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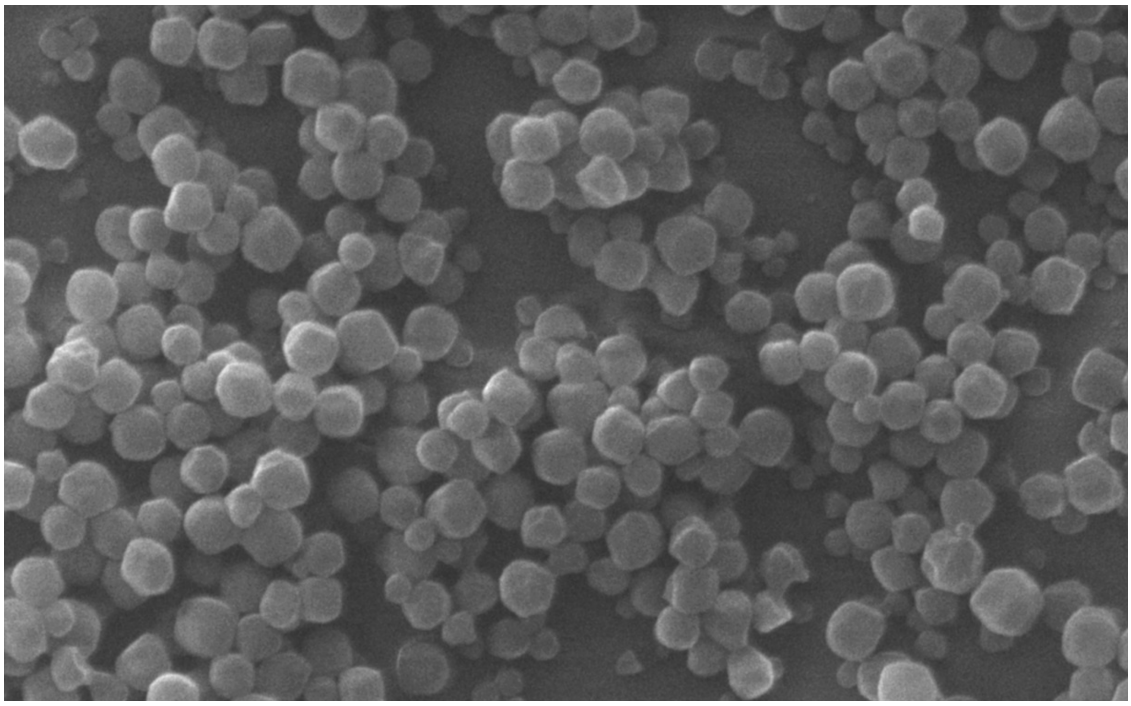

Encapsulation of carbonic anhydrase in metal-organic frameworks to facilitate CO_2 capture

Master's thesis in Innovative and sustainable chemical engineering

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Cover: Micrograph of SazCA encapsulated in ZIF-8.

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Abstract

Global warming is a growing problem because of increasing temperatures and greenhouse gases. Therefore, to reduce the amount of CO_2 in the atmosphere this project explores the encapsulation of the protein SazCA into the Metal organic framework ZIF-8. Because SazCA is a protein that can transform CO_2 into bicarbonate which in later stages can form into a solid material to be used for long term storage or other applications. The activity of SazCA have been measured with Wilbur-Anderson units.

Previous work on this project have shown some problems with the encapsulation of SazCA, namely low activity at 3 % of the free SazCA and low protein coverage. Results found that the reducing of free zinc did not improve the activity of the SazCA@ZIF-8. However it was found that with increasing amount of BSA mixed with SazCA in the ZIF-8 synthesis, the BSA protected the SazCA and increased the activity to about 32 % of the free SazCA. The activity of SazCA@ZIF-8 did not recover completely in long term storage after 8 weeks, but is persistently getting better than that of free SazCA in the same condition. In activity measurements in 50, 60 and 70 °C the SazCA@ZIF-8 did not show better activity at higher temperatures but further experiments will have to be performed. It was noted that in the ZIF-8 synthesis the protein coverage reduced with about 50 % and was traced to the 2-MIM being responsible. It was concluded that the 2-MIM did not degrade the protein and it was concluded that the 2-MIM affect the protein measurement in the Bradford protein measurement.

Keywords: zeolitic imidazolate framework-8 (ZIF- 8), carbonic anhydrase (CA), metal-organic framework (MOF), Carbon capture, Carbon dioxide CO_2, Enzyme immobilization, Enzyme encapsulation .

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Jennie Önnermalm, Gothenburg, May 2026

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis listed in alphabetical order:

BCA	Bovine Carbonic Anhydrase
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase
EDTA	Ethylenediaminetetraacetic Acid
IPTG	sopropyl β-D-1-thiogalactopyranosid
LB	Lennox Broth
MOF	Metal Organic Framework
MQ-water	Milli-Q Water
SazCA	CA from *Sulfurihydrogenibium azorense*
SEM	Scanning Electron Microscopy
TRIS-HCl	tris(hydroxymethyl)aminomethane hydrochloride buffer
XRD	X-Ray Diffraction
ZIF-8	Zeolitic Imidazole Framework-8
2-MIM	2-Methylimidazole

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1
Introduction

In society today, a considerable amount of fossil fuels are burned in industry and releases greenhouse gases. A primary gas released is carbon dioxide which contributes to global warming. Which in turn leads to climate change and possibly natural disasters. This highlights the importance of finding new solutions to decrease the carbon dioxide amount in the atmosphere. Some solutions for this problem is for example decreasing the use of fossil fuels and thus new ideas to replace fossil fuels are needed. Another solution is to decrease the amount of CO_2 in the atmosphere by doing carbon capture. This is what will be focused on in this project. [1]

Biomineralization is a process of organisms making minerals. The formation of these biominerals are processes that happens all around in nature. This function have been evolutionary important as it can form structures with multiple functions [2]. The process of biomineralization can be used in carbon capture and is a good alternative to other chemical and physical methods that is not as environmentally friendly. In the biological process of biomineralization an organism or enzyme can capture carbon without producing toxic by-products. However, this method can be expensive with low efficiency, but it has a promising potential. [3]

1.1 Background

To combat global warming, It is important to find new solutions for carbon capture as CO_2 is one of the main contributors. A proposed design for carbon capture can be seen in figure 1.1 which is based on the process of biomineralization. The enzyme carbonic anhydrases (CA) will be encapsulated by a metal organic frameworks (MOF). Which will make the enzyme immobilized and increases its stability and re-usability. In this project the MOF used is Zeolitic imidazolate framework-8 (ZIF-8).

According to the proposed process the compound of MOF-CA will be placed in a scrubber column where CO_2 from waste gas that comes from industries would dissolve. The reaction taking place in the scrubber column is the transformation of CO_2 into bicarbonate HCO_3^- which is converted by the enzyme CA. This process happens naturally but is catalyzed by the CA enzyme. The formed HCO_3^- will then be transported into a chamber to precipitate. First, HCO_3^- will spontaneously convert into CO_3^{2-}. With the added sea water that contributes with calcium anions Ca^{2+}, the CO_3^{2-} will make a $CaCO_3$ precipitate. This solid precipitate could be

used as a long-term storage for CO_2 and also in industry as a construction material which makes the project both economically and environmentally sustainable.

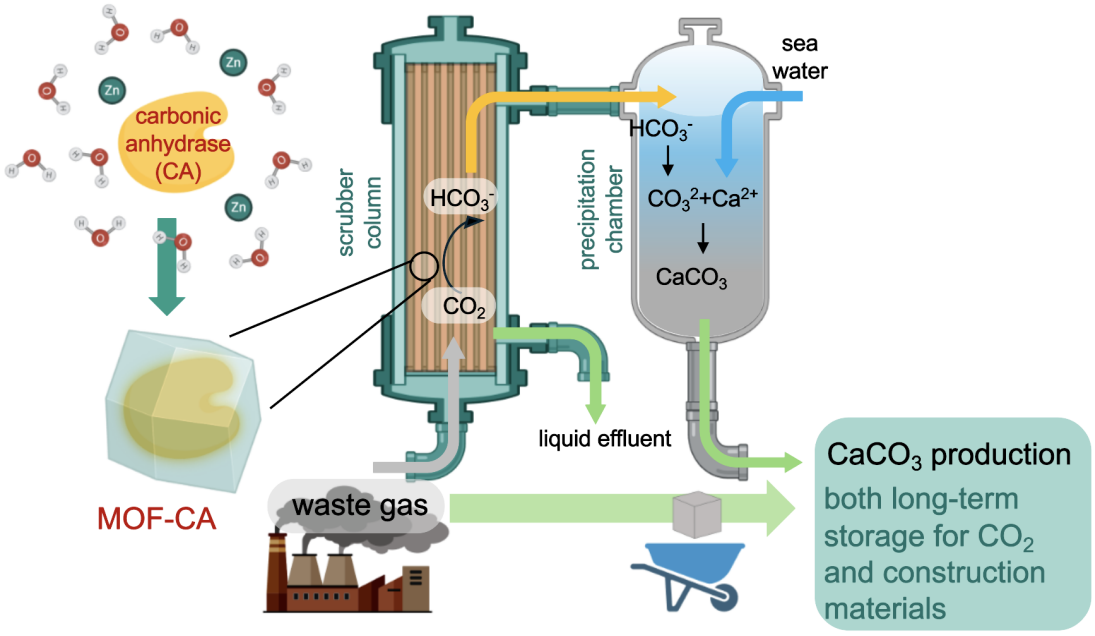

Figure 1.1: Proposed process for carbon capture. Illustration was performed in the assistance of Biorender.

1.1.1 Previous work

Previous master thesis work on this process have investigated the encapsulation of SazCA with the metal organic framework ZIF-8. The parts of this MOF is the organic linker zinc and 2-Methylimidazole (2-MIM) that together construct the ZIF-8 structure. This MOF was synthesized together with SazCA and the encapsulation was successful in making SazCA@ZIF-8 in two of the protocols tested. The project was focused on increasing the thermo stability and activity of the enzyme SazCA which turns CO_2 into HCO_3^-. [4]

A problem found was that the protein coverage is about 50 % when doing the ZIF-8 synthesis. Also, when comparing the free SazCA and SazCA@ZIF-8 it was found that the SazCA@ZIF-8 had a significantly lower activity. This could possibly be explained by the CO_2 limitations in mass transport into the enzyme caused by the ZIF-8 structure or the free Zinc ions having an inhibiting effect. Another reason could be that the SazCA can't rearrange into the active form because the enzyme gets stuck in an inactive form when SazCA@ZIF-8 is produced. It was also found that the activity of the SazCA@ZIF-8 could be increased if sonicated and adding EDTA. The activity was measured with Wilbur-Anderson units assay. The result from the produced SazCA@ZIF-8 was not as good as expected compared to previous studies. In this project the thermostability of the free enzyme and the encapsulated enzyme was measured at different temperatures. It was found that at 85 °C the

SazCA@ZIF-8 had higher activity than the free enzyme. Other temperatures tested (75°C and 95°C) showed to difference between free SazCA and SazCA@ZIF-8.[4]

Some experiments was done to find out what was inhibiting the activity. The first experiment were carried out with free protein of SazCA with different amounts of 2-MIM as seen in table 1.1. The results were that the activity was better in the presence of 2-MIM and therefore it is not the inhibiting factor.

Table 1.1: Effect of 2-methylimidazole concentration on relative activity of SazCA.

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

A second experiment in table 1.2 was carried out with free SazCA and different amounts of Zinc nitrate hexahydrate which is used as the organic linker in the MOF. It was shown that the activity gets lower as the amount of zinc increases and the conclusion was drawn that the zinc is inhibiting the activity of the protein. Other metal salts such as $ZnCl_2$ and $MgCl_2$ was tested to see if other compounds showed similar results.

Table 1.2: Effect of different metal salts on the relative activity of SazCA.

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

A third experiment with both the free SazCA and the organic linker zinc together with Ethylene diamine tetraacetic acid (EDTA) was performed, see table 1.3. With the addition of EDTA the activity came back for the protein. This indicates that the zinc does not permanently damage the SazCA protein because EDTA rescue SazCA.

Table 1.3: Effect of EDTA concentration on the relative activity of SazCA in the presence of $Zn(NO_3)_2$.

Sample	Concentration 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

1.2 Aim

In this master thesis, the encapsulation part will be the main focus as this study will continue the previous work made on this project. The overall goal of this project is to find out why the encapsulation of the SazCA in the ZIF-8 failed in terms of activity and stability of the SazCA. The project investigated the underlying causes to why the activity got low when encapsulated. From the previous work on this project there were some questions left to be addressed which this master thesis have focused on. These questions are the following:

- Low activity in SazCA@ZIF-8 - about 3% of free protein
- Low protein coverage - 50% protein disappearance

1.3 Limitations

A limitation in this project is the use of only SazCA. No other variations of the enzyme CA will be tested because in further industrial applications the CA is expected to keep its structure relatively intact. Then other variants of CA should be able to be used in this application and the results from the project can be used as general directions for any variety of CA. The project are also limited to the use of one MOF (ZIF-8) that is suitable for the process and was used in previous work.

This project will also be limited to the use of only one ZIF-8 synthesis protocol to produce the encapsulated protein compound SazCA@ZIF-8. The protocol was used in previous work on this project from the master thesis [4] and the protocol used is from [5]. This protocol was chosen to be used in the continued research in this project because it previously showed the best results.

This project will be limited to the equipment available in the lab and thus this project will be made in a small lab scale. There will be no real CO_2 waste gas but instead water saturated with CO_2 will be used. This could make the results slightly off compared to what will happen in a real industrial setting. Another limitation for this project is that further steps after the SazCA@ZIF-8 synthesis will not be addressed.

As this project will focus on identifying the problems causing the undesirable results from the protein encapsulation. Finding a way to solve the problem will not be addressed.

2
Theory

2.1 Carbonic Anhydrase

Carbonic Anhydrase (CA) is a metalloenzyme that can be found in Bacteria, Archaea and Eukarya [6]. The enzyme have important functions for all life as it catalyzes hydration of carbon dioxide CO_2 which facilitates pH modulation. This is a fundamental reaction for many cell processes as it has an important part in many physiological cell processes such as photosynthesis, cell growth, respiration and calcification. [7]. The catalytic activity of the carbonic anhydrase depends on the active site of the protein which contain a zinc ion. A metal ion needs to be bound to the active site of the protein to be active for its catalytical properties. [8]

The reaction of hydration of carbon dioxide CO_2 into bicarbonate is a reaction that occur naturally in nature. At neutral pH this is a very slow reaction, but it is catalyzed by CA to reach higher efficiency. This reaction is reversible and shown in equation 2.1. [9]

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

2.1.1 Biomineralization

The first step of the biomineralization of CO_2 into a solid mineral is the dissolving of carbon dioxide in water as in Equation 2.2.

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

The dissolved carbon dioxide will form carbonic acid (H_2CO_3) in the presence of water, equation 2.3. The carbonic acid will then be disintegrated into bicarbonate ion HCO_3^- seen in Equation 2.4. This is further dissociated into carbonate ion CO_3^{2-} and water, seen in Equation 2.5. The formation of the solid mineral calcium carbonate $CaCO_3$ in Equation 2.6 is the precipitation of the carbonate ion CO_3^{2-} and an added calcium ion Ca^{2+}.

$$CO_2(aq) + H2O \rightleftharpoons H_2CO_3 \tag{2.3}$$

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

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

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

2.1.2 Inhibition of CA

Some sulfonamide-based zinc compounds can be used to inhibit the activity of CA by binding to the zinc in the active site of the CA. It has also been shown that some heavy metal ions can have an inhibitory effect on CA. A way to inhibit the CA by the blocking of the active site is the use of carboxylic acid. [8]

Ethylene diamine tetraacetic acid (EDTA) is a strong metal chelator that can bind to metal ions and form stable chelates. This compound have been used to remove heavy metal ions water [10].

2.1.3 Industrial carbon capture faciliated by CA

A technique called carbon dioxide Sequestration is a process to store carbon dioxide to get a lower level of carbon dioxide in the atmosphere. To be able to use CA for a biological carbon sequestration in industrial applications it is important that CA is suitable for large scale and long term stability. [9]

For the CA to be used in industry to store high temperature flue gas it is important that the protein is thermostable. It is also important that the CA can thrive in saline and alkali environments as the produced calcium carbonate $CaCO_3$ precipitates in an alkaline conditions. Other limitations to the use of CA in industry is the purification of the protein. This process is costly and difficult to perform in high quality as the protein can have impurities and a low concentration.[9]

Engineering of CA can open new opportunities for immobilization of the protein to reuse and overcome the limitations of alkali stability and thermostability of the CA [9]. To increase stability there are several methods that can be used for example the introduction of mutations or some immobilization technique such as entrapment [8].

In this project a modified CA protein called SazCA will be used which have favorable characteristics for CO_2 Sequestration. An immobilization technique used in this project to increase the thermostability and long term stability is the use of metal organic frameworks (MOFs).

2.1.4 Sulfurihydrogenibium azorense CA (SazCA)

The protein used in this project is the CA from the extremophilic bacterium Sulfurihydrogenibium azorense, called SazCA. This protein is an α-carbonic anhydrase whose properties are well suited for carbon dioxide capture in industrial processes. Because this protein has shown to be thermostable when heated at 100°C for more than 3 hours, while keeping the catalytic activity of the protein. The SazCA is also suitable because of its fast efficiency with a k_{cat}/K_M of $3.5 \times 10^8 M^{-1}s^{-1}$ which makes this CA protein very efficient compared to other CAs. [11]

2.2 Metal organic frameworks

Metal organic frameworks (MOFs) are made of organic linkers and metal-containing nodes which together will form a porous material [12]. This is a lightweight material made with covalent bonds. Because CA have problems with thermostability and are sensitive to changes in its environment, it is desirable to find a way to stablize CA, and a way to do this is by immobilize CA with a metal organic framework (MOF). [13]

The MOFs porous structure is a good choice to protect the enzyme's stability and activity because of the MOFs properties such as high specific surface area, and chemical and thermal stability. Therefore the choice of MOF must be compatible with the CA to improve the reuse and long livety of the protein to reduce cost and make this economically feasible in industries. [13]

2.2.1 Zeolitic imidazolate framework-8 (ZIF-8)

In this project the Metal organic framework called Zeolitic imidazolate framework-8 (ZIF-8) was chosen because it can be synthesized in environments suitable for the SazCA. Such as it can be made in room temperature and with water as a solvent, it can keep stability in higher temperature and have a permanent porosity [13]. The ZIF-8 uses zinc (Zn^{2+}) as the organic linker and 2-methylimidazole (2-MIM) as the metal containing node as can be seen in figure 2.1. [14]

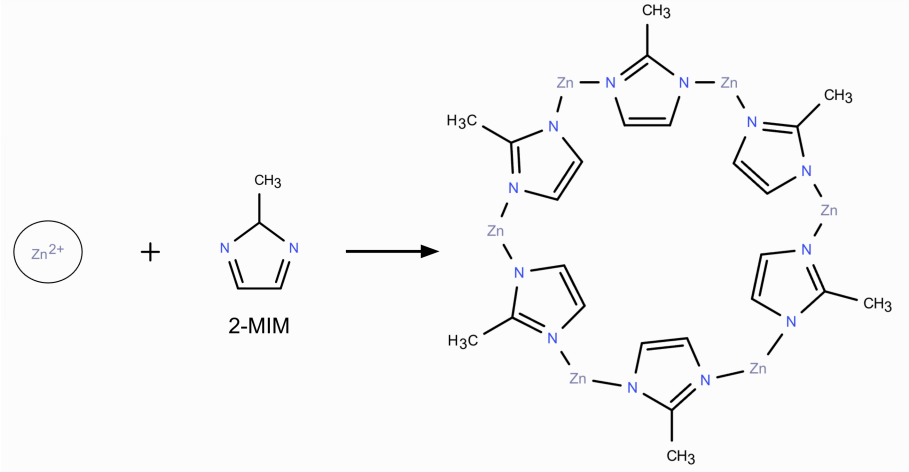

Figure 2.1: Zinc and 2-Methylimidazole form ZIF-8. Figure is made in Freechem-draw.

Both the ZIF-8 and CA protein have a similar structure because of their zinc containing centers. The CA have an active site with a bonded zinc and the ZIF-8 have zinc linkers in its structure. The zinc in ZIF-8 could help facilitate the efficiency of the hydration of carbon dioxide while the imidazole could assist in deprotonation of the water. This have potential to make the hydration process faster and was thus

chosen for this project. The ZIF-8 have also been previously used for encapsulation of other kinds of proteins and is a common MOF used in science. [14]

2.2.2 Enzyme encapsulation

There are four categories of ways for a MOF to immobilize a protein. The first is Surface adsorption where the protein is attached into pores or with weak interactions to the MOF surface. Another way for immobilization is covalent bonding of the protein to the MOF structure. This will make the protein fixed on the surface and can therefore reduce its flexibility and activity. The MOF can also immobilize the protein inside the MOF strucure by entrapment. This way will confine the protein by adsorption through the pores in the MOF. The result is that under harsh environment the protein will be protected and keep its stability. A limit with this technique is that the pore size of the MOF restricts the availability of the protein to diffuse inside. Instead, a similar strategy can be used where the encapsulation occur during the MOF synthesis which is called in situ encapsulation. This technique does not restrict the protein by the pore size and will protect the enzyme by the incapsualtion. However, the choice of linkers and metal nodes must be chosen with care not to disturb the protein and the MOF must be able to be synthesized in favourable conditions to the protein. [13]

In this project the ZIF-8 which is used for encapsulation of the SazCA utilizes the technique of in situ encapsulation. This is because the pore size of ZIF-8 is 1.2 nm which is the diameter of the pore inside the MOF [15]. The pore aperture of the ZIF-8 is 0.34 nm and this is the diameter for the pore entrance into the MOF [15]. An aperture of 0,34 nm is smaller than the SazCA size of 26 kDa which corresponds to about 4-5 nm. Therefore the ZIF-8 must be synthesized in presence of the protein to build the MOF around the protein.

2.3 Previous studies

In a previous study an incapsualted SazCA in ZIF-8 showed promising results concserning thermostability and activity. This research used a membrane hydrolyzed with polyacrylonitrile (HPAN) which was modified by polyethyleneimine (PEI)to let the ZIF-8 grow on because this membrane will have stronger affinity with CO_2. Between the surface on the PEI and the free zinc in the synthesis there are a strong chelation which faciliate the ZIF-8 forming on the surface and accumulates zinc there. The SazCA was placed in the ZIF-8 with an in situ encapsulation and the finished product was a CA@HPAN/PEI/ZIF-8. The result was a that the encapsulated SazCA had 48.7 % and the free SazCA had 18.3 % of the relative original activity after having been in storage for 18 days. It was also seen in the study that the CA@HPAN/PEI/ZIF-8 had a formation of the finished product $CaCO_3$ that was 13.6 times larger than for the free SazCA. [16]

Another study was researching Bovine Carbonic Anhydrase (BCA) encapsulation in ZIF-8. The encapsulated MOF was formed on cross-linked electrospun polyvinyl

alcohol (CPVA). The results show that the activity in temperatures tested from 25 to 65 °C was better for the BCA@ZIF-8. Samples of a BCA@ZIF-8/CPVA and free BCA was also kept for 37 days. It showed that the BCA@ZIF-8/CPVA had a higher activity at 63 % more activity. [17]

A study focusing on encapsulating CA from bovine erythrocytes in ZIF-8 tested the thermostability of the produced CA@ZIF-8 at 60°C. This showed that when placing CA@ZIF-8 and its free enzyme in 60°C for 1 hour the activity of the free CA was about 8%. While for the encapsulated protein in ZIF-8 the activity was about 90 %. This activity was compared to the original activity of the MOF and free protein before the heat treatment.[18]

3
Methods

3.1 Protein production

3.1.1 Protein expression

During the project the protein SazCA was continuously produced from the strain BL21 *E.coli*. The vector pET15b was inserted in the BL21 strain which contained the sequence for SazCA. A 6XHis-tag was connected to the SazCA sequence which facilitated the purification step. The *E.coli* was left to grow on an agar plate and the colonies was inoculated in Lennox Broth (LB) media with added Kanamycin for selection of the vector containing pET15b. When the cultivation reached the end of the exponential growth phase the initiation of the SazCA production was started by adding isopropyl β-d-1-thiogalactopyranoside (IPTG).

3.1.2 Protein purification

To purify the produced protein cell lysis and centrifugation was used to collect cell debris. A 6XHis-tag affinity chromatography was made with Imidazole elution in a solution of 25mM Tris-HCl. Lastly, a desalting column made with PD10 columns was used to remove salts and for buffer exchange. The purified protein was kept in fractions of protein and 25mM Tris-HCl to be used in the MOF synthesis.

A gel electrophoresis was performed using the gel Mini-PROTEAN TGX stain-free gel to confirm the correct protein was synthesized. The molecular weight of the protein was estimated in the gel electrophoresis with the Bio-Rad's Image lab software. The molecular weight of the protein should have been about 26kDa [19] and the protein was thus confirmed.

3.1.3 Bradford Protein assay

Bradford protein assay was used to measure the protein concentration of the produced protein. This method relies on the principle that Coomassie Brilliant Blue G-250 dye binds to protein and can be measured using a spectrophotometer at a wavelength of 595 nm. To measure protein concentration, a protein standard curve was made with Bovine Serum Albumin (BSA) as a standard. The standard curve included BSA concentrations between 2000-125 μg/ml and 20 μl of the each standard was put into 1 ml of Bradford reagent in each cuvette to be measured. The absorbance could then be measured by the spectrophotometer and a standard curve

was plotted. With the help of the standard curve the unknown protein samples could be calculated with equation 3.1. Where a is the absorbance of the unknown sample, b is the intercept of the standard curve and c is the slope. The f is the amount of unknown protein sample added to the Bradford reagent in the cuvette to get the sample in range of the standard curve.

$$Proteinconcentration(\mu g/ml) = \frac{a-b}{c} \times \frac{20}{f} \tag{3.1}$$

3.2 MOF synthesis

Three different ZIF-8 have been made according to the volume and chemical amounts in table 3.1. A pure ZIF-8 in a solution of ethanol and methanol was made according to [5] to be used as a reference for ZIF-8. This pure ZIF-8 was made of a solution of 40 ml with equal amount of ethanol and methanol that was mixed with Zinc nitrate hexahydrate to have a concentration of 120 mM. A second solution of 40 ml of equal amounts of ethanol and methanol were blended with 2-MIM to reach a concentration of 480mM. This solution was stirred for 1 hour and then then washed three times with centrifugation at 7000rpm for 10 min, then resuspended in ethanol between each wash. After washes it was left to dry overnight in fumehood for SEM and XRD measurements.

A protocol for creating ZIF-8 in water was used for making SazCA@ZIF-8. The protocol used is from [20] and was made with BSA and made pure to act as a reference. To make pure ZIF-8 made in water and SazCA@ZIF-8 this protocols method was used for sample 2 and 3 in table 3.1. To make pure ZIF-8 in water the Zinc nitrate hexahydrate and 2-MIM amounts was each suspended in 40 ml of MQ water. These solutions was then mixed together and stirred for 15 minutes, then left in fumehood overnight. The created ZIF-8 was the day after washed 3x in MQ water by spinning down the ZIF-8 in a centrifuge at 7000rpm for 10 min and then resuspended in MQ-water three times.

For the SazCA@ZIF-8 created the method used was similar to the pure ZIF-8 made in water. The protein was mixed with the solution of 2-MIM so that the concentration of protein was 1,25mg/ml and the 2-MIM was the same as in the protocol. This solution was then mixed with the zinc solution so that the final protein concentration was 0,625 mg/ml. Then this solution was stirred for 15 minutes and left overnight. Then The SazCA@ZIF-8 was washed 3x by centrifugation to remove supernatant and resupended in MQ water three times. After the last wash the SazCA@ZIF-8 was divided into 2 tubes, one part was resuspended in 25mM Tris-Cl for activity measurements and long time storage. While the second part was left in a fumehood to dry and then to be used for SEM and XRD measurements.

A modification of the protocol to create SazCA@ZIF-8 was made to remove more Zinc. The SazCA@ZIF-8 was then rolled for 10 min after each resuspension in MQ-water and then centrifuged. This was made in all three of the washes.

A SazCA@ZIF-8 was also made with zinc chloride $ZnCl_2$ by using the same protocol as protocol 3.

Table 3.1: Protocols for different kinds of MOF

Sample	Chemical	Concentration (mM)	Volume (mL)
Pure ZIF-8[1]	2-Methylimidazole	480	40
(ethanol,methanol)	Zinc nitrate hexahydrate	120	40
Pure ZIF-8[2]	2-Methylimidazole	2800	40
(water)	Zinc nitrate hexahydrate	40	40
SazCA@ZIF-8[3]	2-Methylimidazole	2800	40
	Zinc nitrate hexahydrate	40	40

[1] from [5]. [2] and [3] from [20].

3.3 Characterization of SazCA@ZIF-8

3.3.1 X-ray diffraction

X-ray diffraction (XRD) are used for determination of how a material is structured. When X-rays hit a crystalline sample, a pattern take shape because of the different atoms scattering [21]. Peaks with different intensities will be visible on the pattern which correlate to a known structure. This equipment was used to confirm that the correct ZIF-8 and SazCA@ZIF-8 was successfully synthesized. The patterns of the different ZIF-8 was compared to simulated ZIF-8 to confirm ZIF-8 created.

The measurements was done using the equipment Bruker D8 Discover where the samples was put on crystal silicon holders after being crushed to a powder form. The settings for the equipment was 30mA, 40kV and a step speed of 5 per minute with Cu Kα radiation.

3.3.2 Scanning electron microscopy

A Scanning electron microscopy (SEM) was used to examine the samples morphology. The instrument used was JEOL JSM-7800F Prime FEG which sends a high-energy electron beam towards the sample which will give a magnified image [22]. This gives information on the samples size and shape with a magnification up to 300 000 times. This equipment was used to visualize the SazCA@ZIF-8 samples to determine that the particles looks correct and that the size is in an accepted range. The magnification used was 75000x and 30000x in different areas of the samples to then compare the pure ZIF-8 with the SazCA@ZIF-8.

The samples for the SEM was prepared by measuring 1 mg of the dried SazCA@ZIF-8 and ZIF-8 and then dissolve it in 500μl MQ water so the concentration got to about 2mg/ml. These samples was then sonicated for 10 minutes at 100% and 37 kHz and then dropped onto 8mm glass plates to dry. Then a carbon tape was put on and with the Leica EM ACE600 Gold and Chrome Sputter machine the samples was coated with 5nm gold.

3.4 Zinc measurements

To measure the free zinc amount in the SazCA@ZIF-8 and ZIF-8 the Amplite® Fluorimetric Zinc Ion Quantitation Kit was used. According to the protocol [23] a zinc standard curve was made from which the zinc amount of the unknown samples could be calculated from using equation 3.2. In the equation a is the measured fluorescence, b is the intercept and c is the slope of the zinc standard curve. The Volume is the amount of the sample to know how much zinc there is in the whole sample and M is the molar mass.

$$Zinc\,amount\,(mg) = \frac{a-b}{c} \times Volume \times M \tag{3.2}$$

The samples and zinc standards were put 50 μl of each in wells in a 96 solid black plate. The zinc probe reagent was then added 50 μl to each well and incubated for 10 minutes at room temperature. Then the fluorescence was measured in the equipment fluostar omega with the settings at emission 525 nm, emission 525 and the gaines at about 1100.

To determine how well the measurement works with measuring the ZIF-8, zinc standard curves was made with zinc in water, ethanol and 2-MIM.

3.5 Protein encapsulation efficiency

To measure how much protein was encapsulated into the SazCA@ZIF-8 the Bradford protein assay was used in each step of the ZIF-8 synthesis and washing. The concentrations of protein was used to calculate the protein amounts in each step and compared to the added amount of protein. To measure the amount of protein in the supernatants during the washes a special Bradford standard curve was used. This had 0.5 ml of Bradford reagent mixed with 0.5 ml of sample and the standards.

3.6 Catalytic activity testing

To measure the activity of the free protein and the SazCA@ZIF-8 Wilbur-Anderson Units was used [24]. In the method a bottle of MQ water saturated with CO_2 was made with the addition of dry ice to the water. The CO_2 in the water will then act as a substrate for the SazCA active site. A solution of is 0.001% w/v bromothymol blue which will act as a pH indicator in 25mM Tris-Cl buffer solution is the working solution for the measurement. This solution will have a pH of about 8.6 and a

solution of 5mM NaOH was added to get the correct pH.

To make a measurement 3ml of the working solution will be added to a tube and then 10 μl of sample or control will be added. To this solution 3ml of saturated CO_2 water will be added and then the time until the solution changes color are recorded i.e. when the pH changes from about 8.6 to 6.3. The control is 25 mM of Tris-Cl and the samples are free SazCA and SazCA@ZIF-8.

The measured activity for the SazCA@ZIF-8 is a relative activity compared to a measured activity of free SazCA from the same batch of the proteins. This activity is calculated in Wilbur Anderson Units according to equation 3.3. The $t_{control}$ is the time for the control and the t_{sample} is either for the free SazCA or SazCA@ZIF-8 samples.

$$Wilbur - AndersonUnits(WAU) = \frac{t_{control} - t_{sample}}{t_{control}} \tag{3.3}$$

The WAU values are then made into WAU per mg of the sample and the activity of the SazCA@ZIF-8 was calculated by dividing the WAU per mg of SazCA@ZIF-8 by the WAU per mg of the free SazCA. All calculations and statistical analysis was done in excel.

3.7 Long time stability

To make long time stability measurements, SazCA and SazCA@ZIF-8 from the same batch of proteins was stored in a controlled environment. The samples was kept in darkness at 30 degrees and activity measurements was conducted every two weeks. The free SazCA was acting as a control for the SazCA@ZIF-8 to when measuring the activity.

3.8 Thermostability testing

To test the thermostability of the SazCA@ZIF-8 compared to the free SazCA heating blocks was used. A sample of protein and SazCA@ZIF-8 was placed in 50, 60 and 70 for 1 hour. The activity and protein concentration was measured and compared to that of the free SazCA and SazCA@ZIF-8 before the thermostability testing. To make the samples more homogeneous for the activity measurements, they were sonicated for 2 minutes at 37 kHz and 100%.

4

Results and Discussion

4.1 SazCA and SazCA@ZIF-8 produced

During the project the SazCA was produced and purified continously. As well batches of pure ZIF-8 and SazCA@ZIF-8.

4.1.1 Confirmation of SazCA

The SazCA produced in the protein synthesis was confirmed by a gel electrophoresis where the weight of the protein could be compared to a ladder. The expected value for the SazCA was about 26 kDa. As can be seen in figure 4.1 the length of the produced protein is the same as what was expected for the SazCA. This confirmed that the protein SazCA was produced.

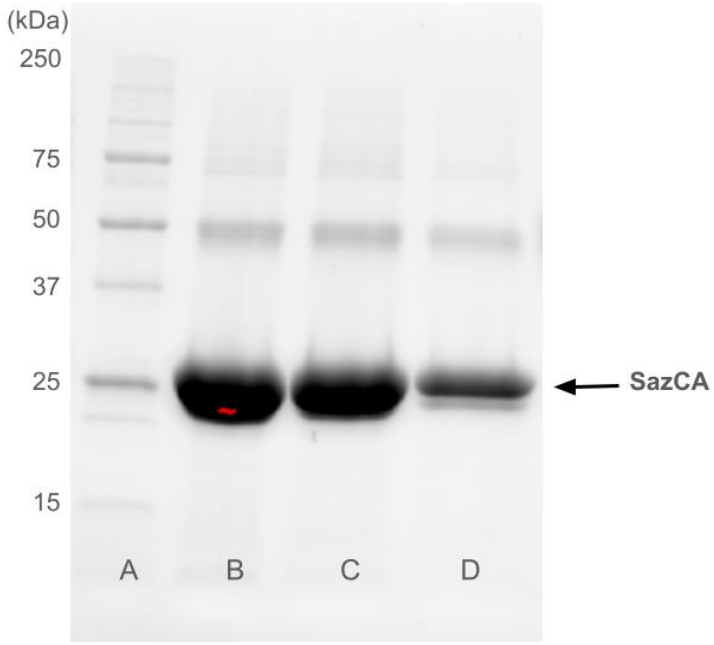

Figure 4.1: Gel with SazCA produced at a lenght of 26 kDa

4.1.2 Bradford protein assay measurement

To measure the amount of protein the Bradford protein assay was used and a new standard curve with BSA was made for each time a protein measurement was needed. An example of the BSA standard curve can be seen in figure 4.2 and a corresponding example of how the SazCA concentration was measured can be seen in equation 4.1. Which show a calculation for a sample with an absorbance of 0.462 nm and a volume of 3 μl mixed with Bradford reagent.

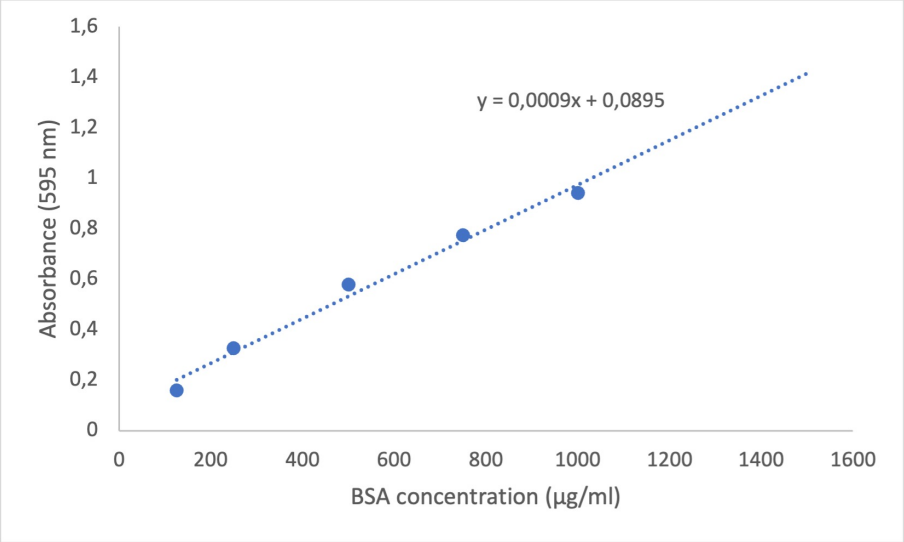

Figure 4.2: BSA standard curve with concentration ranging from 1000-125 $\mu g/ml$. Used for protein measurements.

$$SazCA\ concentration = \frac{0.462 - 0.0895}{0.0009} \times \frac{20}{3} = 2759\ \mu g/ml \tag{4.1}$$

4.1.3 Confirmation of ZIF-8

The confirmation that ZIF-8 was created in the project is shown in figure 4.3. This figure shows X-Ray Diffraction of the different ZIF-8 and SazCA@ZIF-8 samples. When comparing the peaks of the ZIF-8s with the simulated peaks of ZIF-8 it can be noted that the reflections align and the ZIF-8 can therefore be confirmed. The SazCA@ZIF-8 with EDTA does not align with the simulated ZIF-8, indicating that ZIF-8 was not formed.

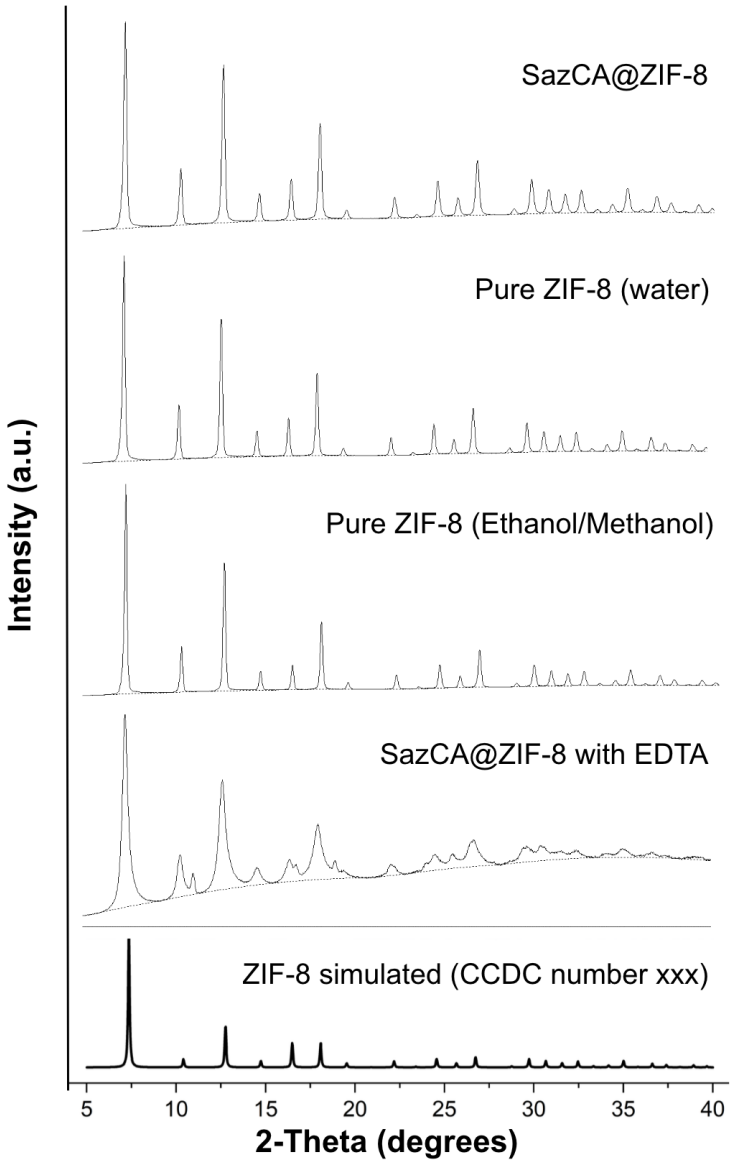

Figure 4.3: XRD on the different MOFs created and simulated intensity peaks of ZIF-8. The simulated ZIF-8 is taken from [4].

4.2 Size distribution

The morphology of a sample of Pure ZIF-8 made in water can be seen in figures 4.4 and 4.5. The first picture has a magnification of 30000 and the second have a magnification of 75000. These pictures show a uniform structure that is the ZIF-8.

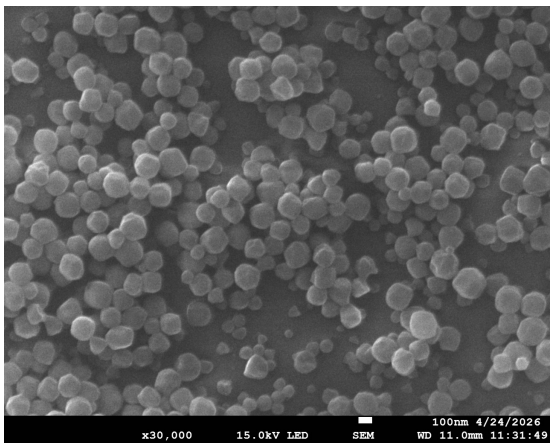

Figure 4.4: SEM picture of pure ZIF-8 made in water. The picture is magnified 30000x.

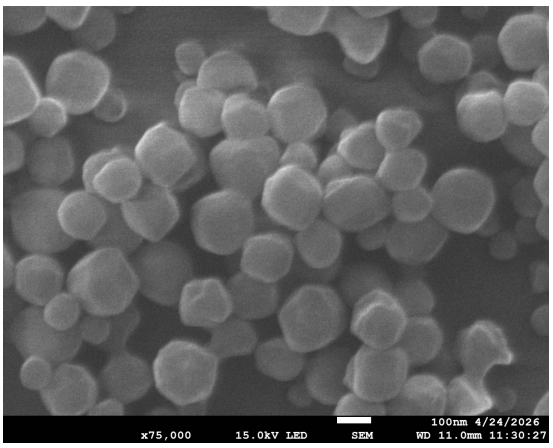

Figure 4.5: SEM picture of pure ZIF-8 made in water. The picture is magnified 75000x.

In figures 4.6 and 4.7 the SEM pictures of a sample of SazCA@ZIF-8 can be seen with a magnification of 30000 and 75000, respectively. These samples also have a uniform structure and are similar in shape to the pure MOF. This is a further confirmation that the ZIF-8 was produced for the encapsulated protein sample. It can also be seen that the size of the SazCA@ZIF-8 and the pure ZIF-8 are about the same and that their morphology are similar.

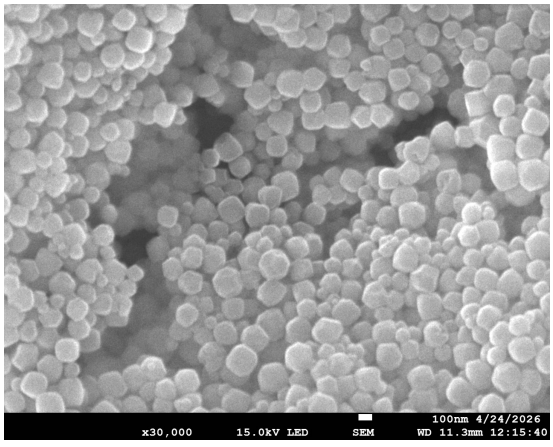

Figure 4.6: SEM picture of SazCA-ZIF-8 made in water. The picture is magnified 30000x.

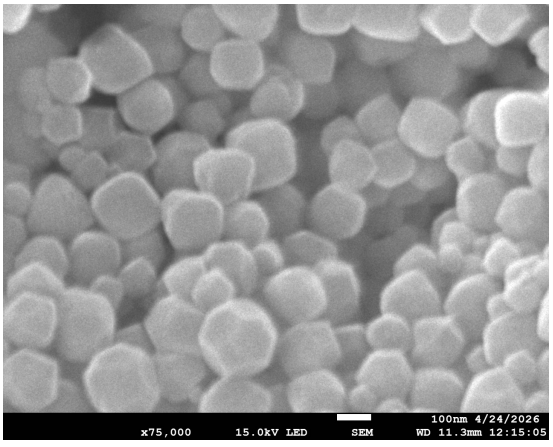

Figure 4.7: SEM picture of SazCA-ZIF-8 made in water. The picture is magnified 75000x.

4.2.1 Quantification of dry weight and ZIF-8 yield

A measurement of the dry weight of SazCA@ZIF-8 and pure MOF in water and in ethanol can be seen in table 4.1. The measurements was carried out with 500 μl of each sample made from 40 ml of ZIF-8 synthesized. The dry weight of SazCA@ZIF-8 are 2.6 mg while the pure ZIF-8 in water is 1.3. These samples had the same amount of 2-MIM and zinc added to the synthesis and the amount of protein added in the total volume of ZIF-8 was 0.625 mg/ml. In 500 μl the amount of protein added will then be $0.625 \times 0.5 = 0.31$mg. As the difference between the dry weight of SazCA@ZIF-8 and pure ZIF-8 (water) is 1.3 which is significantly larger than 0.31, it can be concluded that the adding of the protein facilitates the synthesis of the ZIF-8.

In the ZIF-8 yield vs 2-MIM/Zn amount column which is the ZIF-8 amount produced divided by the amount of 2-MIM and Zinc. It can also be noted that the yield of ZIF-8 here is larger for the SazCA@ZIF-8 than the pure ZIF-8 (water). The yield of the pure ZIF-8 (ethanol) is the largest percent at 30 % and the dry weight at 2 mg, but the protocol for the ZIF-8 made in Ethanol/Methanol have different amounts of 2-MIM and Zinc and therefore the dry weight is not comparable to the other dry weights. However, the yield is much higher than for the ZIF-8 made in water which suggests that the protocol for ZIF-8 made in Ethanol/Methanol is better. Which is reasonable as the ZIF-8 is normally prepared in this way.

Table 4.1: Dry weight of the different ZIF-8 from 500 (μl). With a comparison of the ZIF-8 made compared to the added mixture of 2-MIM and Zinc.

	Dry weight ($500\mu l$)	**ZIF-8 yield vs. 2-MIM/Zn amount**
SazCA@ZIF-8	2.6 ± 0.15 mg	4.2 ± 0.002 %
Pure ZIF-8 (Water)	1.3 ± 0.09 mg	2.5 ± 0.002 %
Pure ZIF-8 (Ethanol	2.0 ± 0.09 mg	30± 0.01 %

4.3 Low enzyme activity in SazCA@ZIF-8

A problem with the encapsulation of the protein is that the activity drops to about 3 % compared to a free protein. This was noted in previous master thesis work and experiments from free SazCA showed that zinc is inhibiting the activity. Even though the fact that SazCA has zinc in its active site which should give the protein some tolerance against zinc. It was also showed that EDTA can rescue CA by making the activity reverse to make the protein regain activity.

4.3.1 First hypothesis: Residual zinc are affecting the activity

Since previous results seen in table 1.2 show that zinc negatively affect the activity of the protein and EDTA can rescue the activity. Based on this an expected outcome

was that 2-MIM can act similarly to EDTA and rescue the SazCA activity if there are no free zinc.

4.3.1.1 Zinc measurements

The free zinc was measured using a standard curve seen in figure 4.8. The standard curve used for measuring the SazCA@ZIF-8 samples was the standard curve in water as all the SazCA@ZIF-8 was prepared in water. If the fluorescence was measured for a sample to be 29492 then the concentration of zinc in mM was calculated according to the example in equation 4.2

$$Zinc\ amount = \frac{29492 - 1006.6}{4 \times 10^6} \times 0,043 \times 65.38 = 0,02\ mg \tag{4.2}$$

From the zinc standard curves in figure 4.8 it was conducted that the zinc measurements was not affected by the Ethanol/Methanol solution. This made the zinc measurements possible to do for the pure MOF made in the ethanol/methanol solution.

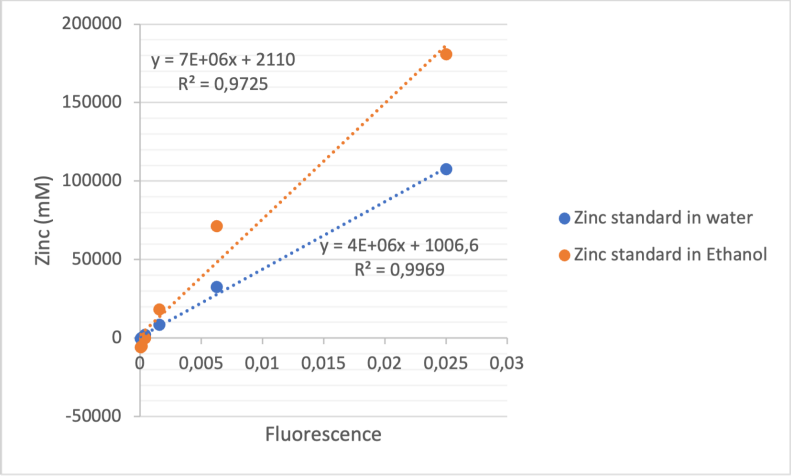

Figure 4.8: Zinc standard curves in water and Ethanol/Methanol solution

A third standard curve was made to see if 2-MIM affected the zinc measurement. This curve can be seen in figure 4.9 and the values are not as expected and seemingly random. The 2-MIM was thus concluded to affect the zinc measurement. The concentration of 2-MIM was the same amount as in the finished SazCA@ZIF-8 produced (1400mM).

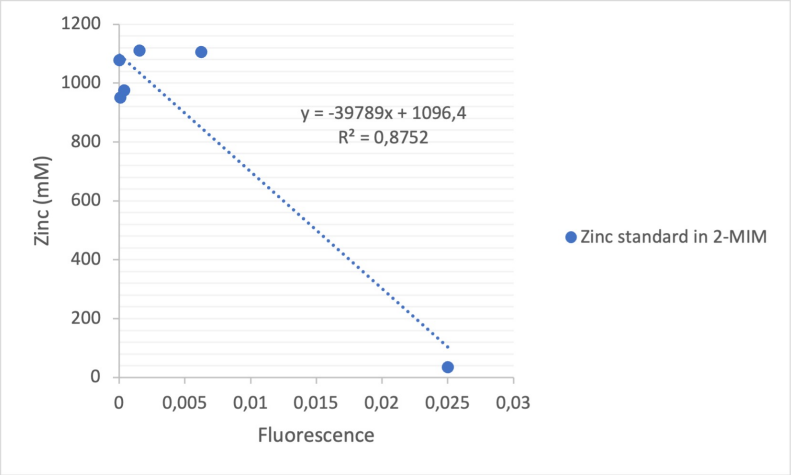

Figure 4.9: Zinc standard curve in 2-MIM solution of 1400mM.

4.3.1.2 Decreasing the free zinc amount does not increase activity

To measure if there are any zinc left in the supernatant of the synthesized SazCA@ZIF-8 the free zinc was measured in all supernatants in the washing steps. In table 4.2, supernatant 1 is the first supernatant taken after the first centrifugation of the synthesized MOF. The supernatant 2-4 is the supernatants after the SazCA@ZIF-8 pellet was suspended in MQ water and centrifuged again. The zinc in the supernatant 1 was measured to be 0, however this sample should still contain a lot of 2-MIM as this sample have not yet been washed. This sample is then not considered reliable but it can be seen with the other supernatants that there is still a lot of free zinc remaining in the SazCA@ZIF-8 after being washed.

Table 4.2: Measured amount of zinc in the supernatant between each wash in the synthesis of SazCA@ZIF-8. The zinc was detected by the Amplite® Fluorimetric Zinc Ion Quantitation Kit. The Wilbur Anderson Units for the activity can be found in Appendix 1.

Sample	**zinc** (μg)
Supernatant 1	0 ± 0.05
Supernatant 2	2,5 ± 0.71
Supernatant 3	44.5 ± 4.5
Supernatant 4	97.1 ± 2.2
Activity	3.0 % ± 0.8

To remove as much free zinc as possible an experiment where the tubes with SazCA@ZIF-8 was rolled for 10 minutes between each wash was done. The result in table 4.3 show a decrease of free zinc in all the supernatants during the washes. The activity got to 3.8 % which is not a significant increase compared to the activity of 3 % for the SazCA@ZIF-8 without rolling. This means that the 2-MIM could not rescue the SazCA even with no free zinc left.

Table 4.3: Measured amount of zinc in the supernatant between each wash in the synthesis of SazCA@ZIF-8 with 10 minutes of rolling in each wash. The zinc was detected by the Amplite® Fluorimetric Zinc Ion Quantitation Kit. The Wilbur Anderson Units for the activity can be found in Appendix 1.

Sample	**zinc** (μg)
Supernatant 1	20.0 ± 0.77
Supernatant 2	4.2± 0.14
Supernatant 3	20.0 ± 0.24
Supernatant 4	0 ± 0.02
Activity	3.8 % ± 1.2

As can be seen in the Theory section 2.3 a study about SazCA being encapsulated in ZIF-8 showed a better activity than the free SazCA after 2.5 weeks. In this study they let the ZIF-8 form and encapsulated the protein on a membrane that had a strong affinity for zinc. This suggest that in this study the zinc could be strongly bonded to the membrane and thus not disturb the activity. Which indicate that it is not only the free zinc that is affecting the activity, which is in line with the results about removing the free zinc in this project.

4.3.1.3 Using EDTA in SazCA washes does not increase activity

Adding EDTA to each wash with MQ water was tested in an amount of 50 mM EDTA. This was then rolled for one hour between each wash. The experiment was not continued as it was noted that the milky white color of the ZIF-8 got transparent which indicate that the ZIF-8 breaks in the vicinity of EDTA.

4.3.2 Second hypothesis: Potential protective effect of other proteins on CA

A SazCA@ZIF-8 was made with a protein that was kept in a freezer at -80 °C for more that 6 months. This sample had an activity of 19.0 % ± 4.6 which is a significant increase of the activity. This result suggest that there might have been other protective structures formed that help protect the activity. A SazCA@ZIF-8 was also made with zinc chloride instead of zinc nitrate hexahydrate. This MOF had a relative activity of 70.4 % ± 8.7 which is a good improvement. From this result some SazCA@ZIF-8 was made with zinc chloride. Wilbur Anderson Units for the frozen SazCA and SazCA@ZIF-8 made can be found in Appendix 1.

Another protein was then introduced to act as a stabilizing factor for the protein during the MOF synthesis. The protein added was Bovine serum albumin (BSA). Different amounts of BSA was mixed with the SazCA according to table A.4 and this MOF was synthesized with Zinc chloride. The protein amount used for calculation of the protein activity was measured by Bradford protein assay and then the added amount of BSA was subtracted of the total amount of protein. For sample 3 the activity was the highest at 32 % which indicates that the BSA did have a protective

effect on the SazCA as the activity gets higher as the amount of BSA gets larger.

Table 4.4: Activity of SazCA@ZIF-8 with part of the SazCA exchanged for BSA. The calculated Wilbur-Anderson Units can be found in Appendix 1.

Sample	**Amount of SazCA/BSA**	Activity
1	100 % SazCA	6.2 ± 1.3 %
2	50 % SazCA, 50 % BSA	16± 2.5%
3	20 % SazCA, 80 % BSA	32 ± 5.4 %

4.3.3 Activity in long term storage

To use SazCA in industry it is of considerable importance that the encapsulated SazCA could keep its stability and activity for a long period of time. An experiment to assess the long term storage was therefore conducted. The SazCA@ZIF-8 and its corresponding protein it was made of was put in storage in 30 degrees with no light exposure to minimize external factors. The activity measured every two weeks can be seen in figure 4.10. Where the activity can be seen to slightly increase as the weeks go by. The largest increase can be seen in the SazCA@ZIF-8 which have increased to almost 12 % after 8 weeks.

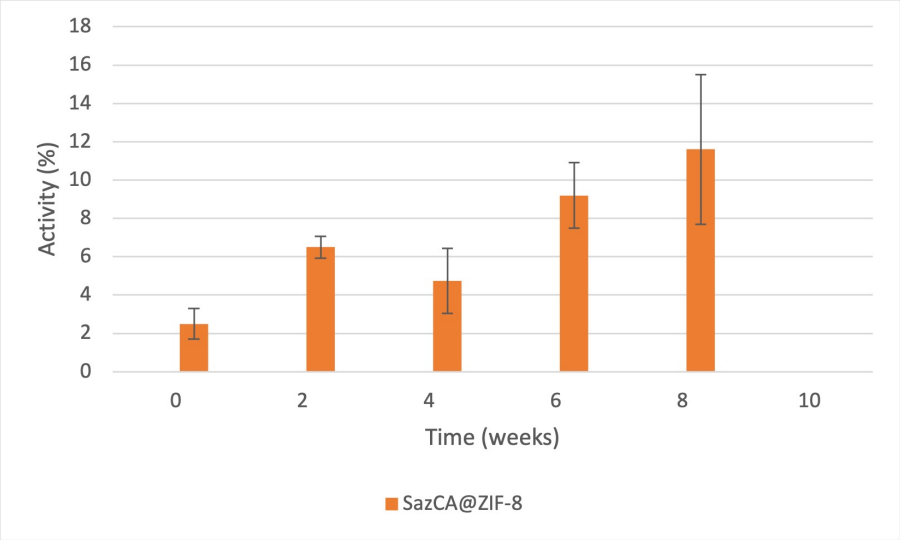

Figure 4.10: Effect of long term storage on activity. Samples stored dark in 30 °C. The calculated Wilbur-Anderson Units can be found in Appendix 1.

4.4 Thermostability

To investigate the thermostability of the SazCA@ZIF-8, a heat treatment was made on a sample of SazCA@ZIF-8 with 20 % SazCA and 80 %BSA. This was the SazCA@ZIF-8 that showed the highest activity and therefore chosen for thermostability experiments. Results from the thermostability measurements can be seen in

table 4.5 where temperatures at 50, 60 and 70 °C was tested. The MOF without heat treatment show the best performance and the activity gets worse for the MOF as the temperature increases. Previous experiments from Theory section 2.3 has showed that the encapsulation of CA in ZIF-8 made the activity higher than for the free SazCA. However, this was a study made on BCA instead of SazCA which might be why the results are different.

Table 4.5: Effect of heat treatment on the activity of SazCA@ZIF-8 with 20 % SazCA and 80 %BSA

Temperature (°C)	**Activity**
No heat treatment	25 ± 8.3 %
50	15 ± 7.3%
60	18 ± 4.4 %
70	2.9 ± 0.6 %

Looking at the WAU/mg for the protein and the SazCA@ZIF-8 separately in table 4.6 it can be seen that the SazCA@ZIF-8 keeps stable during the 50 and 60 °C and then it gets much worse at 70°C. The free SazCA during this temperatures are also very similar to the the no heat treatment sample during the 50 and 60 °C. However at 70°C the WAU/mg for the free SazCA gets increased with about 150%. This indicates that the SazCA works best at 70°C while the encapsulated SazCA get a decreased activity at this temperature.

Table 4.6: Effect of heat treatment on the WAU/mg for of SazCA@ZIF-8 with 20 % SazCA and 80 %BSA, and for the free SazCA.

Sample	**Temperature (°C)**	**WAU/mg**
SazCA@ZIF-8	No heat treatment	52 ± 7.7
SazCA	No heat treatment	208 ± 61.7
SazCA@ZIF-8	50	42 ± 20
SazCA	50	275 ± 29
SazCA@ZIF-8	60	43 ± 8.1
SazCA	60	242 ± 20
SazCA@ZIF-8	70	20 ± 3.2
SazCA	70	624 ± 122

4.5 Low Protein coverage

A problem with encapsulating the protein in the ZIF-8 is the low protein coverage. The table 4.7 shows that from the start of 29 mg added there is only 11.5 mg left after the whole synthesis is done. This means a protein coverage of 40 % which have been a consistent phenomenon during the project when doing SazCA@ZIF-8. The protein coverage have been about 50 % and some possible reasons for this could be that the MOF synthesis is affecting the measurement in some way.

Table 4.7: Measured protein amount of each SazCA@ZIF-8 pellet in the synthesis. The added protein concentration in the SazCA@ZIF-8 was 0.625 mg/ml and measured by Bradford protein assay.

Step in SazCA@ZIF-8 production	SazCA (mg)
Added SazCA to synthesis	29.0 mg
After 15 min stirring	16.5 mg
Overnight	13.6 ± 1.8 mg
Wash 1	13.4 ± 0.4 mg
Wash 2	13.5 ± 1.0 mg
Wash 3	11.5 ± 0.9 mg

The table 4.7 show a reasonably consistent amount in the washing steps, however the largest reduction of protein happens after the 15 minutes of stirring in the ZIF-8 synthesis and when leaving the MOF during the night.To find out if the protein was disappearing through the washes a Bradford protein assay with a lower concentration range was done on the supernatants that were washed away. In table 4.8 Supernatant 1 is the supernatant from the first centrifugation of the SazCA@ZIF-8 which have a protein amount of 4.1 mg. The pellet at the same time show a protein amount of 13.4 mg which added together is approximately the same as the protein amount the day before directly after the stirring with an amount of 16.5. So an amount of protein can be found in the supernatant and washed away. Although in the later supernatants 2-4 which is after the washing steps the protein is zero or close to zero. This indicate that the protein is not released during the harsher conditions of washing in the centrifuge. The amounts of Supernatant 3 and 4 was to low to measure with the assay used and therefore assumed to be zero.

Table 4.8: Measured protein amount in each supernatant in the washing of the SazCA@ZIF-8. The measurements was conducted with Bradford protein assay.

Sample	SazCA (mg)
Supernatant 1	4.1 ± 0.25 mg
Supernatant 2	0.0040 ± 0.008 mg
Supernatant 3	0
Supernatant 4	0

4.5.1 First hypothesis: limited protein accessibility due to encapsulation may affect measurements

There is protein that is unaccounted for between the adding of the protein and after the synthesis is done. An experiment was therefore carried out to find out if the protein could be hiding in the ZIF-8 structure that could lead to a faulty measurement of the protein concentration.

To get a measurement that is not affected by the ZIF-8 structure, the SazCA@ZIF-8 was suspended in 50mM EDTA. The sample was rolled in EDTA for one hour

and three hours to see if the protein measurements were different. The reason for suspending the samples in EDTA was that the EDTA disrupts and destroys the structure. A graphic description of the ZIF-8 destruction can be seen in figure 4.11 which is a SEM picture of SazCA@ZIF-8 that have been suspended in 50mM EDTA for three hours. This figure show a destroyed MOF structure that have lost the structure of the original MOF that can be seen in figure 4.12. When comparing the the both SEM pictures it can be concluded that the morphology of the SazCA@ZIF-8 have been greatly disrupted when EDTA was added.

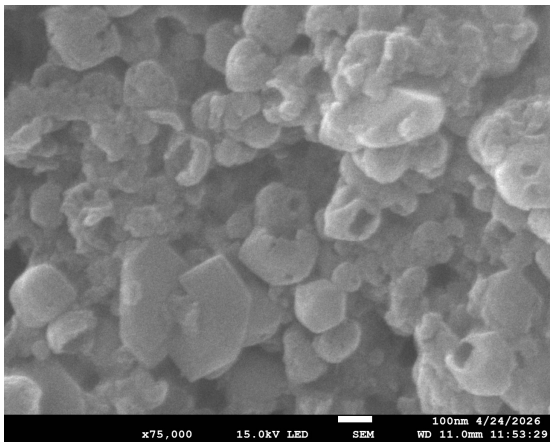

Figure 4.11: SEM picture of SazCA@ZIF-8 suspended and rolled for three hours in 50mM EDTA. With 75000 magnification.

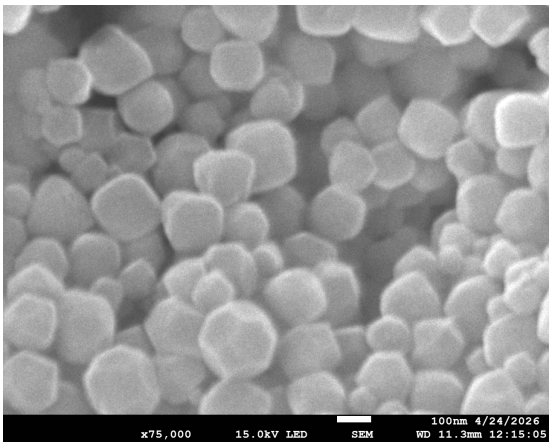

Figure 4.12: SEM picture of SazCA@ZIF-8 with a magnification of 75000

The possible protein hidden in the ZIF-8 structure was expected to be released with the addition of EDTA. The results from having suspended SazCA@ZIF-8 in EDTA can be seen in table 4.9. A protein amount of 1.9 mg was measured before EDTA treatment and after one hour it was 2.1 mg and after three hours it was 2.0 mg of protein. As the amount of protein stays similarly around 2 mg it was concluded that the protein was not hidden in the structure and that the Bradford protein assay was a reliable method. However, it can be noted that this conclusion relies on that the EDTA was successful in releasing hidden protein.

Table 4.9: Measured protein amount in the SazCA@ZIF-8 when suspended and rolled in 50 mM EDTA. The measurements was conducted with Bradford protein assay.

Sample	**SazCA (mg)**
SazCA@ZIF-8	1.9 ± 0.16
SazCA@ZIF-8 + EDTA 1h rolling	2.1 ± 0.05
SazCA@ZIF-8 + EDTA 3h rolling	2.0 ± 0.09

4.5.2 Second hypothesis: 2-MIM may contribute to the low protein coverage

As the protein measurement was concluded to be reliable the focus shifted to the problem that the protein coverage is about halved as soon as the protein synthesis is done. An experiment where every step in the MOF synthesis were measured was carried out, seen in table 4.10. The amount of added protein was 9.4 mg and when mixed with 2-MIM the amount dropped to 4.8 instantly. When zinc was added to the mixture and in the continued measurements during the stirring the amount of protein was consistently around 5 mg SazCA. This indicates that the low coverage of protein can be explained by the addition of 2-MIM where in this example the amount of protein drops to 51 % instantly.

Table 4.10: Measured protein amount in the synthesis of SazCA@ZIF-8.

Step in SazCA@ZIF-8 synthesis	SazCA (mg)
Protein added	9.4 ± 1.1
2-MIM added	4.8 ± 0.02
Zinc added	4.6 ± 0.02
Stirring 5 minutes	4.9 ± 0.02
Stirring 10 minutes	4.9 ± 0.003
Stirring 15 minutes	5.1± 0.02

As 2-MIM is concluded to be the problem with the low protein coverage a test to see if BSA is similarly affected was performed. The results can be seen in figure 4.13 where the difference between the standard curve of water and 2-MIM is not significant. This indicate that the BSA was not affected by the 2-MIM.

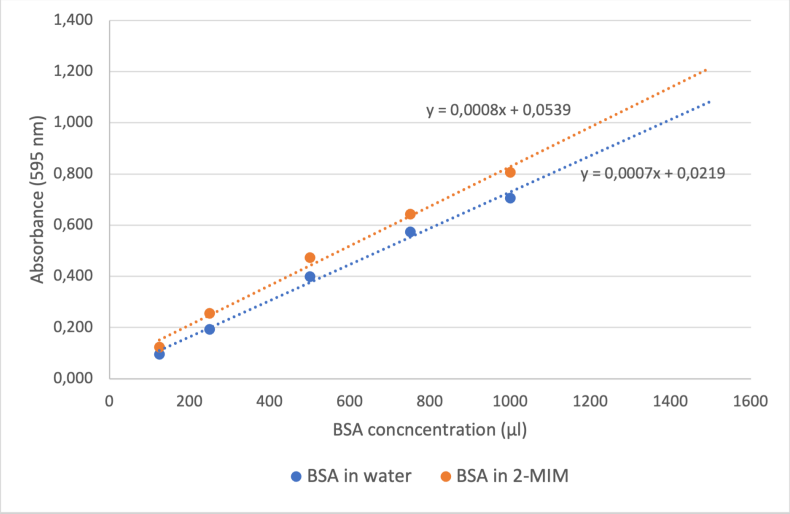

Figure 4.13: BSA standard curves with concentration ranging from 1500-125 $\mu g/ml$. One standard curve with BSA suspended in water and one with BSA suspended in 2-MIM with a concentration of 2800mM.

A gel with BSA and SazCA with water and 2-MIM was made to see how 2-MIM affect the SazCA. In figure 4.14 and 4.15 the sample A is the ladder and sample B-C is the samples for BSA, D-E is samples for a newly produced SazCA protein and the samples F-G is a SazCA that have been frozen for more than 6 months. The samples C, E anf G was suspended in 2800mM of 2-MIM which is the same amount as in the MOF synthesis when 2-MIM and SazCA are mixed. In the BSA samples the 2-MIM shows no noticeable difference which is a further confirmation that 2-MIM does not affect the BSA.

In the figure 4.14 the samples was not boiled before doing the gel. The samples show that the SazCA in water has the dimer form with a molecule weight of about 50 kDa. When 2-MIM is added the SazCA is in its monomer form with about 26 kDa. This is true for both the newly produced and frozen samples of SazCA. However, when the samples are boiled, see figure 4.15, the SazCA in both water and 2-MIM is in its monomer form. When comparing the samples of SazCA none of them showed any degradation of the protein. This indicate that the measurement of the protein is not correct and that the Bradford protein assay is affected by the 2-MIM.

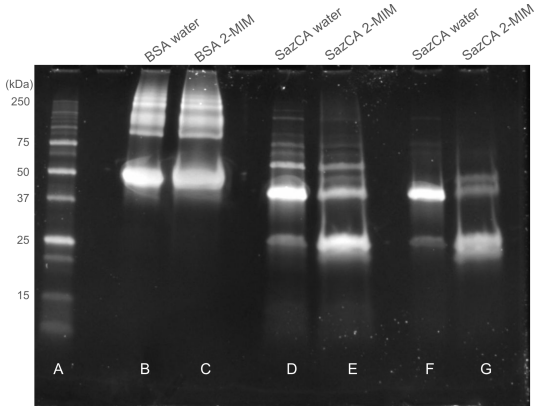

Figure 4.14: Gel picture of not boiled samples of SazCA and BSA. The gel is stained with Coomassie Blue and the 2-MIM concentration is 2800mM.

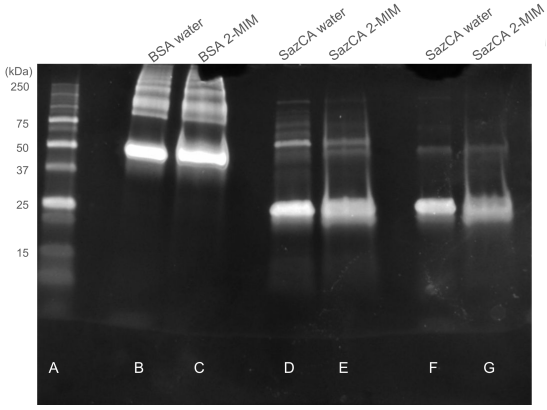

Figure 4.15: Gel picture of boiled samples of SazCA and BSA. The gel is stained with Coomassie Blue and the 2-MIM concentration is 2800mM.

5
Conclusion

During the project, SazCA was confirmed to have been made by a gel electrophoresis. The SazCA@ZIF-8 and pure ZIF-8 was confirmed by SEM pictures and XRD measurements. The evaluated forms of MOF in this project was the encapsulated SazCA@ZIF-8, pure ZIF-8 made in water and pure ZIF-8 made in ethanol/methanol. Through SEM pictures at a magnification of 30000 and 75000 it was noted that the structure and size for the pure ZIF-8 made in water was similar to the SazCA@ZIF-8. A measurement of dry weight showed that the addition of protein to the ZIF-8 facilitates the synthesis of ZIF-8. Although the ZIF-8 made in ethanol/methanol is the most optimal for ZIF-8 yield, but is not possible to use with the protein.

An issue observed in previous work on this project was the low enzyme activity for the encapsulated SazCA to about 3 % of free SazCA. It was also seen that zinc affected the activity negatively and that the activity could be returned by the addition of EDTA. First, it was investigated that if free zinc were removed the 2-MIM could rescue the SazCA activity. It was shown that although the zinc was removed the activity did not return. Also adding EDTA to the SazCA@ZIF-8 did not rescue the protein activity but did destroy the MOF structure.

It was assessed if some other protein could protect the SazCA protein by adding BSA in different amounts. The result was an increase to about 32% activity in the SazCA@ZIF-8 relative the free SazCA. This showed that there is a possibility to protect the enzyme. Looking at a long time storage for the SazCA@ZIF-8 it was showed that the activity of the encapsulated protein got near 12 % relative the free SazCA after about 2 months when starting with an activity of about 3%. This show that encapsulation of SazCA might be a promising way to preserve the activity but it strongly depends on if the activity will hold and increase over a longer period of time. As 12 % activity is not a significantly good increase but depending on the time the protein can be used and stored this might have promise in an industrial setting.

Thermostability measurements at 50,60 and 70 °C was performed and it was seen that the activity of the SazCA@ZIF-8 relative the free SazCA decreased as the temperature increased. It was also noted that the SazCA had its highest activity at 70°C.

Another question addressed in this master thesis was the low protein coverage in the SazCA@ZIF-8 synthesis. It was concluded that the protein was not hidden in

the ZIF-8 structure as the Bradford protein assay showed the same results for when the MOF was destroyed. It was then investigated if the 2-MIM was the issue and if it affected the measurement. It was concluded that the BSA was not affected by the BSA and that the SazCA was affected. When SazCA is mixed with 2-MIM the protein concentration is halved but it was shown that there was no degradation of protein. This indicates that the 2-MIM negatively affect the measurement of protein and that the protein measured are inaccurate.

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A

Appendix 1

Table A.1: Wilbur-Anderson Units of SazCA@ZIF-8 with and withoud rolling between washes from table 4.2 and 4.3

Sample	**rolling/no rolling**	**WAU/mg SazCA**
SazCA@ZIF-8	No rolling	98 ± 20 %
SazCA	No rolling	3323± 614 %
SazCA@ZIF-8	Rolling	26 ± 6.6%
SazCA	Rolling	684± 149%

Table A.2: Wilbur-Anderson Units of SazCA@ZIF-8 and SazCA that have been frozen for more than 6 months in -80 °C.

Sample	**WAU/mg SazCA**
SazCA@ZIF-8 (Zinc nitrate hexahydrate)	45.9 ± 5.6%
SazCA@ZIF-8 (Zinc chloride)	172.2 ± 3.5%
SazCA	243.2 ± 51.2 %

Table A.3: Wilbur-Anderson Units of SazCA@ZIF-8 with part of the SazCA exchanged for BSA.

Sample	**Amount of SazCA/BSA**	**WAU/mg SazCA**
SazCA@ZIF-8	100 % SazCA	149 ± 17 %
SazCA	100 % SazCA	2389 ± 258 %
SazCA@ZIF-8	50 % SazCA, 50 % BSA	160± 11%
SazCA	50 % SazCA, 50 % BSA	1005± 94%
SazCA@ZIF-8	20 % SazCA, 80 % BSA	179 ± 28 %
SazCA	20 % SazCA, 80 % BSA	563 ± 43 %

Table A.4: Wilbur-Anderson Units of long term storage for SazCA@ZIF-8

Sample	**Time (weeks)**	**WAU/mg SazCA**
SazCA@ZIF-8	0	98 ± 20 %
SazCA	0	3323 ± 613 %
SazCA@ZIF-8	2	117± 3.7%
SazCA	2	1810± 149%
SazCA@ZIF-8	4	66 ± 20.5 %
SazCA	4	1378 ± 266 %
SazCA@ZIF-8	6	55 ± 7.8 %
SazCA	6	593 ± 67%
SazCA@ZIF-8	8	101 ± 18 %
SazCA	8	872 ± 250 %

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