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Cloning and Expression of Recombinant Chondroitin AC Lyase Protein of *Flavobacterium columnare* in a Prokaryotic Expression System

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ABSTRACT

Background: Aquaculture, the fastest-growing food production sector worldwide, faces significant challenges due to disease outbreaks, particularly from bacterial pathogens such as *Flavobacterium columnare*. These pathogens cause severe economic losses, necessitating the development of effective diagnostic tools and vaccines.

Methods: This study aimed to express the chondroitin AC lyase gene from *F. columnare*, a key virulence factor in prokaryotic expression vector. The gene was PCR-amplified, cloned into the pET-32a vector and expressed in *Escherichia coli* BL21-D3 cells. Positive clones were confirmed through PCR and restriction enzyme digestion. The expressed protein was analyzed by SDS-PAGE and Western blot, with protein expression tested under varying incubation temperatures. *In silico* analysis was performed to predict secondary and tertiary structures, including identification of key motifs and antigenic sites. Antigenicity was confirmed through an Agar Gel Immunodiffusion (AGID) assay.

Result: The SDS-PAGE and Western blot analysis revealed a prominent band at ~77 kDa, indicating successful expression of the recombinant protein. Expression levels varied with incubation temperature. *In silico* analysis identified significant antigenic sites and the AGID assay showed strong immune reactions, confirming the protein's potential as a vaccine component.

Key words: Chondroitin AC lyase, *F. columnare*, *Labeo rohita*, Recombinant DNA technology.

INTRODUCTION

Fisheries and aquaculture are vital to global food security, with 185 million tonnes of aquatic animal production in 2022. Aquaculture is the fastest-growing food sector (Sandeep *et al*., 2023), shifting to super-intensive farming, which increases disease risks due to high stocking densities and stress, particularly in extreme temperatures. Pathogens like *Aeromonas, Pseudomonas, Edwardsiella and Flavobacterium* severely affect Indian Major Carps, emphasizing the need for better disease management (Kumar *et al*., 1986; Shome *et al*., 1996; Swain and Nayak, 2003; Praveenraj *et al*., 2023; Srinath *et al*., 2024).

India's tropical climate fosters disease outbreaks, particularly columnaris caused by *Flavobacterium columnare* (*F. columnare*), affecting the skin and gills of freshwater fish (Holt *et al*., 1975). This leads to high mortality and economic losses (Austin *et al*., 2016). *F. columnare* has both virulent and non-virulent strains, with virulence influenced by exotoxins, bacteriocins and endotoxins. Chondroitin AC lyase plays a key role in virulence by degrading connective tissue (Bernardet, 2006; Kunttu *et al*., 2011).

Chondroitin AC lyase plays a crucial role in degrading chondroitin sulfate, a key component of the extracellular matrix. This enzyme is valuable for various biotechnological applications, including disease control and vaccine development, where its role in pathogenesis becomes particularly relevant. To enhance production for these applications, cloning the enzyme into *E. coli* has proven to yield higher and more stable amounts, facilitating its use in large-scale biotechnological processes (Zhou *et al*., 2021).

This study focuses on cloning, expressing and characterizing the chondroitin AC lyase gene from *F. columnare* to facilitate the production of recombinant proteins and explore their potential in vaccine development and diagnostic applications. By producing recombinant Chondroitin AC lyase, this research aims to improve early disease detection, enhance immunological tools and strengthen fish disease management efforts. Additionally, it investigates the antigenicity of the recombinant protein, shedding light on its role in stimulating protective immunity against *F. columnare*. With limited commercial vaccines

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available for columnaris disease, the findings of this study offer valuable insights into pathogen-host interactions while contributing to the development of innovative disease prevention and diagnostic strategies to support sustainable aquaculture.

MATERIALS AND METHODS

The research was conducted in the laboratory of the ICAR-Central Institute of Fisheries Education (CIFE), Mumbai, India. Various bacteriological, molecular, enzymatic, cloning and immunoassay experiments were performed in the Aquatic Animal Health Management Laboratory during 2022-2023.

Bacteria

The *F. columnare* strain used in this study was isolated from the kidney of diseased *Labeo rohita* and maintained in the Aquatic Animal Health Management Laboratory, ICAR-CIFE, Mumbai. It was cultured in TYES broth at 28°C with shaking (100 rpm) for 48 hours, then streaked on TYES agar plates and incubated at 28°C for 48 hours, resulting in pale yellow rhizoid colonies. Viable bacterial counts were determined by serial dilution and plating on TYES agar. DNA isolation and species confirmation were done via colony PCR using species-specific primers (Table 1), amplifying a 675 bp product (Darwish *et al*., 2004). PCR conditions were: 94°C for 5 minutes (denaturation), 45°C for 30 seconds (annealing), 72°C for 2 minutes (extension) and a final extension at 72°C for 8 minutes.

Retrieval of chondroitin AC lyase amino acid and protein sequence

The chondroitin AC lyase amino acid and protein sequences from *F. columnare* were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). In silico analysis of the protein's secondary structure and motifs was conducted using various online tools. The Ramachandran plot generated from SWISS-MODEL Interactive Workspace (https://swissmodel.expasy.org/interactive) confirmed the structural integrity of the model. Secondary structure composition was determined using PredictProtein (https://predictprotein.org/), showing â sheets as dominant motifs. The tertiary structure was predicted with SWISS-MODEL (https://swissmodel.expasy.org/) and PDB (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum), which provided a detailed 3D model featuring β sheets, α helices and loops.

Genomic DNA extraction and PCR amplification of chondroitin AC lyase

E. coli BL21-D3 cells (Invitrogen, USA) were transformed with the pET-32a vector (Novagen, Germany). Genomic DNA from *F. columnare* was extracted using DNAzol (Invitrogen, USA) from a 1.5 mL active broth culture, precipitated with ethanol, washed with 95% ethanol and dissolved in nuclease-free water. For directional cloning into the pET vector, the gene sequence was analyzed with the NEBcutter V2.1 tool (http://nc2.neb.com/NEBcutter2/) to identify BamHI (GGATCC) and HindIII (AAGCTT) restriction enzyme sites. Internal primers amplifying a 400 bp segment, based on Luo *et al.* (2016), were synthesized by Eurofins Scientific (Mumbai).

The gene was amplified by PCR with the following conditions: An initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. A final extension at 72°C for 20 minutes was followed by a hold at 4°C. The reaction mixture contained 2.5 µL of 10X Pfu buffer, 0.5 µL of 10 mM dNTPs, 1.25 µL each of forward and reverse primers (10 µM), 1 µL of DNA (400 ng), 0.5 µL of Pfu DNA Polymerase (2.5 U/µL) and nuclease-free water to a final volume of 25 µL.

Restriction enzyme digestion and cloning

A 20 µL digestion reaction for plasmid pET32a contained 1 µL of plasmid DNA (1000 ng/µL), 2 µL of 10X FD buffer, 1 µL each of BamHI and HindIII and 15 µL of nuclease-free water. The mixture was incubated at 37°C for 12 minutes, followed by heat inactivation at 80°C for 10 minutes. PCR amplicons were similarly digested with 1 µL of DNA (200 ng/µL). Both plasmid and genomic DNA were purified using phenol-chloroform extraction, followed by ethanol precipitation with glycogen (20 µg/µL), ammonium acetate and 100% ethanol. After overnight storage at -20°C, the sample was centrifuged and washed with 70% ethanol, then resuspended in 300 µL of TEN buffer.

Ligation of DNA

The ligation reaction, in a 20 µL volume, included 4 µL of vector DNA, 1 µL of insert DNA, 4 µL of 5X ligation buffer, 1 µL of T4 DNA ligase (5 units) and 10 µL of nuclease-free water. Gene PCR products (130 ng) and pET32a vector (100 ng) were mixed at a 3:1 ratio and incubated at 22°C for 10 minutes before transformation.

Transformation by heat shock method

Competent *E. coli* BL21-D3 cells (Invitrogen, USA) were prepared using the standard calcium chloride (CaCl,) protocol (Sambrook *et al*., 1989). For transformation, 200 µL of thawed competent cells were combined with 5 µL (50 ng) of the ligated product, incubated on ice for 10 minutes, heat shocked at 42°C for 90 seconds and cooled on ice for 5-10 minutes. The cells were then incubated in 800 µL of LB broth at 37°C for 2 hours. Finally, 100 µL of the culture was plated on LB agar containing ampicillin (100 µg/mL) and incubated at 37°C for 24 hours to allow colony formation.

Expression induction

The chondroitin AC lyase gene was expressed in *E. coli* BL21-D3 (Invitrogen, USA) cells using isopropyl β-D-1-galactopyranoside (IPTG) induction. Initially, *E. coli* BL21-D3 (Invitrogen, USA) cells containing the pET-32a vector were cultured in 10 mL of LB broth with 50 µg/mL ampicillin and incubated at 37°C overnight with shaking at 180 rpm.

The cells were then transferred to a fresh medium and grown at 37°C for a few hours until an OD of 0.8 at 600 nm was reached. Protein expression was induced by adding IPTG to a final concentration of 1 mM/ml, followed by overnight incubation at 37°C, 32°C and 27°C with continuous shaking at 180 rpm. The cells were harvested 12 hours' post-induction by centrifugation at 6000 rpm for 10 minutes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein visualization

Recombinant chondroitin AC lyase expression was confirmed by SDS-PAGE. Induced cells were harvested and the supernatant discarded. The cell pellet was resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% SDS, 1 mM EDTA and protease inhibitor cocktail). Uninduced cells served as negative controls. The samples were boiled for 10 minutes, loaded into the SDS-PAGE gel and electrophoresis was performed. The gel was fixed in methanol solution for 1 hour and prepared for Western blotting.

Protein purification by immobilized metal ion affinity chromatography (Ni-NTA)

Protein purification was performed using the Ni-NTA Superflow kit (Qiagen, Germany). To generate cleared lysates, 10 mL of Buffer NPI-10 (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and 1 mL of lysozyme solution (10 mg/mL) were added to the cells along with 3 units of Benzonase per mL of original cell culture. The mixture was incubated for 30 minutes at room temperature, then centrifuged at 15,000 × g for 30 minutes at 4°C. The supernatant containing soluble 6xHis-tagged proteins was collected.

For purification, Ni-NTA Superflow Columns (1.5 mL) were equilibrated with 10 mL of Buffer NPI-10. The cleared lysate was loaded onto the column and allowed to drain by gravity. The column was washed twice with 10 mL of Buffer NPI-20 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The 6xHis-tagged proteins were eluted with 3 mL of Buffer NPI-250 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was dialyzed against chilled PBS (pH 7.4) overnight at 4°C, with buffer changes every 3-4 hours.

Protein quantification

Protein concentration was measured using the Bradford assay. The sample (10 µL) was mixed with 1 mL of Bradford reagent (Bio-Rad) and incubated for 5 minutes at room temperature. Absorbance at 595 nm was measured and a calibration curve was constructed using known concentrations of bovine serum albumin to determine the protein concentration.

Antiserum against recombinant chondroitin AC lyase

Two male New Zealand White rabbits (4 months old, weighing 1.3-1.9 kg) were used for antiserum production, as described by Valsalam *et al*. (2023). The rabbits were kept under optimal conditions (temperature: 21±2°C, humidity: 40-60%). The recombinant antigen was administered subcutaneously at 200 µg per rabbit, with Freund's Complete Adjuvant (FCA) used for the initial immunization and Freund's Incomplete Adjuvant (FIA) for subsequent boosters at 14-day intervals. The antigen was emulsified with the adjuvant in a 1:1 ratio. Serum collection was done after confirming a satisfactory immune response.

Agarose gel immunodiffusion (AGID)

For the AGID assay, molten agarose was poured onto a glass slide and allowed to solidify. Wells were created, with recombinant protein added to the first well and rabbit serum to the second. The slide was placed in a humidity box at room temperature for 48 hours. Serum diffusion was observed after the incubation.

Western blot

Western blotting was conducted to confirm protein expression. After SDS-PAGE, the gel was transferred to a pre-wetted nitrocellulose membrane (0.22 µm) using a semi-dry electroblotting apparatus (45 mA for 3 hours). The membrane was washed twice in TBST buffer and blocked overnight at 4°C with 5% non-fat dried milk in PBS-Tween (PBST). It was then incubated with anti-AC lyase serum (1:500) for 1 hour at room temperature, followed by incubation with HRP-conjugated anti-rabbit IgG secondary antibody (1:6000) for 1 hour. The membrane was treated with DAB to visualize the protein bands, as described by Lalruatfela *et al.* (2024).

Table 1: PCR Primers for *F. columnare* and Chondroitin AC Lyase, the underlined sequences indicate restriction enzyme (RE) sites for BamHI (GGATCC) and HindIII (AAGCTT).

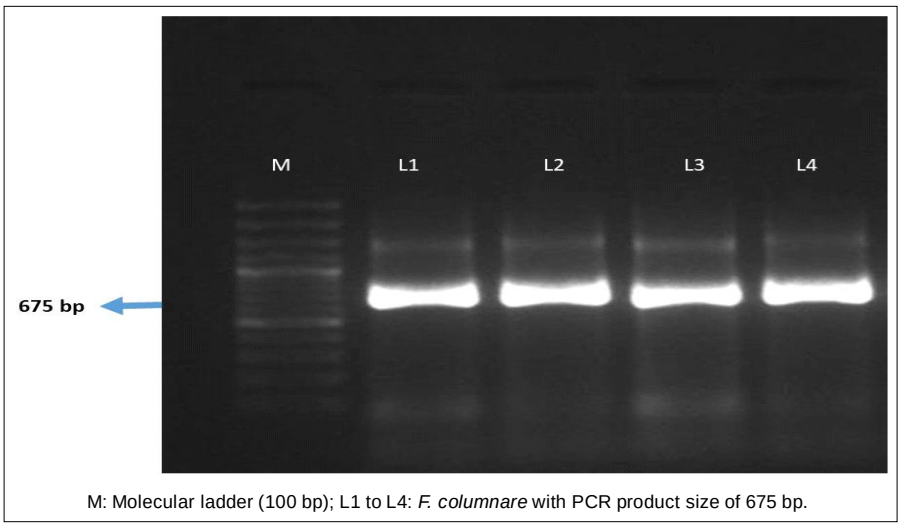
Primer type	Primer name	Primer sequence from 5′ to 3′	Product length (bp)
Species specific forward	SP-F	CAGTGGTGAAATCTGGT	675
Species specific reverse	SP-R	GTCCCTACTTGCGTAGT	
Internal forward primer	I-Fcol-F	CGCGGATCCAAATCCGCATTACAAGATGCCCAT	400
Internal Reverse primer	I-Fcol-R	GAAGCTTCCTGATGCTCCATTTTTCCCATCAG	
Chondroitin AC lyase forward primer	ACL-F	CCCGGATCCAGCACTTATATAGGGCTG	2600
Chondroitin AC lyase reverse primer	ACL-R	CCCAAGCTTAACCTCTTAGTTTGGTCTCTA	

RESULTS AND DISCUSSION

The virulence of *F. columnare* was confirmed through biochemical assays and PCR analysis. The amplification of a 675 bp fragment verified the presence of the target gene (Fig 1), providing a foundational step for further molecular characterizations.

The chondroitin AC lyase gene (AY912281) was successfully sequenced, revealing a 2640 bp gene encoding a 761-amino acid protein with a predicted molecular weight of 75 kDa. The open reading frame (ORF) of this gene spans from 157 to 2442 bp. Structural analysis of the protein revealed significant β turns, β sheets and important motifs such as an alpha-alpha barrel, a beta-plated distorted sandwich and a beta-plated sandwich. These structural components are critical to the protein's potential immunogenic properties, as evidenced by the identification of several ligand-binding sites. The Ramachandran plot (Fig 2) confirmed the presence of anti-parallel β sheets, parallel β sheets and right-handed á helices, indicating a stable protein structure. Tertiary structure modelling further corroborated this, showing a motif-like formation interspersed with loops, β sheets and á helices.

The chondroitin AC lyase gene was PCR-amplified and cloned into the pET-32a vector, yielding a 2600 bp amplicon (Fig 3a). The recombinant vector was transformed into *Escherichia coli* strain BL21-D3 (Invitrogen, USA). Positive clones were selected from LB-ampicillin plates and confirmed through PCR using internal primers, for 400 bp amplicon (Fig 4), verifying successful gene insertion. Further confirmation was achieved through restriction digestion of plasmids, which released the insert as expected (Fig 3b). SDS-PAGE analysis of the induced protein showed a prominent band at approximately 77 kDa (Fig 5), confirming successful expression. Expression levels were highest at



M: Molecular ladder (100 bp); L1 to L4: *F. columnare* with PCR product size of 675 bp.

Fig 1: Molecular confirmation of *F. columnare* using species-specific PCR.

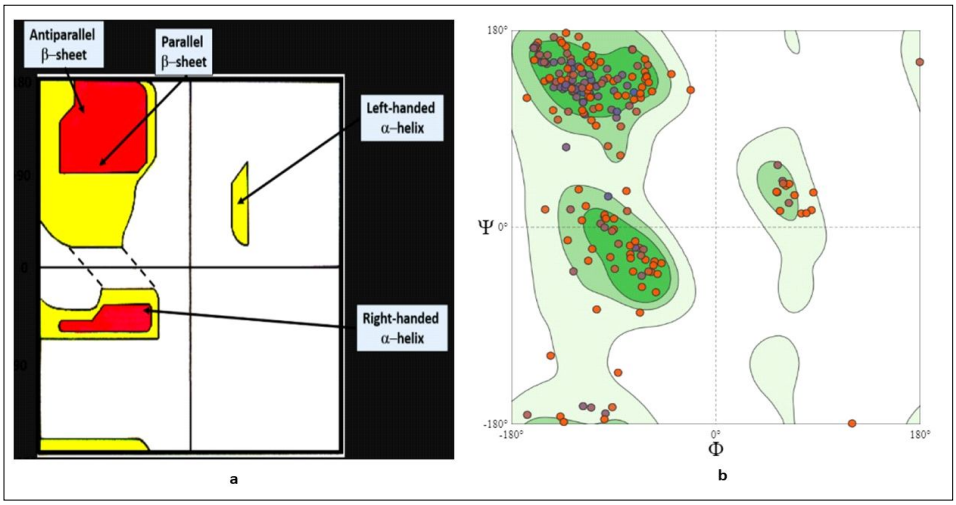


Fig 2: Ramachandran plot for the amino acid sequence of chondroitin AC lyase generated using SWISS-MODEL interactive workspace.

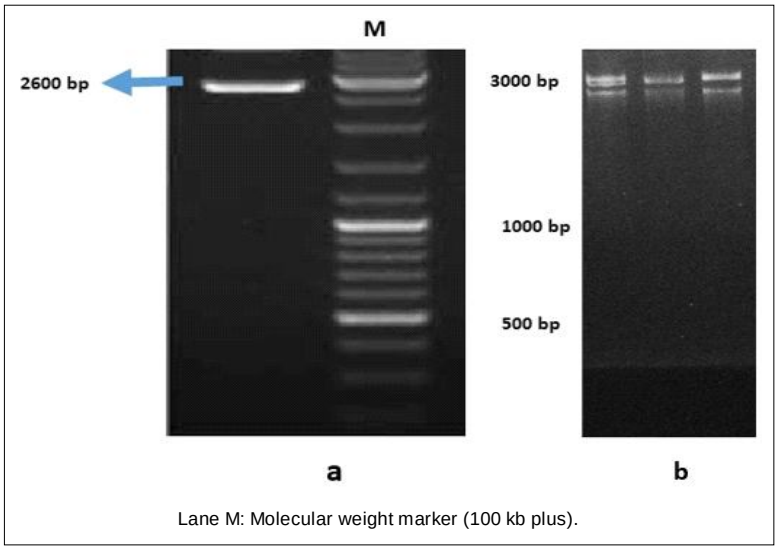


Fig 3: a) Agarose gel electrophoresis (0.8%) showing PCR amplification of the chondroitin AC lyase gene (2600 bp). b) Restriction enzyme-digested plasmid and released insert.

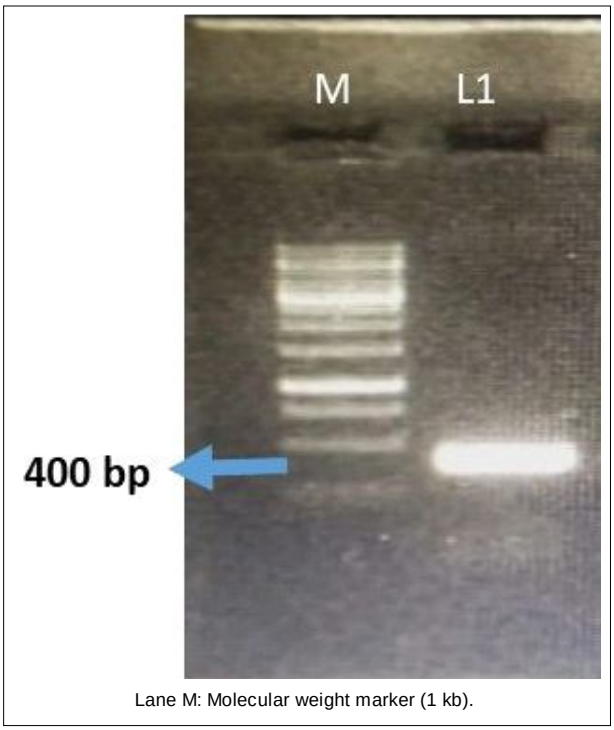


Fig 4: PCR results of recombinant protein amplification, 400 bp internal primer amplification of chondroitin AC lyase.

37°C, with weaker bands observed at lower incubation temperatures (32°C and 27°C), indicating a temperature-dependent expression profile.

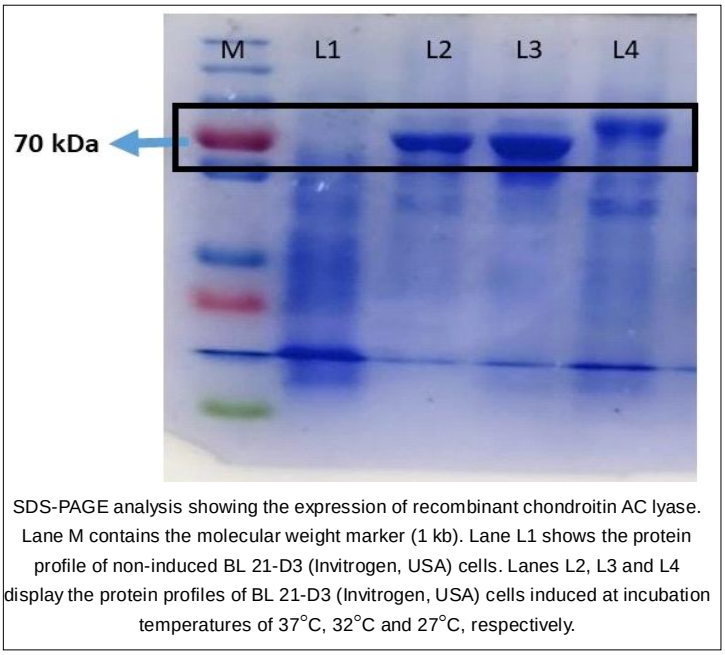
The immunoreactivity of the recombinant protein was confirmed using an Agar Gel Immunodiffusion (AGID) assay (Fig 6). Western blotting further validated the expression, with a clear band at approximately 77 kDa (Fig 7). Following protein purification, 10 mg/L of the recombinant protein was obtained.

The successful cloning, expression and immunological characterization of chondroitin AC lyase highlight its potential role as a virulence factor in *F. columnare* infections. Chondroitin AC lyase is a polysaccharide exolyase that targets glycosaminoglycans, such as hyaluronic acid and chondroitin sulfate A and C, playing a critical role in the pathogenicity of *F. columnare* and contributing to its immunogenicity in fish (Luo *et al*., 2016; Chen *et al*., 2019). The cloned gene exhibited 99.9% similarity with other *F. columnare* strains (TC, PF1 and B245), suggesting its specificity and potential application in developing diagnostic tools for *F. columnare* infections.

The secondary structure of the recombinant protein, composed predominantly of β turns, is typical for enzymes and contributes to protein stability. β turns are often found on the surface of proteins and are known to enhance structural integrity and flexibility (Chen *et al*., 2019). The Ramachandran plot analysis confirmed that the protein's amino acid residues occupy favorable regions, further supporting its stability. The protein's combination of anti-parallel â sheets and á helices is expected to contribute to its rigidity and stability, critical features for enzymatic activity and its potential as a diagnostic or vaccine candidate.

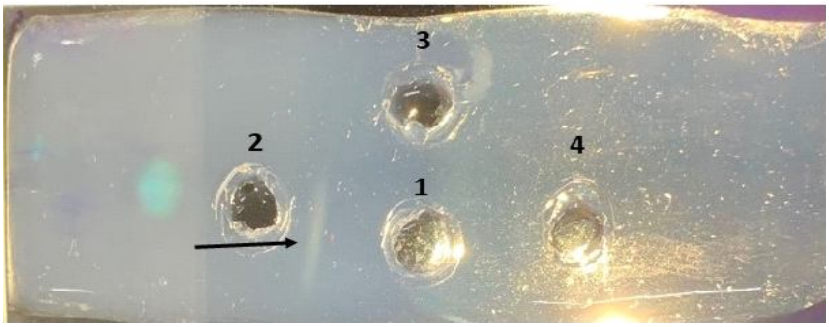
The size of the recombinant chondroitin AC lyase (~77 kDa) is consistent with other reports of similar enzymes, such as ACL from Pedobacter rhizosphaerae (Zhou *et al*., 2021) and Acetobacter (Chen *et al*., 2019). The enzyme's six-domain structure, including N-terminal α helices and C-terminal β sheets, suggests functional complexity, interspersed with β turns, which are crucial for catalytic and binding activities.

Antigenicity prediction confirmed that the protein has strong potential to function as an antigen, with multiple amino acid sequences showing an antigenicity index above 1. The AGID assay further confirmed the immunogenicity of the



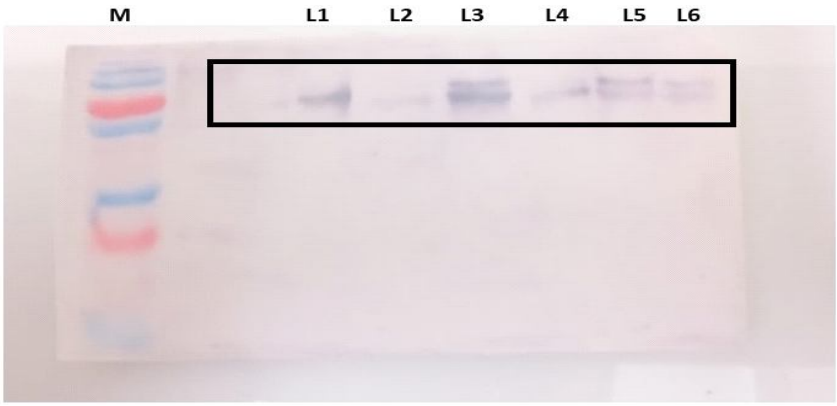
SDS-PAGE analysis showing the expression of recombinant chondroitin AC lyase. Lane M contains the molecular weight marker (1 kb). Lane L1 shows the protein profile of non-induced BL 21-D3 (Invitrogen, USA) cells. Lanes L2, L3 and L4 display the protein profiles of BL 21-D3 (Invitrogen, USA) cells induced at incubation temperatures of 37°C, 32°C and 27°C, respectively.

Fig 5: SDS-PAGE analysis of recombinant chondroitin AC lyase.



Wells 3 and 4 contain prebleed sera and nonspecific rabbit sera, respectively.

Fig 6: Agarose gel immune diffusion assay well 1 (recombinant chondroitin AC lyase protein) and well 2 (PAb).



Western blot analysis for the detection of recombinant chondroitin AC lyase (~~77 kDa) using polyclonal antibodies (PAb). Lane M: Molecular weight marker (ladder). Lanes 1 to 6: Recombinant chondroitin AC lyase protein.

Fig 7: Western blot detection of recombinant chondroitin AC lyase (~~77 kDa) using PAb.

recombinant protein, with a strong immune response observed in fish. These findings indicate that the protein could be utilized as a component in vaccine formulations or as an antigen for diagnostic assays. The immune response observed in these assays supports the protein's potential to provide protective immunity against *F. columnare* infections.

This study demonstrates the successful cloning, expression and immunological characterization of chondroitin AC lyase from *F. columnare*, highlighting its structural integrity, antigenicity and immunogenic properties. Beyond its role as a virulence factor, chondroitin AC lyase has shown promising applications in disease diagnostics and vaccine development. Recent advances confirm its utility as a species-specific diagnostic marker (Mabrok *et al*., 2020) and reinforce its potential as a vaccine candidate, with recombinant formulations eliciting strong protective responses in fish (Luo *et al*., 2016; Declercq *et al*., 2013). These findings align with emerging therapeutic strategies, such as enzyme inhibitors and genomic resistance approaches, offering new avenues for columnaris disease management (Wang *et al*., 2022). This study contributes to the growing body of research advocating for chondroitin AC lyase as both a diagnostic and vaccine target, supporting its continued exploration in aquaculture health interventions and offering a promising strategy for disease management in fish health.

CONCLUSION

In this study, Chondroitin AC lyase, a polysaccharide exolyase from *F. columnare*, was successfully cloned and expressed in the bacterial vector BL21-D3. The enzyme demonstrated high specificity and immunogenicity, making it a promising candidate for developing disease diagnostics and vaccines in aquaculture. The protein's structural analysis revealed a composition favorable for stability and antigenicity, with significant immune response potential confirmed through AGID assays. These findings suggest that recombinant DNA technology can effectively enhance disease management strategies in aquaculture, contributing to the industry's sustainable development.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily reflect the views of their affiliated institutions. The authors are responsible for ensuring the accuracy and completeness of the information presented, but they disclaim any liability for any direct or indirect losses arising from the use of this content.

Informed consent

All animal procedures conducted during the study were approved by the Committee for Experimental Animal Care and handling techniques were reviewed and approved by the University Animal Care Committee.

Conflict of interest

The authors declare that they have no conflicts of interest related to the publication of this article. The design of the study, data collection, analysis, decision to publish and manuscript preparation were not influenced by any funding or sponsorship.

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