinc nitrate hexahydrate	50	20
4[*]	2-Methylimidazole	1660	18
	Zinc nitrate hexahydrate	250	2
5 [12]	2-Methylimidazole	970	5
	Zinc perchlorate hexahydrate	340	5
	Triethylamine	–	0.3

[#]From [16]. Same concentrations used as in the original protocol, but with only 2/3 of the original volume.
[*]From [17]. The ratio was kept the same, but the volume was reduced to 1/5 of the original.

The method used to synthesis protein@ZIF-8 following protocol 1-4 was very similar, with some small deviations from their original method to simplify simultaneous production. All of the solutions were made in Milli-Q water (MQ-water). To start with the protein was mixed with the 2-MIM solution, since it was shown to not impact the activity of SazCA negatively. As a starting point 2.5 mg protein per mL 2-MIM solution was used. This means that the final concentration was 1.25 mg/mL in protocol 1-3 and 2.25 mg/mL in protocol 4. This solution was then mixed with the zinc solution, providing the Zn^{2+}. In protocol 1 and 3 the 2-MIM solution was then added to the zinc solution and in protocol 2 and 4 they were mixed in the opposite order. The solution was then left overnight to give enough time for the formation of ZIF-8. In protocol 3 and 4 it was kept stirred/shaken and in protocol 1 and 2 it as kept still, after an initial brief stirring. It was then centrifuged at around 7 000 rpm for 10 minutes, after which the supernatant was discarded. Afterwards it was washed with MQ-water 3x. However, before the last wash the solution was divided into two tubes. One was resuspended in solution A +, for long term storage and activity testing. The sol. A + was made of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 50 % (V/V) glycerol. The other portion was left to dry completely for a few days, for later characterization with XRD and SEM.

In protocol 5 the production was done slightly differently from the rest. First 5 mL of SazCA (2 mg/mL) in a 25 mM Tris-HCl buffer (pH 8.0) solution was mixed with the 2-MIM and zinc solution, meaning that the final SazCA concentration was 0.67 mg/mL. TEA was then added directly afterwards. The solution was then only stirred for 1 h at 350 rpm and centrifuged at 6 000 rpm for 20 min, before finally being washed 3x with MQ-water or Tris-HCl buffer.

Some of the synthesized SazCA@ZIF-8 was also sonicated for 30 minutes at a frequency of 80 kHz and a power of 100 % before the enzymatic activity assay, in order to improve the separation of particles and increase the diffusion rate. An additional round of sonication was also tried for 10 minutes, with 37 kHz and 100 %.

3.3 Characterization of MOF-CA

3.3.1 X-ray diffraction

XRD can be used to characterize crystal structures as well as atomic spacing in crystalline materials [18]. The technique uses X-rays and the diffracted X-rays after interaction with the sample are detected. XRD was used to confirm if ZIF-8, or some other common ZIF had been synthesized.

The samples were prepared for XRD by grinding to a powder and put on a single crystal silicon holder. The powder XRD was done using a Bruker D8 Discover, with the following settings: 40 kV and 30 mA with Cu Kα radiation and a step speed of 5° per minute. The location of the peaks obtained from the XRD for the different samples were compared with a simulated ZIF-8 pattern to determine if they were ZIF-8. A simulation of ZIF-8 with the CCDC number 602542 was used.

The obtained data was first processed in a program called DIFFRAC.EVA, where the background was subtracted. The data was then imported to Origin where it was normalized (by dividing all the intensity data with the highest intensity) and plotted by stacking the data from multiple samples on top of each other for easier comparison.

3.3.2 Scanning electron microscopy

SEM can be used to investigate inorganic and organic solid morphology and crystallography by generating pictures up to 500 000 times magnification, at higher resolutions than visible-light microscopy [19]. An electron beam is sent at the sample, which creates secondary and backscattered electrons that are captured by detectors. SEM was used to determine the structure of the ZIF-8 complex.

The samples were prepared for SEM by first dissolving around 1-5 mg/mL of the sample powder in MQ-water. They were then sonicated at 37 kHz and 100 % for 10 minutes. Afterwards a few drops were put on a glass plate and left to dry. The plate was then put on a piece of carbon tape and coated with 5 nm gold using the Leica EM ACE600 Gold and Chrome Sputter machine, to prevent charging. The SEM machine JEOL JSM-7800F Prime FEG was then used at 10 kV. Magnifications of around 30 000x to 75 000x were used to investigate a few different locations on every sample and photos were taken.

3.4 Encapsulation efficiency

To investigate how much of the added SazCA was encapsulated in ZIF-8 the protein concentrations were measured with the Bradford protein assay. Both the concentration in the final SazCA@ZIF-8 solution along with all of the supernatants collected from centrifuging during the washing were measured. The calculated amount of SazCA remaining in the synthesized SazCA@ZIF-8 was then compared to the original amount of SazCA added during the synthesis.

At low concentrations the measurements were made with another more sensitive version of the Bradford protein assay, with another standard curve. In this version 500 μL of the Bradford reagent was mixed with up to 500 μL of the sample.

3.5 Catalytic activity testing

The catalytic activity in the free SazCA and in the SazCA@ZIF-8 was tested using the Wilbur-Anderson units assay [20]. In the Wilbur-Anderson units assay the time it takes for the pH to drop from around 8 to 6.3 in a buffer with a CO_2 saturated solution is recorded. This is compared to how long the same drop in pH takes when SazCA is added. However, there is also another common method to test the enzymatic activity in CA, which was considered. This method uses p-nitrophenyl acetate (pNPA) as a substrate. In this method the bioactivity is measured using a

UV/vis 96-well plate reader by loading the wells with the CA samples to an assay buffer with pNPA. pNPA can be used because the active site of CA also has esterase activity [21]. So one could assume that if it can perform this reaction it should also be able to convert CO_2. Compared to the Wilbur-Anderson units assay it is easier to do in a larger scale. However, since the Wilbur-Anderson units assay directly uses CO_2 as a substrate it gives more reliable results for the purpose of this project, and was thus chosen as the preferred method.

The assay was done by first adding a small amount of the protein sample, around 50 μL (usually diluted with MQ-water), to 3 mL of a working buffer. The working buffer contained 0.001% w/v bromothymol blue, which acts as the pH indicator, changing from blue to yellow when the pH is lowered. It also contained 1 mM NaOH and 25 mM Tris-HCl buffer (pH 8.0). 2 mL of a CO_2 saturated solution was then added to the working buffer. This solution was prepared by adding dry ice to a cooled bottle containing MQ-water. The solution was kept cold in an ice box during the experiments. The Wilbur-Anderson units for each sample was calculated using Equation 3.2, where t_{control} is the time it takes for the color to change in the control (no SazCA added) and t_{sample} is the time it takes for it to change when the sample is added, both measured in seconds.

$$\text{Wilbur-Anderson units (WAU)} = \frac{t_{\text{control}} - t_{\text{sample}}}{t_{\text{sample}}} \tag{3.2}$$

The obtained WAU could be converted to WAU/mg SazCA by dividing with the amount of SazCA used. The calculated Wilbur-Anderson units for the samples were then compared to those for the free SazCA from the same batch, since there could be a small variation between batches.

The calculations and statistical analysis were done in Excel. The relative enzymatic activity was calculated by dividing the WAU per mg SazCA in SazCA@ZIF-8 with that of free SazCA. Depending on the amount of replicates used during each experiment, different statistical analyses were done. If two replicates ($n = 2$) were used the mean relative enzymatic activity was calculated and the range given. When at least two ($n \geq 2$) measurements of the time had been made, while not being paired, the mean time was calculated and based on that one activity in WAU was calculated. To plot the data, showing the thermostability in graphs, the program Origin was used.

3.6 Thermostability testing

Lastly, the thermostability was tested by exposing the samples to high temperatures for 1 and 4 hours. The samples were heated using a PCR machine to 75, 85 and 95 °C. This was done with the synthesized SazCA@ZIF-8 and free SazCA, along with pure ZIF-8 to use as a reference.

4

Results and Discussion

4.1 SazCA produced and purified

The production and purification of SazCA was successful throughout the project. An example of a gel image can be seen in Figure 4.1, where the bands showing SazCA have been marked with an arrow. It can be seen that they match the protein weight expected for SazCA in the ladder (around 26 kDa). The purity of the SazCA was always found to be above 95 %. After conducting gel electrophoresis after the first purifications, which consistently showed a high purity of the correct protein, no further gel electrophoresis was necessary for the following productions. The presence of SazCA was instead confirmed from the activity assay.

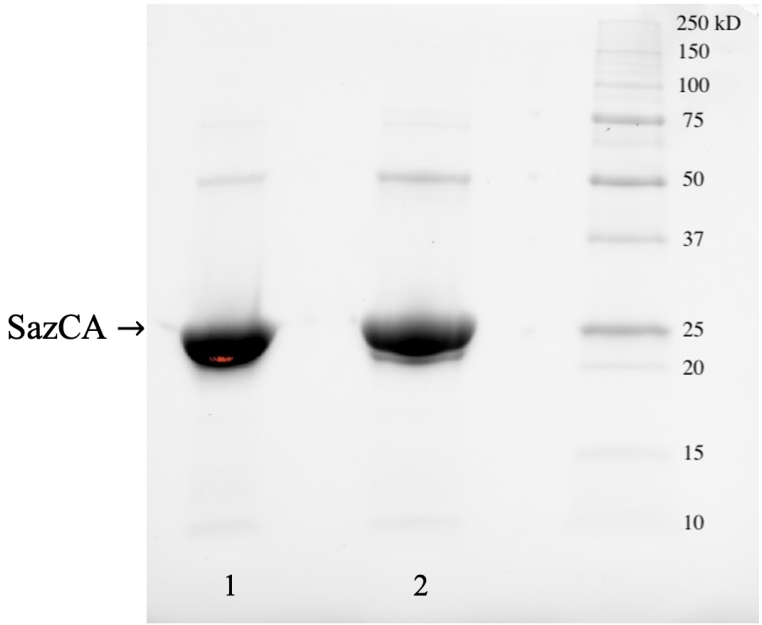

Figure 4.1: Image of a gel with purified SazCA and a protein ladder. Both lane 1 and 2 are from samples with purified SazCA.

The obtained protein concentration was slightly different every time, but it always remained more than high enough for the desired SazCA concentration during ZIF-8 synthesis (0.25-4.5 mg SazCA/mL). An example of a Bradford standard curve created can be found in Figure 4.2.

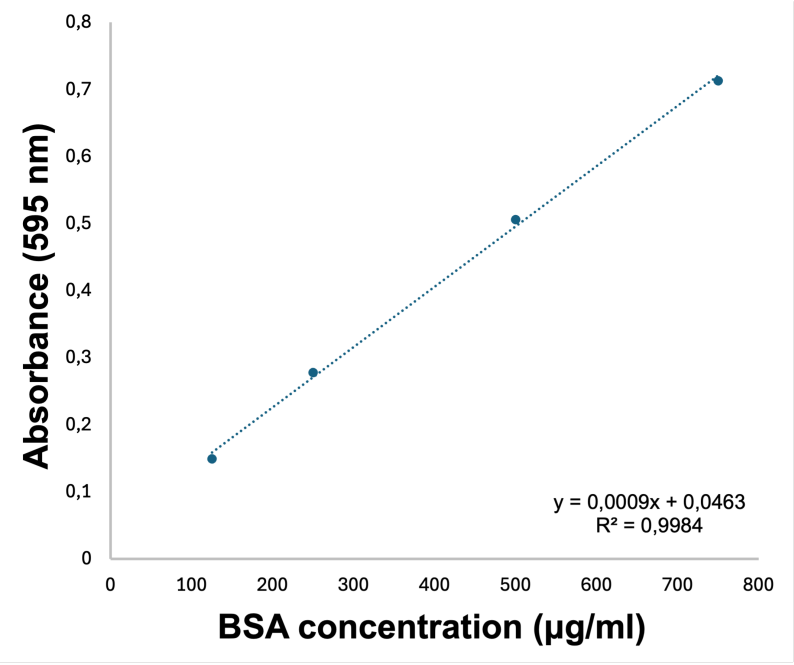

Figure 4.2: Bradford standard curve.

In one sample the absorbance was measured to 0.654 and 1 μL out of the 20 μL added to the Bradford reagent was from this sample, meaning that the fraction was 0.05. From this, the protein concentration was calculated with Equation 3.1 to be 13.5 mg/mL, see Equation 4.1.

$$\text{Protein concentration } (\mu\text{g/mL}) = \left(\frac{0.654 - 0.0463}{0.0009}\right) \cdot \frac{1}{0.05} = 13.5 \text{ mg/mL} \quad (4.1)$$

4.2 Evaluation of SazCA@ZIF-8 synthesized by protocol 1-5

Protocol 1-5 were evaluated through XRD, followed by SEM and catalytic activity testing for those indicating presence of ZIF-8.

4.2.1 Identification of ZIF-8 in protocol 2, 4 and 5

As can be seen in Figure 4.3 the position of the peaks of the pure ZIF-8, synthesized as explained in Materials and Methods 3.2.1, match perfectly with the simulated ZIF-8. The XRD of protocol 1-4 using 2.5 mg BSA/mL 2-MIM solution, following the method described in Materials and Methods 3.2.2, shows that protocol 2 and 4 were successful in producing ZIF-8, since the location of their peaks aligns with those of the simulated ZIF-8. However, the XRD data following protocol 1 and 3 did not seem to match any known ZIF.

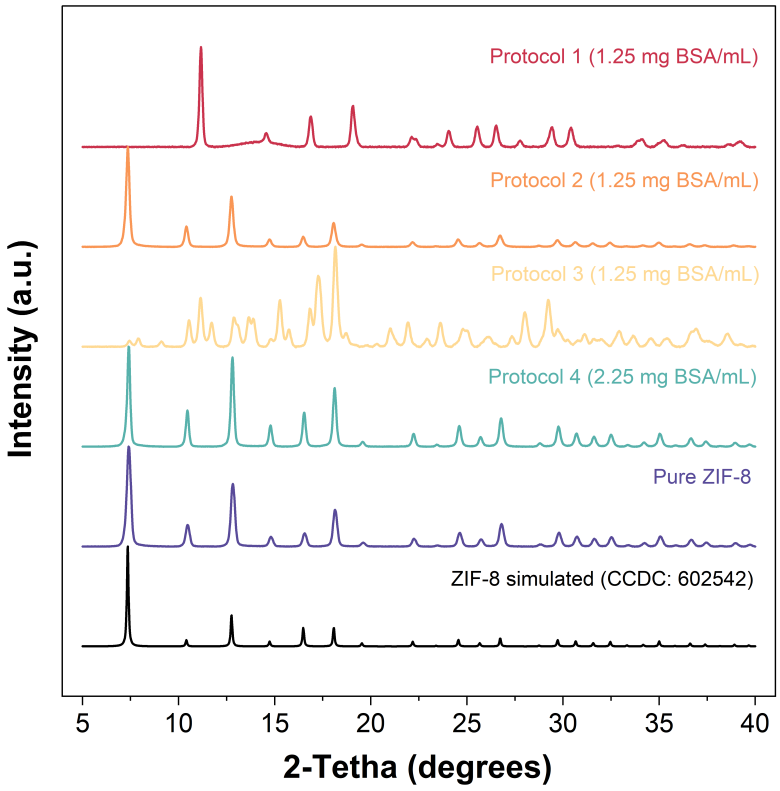

Figure 4.3: Powder XRD patterns of simulated ZIF-8, along with pure ZIF-8 and samples synthesized following protocol 1-4 with BSA.

In Figure 4.4 the XRD of the samples synthesized with SazCA can be found. It can be seen that the pattern in protocol 2 and 4 matches that of the pure and simulated ZIF-8, whereas the pattern from protocol 1 and 3 are very similar to the same ones made with BSA, which did not align with the ZIF-8 pattern. Protocol 5 was only used with SazCA and shows a lot of peaks in the same position as the simulated and pure ZIF-8. However, it also has a few extra peaks. A likely explanation is that some ZIF-8 was synthesized, but it is not as pure as that in protocol 2 and 4.

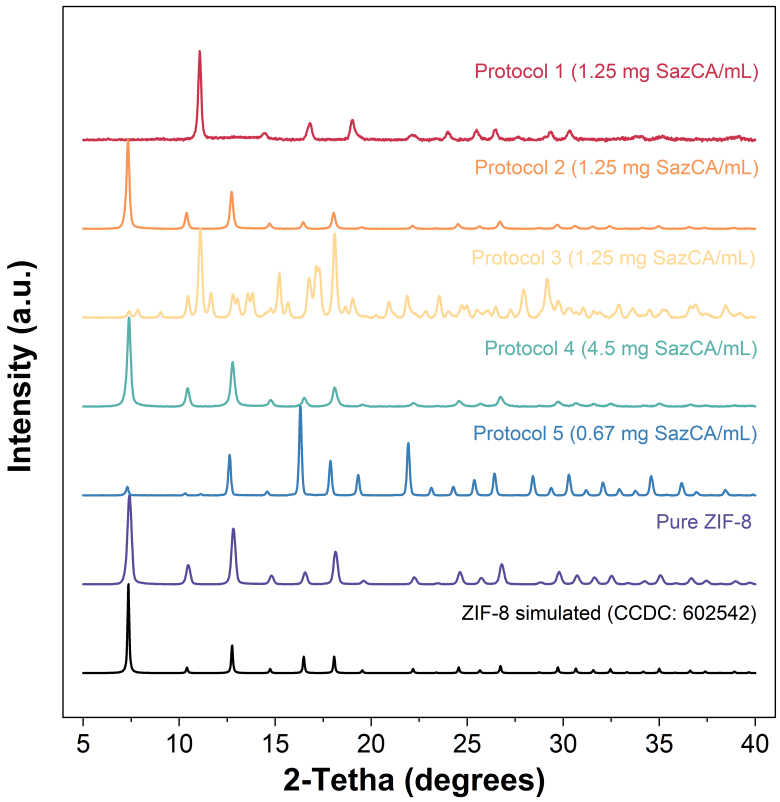

Figure 4.4: Powder XRD patterns of simulated ZIF-8, along with pure ZIF-8 and samples synthesized following protocol 1-5 with SazCA.

When it comes to encapsulating BSA or SazCA in ZIF-8 it seems like a higher ratio of 2-MIM to zinc ions is to be preferred, since protocol 2 and 4 used the highest ratio out of the evaluated protocols.

It is also worth mentioning that during the synthesis with SazCA the volume for some protocols deviated from the volumes mentioned in Materials and Methods 3.2.2, because of difficulties obtaining enough protein during the production and purification. However, the same concentrations were still used and since the results were consistent with those using BSA, this suggests that the volume does not make any difference in these cases. Throughout the rest of the experiments, even if the volume deviates from the one stated in Materials and Methods 3.2.2, it will still just be referred to as coming from that protocol, as long as the concentration was kept the same. Other bigger changes to the standard protocol will however be mentioned.

4.2.2 Visualization of nanoparticles with varied sizes and shapes

Because only protocol 2 and 4 resulted in pure SazCA@ZIF-8 only these, along with pure ZIF-8 as a reference, were inspected with SEM. The SEM micrographs of pure ZIF-8, can be found in Figure 4.5. It can be noted that the particles are very uniform in size and shape, with most exhibiting a cubical shape.

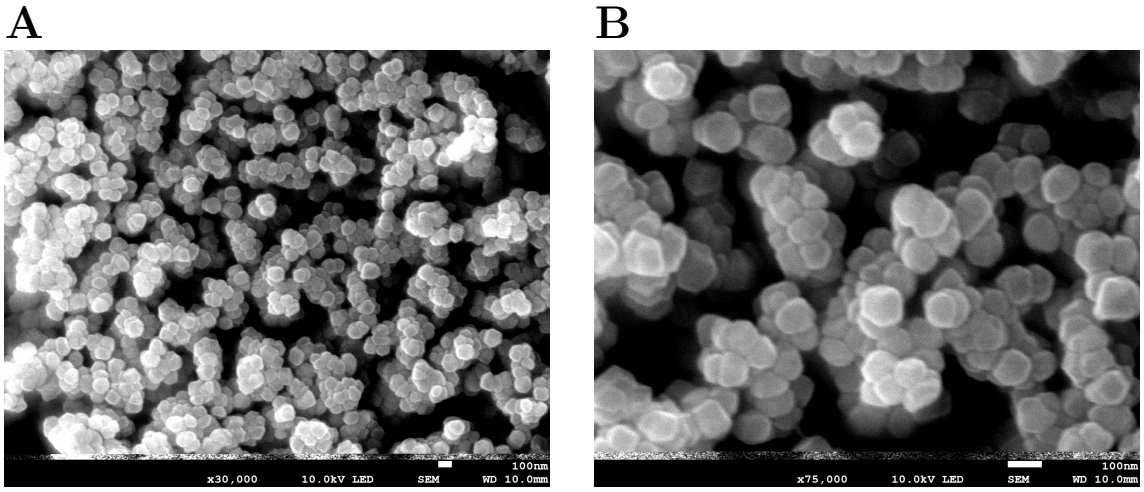

Figure 4.5: SEM micrographs of ZIF-8 synthesized with the protocol for pure ZIF-8 at 30 000× magnification (**A**) and 75 000× magnification (**B**).

SEM micrographs of SazCA@ZIF-8 synthesized following protocol 2 (1.25 mg SazCA/mL) show that most of its particles have a hexagonal, cubical and spherical shape, see Figure 4.6. They look similar to those from pure ZIF-8 in Figure 4.5. They are mostly uniform in size, with some variation. However, the particles seem to be considerably larger than those in Figure 4.5.

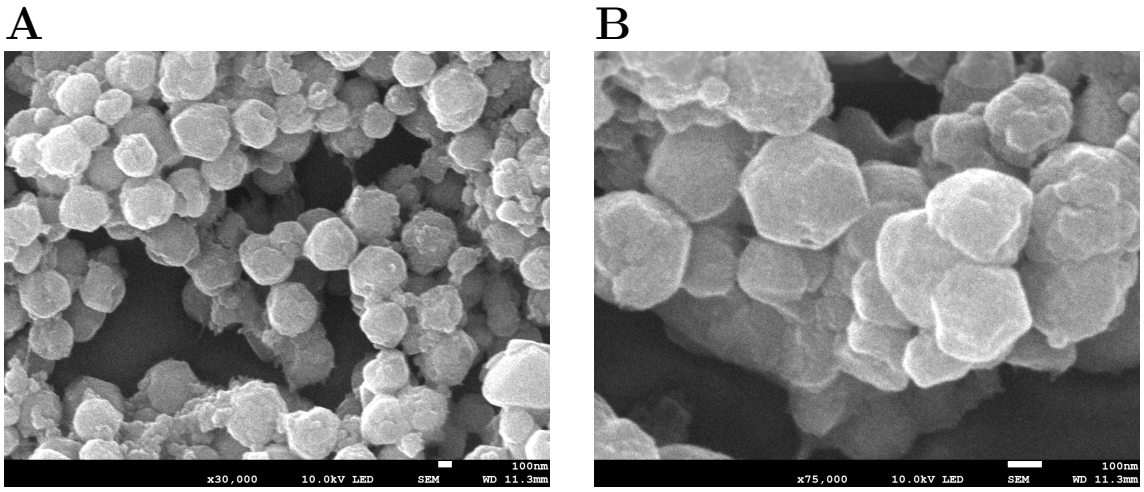

Figure 4.6: SEM micrographs of SazCA@ZIF-8 synthesized with protocol 2 at 30 000× magnification (**A**) and 75 000× magnification (**B**).

It can be seen in the SEM micrographs of SazCA@ZIF-8 synthesized using protocol 4 (4.5 mg SazCA/mL), found in Figure 4.7, that the size of its particles varies a lot. The shape of the particles also varies, with some looking more cubical and round and others looking more porous. The shape of these particles deviates more from those in the pure ZIF-8, see Figure 4.5, compared to those synthesized with protocol 2.

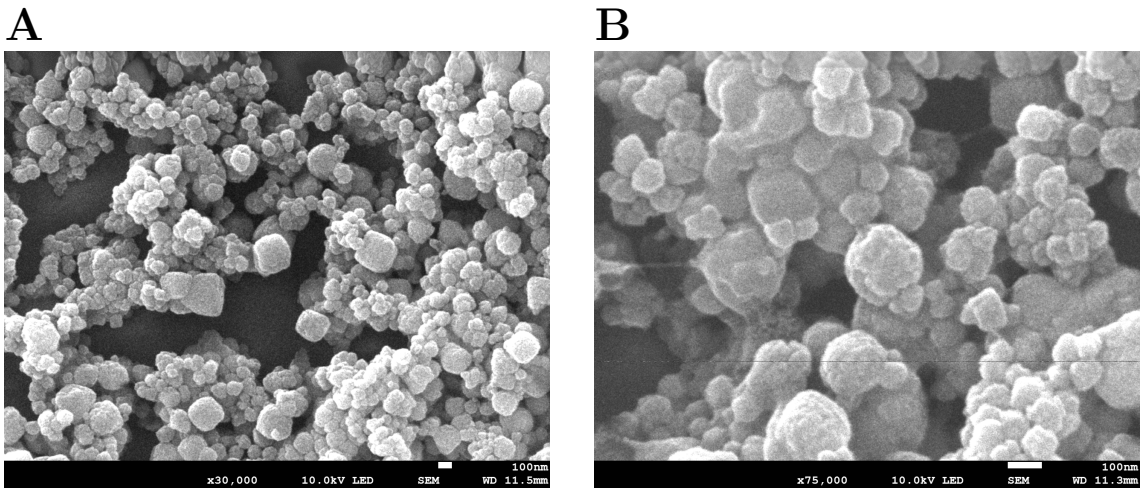

Figure 4.7: SEM micrographs of SazCA@ZIF-8 synthesized with protocol 4 at 30 000× magnification (**A**) and 75 000× magnification (**B**).

4.3 Catalytic activity testing

4.3.1 Activity in SazCA@ZIF-8 from protocol 2, 4 & 5

The relative enzymatic activity retained in SazCA@ZIF-8 synthesized with protocol 2 (2.5 mg SazCA/mL), 4 (4,5 mg SazCA/mL) and 5 can be found in Table 4.1. It is worth mentioning that the SazCA@ZIF-8 synthesized with protocol 2 was by accident made using 3.0 M 2-MIM solution instead of 2.8 M and that the SazCA@ZIF-8 synthesized with protocol 2 and 4 were resuspended in sol. A +, while the sample synthesized with protocol 5 was resuspended in sol. A.

Table 4.1: Relative enzyme activity of SazCA@ZIF-8 synthesized with protocol 2, 4 and 5, compared to free SazCA. The free SazCA was kept in sol. A for protocol 2 & 4 and in 25 mM Tris-HCl for protocol 5. Based on calculated Wilbur-Anderson units found in Table A.1 and A.2 in Appendix A.1 (n = 1).

Sample	**Relative activity (%)**
Free SazCA	100
Protocol 2 (2.5 mg/mL)	5.08
Protocol 4 (4.5 mg/mL)	2.75
Protocol 5 (0.67 mg/mL)	11.82

The Wilbur-Anderson units were calculated as explained in Equation 3.2 in Materials and Method 3.5. For example, one time using free SazCA the time it took for the color change to occur was 21 seconds, while for the control it took 69 seconds. From this, the Wilbur-Anderson units could be calculated in Equation 4.2 to 2.29 WAU.

$$\text{Wilbur-Anderson units (WAU) for free SazCA} = \frac{69 - 21}{21} = 2.29 \tag{4.2}$$

Since 0.0011 mg SazCA was used, the activity per mg SazCA could then be calculated to 2056.27 WAU/mg SazCA by simple division.

The results show that the highest maintained activity was achieved following protocol 5. However, even for protocol 5 the enzymatic activity is only 11.82 % of that in free SazCA. When comparing these results to those in the older studies mentioned in Theory 2.2.1.2, it can be found that the activities achieved in this experiment are a lot lower. The lower activity compared to free SazCA could partly be because the structure of the MOF itself limits the mass-transport of CO_2 into the enzyme, as was suggested by another research group. Another explanation could be that during the synthesis of ZIF-8 the SazCA gets trapped in its inactive conformation in a way that also hinders its conformational change to its active form, which is when the CO_2 can reach the active site. Additionally, one or more of the chemicals used during the synthesis could be inhibiting the enzyme, which will be investigated in the next section. The reason for the activity being even lower than in the previous studies could be because the type of CA used in this project is different and might be more sensitive than the other ones tested.

4.4 Factors affecting enzyme activity

To determine if any of the chemicals used during the synthesis or if the conditions, such as the temperature, influence the activity, additional enzymatic activity assays were performed. The protein concentration was kept at 2.5 mg/mL in the stated concentration of metal ions or organic linker. This mix was then diluted, before 50 μL was finally added to the buffer solution. This means that the actual final concentration was significantly lower than the stated one, but the ratio between the SazCA and the investigated chemical was still the same.

4.4.1 Zinc ions inhibit the activity

It can be seen in Table 4.2 that the activity decreases severely compared to free SazCA in all 25 mM zinc solutions. It is most noticeable in zinc acetate dihydrate ($Zn(CH_3COO)_2$) and zinc nitrate hexahydrate, where only about 22 % of the original activity remains, but it can also be observed in $ZnCl_2$. Additionally, magnesium chloride ($MgCl_2$) was tested as a reference to establish if it is the zinc ion inhibiting the enzyme or its anion. When comparing the activity in $ZnCl_2$ and $MgCl_2$ it is clear that even if the activity is also lower in $MgCl_2$ compared to the free SazCA, it is more than twice of the activity in $ZnCl_2$. This implies that it is mainly the zinc

ion that is inhibiting the enzyme.

Even as low concentrations of zinc nitrate hexahydrate as 0.5 and 1 mM were tested and showed a decrease in activity, see Table 4.2. This is in line with a previous study, mentioned in Theory 2.1.3, which also found that the activity of SazCA was negatively affected by Zn^{2+}. This is despite the fact that the enzyme has zinc ion in its active site, which should suggest that the enzyme has some tolerance for zinc ions. However, the zinc ion only takes up a very small portion of the entire enzyme, making it reasonable that its activity could be inhibited when the outer environment contains zinc ions, even at low concentrations. This is contrary to the conclusion another study, which was mentioned in Theory 2.2.1.2, came to. They claimed that it was the nitrate causing the loss in activity, whereas the results in this project indicate that it was at least partly the zinc ions. However, they used BCA and not SazCA, which might have a different tolerance.

Table 4.2: Relative enzyme activity of SazCA exposed to different zinc and magnesium compounds, compared to free SazCA in sol. A. Based on calculated Wilbur-Anderson units found in Table A.3 and A.4 in Appendix A.1 with n = 2, but not paired data.

Sample	**Concentration (mM)**	**Relative activity (%)**
Free SazCA	-	100
$ZnCl_2$	25	31.59
$MgCl_2$	25	69.42
$Zn(CH_3COO)_2$	25	21.58
$Zn(NO_3)_2$	25	21.68
$Zn(NO_3)_2$	1	26.93
$Zn(NO_3)_2$	0.5	55.08

However, when testing the activity of SazCA in the zinc nitrate solution after the addition of Ethylenediaminetetraacetic acid (EDTA), it can be seen in Table 4.3 that at 5 mM almost all of the activity returns. At the higher concentrations of 10 mM and 20 mM the activity is even higher than in free SazCA. This demonstrates that the inhibition is reversible, which means that even if the activity is initially lowered when mixing SazCA with zinc during ZIF-8 synthesis, if all free zinc ions are removed in the end, the activity might be restored. The most probable explanation for EDTA's ability to restore the activity is that it binds directly to the zinc ions and removes them from the SazCA.

Table 4.3: Relative enzyme activity of SazCA exposed to 25mM zinc nitrate hexahydrate with different concentrations of EDTA, compared to free SazCA in sol. A. Based on calculated Wilbur-Anderson units found in Table A.4 in Appendix A.1 with n = 2, but not paired data.

Sample	Concentration of EDTA (mM)	Relative activity (%)
Free SazCA	-	100
$Zn(NO_3)_2$ + EDTA	20	185.41
$Zn(NO_3)_2$ + EDTA	10	232.5
$Zn(NO_3)_2$ + EDTA	5	89.67
$Zn(NO_3)_2$ + EDTA	1	6.82

4.4.2 2-Methylimidazole increases the activity

The activity of SazCA can be noted in Table 4.4 to increase with higher 2-MIM concentrations. This is in line with what a previous study, mentioned in Theory 2.2.1.2, found when mixing BCA with 2-MIM. An explanation for this increase in activity could be that the 2-MIM binds to some location on the enzyme that changes its confirmation in a way that makes it easier for the substrate (CO_2) to reach the active site.

Table 4.4: Relative enzyme activity of SazCA exposed to different concentrations of 2-MIM, compared to free SazCA in sol. A. Based on calculated Wilbur-Anderson units found in Table A.3 in Appendix A.1 with n = 2, but not paired data.

Sample	Concentration (M)	Relative activity (%)
Free SazCA	-	100
2-MIM	1.7	292.84
2-MIM	0.6	261.32
2-MIM	0.3	201.43

4.4.3 Temperature during synthesis does not significantly affect the activity

It can be seen in Table 4.5 that the activity is almost completely unaffected after the free SazCA was stored either 2 days in room temperature or 1 day in 37 °C, compared to SazCA that had been stored as normally on ice in a 4 °C room. These results are not completely unexpected, since the enzyme is known for its high thermostability.

Table 4.5: Relative enzyme activity of free SazCA in sol. A stored at different temperatures, compared to free SazCA stored on ice in 4 °C. Based on calculated Wilbur-Anderson units found in Table A.5 in Appendix A.1 (n = 1).

Sample	Relative activity (%)
Free SazCA on ice in 4 °C	100
Free SazCA room T	95.11
Free SazCA 37 °C	95.11

4.4.4 Additional metal ions and organic linkers

The activity was also tested after mixing SazCA with the metal ions Ca^{2+} and Fe^{3+}, as well as the organic linker fumaric acid. Fe^{3+} and fumaric acid were evaluated, since they can be used to synthesize another MOF material, called MIL-88A. It can be seen in Table 4.6 that the iron(III) chloride hexahydrate ($FeCl_3 \cdot 6\,H_2O$) almost completely inhibits the SazCA. Fumaric acid and calcium chloride dihydrate ($CaCl_2 \cdot 2\,H_2O$) only slightly inhibits the SazCA, since more than half of their original activity remains. Based on these findings it is also probable that synthesizing MIL-88A instead of ZIF-8 would involve the same challenges with inhibition from the metal ion. It is clear that out of the tested metal ions, Ca^{2+} would be the best choice for MOF synthesis to retain the highest activity.

Table 4.6: Relative enzyme activity of SazCA exposed to calcium ions, iron ions and fumaric acid, compared to free SazCA in 25 mM Tris-HCl. Based on calculated Wilbur-Anderson units found in Table A.6 in Appendix A.1. All except the free SazCA (with n = 1) had n = 2, but not paired data.

Sample	Concentration (mM)	Relative activity (%)
Free SazCA	-	100
$FeCl_3$	25	0.89
Fumaric acid	25	64.17
$CaCl_2$	25	70.92

4.5 Optimization of the protocol

A few different measures were taken to see if the enzymatic activity of the SazCA@ZIF-8 could be improved.

4.5.1 Powder form decreases the activity

Instead of directly resuspending the synthesized SazCA@ZIF-8 after centrifugation, an alternative approach was tried, in which the SazCA@ZIF-8 was left to dry completely and was then resuspended before the activity assay. This method seemed to have been used in some of the previous studies and the belief was that by letting the sample dry first this might impact the structure and make the free zinc ions more tightly bound to the MOF.

This approach was tested using SazCA@ZIF-8 synthesized with protocol 2 (1.25 mg SazCA/mL). The sample was dried by incubating it in 37 °C overnight and 10 mg was then resuspended in 1 mL MQ-Water. The activity remaining was only measured to be 0.62 % compared to free SazCA in sol. A. The calculated Wilbur-Anderson units can be found in A.5 in Appendix A.1 (n = 1).

It is worth mentioning that the dry particles were noticed to not fully dissolve, which could both affect the measurement of the concentration and the activity assay, so

these results might not be as reliable. However, it should have still been observed if the activity had been significantly improved.

4.5.2 Adding SazCA after mixing marginally improves the activity

Another approach that was investigated was to add the SazCA after mixing the 2-MIM and zinc solutions, to avoid exposing the SazCA to very high concentrations of free zinc ions. The synthesis was done following protocol 2 (1.64 mg SazCA/mL), except that SazCA in 25 mM Tris-HCl was added 15-30 min after the 2-MIM and zinc solutions were mixed. Zinc chloride was also used instead of zinc nitrate hexahydrate. The mix was stirred for 1 h and 30 min in total and centrifuged at 7 000 rpm for 20 min the first time and then washed with MQ-water at 7 000 rpm for 10 min, each time. It was then stored in sol. A for activity testing.

The Wilbur-Anderson unit assay revealed that 6.98 % of the activity remains compared to free SazCA in 25 mM Tris-HCl. The calculated Wilbur-Anderson units can be found in A.2 in Appendix A.1 (n = 1). This is somewhat more than the 5.08 % achieved before, but it is not high enough to make any significant difference and it is hard to tell which of the changes contributed the most to this slight increase.

4.5.3 Addition of EDTA slightly improves the activity

EDTA was added during the synthesis of SazCA@ZIF-8 with protocol 2 (1.25 mg SazCA/mL), because of EDTA's proven ability to restore the activity in SazCA after it was mixed with zinc in Results and Discussion 4.4.1. Additionally, this time the SazCA in sol. A was first added to the zinc solution instead of the 2-MIM solution. After mixing with the 2-MIM solution the EDTA was added, so that the final concentration reached 10 mM. During washing it was washed with solution A and finally, the synthesized SazCA@ZIF-8 was resuspended in sol.A +.

The activity assay shows that 10.77 % of the activity compared to free SazCA in sol. A, remains. The calculated Wilbur-Anderson units can be found in A.5, see Appendix A.1 (n = 1). Even though it is a low number, it is the highest activity measured for SazCA@ZIF-8 synthesized with protocol 2. However, there is a risk that the addition of EDTA could affect the synthesis of ZIF-8 and destabilize the MOF structure. This theory can't be confirmed or discarded, since this sample was never characterized with XRD or SEM.

4.5.4 Sonication doubles the activity retained

Sonication was tried on a sample synthesized following protocol 5. After the synthesis the sample had been put in a solution with EDTA in 25 mM Tris-HCl buffer. The final concentration of EDTA was 50 mM. It was then stirred by a magnetic stirrer at 350 rpm overnight. This was done to allow the free zinc ions to diffuse out into the solution, which might be facilitated by EDTA. The next day the mixture was

centrifuged and then washed 1x with MQ-water, before being resuspended in sol. A.

After sonication for 30 minutes as described in Materials and Methods 3.2.2 the activity improved with 122.41 % and after one additional sonication for 10 minutes, the improvement was slightly smaller (94.08 %). The calculated Wilbur-Anderson units can be found in A.7 in Appendix A.1 with n = 3, but no paired data. The improvement is likely due to an increase in the dispersion of the particles, which increases the diffusion rate, by making it easier for the CO_2 to reach the SazCA located inside the MOF.

Even though the material tested was not entirely made up of pure ZIF-8, since it was made following protocol 5, one can still assume that sonication of the SazCA@ZIF-8 would lead to an improved activity for any synthesis protocol followed. This makes it the most promising addition for improved enzymatic activity.

4.6 Evaluation of SazCA@ZIF-8 from protocol 2

Protocol 2 was decided to be used to synthesize the SazCA@ZIF-8 used to test the thermostability. This protocol was chosen because out of the protocols that had confirmed pure SazCA@ZIF-8 from the XRD, its SEM micrographs showed the most desirable structure. It also had a somewhat higher relative activity. Samples synthesized with protocol 2 at 0.25 and 1.25 mg SazCA/mL, as well as without any protein, were used. However, the synthesis method deviated somewhat from the standard one, since they were stirred for 1 h at 600 rpm after mixing and then centrifuged. They were then resuspended and stored in 25 mM Tris-HCl.

4.6.1 Confirmation of ZIF-8 through X-ray diffraction

The XRD pattern for the samples synthesized with protocol 2 using 1.25 mg SazCA/mL and without any protein can be found in Figure 4.8. It can be seen that their pattern matches that of pure and simulated ZIF-8 very well.

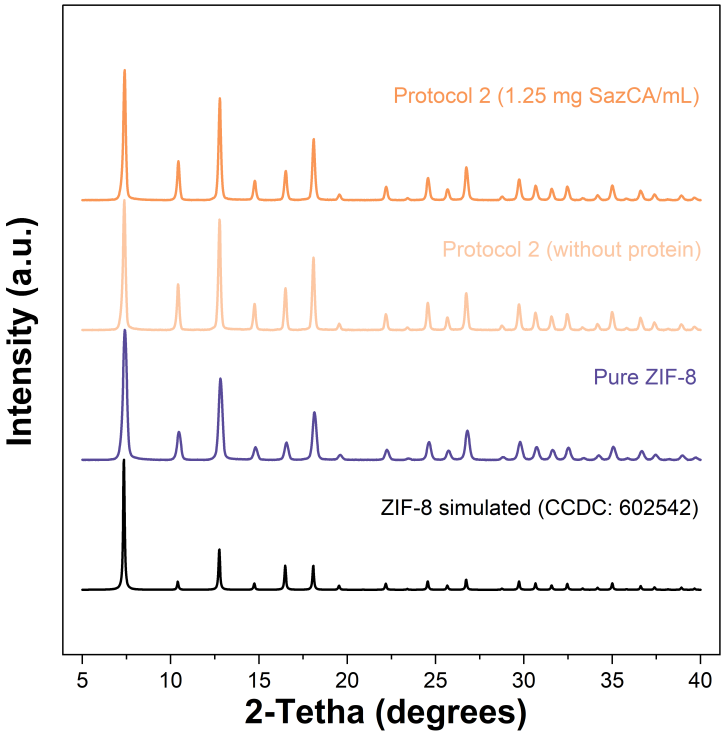

Figure 4.8: Powder XRD patterns of simulated ZIF-8, along with pure ZIF-8 and samples synthesized with protocol 2.

4.6.2 Visualization of the ZIF-8 nanoparticles

The micrographs of ZIF-8 synthesized with protocol 2 without any protein can be found in Figure 4.9. It can be noted that the particles are of mostly uniform size and shape. They have mostly a cubical shape, similar to those of pure ZIF-8 shown in Materials and Methods 4.2.2.

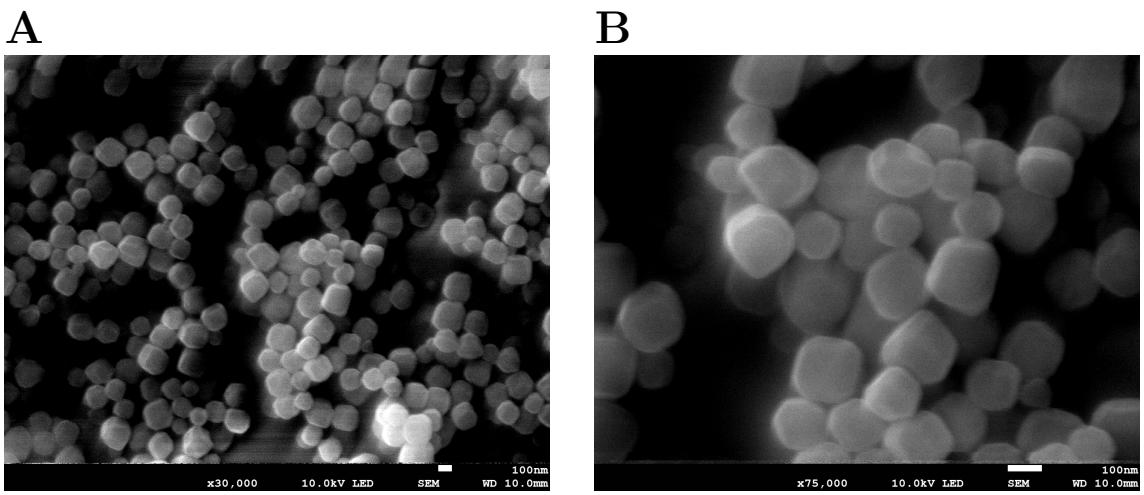

Figure 4.9: SEM micrographs of ZIF-8 synthesized with protocol 2 without protein at 30 000× magnification (**A**) and 75 000× magnification (**B**).

The particles of SazCA@ZIF-8 synthesized with protocol 2 (1.25 mg SazCA/mL) can be seen in the micrographs in Figure 4.10. It can be noted that the particles here are also mostly uniform in size and shape. The shapes are mostly round and cubical. The size variation is possible slightly larger compared to the particles in Figure 4.9, which could be due to the effects of SazCA.

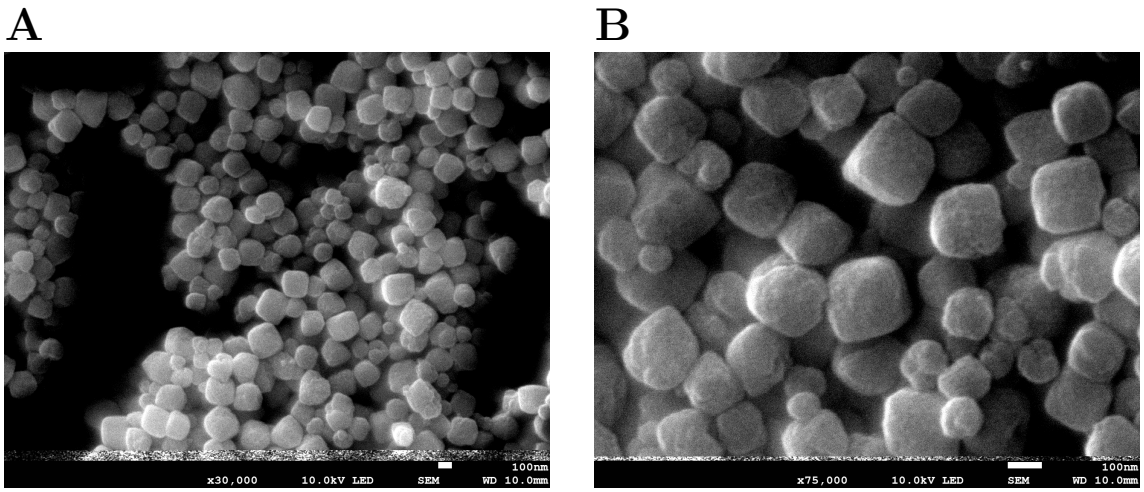

Figure 4.10: SEM micrographs of SazCA@ZIF-8 synthesized with protocol 2 (1.25 mg SazCA/mL) at 30 000× magnification (**A**) and 75 000× magnification (**B**).

4.6.3 Low encapsulation efficiency of SazCA

Samples synthesized with protocol 2 at 0.25 and 1.25 mg SazCA/mL, as well as without any protein, were used to determine the encapsulation efficiency. In the synthesized SazCA@ZIF-8 with 1.25 mg/mL the final amount of SazCA was measured to be 5.17 mg through the Bradford protein assay. However, since 1/4 of the synthesized SazCA@ZIF-8 was dried for analysis with XRD, the actual total should have been close to 6.89 mg. The original amount added was 50 mg. This means that the encapsulation efficiency is 6.89/50 = 13.78 %. However, the total amount measured in all of the discarded supernatants, which can be seen in Table 4.7, only equals 3.71 mg. This means that 50 - 6.89 - 3.71 = 39.4 mg SazCA remain unaccounted for.

Table 4.7: Amount of protein detected by the Bradford protein assay during the synthesis of SazCA@ZIF-8 following protocol 2 (1.25 mg/mL). Supernatant 1 was collected after the first centrifugation. Supernatant 2, 3 and 4 were collected after the first, second and third centrifugation during the washing.

Sample	Concentration (mg/mL)	Volume (mL)	Amount (mg)
Supernatant 1	0.090	37.5	3.38
Supernatant 2	0.0085	9.25	0.079
Supernatant 3	0.011	8.75	0.096
Supernatant 4	0.015	10.25	0.15
Total			3.71

If the same calculations are done for the SazCA@ZIF-8 synthesized with 0.25 mg/mL, where 10 mg was originally added, whose amount in the supernatants can be seen in Table 4.8, the encapsulation efficiency is 14.1%, while 4.95 mg remain unaccounted for.

Table 4.8: Amount of protein detected by the Bradford protein assay during the synthesis of SazCA@ZIF-8 following protocol 2 (0.25 mg/mL). Supernatant 1 was collected after the first centrifugation. Supernatant 2, 3 and 4 were collected after the first, second and third centrifugation during the washing.

Sample	Concentration (mg/mL)	Volume (mL)	Amount (mg)
Supernatant 1	0.089	37.5	3.35
Supernatant 2	0.010	8	0.081
Supernatant 3	0.015	9.5	0.14
Supernatant 4	0.0069	10.25	0.071
Total			3.64

When it comes to the ZIF-8 synthesized following protocol 2 without SazCA it was measured that 0.051 mg protein was in the resuspended sample. However, this number should naturally be 0, since no SazCA or any other protein was added during the synthesis. This low amount might be attributed to a small acceptable error for the Bradford protein assay. However, the measurements of the supernatants, which can be found in Table 4.9 also indicate a detection of protein, most notably in the first supernatant (3.05 mg). In the other supernatants it is under 0.026 mg, which might be considered negligible.

Table 4.9: Amount of protein detected by the Bradford protein assay during the synthesis of SazCA@ZIF-8 following protocol 2 (no SAzCA). Supernatant 1 was collected after the first centrifugation. Supernatant 2, 3 and 4 were collected after the first, second and third centrifugation during the washing.

Sample	Concentration (mg/mL)	Volume (mL)	Amount (mg)
Supernatant 1	0.080	38	3.05
Supernatant 2	0.0035	7.5	0.026
Supernatant 3	0.00065	8	0.0052
Supernatant 4	0.00097	9.25	0.0089
Total			3.09

From these results, it is clear that the chemicals seem to somehow interact with the Bradford reagent, which was not as noticeable during the normal Bradford protein assay, when no more than 20 μL of the total 1000 μL came from the sample. In the Bradford method used for these measurements, up to half of the 1000 μL could come from the sample. This means that any effect the chemicals have would have become more prominent at higher concentrations. Presumably, in the normal Bradford protein assay most of the detected color change should have been a result of the protein interacting with the Bradford reagent, since no significant change could

be detected with just pure ZIF-8.

The results from both protocol 2 with 1.25 and 0.25 mg/mL show that a lot of the original SazCA added remain unaccounted for. One explanation could be mass-transport limitations decreasing the Bradford reagents ability to reach the SazCA inside the MOF. However, they were given at least 10-30 minutes to interact. An additional explanation could be that the amount of enzymatically active SazCA decreased with time or was degraded, especially in the supernatants with just MQ-water. This is a likely explanation, since the concentration in the supernatants was first measured days after the MOF synthesis. This theory is strengthened by the fact that one time when the activity of SazCA was measured in the same solution only one day in-between the concentration had decreased to 5.94 mg/mL from 10.41 mg/mL, meaning that only 57.07 % remained, despite being kept in 25 mM Tris-HCl buffer. It is also possible that some of the protein was absorbed on the surface of the plastic tubes used, which would lead to it not being detected in the solution. These observations suggest that the Bradford protein assay might not be the most accurate method to measure the protein concentration in this project. However, the accuracy might be improved by doing the measurements directly and sonicating the sample beforehand, to reduce the mass-transport limitations.

These findings could thus lead one to question the relative enzyme activity calculations, which were based on the protein concentration measurements, when comparing per mg SazCA. However, one could also argue that the amount of SazCA able to react with the Bradford reagent should also be able to convert CO_2, which would mean that the activity measurements done based on the concentration calculated from the Bradford protein assay would be accurate to the SazCA that would actually be able to react. However, they were given longer time to react with the Bradford reagent than with CO_2 during the Wilbur-Anderson units assay, so the amount that could convert CO_2 would probably be lower. If that is taken into account the actual activity of the SazCA that could react in the Wilbur-Anderson units assay might have been higher than what was measured. Nevertheless, even if this is the case, it might not have any practical relevance.

4.6.4 Higher thermostability in SazCA@ZIF-8 at 85 °C

The thermostability of SazCA@ZIF-8 synthesized with protocol 2 (1.25 mg/mL) was investigated and compared to free SazCA in 25 mM Tris-HCl. Before the activity assay, after being incubated at the chosen temperatures, the SazCA@ZIF-8 samples were sonicated. The relative activity of the synthesized SazCA@ZIF-8 resuspended in 25 mM Tris-HCl compared to free SazCA exposed to the same temperature, after incubation at 75 , 85 and 95 °C for 1 and 4 hours, can be found in Figure 4.11 A.

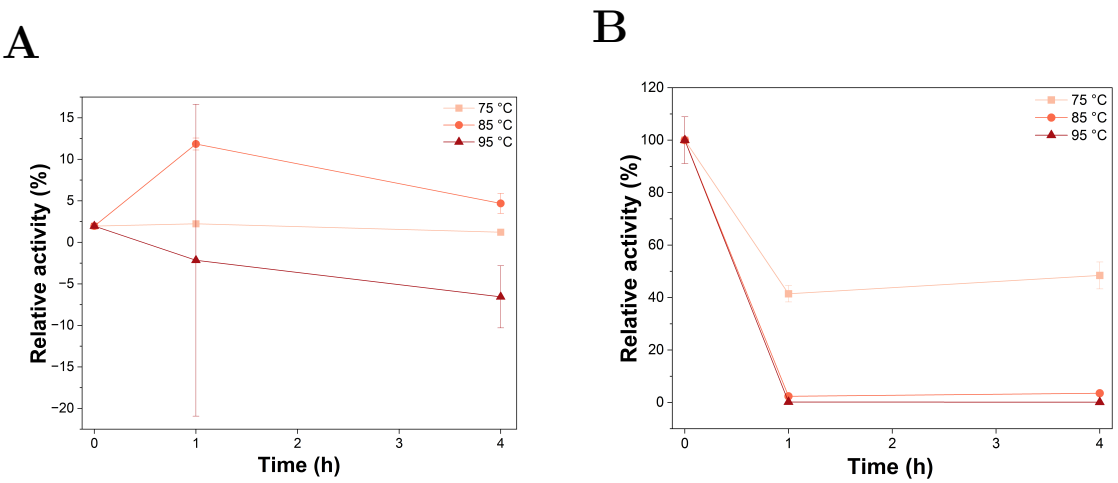

Figure 4.11: The relative activity after incubation at 75, 85 and 95 °C for 1 and 4 hours. Based on calculated Wilbur-Anderson units found in Table A.8 and A.9 in Appendix A.1. Data reported as mean ± range (n = 2). **A** Shows the activity of SazCA@ZIF-8 compared to free SazCA at the same conditions. **B** Shows the activity of free SazCA compared to its initial activity.

At 75 °C the free SazCA and SazCA@ZIF-8 both decrease proportionally with about the same amount of activity during the whole period, keeping the relative activity around its starting value at 1.97 ± 0.30 %. It can be seen in Figure 4.11 B that the activity the free SazCA retains is a bit less than half of its original activity, even after 4 h. This is likely because SazCA comes from a thermophilic bacterium. However, the previous research, mentioned in Theory 2.1.3, found that it could keep its activity after hours of being exposed to temperatures of 90 to 100 °C, which is contrary to the findings in this report. A difference could be that in this project a very small volume of just 50 μL of each sample was used in a PCR machine, which is exceptionally good at distributing the temperature throughout the whole sample. If a larger volume was used in the previous study, this could have protected the SazCA more.

The only noticeable improvement of the thermostability in SazCA@ZIF-8 compared to free SazCA can be observed at 85 °C, after both 1 and 4 hours. The biggest difference was after just 1 hour, when the relative activity was 11.85 ± 0.72 %. Already after one hour the free SazCA loses almost all of its activity and stays below 4 %. However, when it comes to the thermostability found in the previous research, mentioned in Theory 2.2.1.2, their results showed that the CA@ZIF-8 retained a lot more of its original activity (compared to free CA) than this. This could be because the free CA they used was not as thermostable in itself as SazCA, which made the difference bigger. They also only tested at lower temperatures, where the CA@ZIF-8 is probably more stable.

At 95 °C it can be seen in Figure 4.11 A that SazCA@ZIF-8 loses more of its original activity than free SazCA. However, practically both lose their activity almost completely after only 1 hour. The activity of the free SazCA is only 0.20 ± 0.068 %

compared to its initial value after 1 hour and even less after 4 hours. Even though MOFs have previously been shown to increase the thermostability of enzymes, there must be a limit. It could be that this limit is reached at 95 °C for SazCA@ZIF-8. Out of the previous studies mentioned in Materials and Methods 2.2.1.2 none tested the thermostability of CA@ZIF-8 beyond 65 °C.

It is also worth mentioning that the Wilbur-Anderson units calculations should have ideally been done using the reference ZIF-8 produced following protocol 2 without any protein as a control, to role out any of the measured activity for SazCA@ZIF-8 coming from the ZIF-8 itself, since it can also absorb CO_2. However, using this as a control did not work very well, because when the volume used from the ZIF-8 sample was too high, the color change in the buffer solution occurred almost immediately, even before the CO_2 saturated water was added.

5
Conclusion

SazCA@ZIF-8 was successfully produced and confirmed with XRD. The best protocol was determined to be protocol 2, which used 2-MIM and zinc nitrate hexahydrate with a ratio of 70:1. It gave both pure ZIF-8 and the most uniform particles, with mostly cubical and hexagonal shapes, identified with SEM. It was also found that the temperatures used during the synthesis were not harmful for the enzyme and that 2-MIM can increase its activity. However, it was also clear that free zinc ions severely inhibits the enzyme, even at low concentrations. Although, the activity could be restored with a high enough concentration of EDTA.

The activity in the synthesized SazCA@ZIF-8 was found to be considerably lower than in free SazCA, which could be explained through the inhibiting effect of zinc, but also mass-transport limitations introduced by the MOF structure. Resuspending the dried SazCA@ZIF-8 before measuring the activity did not improve it. However, adding the SazCA after mixing the 2-MIM and zinc solution and adding EDTA during the synthesis showed a slight improvement in the activity, but might negatively impact the quality of the ZIF-8. Introducing sonication resulted in a doubling of the activity, making it the most important measurement taken for increasing the activity. While determining the encapsulation efficiency, problems regarding the accuracy of the method used to measure the protein concentration were noticed, which could introduce uncertainties to some of the activity calculations. This would need to be addressed in the future for improved accuracy.

The thermostability tests showed that SazCA@ZIF-8 maintained more of its activity at 85 °C compared to the free SazCA, while the relative activity was roughly the same at 75 °C, where the free SazCA still maintained almost half of its original activity. At 95 °C the enzyme seems to be almost entirely degraded, even if it is encapsulated in ZIF-8. Further tests at temperatures around 85 °C during longer times should be conducted to investigate the possibility for long-term industrial applications.

Overall the results deviated a lot from those obtained in previous studies on CA@ZIFs. Firstly, less of the enzymatic activity was maintained after the encapsulation. Secondly, the thermostability was not improved as much in the CA@ZIF-8 synthesized in this project. This could be because of multiple reasons. Most importantly is the difference in type of CA used. The SazCA and the PmCA, which was used in one of the previous studies, both maintained less of their initial activity, compared to BCA. SazCA and PmCA have in common that they are both thermostable CAs, so

it could be that the structure causing its thermostability also makes it more sensitive to zinc ions or any of the other conditions the CA was exposed to during the synthesis. The difference in the thermostability of CA@ZIF-8 is harder to compare to the previous studies, since different temperatures were evaluated.

In conclusion, the synthesized SazCA@ZIF-8 did not perform as expected when it comes to retaining the enzymatic activity and substantially increasing the thermostability. However, a thorough investigation of SazCA@ZIF-8's potential for CO_2 capture for industrial applications was conducted and through the work important design principles were identified, which could provide support for future experiments within the field. Investigating other types of MOFs in the future, where the metal ion used is more compatible with SazCA, could lead to more promising results.

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A

Appendix 1

A.1 Data from Wilbur-Anderson units assays

Table A.1: Activity in Wilbur-Anderson units of free SazCA and SazCA@ZIF-8 synthesized with protocol 2 and 4 (n = 1).

Sample	**Activity (WAU/mg SazCA)**
Free SazCA	2056.27
Protocol 2 (2.5 mg/mL)	104.44
Protocol 4 (4.5 mg/mL)	56.65

Table A.2: Activity in Wilbur-Anderson units of free SazCA and SazCA@ZIF-8 synthesized with protocol 2 and 5 (n = 1).

Sample	**Activity (WAU/mg SazCA)**
Free SazCA	2782.61
Protocol 2 (1.64 mg SazCA/mL) with SazCA added 15–30 min after mixing	194.29
Protocol 5 (0.67 mg/mL)	328.89

Table A.3: Activity in Wilbur-Anderson units of SazCA exposed to different zinc, magnesium and 2-MIM concentrations with n = 2, but not paired data.

Sample	**Concentration (mM)**	**Activity (WAU/mg SazCA)**
Free SazCA	-	941.18
$ZnCl_2$	25	297.30
$MgCl_2$	25	653.33
$Zn(CH_3COO)_2$	25	203.13
$Zn(NO_3)_2$	25	204.08
2-MIM	1700	2756.10
2-MIM	600	2459.46
2-MIM	300	1895.83

Table A.4: Activity in Wilbur-Anderson units of SazCA exposed to different zinc nitrate and EDTA concentrations with n = 2, but not paired data.

Sample	**Activity (WAU/mg SazCA)**
Free SazCA	1774.19
0.5 mM $Zn(NO_3)_2$	977.27
1 mM $Zn(NO_3)_2$	477.88
25 mM $Zn(NO_3)_2$ + 20 mM EDTA	3289.47
25 mM $Zn(NO_3)_2$ + 10 mM EDTA	4125
25 mM $Zn(NO_3)_2$ + 5 mM EDTA	1590.91
25 mM $Zn(NO_3)_2$ + 1 mM EDTA	121.02

Table A.5: Activity in Wilbur-Anderson units of SazCA exposed to different temperatures and SazCA@ZIF-8 synthesized with protocol 2 (n = 1).

Sample	**Activity (WAU/mg SazCA)**
Free SazCA on ice in 4 °C	2137.93
Free SazCA room T	2033.33
Free SazCA 37 °C	2033.33
Protocol 2 (1.25 mg SazCA/mL) as powder	13.25
Protocol 2 (1.25 mg SazCA/mL) + 10 mM EDTA	230.30

Table A.6: Activity in Wilbur-Anderson units of SazCA exposed to calcium ions, iron ions and fumaric acid with n = 2, but not paired data, except for the free SazCA (n = 1).

Sample	**Concentration (mM)**	**Activity (WAU/mg SazCA)**
Free SazCA	-	2782.61
Iron(III) chloride hexahydrate	25	24.88
Fumaric acid	25	1785.71
Calcium chloride dihydrate	25	1973.33

Table A.7: Activity in Wilbur-Anderson units of SazCA@ZIF-8 synthesized with protocol 5 after an EDTA "bath" and sonication. Activities calculated from the mean times (n = 3), because of no paired data.

Sample	**Activity (WAU/mg SazCA)**
Protocol 5 + EDTA bath	193.77
Protocol 5 + EDTA bath + sonication 1x	430.96
Protocol 5 + EDTA bath + sonication 2x	376.07

Table A.8: Activity in Wilbur-Anderson units of free SazCA and SazCA@ZIF-8 synthesized with protocol 2 after incubation for 1 hour in 75, 85 and 95 °C. Data reported as mean ± range (n = 2).

Sample	**Temperature (°C)**	**Activity (WAU/mg SazCA)**
Free SazCA	-	2518.49 ± 224.37
Protocol 2 (2.5 mg/mL)	-	48.85 ± 3.23
Free SazCA	75	1037.27 ± 14.01
Protocol 2 (2.5 mg/mL)	75	23.23 ± 0.50
Free SazCA	85	59.03 ± 0.058
Protocol 2 (2.5 mg/mL)	85	7.00 ± 0.43
Free SazCA	95	4.90 ± 1.27
Protocol 2 (2.5 mg/mL)	95	0.13 ± 0.89

Table A.9: Activity in Wilbur-Anderson units of free SazCA and SazCA@ZIF-8 synthesized with protocol 2 after incubation for 4 hours in 75, 85 and 95 °C. Data reported as mean ± range (n = 2).

Sample	**Temperature (°C)**	**Activity (WAU/mg SazCA)**
Free SazCA	75	1406.25 ± 93.75
Protocol 2 (2.5 mg/mL)	75	17.21 ± 0.38
Free SazCA	85	102.86 ± 13.21
Protocol 2 (2.5 mg/mL)	85	4.99 ± 1.86
Free SazCA	95	3.63 ± 0.30
Protocol 2 (2.5 mg/mL)	95	-0.25 ± 0.16

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