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Bacteriophage Endolysin: Isolation, Cloning, and Antibacterial Activity

Fatma Abdelrahman[1], Gamal El-Didamony[2], Ahmed Askora[2*], and Ayman El-Shibiny[1]
[1]Center for Microbiology and Phage Therapy, Zewail City of Science and Technology, 6th of October City 12578, Egypt
[2]Department of Microbiology and Botany, Faculty of Science, Zagazig University, Zagazig 44519, Egypt
Corresponding author: E-mail: ahmedaskora@gmail.com

ABSTRACT **: Antimicrobial resistance (AMR) stands as a formidable global crisis, casting a shadow over the efficacy of our efforts in preventing and managing the persistent flow of infectious diseases, thereby posing a grave threat to the health of humanity at large. As a response to this growing crisis, the search for viable alternatives to antibiotics has become imperative. Among these alternatives, bacteriophages, and their associated enzymes, particularly endolysins, have emerged as an attractive avenue of exploration. In this study, we successfully conducted the cloning, expression, and purification of LysZC1 endolysin. Notably, our findings revealed that LysZC1 exhibited significant antibacterial activity against *Bacillus cereus*. These results strongly indicate that LysZC1 holds considerable promise as a prospective therapeutic agent for combating bacterial infections and as a valuable tool for biocontrol against bacterial pathogens. A deeper understanding of the mechanisms underlying LysZC1's antibacterial activity could pave the way for enhancing its efficacy. For instance, exploring how LysZC1 interacts with bacterial membranes, and the specific pathways it disrupts, can provide vital knowledge.**

KEYWORDS: **Phage, Endolysin, Isolation, Cloning**

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I. INTRODUCTION

The rising challenge of multidrug-resistant superbugs has cast a shadow over the field of healthcare, posing remarkable hurdles in the treatment of infectious diseases (**Aslam *et al.*, 2018; Neill, 2014**). This alarming trend has elevated multidrug-resistant bacteria to a top threat to global public health (**Marston *et al*., 2016; Woolhouse & Farrar, 2014**). Among these concerns, *Bacillus cereus*, a Gram-positive, spore-forming bacterium ubiquitous in the environment, has come into focus. This opportunistic foodborne pathogen is known for causing both local and systemic infections **(Bottone, 2010; Stenfors Arnesen *et al.*, 2008)**. Within the food industry, *B. cereus* ranks as a leading cause of food poisoning, leading to severe gastrointestinal disorders (**Ceuppens *et al.*, 2011)**. The bacterium produces two types of toxins, a diarrheal toxin and an emetic toxin, which contribute to its pathogenicity and the severity of diseases it can induce **(Ceuppens *et al.*, 2011)**. Consequently, there is an imperative need for the development of new antimicrobial agents to control, prevent, and treat *B. cereus* infections. In recent times, the potential of phage therapy has captured the imagination of researchers and healthcare practitioners as a formidable strategy to counteract multidrug-resistant bacteria, offering a glimmer of hope in the face of escalating clinical infection challenges **(Jun *et al..*, 2014; Taati Moghadam *et al.*, 2020)**. Bacteriophages, are viruses that infect and exterminate bacteria (**Abdelrahman *et al..*, 2022; Sharp, 2001)**. These microscopic entities, naturally occurring in the environment, have emerged as potent allies in the ongoing battle against antibiotic resistance (**Drulis-Kawa *et al..*, 2015**). Beyond the use of complete phage particles to combat antibiotic-resistant bacteria, the focus has shifted towards harnessing the potential of phage-encoded endolysins as viable candidates for the treatment of bacterial infections. Endolysins, also known as phage lysins, constitute a class of peptidoglycan hydrolases (PGHs) that play a pivotal role in the phage replication cycle, culminating in the breakdown of the bacterial cell wall's peptidoglycan layer(**Loessner, 2005; Nelson *et al..*, 2012**). Endolysins are classified into four categories based on their lytic activities: (A) N-acetylmuramoyl-L-alanine amidases, which break amide bond between N-acetylmuramic acid (MurNAc) and L-

alanine. (B) N-acetyl-β-D-muramidases, commonly known as muramidases or lysozymes, which cleave β-1,4 glycosidic bonds linking N-acetylmuramic acid (MurNAc) and N-acetyl-D-glucosamine (GlcNAc) residues. (C) Endo-β-N-acetylglucosaminidases (glucosaminidases), catalyze the hydrolysis of the glycosidic bond within peptidoglycan. (D) Endopeptidases, which are responsible for cleaving the peptide bond between two amino acids within the stem peptide **(Abdelrahman *et al.*, 2021**).

This study highlights the potential of the endolysin LysZC1 as an effective antibacterial solution. Our results demonstrate that LysZC1 exhibits strong antibacterial properties against *B. cereus*, indicating its potential use as an antibacterial agent for preventing *B. cereus*-related food poisoning.

2. MATERIALS AND METHODS

2.1. Plasmids, Bacterial Strains, and Growth Conditions

pET 28b expression vector was obtained from Novagen. Cloning and screening were done in *Escherichia coli* strain DH5α, whereas protein expression was carried out in *E. coli* strain BL21 (DE3). For all antimicrobial activity assays, the standard strain of *B. cereus* ATCC 11778 was acquired from the American Type Culture Collection (Manassas, VA, USA). The stain was grown in tryptic soya broth (TSB) aerobically at 37 C and under shaking conditions at 120 rpm. The stocks of bacterial strain were kept in 20 % [vol/vol] glycerol at -80 until needed.

2.2. *In silico* Whole Genome Analysis and Identification of Phage Endolysin, LysZC1

ZCPS1 phage genome was annotated with the Rapid Annotation using Subsystem Technology Toolkit (RASTtk) algorithm **(Aziz *et al.*, 2008)** and PHASTER, and the alternative start codons were manually evaluated. The functional domain analysis of phage ZCPS1 and LysZC1 was performed by blastp search in NCBI **(Altschul *et al.*, 1990).** SnapGene Viewer v. 5.1.3.1 software was used to analyze the position, base composition, and GC content of the LysZC1 coding gene; NEBcutter (http://nc2.neb.com/NEBcutter2) was used for the prediction of restriction enzymes. LysZC1 protein's properties, including isoelectric point (pI), and molecular weight, were predicted using ExPASY prot param tool.

2.3. PCR Amplification of Endolysin Gene from Phage DNA

Polymerase Chain Reaction (PCR) was conducted to amplify the Endolysin Gene from Phage DNA. The PCR reaction was carried out using forward primer: 5′GGCCATGGGCATGAAGTTCGACGAATACTC and reverse primer: 5′-CCTCGAGAGTCACCGTGCTACCTATC), in a total volume of 25 µL. The reaction mixture included 12.5 µL of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1.25 µL of each primer at a concentration of 10 µM, 4.5 µL of water, and 5 µL of DNA template. The reactions were conducted in Thermal Cycler PCR (Bio-Rad, USA). The PCR amplification was performed using a specific cycling profile. The reaction started with an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 60 seconds. A final extension step at 72 °C for 7 minutes ensured complete extension of PCR products. The reaction was then held at 4 °C. After PCR amplification, the resulting products were separated by electrophoresis on a 1% agarose gel (Sigma Aldrich, USA) in 1x TAE buffer at room temperature.

2.4. Cloning and Transformation of Endolysin Gene

Cloning was performed to construct customized plasmids carrying the amplified endolysin gene for expression. The ligation reaction involved mixing approximately 200 ng of vector and insert in a 1:3 ratio, along with 1x ligation buffers, 1.5 µL of T4 DNA ligase enzyme, and nuclease-free water. A negative control reaction was included without the insert. The ligation reactions were incubated overnight at 16 °C. For the transformation of recombinant plasmids into *E. coli*, competent cells were thawed on ice and mixed with 5 µL of the ligation product. The mixture was incubated on ice for 30 minutes before subjecting it to a heat shock at 42 °C for 1 minute. After the heat shock, the tubes were transferred back to ice for 5 minutes. The transformed cells were then transferred to a tube containing LB medium and incubated at 37 °C for 1 hour at 180 rpm. Following incubation, different volumes of the transformed cells were spread onto LB agar plates supplemented with kanamycin and incubated overnight at 37 °C.

2.5 Clone Selection, Screening, and Analysis

Following cell transformation, single colonies were selected from overnight-incubated petri dishes and then cultured in LB broth with kanamycin at 37 °C and 150 rpm, followed by growth on kanamycin-supplemented LB agar plates at 37 °C. Confirmation of successful cloning involved two methods. Firstly, colony PCR was performed using a vector-specific forward primer (T7 promoter) and an insert-specific reverse primer (LysZC1). This technique amplified fragments specific to the LysZC1 gene, indicating its presence in the clones. Positive clones from colony PCR were further confirmed by Sanger sequencing. This method validated the sequence of the inserted gene within the plasmid. Sanger sequencing provided definitive confirmation of the nucleotide sequence of the integrated LysZC1 gene.

2.6 Sodium Dodecyl Sulphate Polyacrylamide Gel (SDS-PAGE)

After expression and purification, protein was visualized using SDS-PAGE on a Mini-PROTEAN 4 apparatus. A 15% resolving gel and a 4% stacking gel were prepared, polymerized, and poured into the glass plate cassette. Protein samples, including a molecular weight marker (Nippon Genetics, Germany), were loaded into the wells after denaturation. Electrophoresis was conducted starting at 100 V for 15 minutes, followed by 120 V for about 1.5 hours. After electrophoresis, the gel was fixed, stained with Coomassie Blue, and destained. Gel images were captured using the ChemiDOC™ MP imaging system.

2.7 Assessment of Endolysin Lytic Activity

The lytic activity of each expressed endolysin was determined by the spot test method as previously described **(Mishra *et al.*, 2013)**.with some modification. Briefly, A liquid culture *B. cereus* was grown until it reached the exponential growth phase. Then ~100 µl of the culture were spread onto TSA agar plates. Subsequently, the plates were allowed to air-dry for about 10 min. Subsequently, 5 µl of purified LysZC1 endolysin were carefully spotted onto the ager surface. The plates were incubated overnight at 37°C. After incubation overnight visualization of inhibition zone on agar plates indicated the presence of endolysin activity.

SEM (Scanning Electron Microscopy) was employed to observe the potential impact of LysZC1 on bacterial cells. The logarithmic phase of the reference bacterial strain, *B. cereus*, was collected by centrifugation, washed, and then resuspended in 20 mM Tris-HCl (pH 7.5). A mixture of 800 µL of bacterial suspension and 200 µL of LysZC1 (500 µg/ml) or Tris-buffer (as a control) was incubated for 2 hours at 37°C. The bacterial cells were subsequently centrifuged, washed twice with 20 mM Tris-HCl (pH 7.4), and fixed with 2.5% glutaraldehyde. After washing with PBS, the samples underwent ethanol dehydration, gold coating using a JEOL JFC-100 sputter, and examination of the morphology of the aggregated bacterial pellets using SEM (JEOL JSM-5300, UK).

3. RESULTS

3.1. *In silico* Whole Genome Analysis and Identification of Phage Endolysin, LysZC1

The bioinformatics analysis of *Pseudomonas* phage ZCPS1 revealed the presence of a lysis module that includes genes responsible for bacterial lysis, such as Endolysin (lysozyme) and holin genes (Fig.1). The putative endolysin gene was identified in the genome of the bacteriophage ZCPS1 and named LysZC1. LysZC1was identified through a BLAST search against the NCBI non-redundant protein sequence database. LysZC1 position ranges from 1320 - 1817 bp with 498 bp length within the ZCPS1 whole genome (Fig.2). Notably, LysZC1 exhibits a significant similarity with known *Pseudomonas* phages and other phage lysozyme sequences (Fig.3). Furthermore, both the NCBI's conserved domain database search and InterProScan servers confirmed the presence of a lysozyme-like domain within LysZC1 (Fig.4). Consequently, it can be concluded that LysZC1 is indeed a lysozyme encoded by *Pseudomonas* phage ZCPS1, encoding a protein comprising 165 amino acids with an estimated molecular weight of 18,870 Da and an isoelectric point (pI)of 8.43.

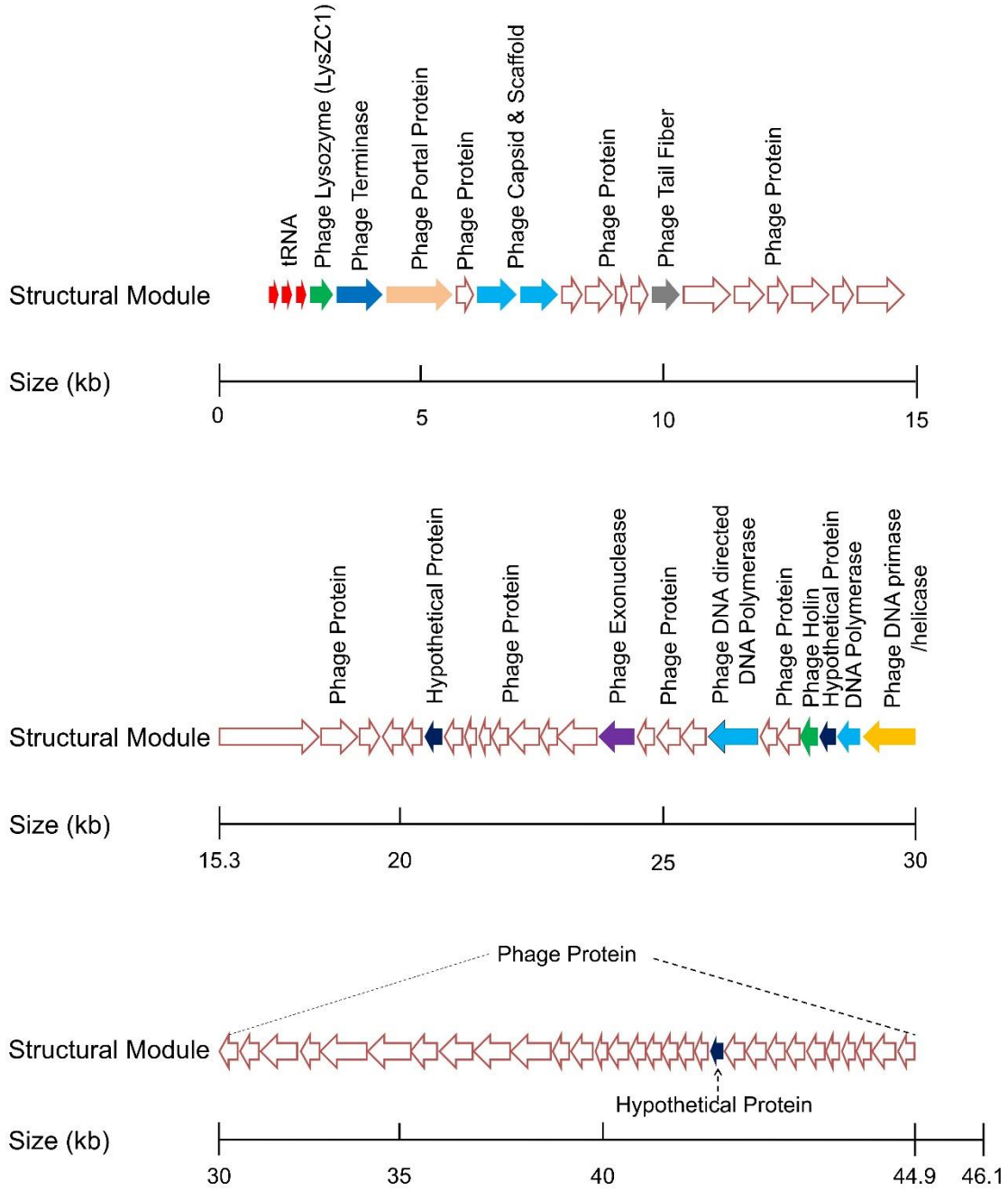

Figure 1. Schematic representation of the annotated genes of the structural module of ZCPS1 phage.

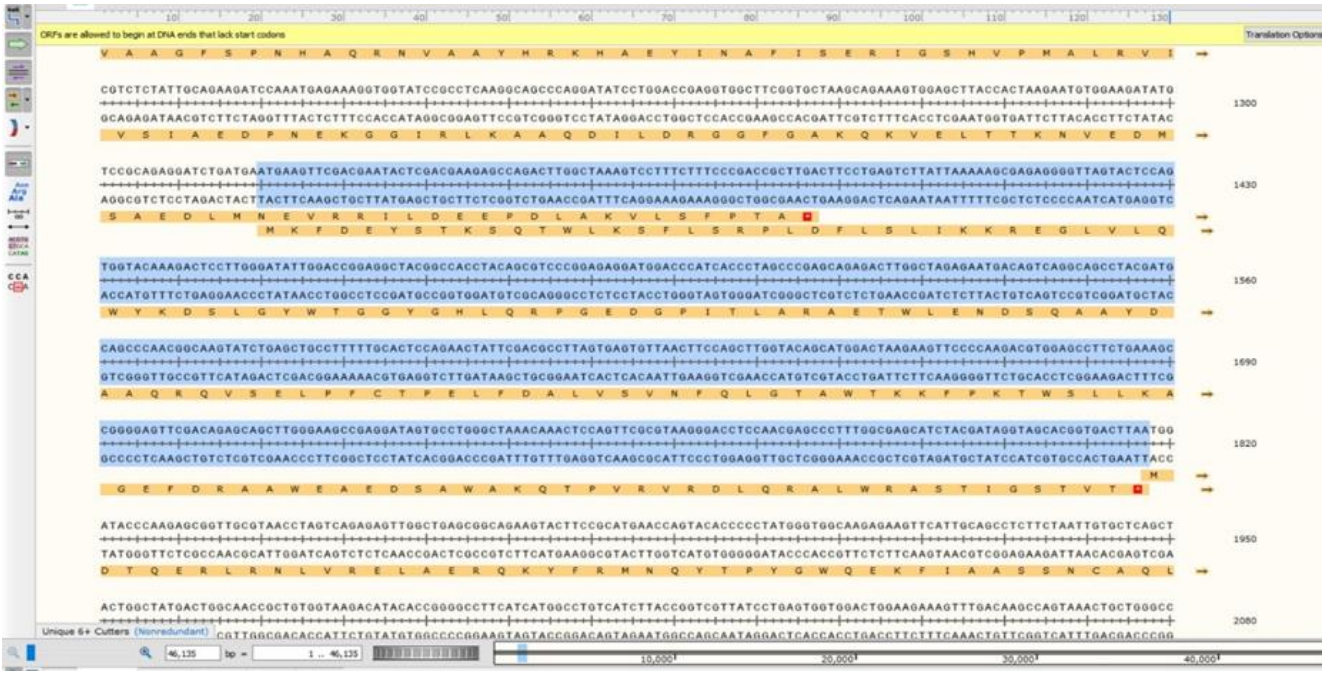

Figure 2. Coding sequence for Lysozyme-like domain (LysZC1), which ranges from 1320 - 1817 bp within the whole genome sequence of ZCPS1 phage.

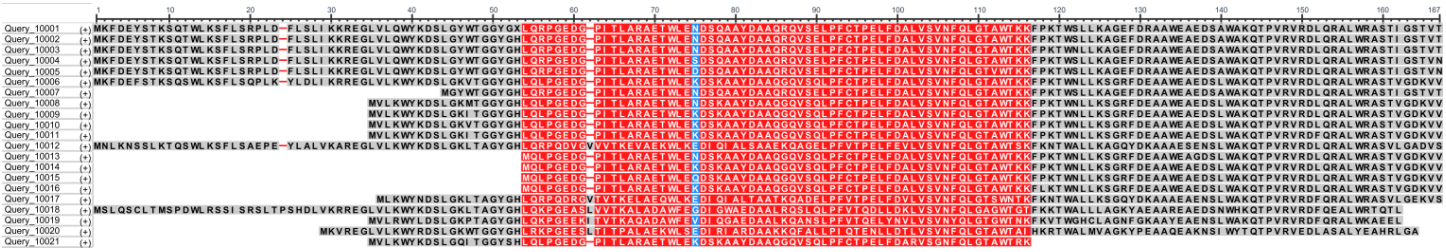

Figure 3. Multiple sequence alignment of LysZC1 with various phage lysin proteins showing high sequence similarity. The highly and partially conserved residues are highlighted with red and blue colors, respectively.

Figure 4. Phage ZCPS1 endolysin domain. InterProScan (A) and Conserved Domain Database (B).

3.2. PCR Amplification of Endolysin Genes from Phage DNA

The PCR was performed to amplify the sequence of endolysin. The results are shown in Fig.5, which displays a successful amplification step. The visualized bands correspond to the expected length of endolysin LysZC1 with a 512 bp. The generated products were purified for further utilization in and restriction digestion and ligation steps.

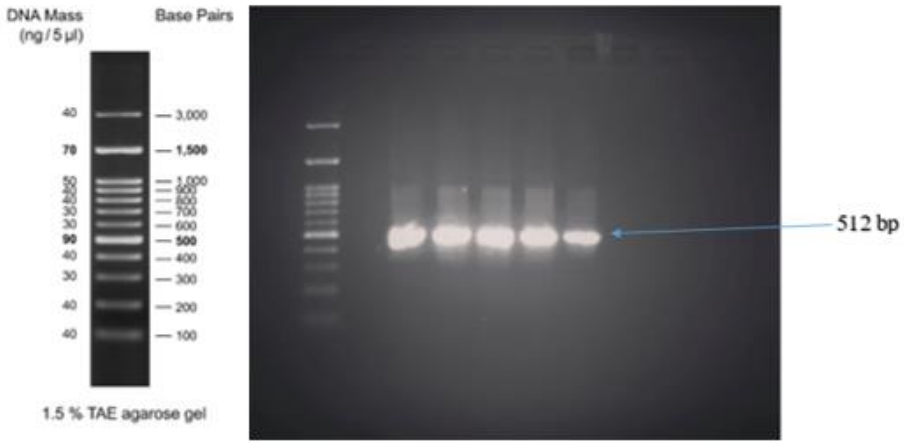

Figure 5. PCR of gene of the interest (LysZC1) with 512 bp size product.

3.3. Plasmid linearization, Molecular Cloning and Transformation of Endolysin Gene

Prior to ligation step, cloning vector pET28b and LysZC1 purified amplicon were digested using NcoI and XhoI restriction enzymes. This enzymatic treatment yielded a linearized plasmid and the gene of interest, both possessing sticky ends conducive to subsequent ligation (Fig.6).

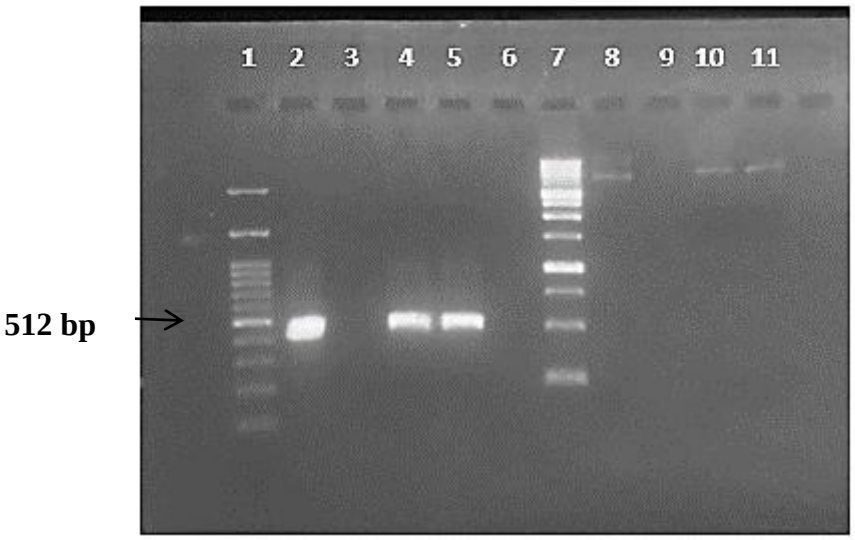

Figure 6. Double digestion of pET28b cloning vector and LysZC1 amplicon in agarose gel 1%. Lane 1: GeneDireX 100bp DNA Ladder, Lane 2: LysZC1 amplicon (Undigested), Lanes 4&5: LysZC1 amplicon digested with NcoI and XhoI, Lane 7: GeneDireX 1Kb DNA Ladder, Lane 8: uncut pET28b plasmid, Lanes 10 &11: pET28b plasmid digested with NcoI and XhoI.

After linearization the plasmid and the digested LysZC1 were extracted from agarose gel and purified. Then both digested plasmid and endolysin LysZC1 were cloned using T4 DNA ligase.

Following the ligation step, the cloned vector (pET28b-LysZC1) was initially transformed by heat shock onto *E. coli* DH5α competent cells. Subsequently, it was transformed into *E. coli* DE3 for further utilization in expression (Fig.7). The efficiency of transformation was evaluated by colony-PCR to identify the cells that contain the desired insert. Subsequently, plasmid DNA was isolated from resulting colonies and selected based on band size (596 bp) on gel electrophoresis (Fig.8), then the presence and orientation of insert were verified by sequencing.

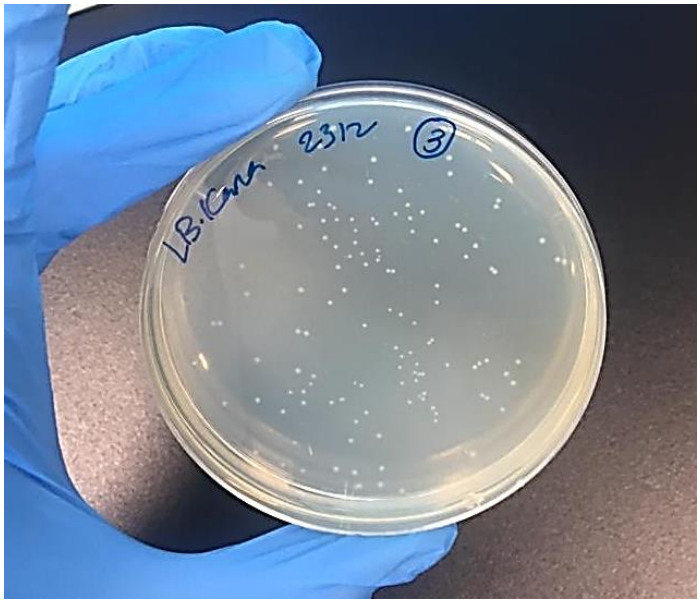

Figure 7. Transformation of cloned vector (pET28b-LysZC1) into *E. coli* competent cells.

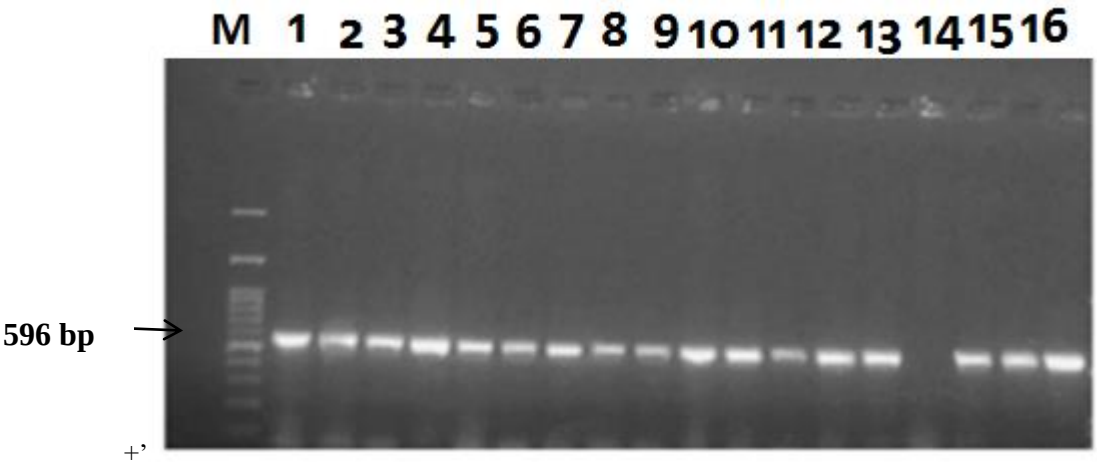

Figure 8. Colony-PCR results after "heat-shock" transformation using *E. coli* DH5α competent cells. Lane M: GeneDireX 100bp DNA Ladder, Lane 1-16 PCR PRODUCT(596 bp) results after "heat-shock" transformation using *E. coli* DH5α competent cells

3.4. Overexpression, Purification and SDS-PAGE Analysis of Purified Expressed Endolysin

To confirm the functionality of LysZC1 as a bactericidal lysozyme capable of degrading bacterial cell walls, a series of critical steps were undertaken. Initially, Plasmid harboring endolysin gene with the right orientation was successfully transformed into *E. coli* BL 21 (DE3) cells for expression and purified from *E. coli* cells. Analysis of the SDS-PAGE profile of the purified protein revealed a single distinct band, corresponding to the expected molecular weight of recombinant lysin LysZC1 with approximately 20 kDa (Fig.9).

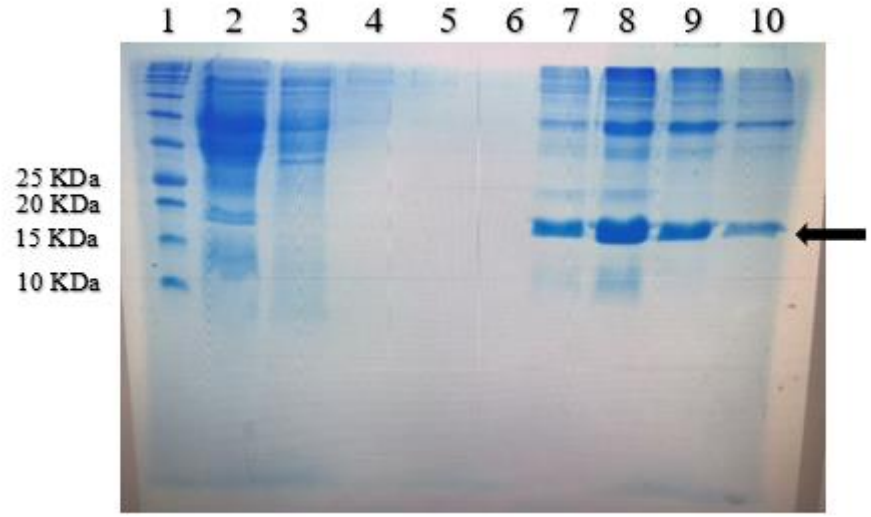

Figure 9. Expression of LysZC1 analyzed by SDS-PAGE. Lane 1, pre-stained protein marker; Lane 2, flow-through; Lane 3: wash 1; Lane 4: wash 2; Lane 5: wash 3; Lane 6: wash 4; Lanes 7-10: elution fractions. the arrow denotes the target protein.

3.5. LysZC1 Shows a Lytic Activity against *B. Cereus*

The lytic activity was measured by a formation of a clear lytic zone of inhibition over *B. cereus* bacterial lawn (Fig. 10A). Furthermore, the antimicrobial effect of LysZC1 on the morphology of *B. cereus* strains was visualized using SEM on 1µm sections. Exposure of *B. cereus* strains to 100 µg/ ml of lysin (LysZC1) for 2 h caused severe damage and disruption of the cell wall and cell membrane compared to untreated bacterial cells, which showed no evident cell disintegration (Fig.10B).

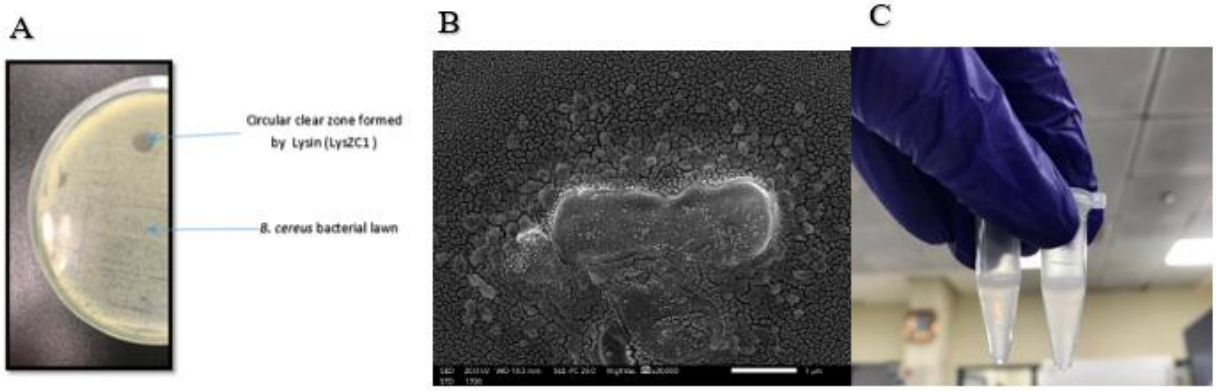

Figure 10. Lytic activity of LysZC1. (A) Plate lysis (spot) assay of LysZC1, the formation of clear zone due to the lytic activity of the lysin LysZC1 against *B. cereus* bacterial cells. (B) Scanning Electron Micrograph (SEM) image of *B. cereus* strains treated with LysZC1, revealing morphological changes indicative of bacterial cell lysis. (C) Turbidity reduction assay illustrating the impact of LysZC1 on *B. cereus*. The image highlights a decrease in turbidity in the Eppendorf tube containing LysZC1-treated cells (left) after exposure to LysZC1 at a concentration of 100 µg/mL for 2 hours compared to the control (right).

4. DISCUSSION

A close international cooperation is now imperative in order to ensure that future antimicrobial remedies remain effective since the antimicrobial resistance (AMR) has become a growing global concern **(Scarafile, 2016).** With antibiotic resistance causing an estimated 700,000 deaths worldwide yearly, it is predicted that 10 million people will be at risk of death by 2050 **(Scarafile, 2016**). According to a study published by the European Centre for Disease Prevention and Control (ECDC), approximately 33,000 deaths are caused by antibiotic-resistant bacteria per year **(Cassini *et al.*, 2019)**. Furthermore, AMR has marginally increased the costs of healthcare due to the rise in antibiotic imports coupled with the generalized price and tax inflation (**Iskandar *et al.*, 2021)**. Despite significant advances in food sanitation procedures and pathogen tracking, foodborne diseases remain among the leading causes of hospitalization and death globally (**Kirk *et al.*, 2015**).

Bacillus cereus is recognized as one of the most prevalent foodborne pathogens globally, poses a significant health risk due to its release of enterotoxins and the production of the toxic cereulide upon ingestion of contaminated food (**Bennett *et al.*, 2013; WHO, 2015**). The conventional approach to reducing microbial infections in livestock and food production involves the use of antibiotics in feed and the application of disinfectants and biocides on farms, a process known as primary production. This process ending in antimicrobials potentially reaching consumers through the food chain **(Endersen *et al.*, 2014; Oniciuc *et al.*, 2019).**

Unfortunately, the frequent use of antimicrobials at sub-lethal and lethal doses has precipitated the emergence of antimicrobial resistance in these pathogens. For instance, various bacteria, including *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Salmonella* spp., and *Bacillus* spp., have developed resistance to antibiotics, creating a pressing need for alternative approaches to bacterial controlling **(Whitehead et al., 2011; Gadea *et al.*, 2017; Wand *et al.*, 2017; Lu *et al.*, 2018; Kim *et al.*, 2018).** In response to this escalating crisis, there is an urgent call for conglomerated efforts to explore alternative antimicrobial agents to replace traditional antibiotics. One promising avenue of research focuses on phage lytic enzymes, often referred to as "enzybiotics" **(Danis-wlodarczyk *et al.*, 2021)**. These enzymes act on bacterial cell walls either post-adsorption (Tail lysozymes) or during bacterial lysis for phage progeny release (endolysins). Notably, they have been investigated as potential biocidal agents in various food products. For example, Ply511, an anti-Listeria endolysin, has been expressed in *Lactococcus* spp. in fermented products to serve as biopreservatives against *Listeria monocytogenes* and other foodborne pathogens **(Gaeng *et al.*, 2000)**. Other examples include the reduction of *Salmonella* spp. in chickens through the oral administration of purified truncated *Salmonella* phage tail spike endoglycosidase **(Waseh *et al.*, 2010)** and the use of phage endolysin CHAP8H3b to eliminate *S. aureus* in raw milk **(Rodríguez-Rubio *et al.*, 2013).**

This study contributes to this growing body of research. Our findings strongly suggest that LysZC1 possesses the potential to combat one of the most virulent foodborne pathogens, *B. cereus*, thereby laying the foundation for future research aimed at enhancing and exploiting LysZC1 to control multidrug-resistant pathogens commonly found in food products. Furthermore, our investigation reveals that LysZC1 belongs to the group of Gram-negative phage endolysins, characterized by a modular structure that may include one or two cell wall binding domains (CBDs) at the N-terminus, while the enzymatically active or catalytic domain (EAD) is situated at the C-terminus (**Briers *et al.*, 2007**). Our study provides compelling evidence that LysZC1 exhibits a robust lytic effect on *B. cereus* ATCC 11778 bacterial strains. It is worth noting that previous research has shown that endolysins tend to be more active against bacteria in the logarithmic growth phase compared to those in the stationary phase (**Yang *et al.*, 2015**).Moreover, our SEM analysis demonstrates that *B. cereus* cells subjected to LysZC1 treatment exhibit significant morphological changes indicative of cell lysis. This features LysZC1's potent bactericidal activity against *B. cereus* bacteria. Importantly, the study reveals that LysZC1 employs a lysozyme-like domain, which contributes to its broad-spectrum activity. This ability to target both Gram-positive and Gram-negative bacteria may be attributed to similarities in the structure of their cell walls, characterized by the presence of N-acetyl glucosamine and N-acetyl muramic acid.

In conclusion, the findings of this study highlight LysZC1 as a challenging antibacterial agent with substantial bactericidal activity. LysZC1 presents itself as a promising candidate for therapeutic applications in the fight against bacterial infections and as a valuable tool for preserving food products through biocontrol. This research sets the stage for further investigations aimed at optimizing and harnessing the potential of LysZC1 in the battle against multidrug-resistant pathogens commonly encountered in the food industry.

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