I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
ENVIRONMENTAL BIOTECHNOLOGY

IMMOBILIZATION OF CARBONIC ANHYDRASE FROM Sulfurihydrogenibium azorense ONTO IMMOBEAD 350 FOR CO_2 CAPTURE

Ravena C. Oliveira[1], Guilherme A. Lobo[1*], Talita C. E. S. Nascimento[2], Denise C. Hissa[2], Vânia M. M. Melo[2] & Luciana R. B. Gonçalves[1*]

[1] *Department of Chemical Engineering, Federal University of Ceará, Fortaleza, Brazil.*
[2] *Department of Biology, Federal University of Ceará, Fortaleza, Brazil.*
** Corresponding author's email address: Lrg@ufc.br*

ABSTRACT

Carbon emissions increase yearly, breaking a record of 35.7 billion tons released into the atmosphere in 2022. In this scenario, the enzyme carbonic anhydrase (CA) has been a promising alternative to carry out the bioprocess of carbon capture and storage. Within biotechnological processes, one can highlight the obtaining of CA from heterologous expression in a simple microorganism such as *Escherichia coli*. Therefore, considering the importance of obtaining the carbonic anhydrase enzyme for use in CO_2 mitigation technologies and for improving CA's industrial applicability, this work aims to produce a recombinant thermostable carbonic anhydrase expressed in *Escherichia coli* and evaluate its immobilization on support Immobead 350. For this, CA from *Sulfurihydrogenibium azorense* was obtained by heterologous expression in *Escherichia coli* ArcticExpress (DE3) using IPTG as an inducer. The extracts obtained were characterized, purified, and immobilized on Immobead 350. The purified extract showed a 379.783 ± 4.759 WAU/mg. The best result of immobilization was obtained with a load of 5.0 mg/g, showing an activity of 16.91 U/g. This study presented promising and innovative protocols for effectively expressing carbonic anhydrase from *Sulfurihydrogenibium azorense* and its immobilization for CO_2 capture.

Keywords: Decarbonization. Biocatalyst. Carbon capture.

1 INTRODUCTION

Over the last centuries, industrialization has steadily raised greenhouse gas levels, leading to notable shifts in the global climate. Carbon dioxide (CO_2) stands out as the most frequently emitted greenhouse gas, and fossil CO_2 emissions have surged considerably from 22,683 Mt/yr in 1990 to 38,016 Mt/yr in 2019.[1] Therefore, the reduction of CO_2 emissions and their conversion into usable materials are crucial for the advancement of sustainable development. A series of technologies that can capture CO_2 have been developed, such as physical absorption [2] and chemical absorption [3]. Although these methods can effectively capture CO_2, they also have problems such as the generation of by-products, high energy consumption, and secondary pollution [4]. The use of biological processes for carbon capture and storage (CCS) is an eco-friendly means to reduce the concentration of CO_2 in the atmosphere and reduce CO_2 emissions at the source, which can avoid the problem of secondary pollution during CO_2 capture.

Carbonic anhydrase (CA) is an enzyme that catalyzes the conversion of CO_2 into bicarbonate ($HCO3^-$), and it is involved in crucial physiological processes [5]. The use of CA to capture CO_2 has attracted increasing attention due to its mild reaction conditions, lack of secondary pollution, and simplicity [6]. Due to the fact that biomimetic carbon sequestration is frequently carried out in challenging environments characterized by elevated temperatures and high levels of organic ions and metals, the use of thermostable carbonic anhydrase emerges as a sensible option[5]. Among thermostable CA, an α-CA from thermophilic archaea *Sulfurihydrogenibium azorense* (SazCA) stands out because it is one of the fastest CA known[7] with a k_{cat} of $4.4 \times 106\ s^{-1}$. In order to improve the industrial applicability of CA, immobilization has been investigated as a potential method for large-scale application of CA because it not only enhances the tolerance of CA under extreme conditions but also enables the reuse and recycling of CA so that the cost of the entire process can be reduced[8].

Thus, this work aims to produce recombinant thermostable carbonic anhydrase in *Escherichia coli* by heterologous expression and evaluate its immobilization to support Immobead 350 for its use in enzymatic technology for CO_2 capture.

2 MATERIAL & METHODS

Bacterial Strain and Construction of the Recombinant Carbonic Anhydrase (rCA): *Escherichia coli* ArcticExpress (DE3) was transformed with the vector harboring the recombinant gene of carbonic anhydrase from *Sulfurihydrogenibium azorense* by the electroporation method and enzyme production was induced through the lac operon. The recombinant protein has 6 histidine residues (His) in the N-terminal portion of its structure.

Culture Medium and Assay Conditions: For the expression of rCA, enzyme production was induced with 0.5 mM of IPTG final concentration as an inducer agent. A volume of 1 mL of reactivated culture, 1% (v/v) inoculum, was transferred to 100 mL of Luria Bertani medium containing 50 μg/mL of kanamycin and incubated at 30 °C, 250 rpm until it reached an OD_{600nm} from 0.6 to 0.8. Immediately, the culture was induced for CA expression by adding IPTG. The expression occurred at 12 °C, 250 rpm and 24h. At the end of the assay, cultures were centrifuged at 7000×g at 4 °C for 20 min. The supernatant was discarded, and cells were subsequently washed with 0.1 M NaCl solution followed by centrifugation at 7000×g at 4 °C for 30 min. Cell pellets were stored at − 20 °C for further protein expression studies and enzyme activity assays.

Cell Lysis and Extraction of rCA: Cell pellets were resuspended in 20 mM Tris HCl pH 8.3 and cell lysis was conducted by sonication (Sonicator Qsonic, Q500 Sonicators, USA) at 30% amplitude for 4 min (working time and interval of 3 s) at 4 °C. Cell suspension was sonicated until a transparent pale yellow color was obtained. After cell disruption, samples were centrifuged at 5000xg, 4 °C for 30 min, and supernatant (crude CA extract) was stored at − 20 °C and further used to determine enzyme activity, protein concentration, and SDS-Page.

Protein Quantification: Protein concentration was determined by the Bradford method [9], using bovine serum albumin as a standard for building the calibration curve.

Analysis of rCA Expression by SDS–PAGE: Protein expression levels were determined by SDS–PAGE electrophoresis, employing Coomassie Brilliant Blue R-250 as a staining agent, as described by Laemmli [10].

Enzyme Purification: Purification of crude enzyme extracts was performed by immobilized metal affinity chromatography (IMAC) using Ni-Sepharose 6 Fast Flow matrix with a degree of activation of 15 µmol of Ni^{2+} ion per ml of wet resin (GE Healthcare). The chromatography column (1.5 cm high per 1.5 cm wide; volume column: 3 mL) was equilibrated with five column volumes of 20 mM Tris HCl pH 8.3 and 150 mM NaCl buffer. Then, 2 mL of enzymatic extract was applied to the column. Non-retained proteins were recovered and immediately washed with 20 mM Tris–HCl pH 8.3, 150 mM NaCl, and 6 mM imidazole buffer to remove the weakly bound proteins. After that, five column volumes of elution buffer (20 mM Tris HCl pH 8.3, 150 mM NaCl, and 250 mM imidazole) were applied to the column in order to collect the purified protein. Finally, purified rL-AI was dialyzed for 24 h against 20 mM Tris–HCl pH 8.3 for imidazole remotion and further analyzed by SDS–Page.

Enzymatic Activity Assay: The rCA activity was assayed by its esterase and hydratase activity. The assay procedures were the same as those reported by others [11]. For hydratase activity, one Wilbur–Anderson unit (U) is defined as $(T_0 - T) / T$, where T_0 and T are the time needed for pH changing from 8.3 to 6.3 in the absence and presence of CA, respectively. Esterase activity was performed using *p*-nitrofenyl acetate as substrate.

Immobilization on Immobead-350: Preliminary studies of rCA immobilization were conducted with macroporous acrylic polymer support, Immobead-350 (IB-350). Firstly, 20 g of IB-350 was incubated under gentle stirring in 25 mL of ethanol (95%) for 4 h at 25 °C. Then, the support was filtered and washed with distilled water. After, 1 g of support IB-350 was suspended in 10 mL of enzyme solutions (protein concentration of 0,5, 1,0 and 5,0 mg/ml) in 20 mM Tris-HCl buffer at pH 7,2 and 25 °C under gentle stirring for 2 h. The immobilized biocatalysts were incubated in 1 M EDA at pH 10 and 25 °C for 24 h to block the remaining reactive groups in the support. The immobilization parameters were determined as described by Silva and colleagues. [12]

3 RESULTS & DISCUSSION

Carbonic anhydrase expression: According to Figure 3, a strong band close to 26 kDa after induction can be seen, the size reported for the monometer of the carbonic anhydrase enzyme from *Sulfurihydrogenibium azorense*, indicating the expression of the enzyme of interest. Besides that, it is important to notice that most of the protein produced is in the soluble fraction after lysis cellular.

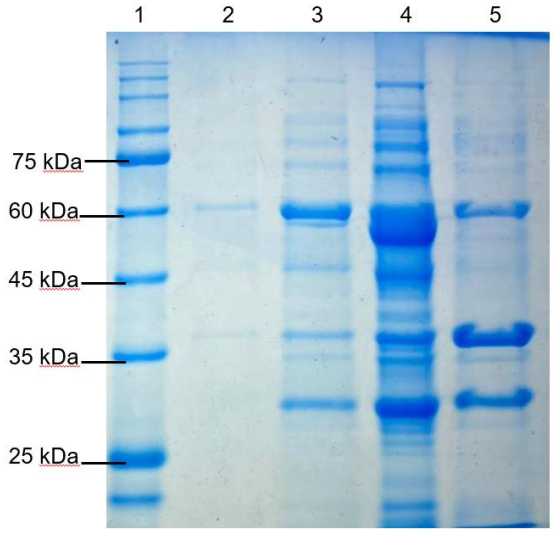

Figure 1 Electrophoretic profile for carbonic anhydrase from *Sulfurihydrogenibium azorense* expression in *E. coli* ArcticExpress (DE3). (1) Molecular weight standards, (2) culture medium before induction, (3) culture medium after 24 h of induction, (4) crude enzymatic extract, and (5) insoluble fraction.

Purification and Characterization of recombinant Carbonic Anhydrase: Purification of recombinant carbonic anhydrase was performed by immobilized nickel affinity chromatography, and the results obtained can be seen in Table 1. Purified CA extracts showed an enzyme specific activity of 379.78 WAU/mg, which turned out to be 3.66-fold higher than the specific activity of the crude extract (110.53 WAU/mg), which indicates the presence of higher concentrations of target carbonic anhydrase.

Table 1 Purification parameters of carbonic anhydrase obtained from heterologous expression.

Enzymatic extract	Protein concentration (mg/mL)	Enzymatic activity (WAU/mL)	Specific activity (WAU/mg)	Purification Factor
Crude	1.07 ± 0.02	110.53 ± 7.02	103.68 ± 7.03	1.00
Purified	0.49 ± 0.01	185.71 ± 4.76	379.78 ± 4.76	3.66

Carbonic anhydrase immobilization on Immobead 350: Table 2 presents the immobilization parameters of CA onto Immobead 350. According to these results, the 5.0 mg/g load produced a biocatalyst with the highest activity (16.91 ± 1.10 U/g). Furthermore, it is possible to see that higher loads lead to lower recovered activity and immobilization yields, which can be explained by a high protein load favoring the enzymatic cluster in the support, thus increasing its proximity. This can cause diffusional limitations in the system, in addition to incorrect positioning of the enzyme in the support and the distortion of the active site.[13]

Table 2 Immobilization parameters were obtained in the process at 25 °C in 20 mM Tris-Hcl buffer pH 7.2 under gentle agitation for 2 h.

Load (mg/g)	Immobilization yield (%)	Activity offered (U/g)	Biocatalyst activity (U/g)	Recovered activity (%)
0.5	68.17 ± 2.83	11.06 ± 0.31	7.15 ± 0.28	95 ± 2.05
1.0	54.95 ± 1.36	35.10 ± 2.77	15.86 ± 1.17	82 ± 8.56
5.0	38.07 ± 4.24	65.17 ± 3.85	16.91 ± 1.10	74 ± 8.06

4 CONCLUSION

This work shows that it is possible to obtain an enzymatic extract of carbonic anhydrase from *Sulfurihydrogenibium azorense* by heterologous expression in *E. coli* ArcticExpress (DE3), presenting a high value of hydratase activity (379.783 ± 4.759 WAU/mg). The preliminary results of CA immobilization have shown that the immobilization strategies using Immobead-350 as support were efficient, allowing obtaining a biocatalyst with high activity and recovered activity. Finally, this study presented promising and innovative protocols for effectively expressing carbonic anhydrase from *Sulfurihydrogenibium azorense* and its immobilization for its use in enzymatic technology for CO_2 capture.

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ACKNOWLEDGEMENTS

The authors acknowledge the Brazilian Research Agency CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico and the the Instituto Nacional de Ciência e Tecnologia em (Bio/Foto)Catálise, Adsorção e Intensificação de Processo para avançar na Captura e Utilização de CO2 (INCT-CAPICUA) for the financial support.