HETEROLOGOUS EXPRESSION OF A THERMOSTABLE CARBONIC ANHYDRASE FROM *SULFURIHYDROGENIBIUM AZORENSE* IN *E. COLI* ARCTICEXPRESS (DE3) FOR OPTIMIZING INDUSTRIAL CO_2 ABSORPTION

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ABSTRACT

This study explores innovative solutions for addressing global warming through enzymatic carbon capture technologies. Our primary goal was to produce the thermostable carbonic anhydrase enzyme, *Saz*CA, from *Sulfurihydrogenibium azorense*,by heterologous expression on *Escherichia coli* ArcticExpress(DE3) strain. After gene cloning and extensive testing of expression conditions, the results, depicted in an electrophoresis SDS-PAGE gel, validated the success of the process. Furthermore, 0.5mM of IPTG yielded 0.69mgP/mL in a soluble fraction. This achievement opens new doors for developing industrial-scale carbon capture utilization and storage (CCUS) technologies and inspires a future with significantly reduced CO_2 emissions.

Keywords: Carbon capture. Heterologous expression. Carbonic anhydrase. Mutant. Environmental biotechnology.

1 INTRODUCTION

The Sustainable Development Goals, proposed by the United Nations in 2014, is an example of international concerns over global warming. Unfortunately, recent research has shown little to no positive chances against global heating[1]. The National Centers for Environmental Information (NCEI) declares that 2023 was the warmest year up to date[2] and the consequences such as ocean heat content, snow cover, sea ice reduction, and also tropical cyclones can be catastrophic for the environment and humanity. Among the gases responsible for this, carbon dioxide, present in abundance in the atmosphere due to its emission by energy production from carbon sources, is associated with infrared absorption and other undesirable chemical reactions with the ozone layer[3], being the most related to global warming. This study, therefore, is a significant step towards addressing this issue, as it explores innovative solutions for addressing global warming through enzymatic carbon capture technologies.

It is the scientific community's responsibility to develop new technologies capable of reducing CO_2 emissions, which can be either finding new completely green energy sources or a way to capture de emitted CO_2. The carbon dioxide capture, utilization and storage (CCUS) has developed enough to three possible pathways: the physical one, which has not shown scale-up advances; the chemical, which generates harmful by-products, and the biological, which does have the possibility of contamination due to microorganisms, but that can be bypassed by the possible application of enzymes[4], which need to be studied and appraised.

Furthermore, carbonic anhydrase is one of the most efficient enzymes for carbon capture, and the one produced by *Sulfurihydrogenibium azorense*[5] is an example. This class of lyases can physiologically catalyze the carbon dioxide in the presence of water to the ion CHO_3^- and H^+. Associated with the genetic engineering and other biotechnology tools available, such as heterologous production on many different cellular platforms and enzymatic immobilization, it is possible to optimize this enzyme application[6]. The heterologous expression has shown many possibilities of strains to achieve specific chemical characteristics, such as BL21 (DE3), which is deficient in the *lon* and *ompT* proteases and has the *lac* promotor for Isopropyl-ß-D-thiogalactopyranoside (IPTG) induction; or the Rosetta(DE3) that has extra tRNAs for rare codons, and even the ArcticExpress(DE3), with suitable chaperonins Cpn10 and Cpn60, adapted from 10°C to 12°C expression temperatures to reduce insolubility[7].

The main objective of this study is to evaluate the heterologous production of the thermostable carbonic anhydrase enzyme dimer *Saz*CA by the Escherichia coli ArcticExpress(DE3) strain. This evaluation is crucial as it will determine the enzyme's potential for large-scale application in the CCUS enzymatic pathway. To achieve this, a series of steps were conducted, including gene cloning, expression testing, and analysis of the results using electrophoresis SDS-PAGE.

2 MATERIAL & METHODS

The carbonic anhydrase sequence was acquired on the Protein Data Bank, under PDB ID: 4X5S[8], and bought within the vector pET-28a(+) with kanamycin resistance, lac promoter and his tag. from FastBio Ltda. The ArcticExpress(DE3) strain was acquired from Agilent Technologies and put under electrocompetent protocol to further be electroporated in cloning protocol[9] and observed grown in a plate containing selection antibiotic kanamycin.

The first step after transformation according to the manufacturer's protocol for expression was observed: 50ml LB broth were added 1ml inoculates, containing 20µg/ml of gentamycin and 50µg/ml of kanamycin, from single colonies selected on the transformation plates. Then, it was incubated at 37°C, shaking at 220–250 rpm overnight. More than one clone was tested, as variations in protein expression may occur among colonies. After that, 60µl of each culture was pipetted into 3 ml of LB broth in 15ml centrifuge tubes, containing no selection antibiotics, and incubated at 30°C with shaking at 220–250 rpm for 3 hours until optical density between 0.6-0.8 at 600nm. Transferred 100µl of each culture into a clean microcentrifuge tube and placed the tubes on ice until needed for gel analysis as the non-induced control samples. The primary cultures were then put in 10–13°C condition and incubated with shaking at 220–250 rpm for 10 minutes. After it was equilibrated to 10–13°C, added IPTG to each 15 ml tube to a final concentration of 1 mM and incubated at 10–13°C, with shaking at 220–250 rpm, for 24 hours.

The manufacturer also indicates the sampling of 20µl of the final grown culture after expression in centrifuge tubes for Electrophoresis SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel) which was carried in a Biorad system, with a 12% polyacrylamide gel and SIGMA BLUeye Prestained Protein Ladder. For each sample, 20µl of SDS 2X sample buffer was added and prepared under 5 minutes of 95° heat. The best clone was tested with IPTG concentrations of 0.5mM and 1.5mM, and their soluble and insoluble fraction after the ultrasonic cell disruptor procedure was also analyzed with electrophoresis SDS-PAGE. Then the best condition was expressed with expression time study and protein concentration was determined by the Bradford method using bovine serum albumin as reference[10] and Bicinchoninic acid (BCA) assay for insoluble proteins[11].

3 RESULTS & DISCUSSION

After the expression, SDS-PAGE gels were prepared, registered and gathered in Figure 1 for analysis. Initially, three different clones were tested according to the manufacturer's protocol. It is important to highlight that the expressed protein band appeared near 27kDa, an unexpected result considering the PDB data indicated *Saz*CA has 54.61kDa. This discrepancy could be due to the denaturized methodology for electrophoresis SDS-PAGE gel, which may have caused the dimer to separate into its monomer. Another possibility is that the crystal structure of *Saz*CA forms a dimer despite its natural form being a monomer. In Figure 1A, it can be seen that expression levels are similar in the three clones. The subsequent experiments were carried out with clone number one. Figure 1B shows the evaluation of different concentrations of the inductor. Interestingly, it was found that 0.5mM of IPTG was not only sufficient but also efficient in producing the same results as higher concentrations. The observed soluble fraction showed the presence of the enzyme of interest, indicating the positive functionality of the produced enzyme as it stays soluble after expression. Figure 1C represents the expression in 50mL of LB broth media follow-up through expressing time, and it was observed that 20 hours of expression reached a similar band aspect to the 24 hour expression in the gel. This finding opens up interesting possibilities for further study and optimization of the expression duration, a potential area of significant advancement in our field.

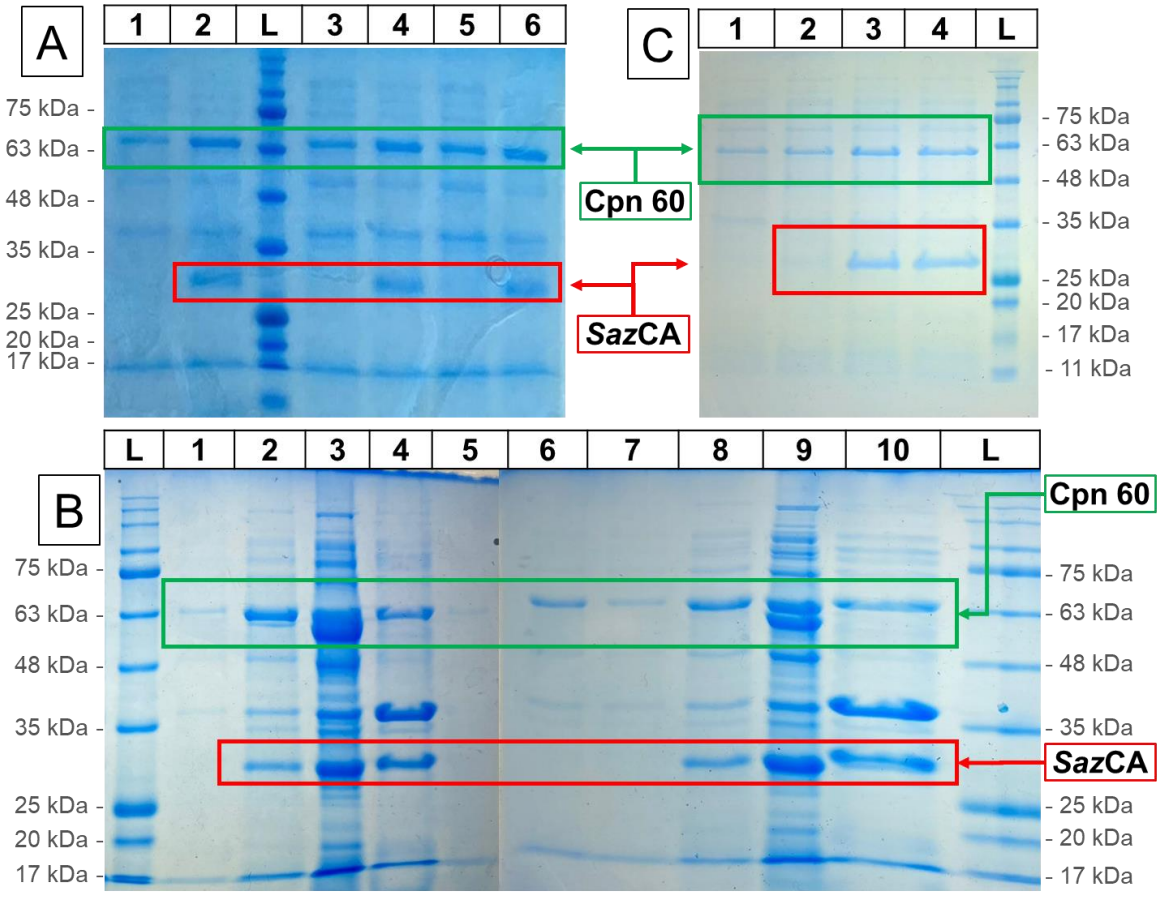

Figure 1 A Coomassie-stained SDS polyacrylamide gel of 12% analyzing protein expression. Each capital L refers to the Protein Ladder. **A:** A1: Clone 1 pre-induction. A2: Clone 1 under IPTG 1.0mM 24h induction. A3: Clone 2 pre-induction. A4: Clone 2 under IPTG 1.0mM 24h induction. A5: Clone 3 pre-induction. A6: Clone 3 24h under IPTG 1.0mM 24h induction. **B:** B1: Clone 1 pre-induction. B2: Clone 1 under 0.5mM IPTG 24h induction. B3: Soluble fraction of Clone 1 under 0.5mM IPTG 24h induction. B4: Insoluble fraction of Clone 1 under 0.5mM IPTG 24h induction. B5: Non-transformed ArcticExpress(DE3) pre-induction. B6: Non-transformed ArcticExpress(DE3) under IPTG 1.0mM 24h induction. B7: Clone

1 pre-induction. B8: Clone 1 under 1.5mM IPTG 24h induction. B9: Soluble fraction of Clone 1 under 1.5mM IPTG 24h induction. B10: Insoluble fraction of Clone 1 under 1.5mM IPTG 24h induction. **C:** C1: Clone 1 pre-induction. C2: Clone 1 under 0.5mM IPTG 4h induction. C2: Clone 1 under 0.5mM IPTG 20h induction. C2: Clone 1 under 0.5mM IPTG 24h induction.

The most favorable conditions for enzyme expression were determined by quantifying protein concentration using the Bradford and BCA assays. The beneficial effect of ArcticExpress(DE3) heterologous expression is evident when the soluble and insoluble fraction concentrations are compared. Although there is still room for improvement to achieve higher concentrations, it is also crucial to thoroughly characterize the enzyme through activity tests and further investigate the purification protocol. These steps are crucial for the success of our research.

Table 1 Protein concentration of soluble, with Bradford essay, and insoluble, with BCA essay, fractions of ruptured cells from clone 1 expression under 0.5mM IPTG induction during 24h.

Sample	Concentration (mgP/ml)	Standard deviation
ArcticExpress(DE3) Clone 1 soluble fraction	0.69	±0.016
ArcticExpress(DE3) Clone 1 insoluble fraction	0.002	±0.003

4 CONCLUSION

In conclusion, the heterologous expression of the thermostable *S. azorense* carbonic anhydrase in the ArcticExpress(DE3), a laboratory *Escherichia coli* strain, was successful. The soluble fraction indicated the presence of the enzyme of interest, which presented CO_2 and esterase activity. The recent discoveries broaden new opportunities in environmental biotechnology for improving the production and effectiveness of enzymatic carbon capture on an industrial level. This could lead to better conditions for reducing CO_2 emissions globally.

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