A new strategy for the high-yield production of recombinant *Clostridium septicum* alpha-protoxin: Periplasmic expression in *Escherichia coli* C43 followed by extracellular release via osmotic shock

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Abstract:

Introduction: Given the challenges associated with producing native toxin from *Clostridium* bacteria, including biosafety risks, impurity and low yield, recombinant expression in hosts such as *E. coli* has emerged as a safe, pure and cost-effective approach. **Materials & Methods:** The codon-optimized sequence of the alpha protoxin of *Clostridium septicum* was synthesized and cloned into the pET22b+ vector for expression in *E. coli*. The recombinant plasmid was transformed into the *E. coli* strain C43 via heat shock and induced with 0.2% lactose in 5 liter 2YT media at 22°C. Using the pelB leader signal, the protoxin was expressed in the periplasmic space. Osmotic shock was employed to release the protein extracellularly. The protoxin was purified from the osmotic shock buffer containing the recombinant toxin using nickel resin affinity chromatography. **Results:** The yield of purification estimated 10.4mg per one liter of bacterial culture. After dialysis, protein concentration was performed using the Bradford assay. The recombinant protoxin was confirmed by Western blotting and specific NIBSC antibody by indirect ELISA. Furthermore, the produced recombinant toxin successfully detected antibodies generated in rabbits immunized with the quadrivalent clostridial vaccine of Razi institute using indirect ELISA assay. **Conclusion:** This recombinant toxin might be used for the development of recombinant vaccine or as a standard antigen for quality control of the traditional vaccine.

1-Introduction

Clostridium septicum is a Gram-positive, spore-forming bacillus that grows only in anaerobic condition and is known to cause severe illnesses in both humans and animals. It is responsible for life-threatening conditions such as gas gangrene (myonecrosis) in human and braxy in sheep [1]. These infections develop rapidly and often lead to death, especially in individuals with weakened immune systems or underlying issues like colorectal cancer [2].

The main virulence factor of C. septicum is the alpha toxin, a 46 kDa single-chain protein that belongs to a family of pore-forming toxins [3]. In first step, the alpha toxin attaching tothe GPI anchored receptors on the outer surface of host cells. It then forms heptameric complexes that create pores in the cell membrane, causing the cell to swell, lose its structural integrity, and eventually die through necrosis [4]. Several amino acid residues which were located in domain 3 of alpha toxin are essential for effective oligomerization [5].

Understanding how this toxin works is critical for discovering new treatments and creating fast diagnostic tools. Alpha toxin is also a key target for vaccine development against *C. septicum*, and neutralizing antibodies against the toxin can offer strong protection [6]. Moreover, the toxin serves as a valuable tool in research on membrane pore formation and cell signaling.

C. septicum culture is difficult and expensive due need to strict anaerobic and high biosafety level conditions. Extracting the natural toxin from culture fluids often results in low purity,

low yield, and contamination with other bacterial products. Therefore, producing the alpha toxin recombinant in a harmless host like *E. coli* is an effective way to overcome these challenges [7]. This approach allows for large-scale production of pure, consistent toxin for structural and functional studies, as well as providing a safe, high-quality antigen for subunit vaccines and the production of highly specific monoclonal antibodies [8].

In this study, we attempted to express the soluble form of alpha toxin in periplasmic space and release extracellularly. Obtaining this recombinant protein will be a fundamental step towards future research for designing new vaccines against this lethal pathogen.

2-Materials and Methods:

2-1 Design a DNA construct for periplasmic expression:

To achieve periplasmic expression of the target protein in *E.coli*, a dedicated genetic construct was designed and synthesized. The alpha-protoxin coding sequence was codon-optimized for enhanced expression in *E. coli* and cloned into the pET22b+ expression vector. Crucially, the sequence encoding the pelB signal peptide was incorporated at the 5´ region of the alpha-protoxin. This leader peptide facilitates its transport across the inner membrane and subsequent cleavage by signal peptidases in the periplasmic space. The integrity of the final recombinant plasmid, designated pET-pelB-protoxin-6His, was confirmed by restriction enzyme digestion and DNA sequencing, ensuring accurate assembly of all genetic elements for precise periplasmic targeting.

2-2 Periplasmic expression of protoxin in *E.coli* C43 in a 5 L Fermentor

Initially, a bacterial suspension expressing the recombinant alpha-toxin, derived from a master plate, was inoculated into 5 ml of 2YT medium containing 5 µl of ampicillin. The culture was incubated at 37°C with shaking at 110 RPM. After 8 hours, the bacterial suspension was transferred to an Erlenmeyer flask containing 50 ml of 2YT medium supplemented with 50 µl of ampicillin and incubated overnight at 37°C until the OD reached approximately 0.6.The following day, this 50 ml culture was transferred into the fermentor containing 5 L of 2YT culture medium, which had been prepared the previous day and verified to be free of contamination. Sampling and OD measurements were performed every 2 hours. After 4 hours, 50 ml of a 20% lactose stock solution was added to achieve a final concentration of 2 g/L (0.2% w/v). The culture was then transferred to a 22°C incubator. At this point (t_0), a 5 ml sample was collected, and its absorbance was measured. To check for contamination, the sterility test was streaked onto blood agar. Additionally, 1 ml of the sample was centrifuged at 5,000 RPM for 5 minutes at 4°C. The supernatant was discarded, and the pellet was stored at -20°C. This procedure was repeated for subsequent time points (t1 to t3). The following day, the entire bacterial culture was harvested, after an induction period of 20 hours, and stored in a cold room.

2-3 Extracellular release of recombinant protoxin by Osmotic shock:

The overnight bacterial culture was diluted to 1:100 in fresh LB/ampicillin and grows at 37^{o}C with 250 RPM shaking incubator until OD600 reached to 0.6. The culture move into 18^{o}Cincubator with 180 RPM until OD600 reached to 1. The expression was induced by adding 0.2 % lactose and incubation for 20 hours at 18^{o}Cwith 180 RPM. The culture was spun down at 8000 x g at 4^{o}C for 20 minutes and the supernatant discarded. The pellet was

re-suspended in 3ml of TSE buffer (200mM Tris-HCl pH8, 500mM sucrose, 1mM EDTA) per gram of cells. The suspension incubated at room temperature for 10 minutes. Cold-shock was done by adding 3ml of ice-cold MQ water per gram of cells. The suspension was incubated on ice for 10 minutes, centrifuged at 8000 x g, at 4^{o}C for 20 minutes, and then the supernatant was collected.

2-4 **Purification of protoxin by affinity method:**

The recombinant alpha protoxin, fused with a 6His-tag was purified by using immobilized metal affinity chromatography (IMAC). The 200µl Nickel resin was subsequently loaded onto secreted recombinant toxin in osmotic shock buffer and incubated for 1hour at 22^{o}C. Resin was pre-equilibrated with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and then washed with 300 µl of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 25 mM imidazole, pH 8.0) to remove non-specifically bound proteins. The bound target protein was eluted using of imidazole 300 mM in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The eluted fractions were analyzed by SDS-PAGE and those containing the protein of sufficient purity were pooled and dialyzed against PBS, pH 7.4 for further analysis.

2-5 Micro dialysis and Bradford protein assay:

For micro dialysis, the purified protein was subjected to dialysis against PBS buffer pH 7.5 using a dialysis membrane. The dialysis was performed at 4°C under constant stirring for 4 hours, with multiple changes of the dialysis buffer to ensure efficient removal of salts, imidazole and other small molecules.

Following purification, the concentration of the recombinant protein was accurately determined using the Bradford protein assay. Briefly, a standard curve was created using known concentrations 1 to 10 µg/ml of bovine serum albumin (BSA). A 2µl of the purified protein sample was mixed with Bradford reagent and incubated at room temperature for 10 minutes. The absorbance was then measured at 595 nm using a spectrophotometer. The protein concentration of the unknown sample was subsequently calculated by interpolating its absorbance value against the standard curve.

2-6 **Evaluation of protoxin by western blotting and ELISA**

Western blot analysis was carried out. The purified protein was separated by 10% SDS-PAGE, transferred onto a PVDF membrane and probed with An HRP-conjugated antiHis-tag antibody. Additionally, to confirm the identity and antigenicity of the purified protein (250ng/well), enzyme-linked immunosorbent assay (ELISA) was performed using a NIBSC standard specific antibody and antibodies generated in rabbits immunized with clostridia vaccine (quadrivalent) of Razi vaccine and serum Research Institute. Briefly, micro plates were coated with the recombinant protoxin, followed by blocking with 2% BSA and incubation with the NIBSC specific antibody for 1hour. Bound antibodies were detected using an HRP-conjugated secondary antibody with a 4-Chloro-1-naphthol colorimetric substrate and H_2O_2.

3- **Results**:

A gene construct was designed in such a way that the pelB signal peptide was placed at the N-terminal of the protoxin (Fig.1). This prevents the addition of an extra amino acid at the N-terminal of the toxin after purification. Furthermore, a 6xHis-tag was incorporated at the N-terminal so that after cleavage by furin on the cell surface, this sequence along with the pro-peptide would be removed, causing no alteration to the structure of the toxin (Fig.2).

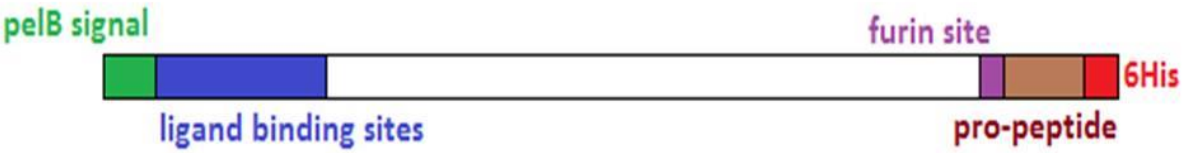


Figure.1 Schematic of designed protoxin for periplasmic expression in *E.coli*

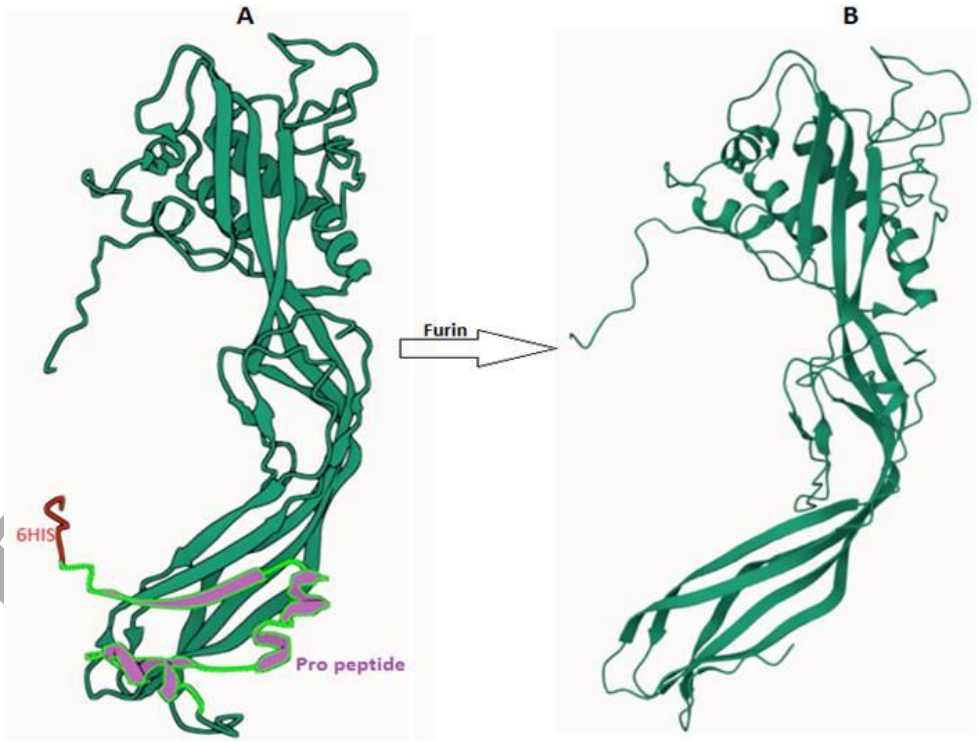


Figure. 2 Comparing 3D structure of alpha toxin active form (Left), protoxin with 6His-tag sequence (Right)

Consequently, the structure of conformational epitopes remains unchanged, and the recombinant alpha protoxin structure can maintain a monomeric form, leading to better performance of immunogenicity.

The insilico designed oligomerized structure of the recombinant protoxin was investigated before and after digestion with furin enzyme, and the results showed that after digestion with furin, the pro-peptide region and the His-tag sequence are removed, and the natural heptamer structure is formed (Fig.3).

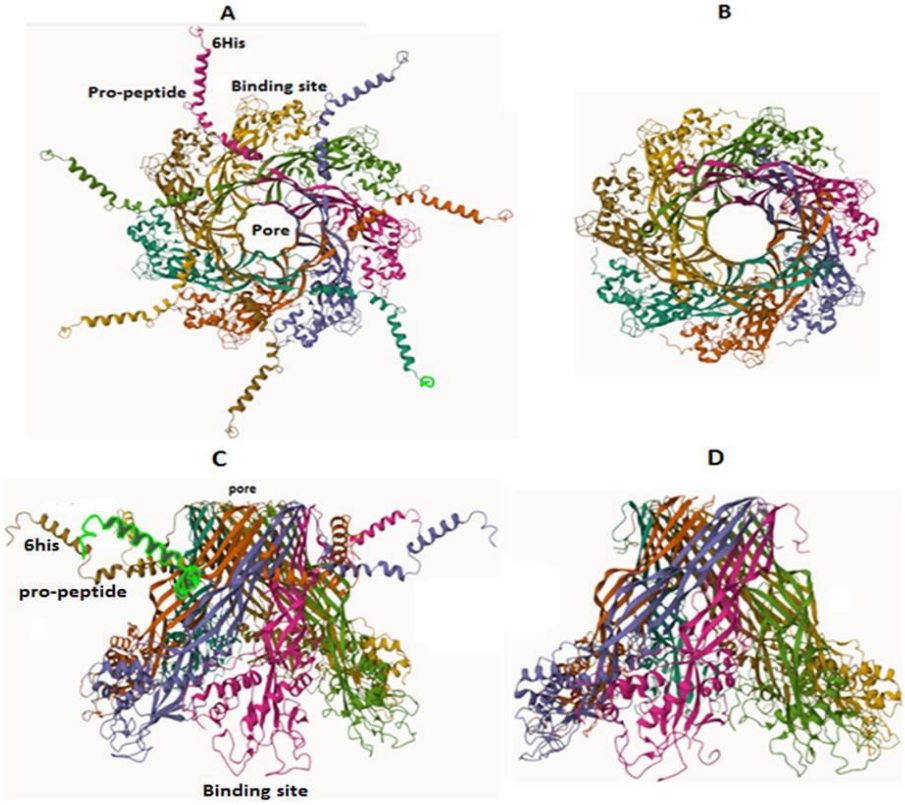
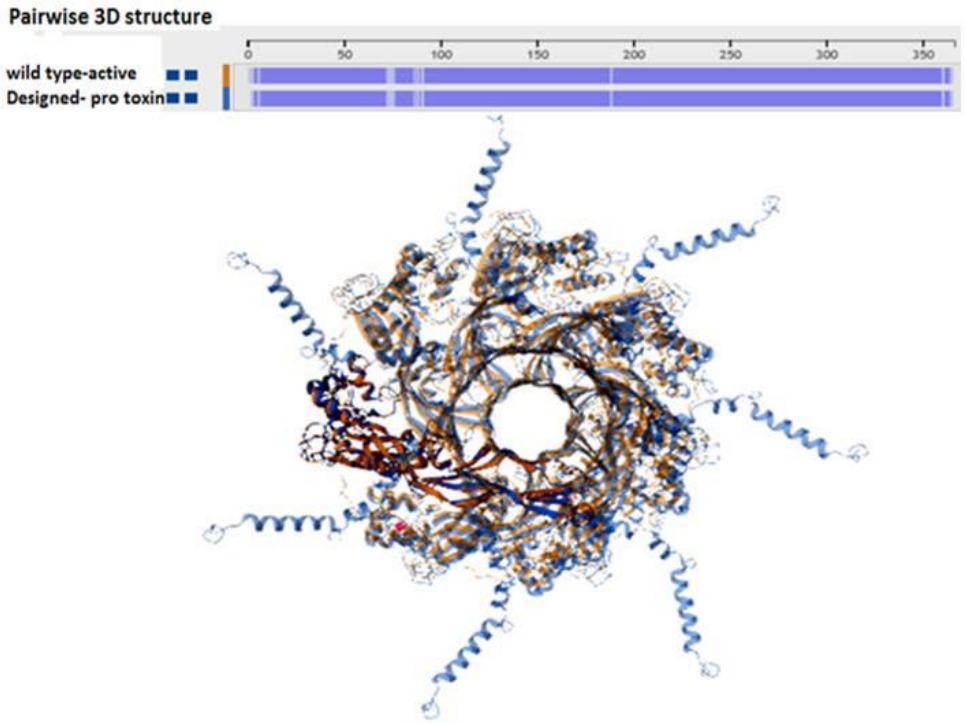


Figure.3. Heptamer form of designed alpha protoxin before (A, C) and after activation (B, D)

This designed construct of recombinant toxin was compared to the three-dimensional structure of the wild-type active toxin, and the results showed 100% similarity with a 0.95 TM-score (Fig.4).



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155 Figure.4. Comparing 3D structure of inactive and active forms of engineered protoxin.
156 TM-score 0.95, 100% identity.

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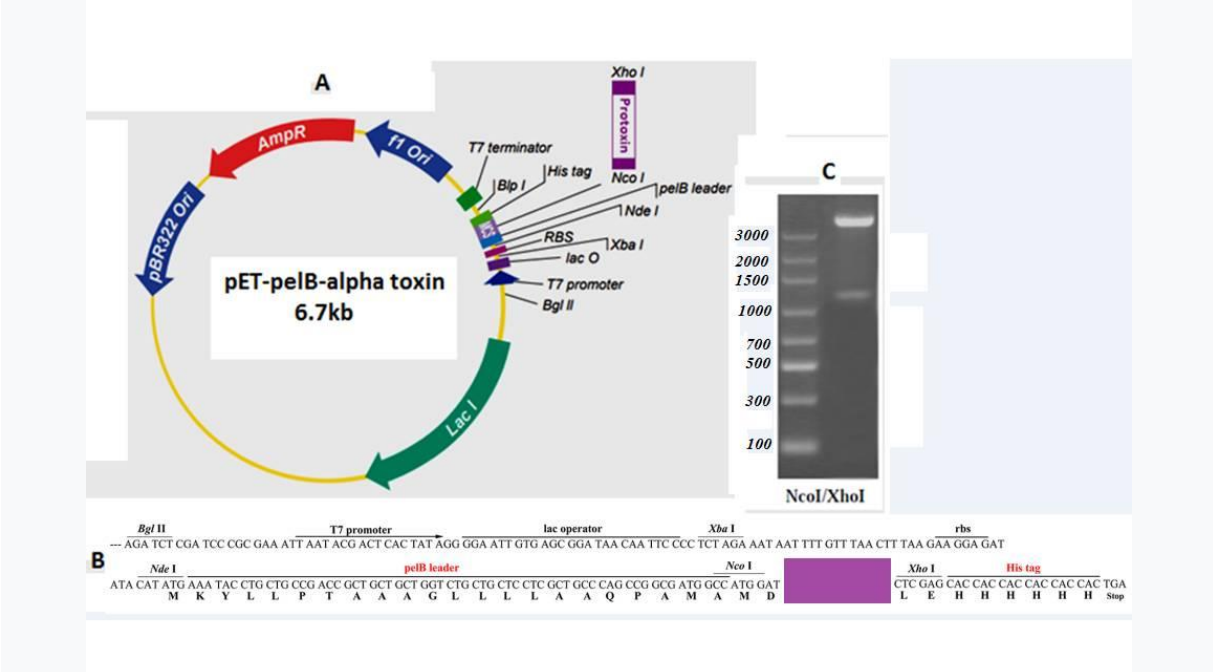
158 The codon-optimized sequence was synthesized for expression in E. coli and cloned between
159 NcoI and XhoI recognition sites in multi cloning site of pET22b+ expression vector (Fig.5).



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161 Figure.5.A, B: Map of the genetic construct designed for expression of protoxin and C:
162 enzymatic digestion pattern of the vector with *Nco*I and *Xho*I restriction enzymes

The genetic construct, harboring the protoxin sequence fused to a pelB signal sequence, was successfully transformed into the *E.coli* strain C43 host. Expression of protoxin successfully translocated into the periplasmic space. This approach enabled the production of the protoxin in a soluble form, which was subsequently confirmed by SDS-PAGE and western blotting (Fig.6).

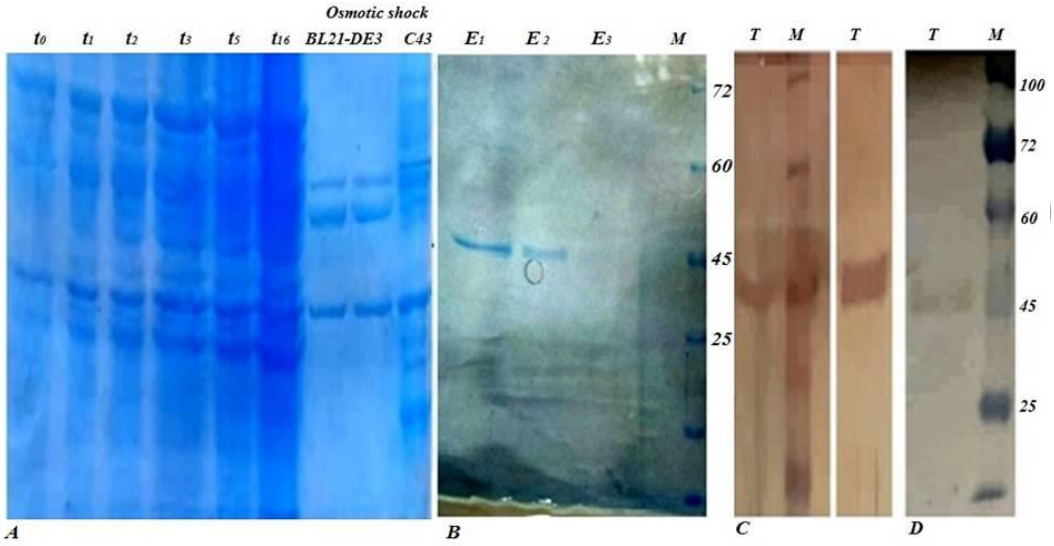

Figure.6A: Expression of protoxin and secretion in osmotic buffer, B: purification of protoxin by affinity chromatography, C: Silver staining of purified protoxin, D: Western blotting, Protein ladder PM1700

Periplasmic extraction via osmotic shock provided a high yield of purified product (10mg per liter) and reduced contamination from cytoplasmic proteins, which facilitating downstream purification. As shown in Figure 7b and Table 1, the purification yield of the recombinant protoxin in the DE3 strain ranged from 1.796 to 4.421 mg/L. The highest purification yield in this strain was obtained using the osmotic shock method.

Similarly, in the C43 strain, the amount of purified toxin was calculated to range from 2.264 to 11.2 mg/L. The highest toxin production was obtained from the C43 strain using the osmotic shock method. The amount of toxin recovered after dialysis showed a 10% reduction; in other words, 90% of the purified toxin was retained after the dialysis step.

The identity of the recombinant protein was successfully confirmed by western blotting (Fig.6) and ELISA (Fig.7C).

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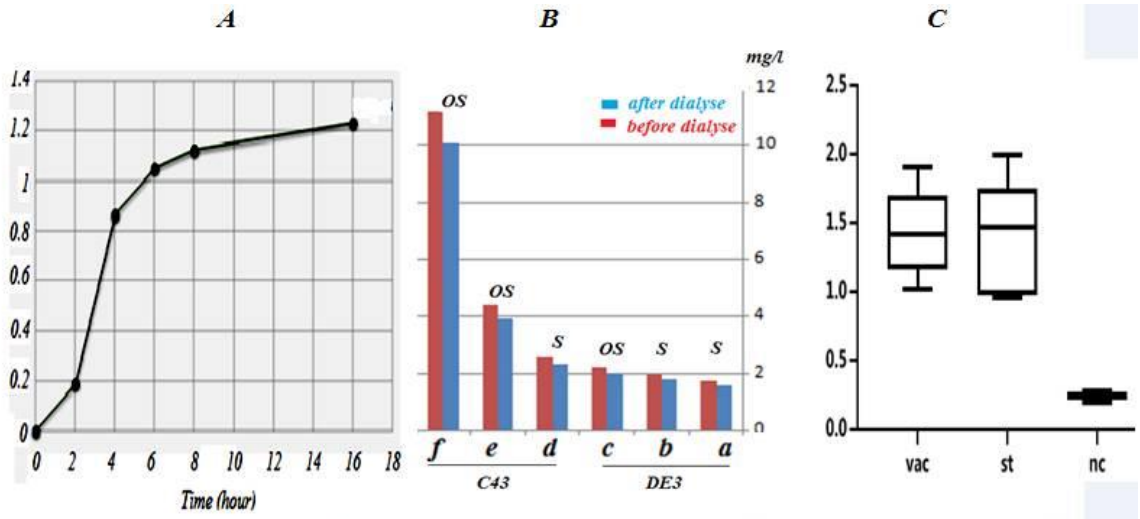

D

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
st vs. vac	-0.0460	-0.588 to 0.496	No	ns	0.99398
nc vs. st	-1.14	-1.68 to -0.596	Yes	***	0.00025
nc vs. vac	-1.18	-1.73 to -0.642	Yes	***	0.00017

Figure.7 A: bacterial growth curve in the fermentor for the expression of the recombinant toxin B: yield of affinity purification of recombinant protoxin: The three samples a, b, and c represent the protein expression in DE3 cells**,** while the three samples d, e, and **f** represent the protein expression in C43 cells**.** Furthermore, samples a, b, and d show protein purification from the pellet fraction after sonication**,** whereas samples c, e, and f show protein purification after osmotic shock without sonication**.** The amount of protein after dialysis is indicated in blue., C: Indirect ELISA with standard antibody from NIBSC and serum of immunized rabbit with quadrivalent clostridial vaccine, D: Statistical analysis of data collected from indirect ELISA using Sidak's multiple comparison test.Table 1: Yield of the recombinant toxin obtained from two different *E. coli* strains under different conditions.

ELISA produced a strong positive reaction when the recombinant protein was incubated with the NIBSC specific antibody, indicating specific antigen-antibody recognition (Fig.7C, D). Furthermore, western blot analysis revealed a single immune-reactive band at the expected molecular weight, with no detectable non-specific or dimer bands (Fig.6D). These results collectively confirmed that the expressed and purified protein was indeed the target recombinant protoxin with preserved antigenic epitopes.

4- Discussion:

The alpha-toxin from *C. septicum* is a pore-forming protein that likely depends on precise folding. In this regard, the periplasmic space offers a better environment for maturation than the cytoplasm, which has reductive environment. Expressing this toxin as a protoxin (an inactive precursor) in *E. coli* marks an important step forward in creating safe and effective vaccines against clostridial diseases. *Clostridium septicum* is a dangerous bacterium that

causes serious illnesses like malignant edema (gas gangrene) in animals and sometimes in humans with its alpha-toxin being the main factor of virulence [9].

Using an E.coli expressionsystem to produce the recombinant protoxin is especially valuable for vaccine development. Since the protoxin lacks toxicity compared to the active toxin, there's no need for aggressive chemical inactivation that might damage its antigenic properties. This method allows for the production of a structurally natural antigen that still carries the conformational immunogenic sites needed to trigger strong protective immunity [9].

Directing expression to the periplasm is a smart move for ensuring the correct three-dimensional structure of epitopes in recombinant vaccine products. The oxidizing condition in the periplasm space support proper disulfide bridging is critical for the toxin's structural integrity and biological behavior [10]. Moreover, our successful recovery using osmotic shock confirms that the protoxin reached the right location and was extracted cleanly with little contamination from cytoplasmic proteins. This stands in contrast to earlier efforts where cytoplasmic expression often led to inclusion bodies, requiring tricky denaturation and refolding steps that frequently undermined biological activity [11].

Having a dependable source of recombinant, active alpha-toxin opens many research possibilities. This recombinant protoxin serves as an ideal antigen for designing new vaccines [12]. Our work shows that targeted periplasmic expression combined with osmotic shock is an effective approach for producing high yields of protoxin in *E. coli*, improving solubility and streamlining recovery.

Producing *C. septicum* alpha protoxin in *E.coli* brings several key benefits. Most importantly, the protoxin is biologically harmless, making it safe to handle without toxicity risks. Additionally, *E. coli* allows for high-output, scalable, and inexpensive production, which is a clear economic advantage over purifying native toxins from hazardous cultures [13]. Recombinant methods also ensure batch-to-batch consistency, which is essential for regulatory approval and commercial vaccine production. Regarding immune response, upon controlled activation (e.g., with trypsin), the protoxin can be turned into an active or used directly as a genetically inactivated vaccine component, both capable of inducing strong neutralizing antibodies [14]. This fits well with modern "next-generation" vaccine strategies, replacing traditional formaldehyde-treated toxoids with safer and better-defined recombinant antigens [15]. Ultimately, this research lays the groundwork for a subunit vaccine against *C. septicum* infections, protecting vulnerable animals (like sheep, cattle and poultry) and possibly even humans from this pathogen [16].

In this study, the recombinant alpha protoxin was successfully produced in the periplasm of *E. coli*. This expression approach offers clear benefits over cytoplasmic production. Targeting the periplasm aids proper disulfide bond formation and folding, which is especially important for the protoxin's structural stability and antigenicity. Also, periplasmic localization shields the recombinant protein from cytoplasmic proteases and lowers any toxicity to the host cells, boosting overall yield [9]. Studies indicate that expressing clostridial toxins in *E. coli* often requires fine-tuning, and the host strain selection can greatly affect soluble protein production [13].

To avoid proteolytic breakdown of the recombinant protoxin during expression and production, a soybean-derived peptone was used as the nitrogen source in the culture medium. This peptone created a trypsin-free environment, ensuring the production of full-length, intact recombinant toxin. In this work, lactose-a natural, nontoxic, and metabolizable

inducer-was used in place of IPTG. This lactose-based induction strategy is not only more economical but also better suited for the safe production of the recombinant alpha protoxin.

After expression, the protoxin was efficiently released from the periplasm using gentle osmotic shock, a mild lysis technique that preserves protein structure and activity while leaving most cytoplasmic contaminants behind [17]. This approach greatly enriches the target protein early on, simplifying downstream processing. Traditional toxin purification methods often rely on sonication or other harsh disruption techniques that can denature the protein and introduce endotoxin contamination, which is problematic for vaccine development [13].

The final purified protoxin retains its natural immunogenic epitopes, making it an excellent candidate for subunit vaccine development against clostridial infections [15]. Research has shown that recombinant alpha toxin produced in *E. coli* can be expressed as a soluble protein and purified using affinity methods like Ni-NTA resin, yielding a product with good immunogenicity [16]. The combination of periplasmic expression, osmotic shock and affinity purification provides a scalable, cost-effective and chromatography-friendly production platform. Importantly, this process avoids harsh denaturation or sonication steps that could affect the antigen. Overall, this expression and purification strategy offers a gentle and efficient way to produce high quality *Clostridium septicum* alpha protoxin for veterinary or human vaccine use. In the next phase of this project, the in vitro and in vivo toxicity and the assessment of the immune response, of recombinant alpha protoxin will be evaluated.

5-Conclusion

In conclusion, we established an efficient system for the production of recombinant alpha-toxin of *C. septicum* in *E.coli*. The periplasmic expression and secretion using an osmotic shock strategy proved effective in yielding a soluble form. To ensure that the expressed protoxin was produced in the periplasmic space with a proper structure, the soluble toxin was secreted extracellularly using osmotic shock. This method allows for the purification of the protoxin with minimal manipulation and contamination.

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7-Authors' Contribution:

Writing the original draft: M.E.; Review and editing: M.N., S.S., S.A.A., A.N., A.E., S. k., M.s.

8- Conflict of interests:

The authors declared no conflict of interest

9-Ethics approval:

This project received ethics approval no. IR.RVSRI.REC1404.005 from Research Ethics Committees of Razi Vaccine and Serum Research Institute.

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6- Data Availability:

All produced data are included in this article.

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