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A Simplified and Robust Approach for Recombinant Lysostaphin Production in Buffalo Fibroblast Cells

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ABSTRACT

Background: Mastitis caused by *Staphylococcus aureus* remains a significant challenge in dairy livestock due to the emergence of multidrug-resistant strains and the limited effectiveness of conventional therapeutic approaches. Lysostaphin, a staphylolytic enzyme with high specificity against *Staphylococcus* species, has emerged as a promising alternative antimicrobial agent. In this study, we aimed to establish a preliminary mammalian expression strategy for production of biologically active recombinant lysostaphin in buffalo fibroblast cells.

Methods: A modified lysostaphin gene, designed to eliminate predicted N-glycosylation sites, was synthesized and initially cloned into the pUC57 vector followed by subcloning into the pcDNA3.1/CT-GFP-TOPO expression vector. Recombinant constructs were confirmed by colony PCR and Sanger sequencing before transfection into buffalo fibroblast cells. Recombinant expression was qualitatively assessed using GFP fluorescence and antibacterial activity was evaluated using a qualitative plate inhibition assay against *Staphylococcus aureus*.

Result: The lysostaphin gene (~798 bp) was successfully amplified and cloned into the mammalian expression vector. Screening and sequencing confirmed the correct insertion and orientation without mutations. Transfected buffalo fibroblast cells exhibited GFP fluorescence, indicating successful expression of the recombinant construct. Lysates from transfected cells produced visible zones of inhibition against *S. aureus*, indicating antibacterial activity of the expressed lysostaphin.

Key words: Buffalo fibroblast cells, Lysostaphin, pcDNA3.1 vector, Recombinant expression, *Staphylococcus aureus*.

INTRODUCTION

Mastitis occurs in both clinical and subclinical forms and is mainly caused by pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli*, with *S. aureus* being strongly associated with persistent and recurrent infections (Sekhri *et al*., 2021; Srujana *et al*., 2022). Although antibiotics, chemotherapeutics and vaccines are commonly used for disease control, their effectiveness is limited due to the emergence of multidrug-resistant (MDR) strains, vaccine-associated side effects and inadequate immune responses (Sekhri *et al*., 2021). Consequently, alternative antimicrobial strategies are being explored, among which lysostaphin has emerged as a promising staphylococcal bacteriolysin with significant therapeutic potential against MDR *S. aureus* (Wall *et al*., 2005). Lysostaphin was first discovered by Schindler and Schuhardt in 1964 from bacterial strain K-6-WI, which exhibited lytic activity against *S. aureus* and *S. epidermidis* (Bastos *et al*., 2010). It was later isolated from *Staphylococcus simulans* biovar *staphylolyticus* and identified as a 493-amino acid preproenzyme encoded by the plasmid pACK1 (Schindler and Schuhardt, 1964; Sloan *et al*., 1982). The structural organization of lysostaphin includes a leader sequence, tandem-repeat region, catalytic domain, linker region and cell wall-targeting domain, as shown in Fig 1.

Lysostaphin is a 25 kDa zinc metalloenzyme that exhibits glycylglycine endopeptidase activity, hydrolyzing the polyglycine interpeptide bridges of the *Staphylococcus aureus* cell wall and causing bacterial lysis (Heath *et al*., 1989). Due to its highly specific antibacterial mechanism, lysostaphin has gained attention as a potential therapeutic agent against multidrug-resistant (MDR) *S. aureus* infections (Huang *et al*., 2013). Several studies have demonstrated its efficacy in different experimental models (Kokai-Kun *et al*., 2003; Dajcs *et al*., 2000). Lysostaphin, either alone or in combination with vancomycin, was found to be more effective than vancomycin alone in treating experimental MRSA-induced aortic valve endocarditis in rabbits without inducing

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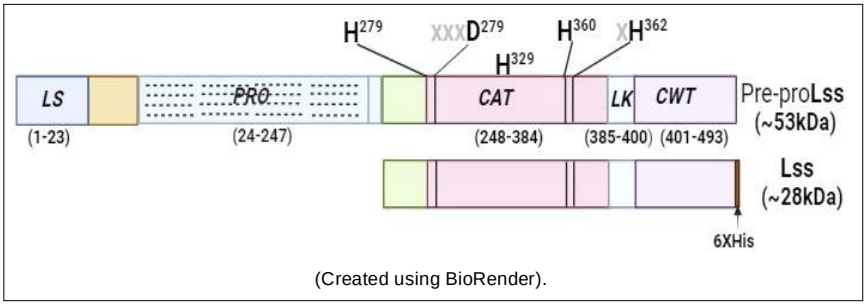


Fig 1: Schematic diagram of the ~53-kDa full length pre-proLss showing leading sequence (LS), proregion (PRO), catalytic domain (CAT), linker (LK) and cell wall targeting domain (CWT).

significant immune reactions (Heinrich *et al*., 1987). Its ability to eradicate *S. aureus* from infected tissues and blood in mice has also been reported (Climo *et al*., 1998). Moreover, lysostaphin-delivering hydrogels have shown promise in reducing *S. aureus* infection while promoting bone regeneration in infected radial defects in mice (Kokai-Kun *et al*., 2007; Johnson *et al*., 2019). However, despite its promising therapeutic potential, recombinant expression of biologically active lysostaphin in mammalian expression systems remains challenging due to possible post-translational modifications such as glycosylation, which may affect protein structure and function. To address this, the lysostaphin gene was modified by substituting predicted N-glycosylation-associated residues to improve compatibility with mammalian expression. Therefore, this study aimed to perform a preliminary qualitative proof-of-concept assessment of modified lysostaphin expression in buffalo fibroblast cells and to evaluate whether biologically active lysostaphin could be expressed in this mammalian host system.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents

The experimental work was conducted during the period 2023-2025 at the ICAR-National Dairy Research Institute (ICAR-NDRI), Karnal, Haryana, India, in association with the ICAR-National Research Centre on Yak (ICAR-NRCY), Dirang, Arunachal Pradesh, India. Chemically competent *Escherichia coli* TOP10 cells (Invitrogen, USA) were used for cloning and propagation of recombinant plasmids. The mammalian expression vector pcDNA3.1/CT-GFP-TOPO was used for cloning and expression of the lysostaphin gene (Fig 2). Plasmid purification and gel extraction were performed using the QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit (Qiagen, Germany). The synthesized lysostaphin gene in the pUC57 cloning vector was first propagated in competent *E. coli* TOP10 cells to obtain sufficient plasmid DNA for downstream cloning experiments and transformed colonies were selected on LB agar plates containing ampicillin (100 µg/mL).

Preparation of competent *E. coli* cells

Chemically competent *E. coli* TOP10 cells were prepared using the calcium chloride method based on the protocol described by Sambrook and Russell (2001) with minor modifications. An overnight culture of TOP10 *E. coli* cells was inoculated into fresh SOB medium and incubated at 37°C with shaking at 250 rpm until the optical density reached approximately O.D. 600 ≈0.6, corresponding to the mid-logarithmic phase of bacterial growth. The culture was chilled on ice for 5 min and centrifuged at 4000 rpm for 10 min at 4°C. The resulting bacterial pellet was resuspended in 0.1 M $CaCl_2$, containing 15% glycerol and incubated on ice for 1 h to enhance competency. Aliquots of 250 µL were prepared and stored at -80°C until further use.

Primer design

Primers specific for amplification of the lysostaphin gene were designed using Primer3 software (Untergasser *et al*., 2012) based on the published lysostaphin gene sequence. The primers were synthesized commercially (Sigma-Aldrich, Bengaluru, India). The sequences of primers used in this study are presented in Table 1.

PCR amplification of lysostaphin gene

Amplification of the lysostaphin gene was performed using DreamTaq 2× PCR master mix (Thermo scientific) following standard PCR protocols (Saiki *et al*., 1988). Each PCR reaction was carried out in a final volume of 20 µL, containing 10 µL of DreamTaq master mix, 0.4 µL of forward primer (10 µM), 0.4 µL of reverse primer (10 µM), 1 µL of template DNA and 8.2 µL of nuclease-free water. PCR amplification was performed in a Bio-Rad S1000 thermal cycler under the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. A final extension step was carried out at 72°C for 5 min, after which the reactions were held at 4°C until further analysis. The amplified PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide (0.5 µg/mL) and DNA bands were visualized under ultraviolet illumination using a Bio-Rad gel doc XR imaging system. The PCR products were purified using QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified DNA was quantified using a NanoQuant spectrophotometer and stored at -20°C until further use.

Cloning of lysostaphin gene into pcDNA3.1 vector

The purified PCR product containing the lysostaphin gene was cloned into the pcDNA3.1/CT-GFP-TOPO expression vector according to the manufacturer's protocol. The cloning reaction was prepared in a total volume of 6 µL, consisting of 2 µL purified PCR product (~150 ng DNA), 1 µL salt solution, 1 µL pcDNA3.1/CT-GFP-TOPO vector and 2 µL nuclease-free water. The reaction mixture was gently mixed and incubated at room temperature to facilitate insertion of the lysostaphin gene into the vector.

Transformation of recombinant plasmid

The recombinant plasmid was introduced into competent *E. coli* TOP10 cells using the heat-shock transformation method (Froger and Hall, 2007). Competent cells were mixed with the ligation mixture and incubated on ice for 30 min, followed by heat shock at 42°C for 90 s and immediate cooling on ice for 2 min. Subsequently, 500 µL SOC medium was added and the cells were incubated at 37°C for 1 h with shaking at 200 rpm. The transformed cells were plated onto LB agar containing ampicillin (100 µg/mL) and incubated overnight at 37°C.

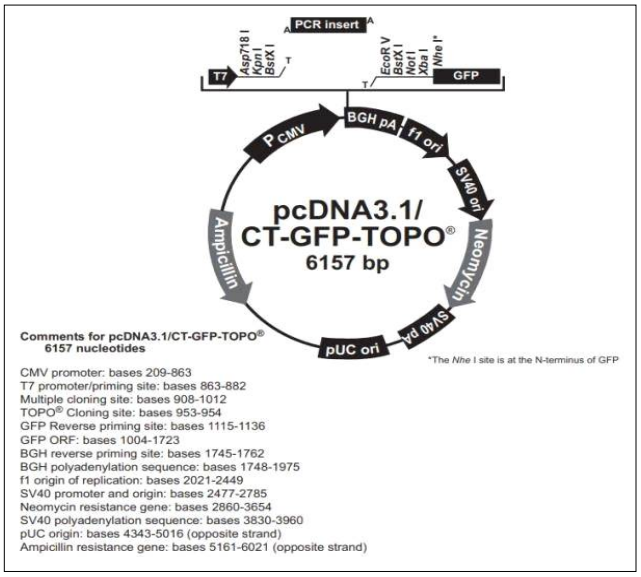


Fig 2: Map of the pcDNA3.1/CT-GFP-TOPO expression vector.

Screening of recombinant clones by colony PCR

Transformant colonies were screened using colony PCR to confirm the presence of the lysostaphin gene. Individual colonies were suspended in 10 µl of nuclease-free water in PCR tubes and heated at 95°C for 10 min to lyse the cells. The tubes were then centrifuged and 3 µl of the supernatant containing plasmid DNA was used as template for PCR amplification using lysostaphin-specific primers. The PCR reaction was carried out in a 20 µl reaction mixture containing 10 µl of 2× DreamTaq buffer, 0.5 µl of forward primer, 0.5 µl of reverse primer, 3 µl of bacterial lysate and 6 µl of nuclease-free water. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis along with a 100 bp DNA ladder and a negative control to confirm the presence of the expected lysostaphin gene amplicon.

Isolation of recombinant plasmid DNA

Positive clones identified by colony PCR were cultured overnight in LB medium supplemented with ampicillin (100 µg/mL) at 37°C with shaking. Plasmid DNA was isolated using the QIAprep spin miniprep Kit (Qiagen) according to the manufacturer's instructions. The purified plasmids were quantified using NanoQuant spectrophotometry and stored at -20°C.

Sequence verification

The integrity and correct orientation of the cloned lysostaphin gene were then verified by Sanger sequencing using the T7 forward primer (TAATACGACTCACTATAGGG). Sequence data were analyzed to confirm the correct reading frame and insertion of the gene construct.

Transfection of recombinant constructs into buffalo fibroblast cells

Recombinant pcDNA3.1/CT-GFP-TOPO plasmids containing the lysostaphin gene were transfected into buffalo fibroblast cells as a preliminary qualitative assessment of recombinant expression in a mammalian host system. Transfection was performed using lipofectamine-mediated chemical transfection followingstandard mammalian cell transfection protocols (Strauss, 1996). The pcDNA3.1/CT-GFP-TOPO vector contains a green fluorescent protein (GFP) reporter gene, allowing qualitative visualization of transgene expression

Table 1: Primers used for amplification of the lysostaphin gene.

Set no.	Primer name	Orientation	Primer sequence	Length (bp)
A	LST_TOPO_BuBLGF	Forward	ACCATGAAGTGCCTCCTGCTTGCCCTGG	28
	LST_TOPO_R	Reverse (without stop codon)	GCTTTATAGTTCCCCAAAGAACACC	25
B	LST_TOPO_BuBLGF	Forward	ACCATGAAGTGCCTCCTGCTTGCCCTGG	28
	LST_TOPO_R1	Reverse (with stop codon)	GTCACTTTATAGTTCCCCAAAGAACACC	28
C	LST_TOPO_BuBLGF1	Forward	ACCATGGGGGAAGTGCCTCCTGCTTGCCCTGG	31
	LST_TOPO_R	Reverse (without stop codon)	GCTTTATAGTTCCCCAAAGAACACC	25
D	LST_TOPO_BuBLGF1	Forward	ACCATGGGGGAAGTGCCTCCTGCTTGCCCTGG	31
	LST_TOPO_R1	Reverse (with stop codon)	GTCACTTTATAGTTCCCCAAAGAACACC	28

by fluorescence microscopy. As the present work was designed as a preliminary qualitative proof-of-concept study, quantitative transfection efficiency determination was not included.

Qualitative antibacterial activity assay

The antibacterial activity of lysostaphin produced in transfected buffalo fibroblast cells was preliminarily evaluated using a qualitative plate inhibition assay against *Staphylococcus aureus*. Agar plates seeded with a bacterial lawn of *S. aureus* were prepared and aliquots of lysates obtained from transfected fibroblast cells were spotted onto the plates. Purified lysostaphin was included as a positive control for comparison. Plates were incubated at 37°C for 18-24 h. The appearance of visible zones of inhibition around the lysate spots was considered indicative of staphylolytic activity of the expressed lysostaphin protein (Bastos *et al*., 2010). Since this study was intended as an initial qualitative feasibility assessment, quantitative inhibition zone measurements and replicate-based statistical analysis were not included.

RESULTS AND DISCUSION

Synthesis and verification of the modified lysostaphin gene

For the expression of lysostaphin in mammalian cells, the lysostaphin gene was modified by replacing two predicted N-glycosylation sites located at amino acid positions 125 and 232. At these positions, the original asparagine (ASn) residues were substituted with glutamine (Gln) in order to prevent glycosylation during expression in eukaryotic cells while maintaining the antibacterial activity of the enzyme. Following the modification, the lysostaphin gene was synthesized commercially and delivered in the pUC57 cloning vector. The newly synthesized fragment had a length of 798 bp, corresponding to the complete coding sequence of the modified lysostaphin gene. After that, the obtained sequence was aligned with the reference lysostaphin gene sequence for ensuring the accuracy of the synthesized construct. The alignment confirmed that the intended nucleotide substitutions were correctly incorporated into the gene. The sequence comparison revealed expected agreement with the modified lysostaphin sequence (Fig 3), thereby confirming the successful synthesis of the gene construct.

Propagation of the pUC57 plasmid containing the modified lysostaphin gene

To obtain sufficient plasmid DNA for downstream molecular analysis, the synthesized pUC57 plasmid carrying the modified lysostaphin gene was propagated within chemically competent TOP10 *Escherichia coli* cells. Following transformation, the bacterial suspension was spread onto LB agar plates supplemented with ampicillin to facilitate the selective growth of the transformed cells. After overnight incubation, multiple colonies were observed on the cultured agar plates which indicated successful uptake and propagation of the plasmid within the host cells. Individual colonies were subsequently selected and inoculated into LB broth containing ampicillin to allow further amplification of the plasmid prior to the DNA isolation.

Isolation and quantification of pUC57 plasmid DNA

Plasmid DNA was isolated from overnight grown bacterial cultures using a standard plasmid extraction protocol. The quality and concentration of the extracted DNA were measured using a NanoQuant spectrophotometer. The obtained concentrations of the plasmid DNA were 245.35 ng/µL and 235.41 ng/µL, with A260/A280 ratios of 1.90 and 1.91, respectively. These values indicate good quality plasmid DNA with minimum protein contamination. Therefore, the isolated plasmid DNA was considered suitable for subsequent PCR amplification of the lysostaphin gene.

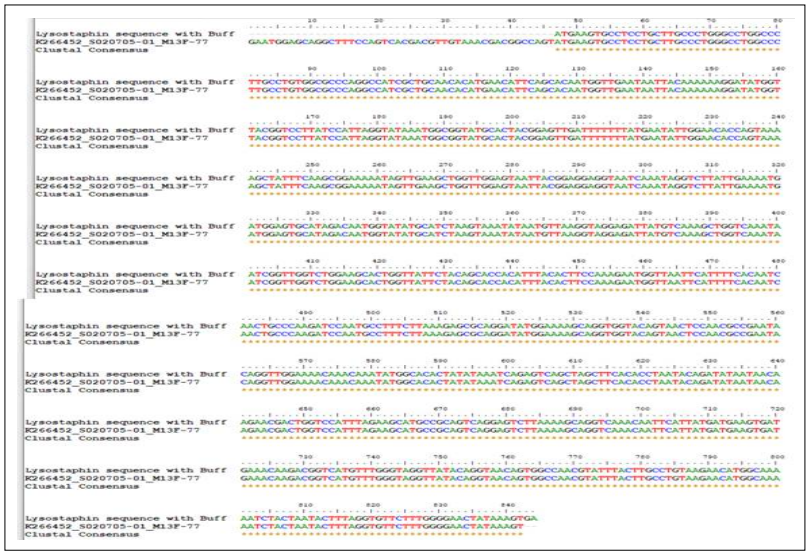


Fig 3: Alignment of the modified lysostaphin gene matching exactly with the reference sequence.

PCR amplification of the lysostaphin gene

The modified lysostaphin gene was amplified from the synthesized pUC57 plasmid by using four primer combinations (Set A, Set B, Set C and Set D) that were designed to facilitate the cloning of the gene into the mammalian expression vector. PCR amplification produced a clear DNA fragment of approximately 798 bp, which corresponds to the expected size of the lysostaphin gene. The agarose gel electrophoresis results clearly showed distinct amplification bands for the different primer sets; these bands can be observed in Fig 4, confirming successful amplification of the target gene.

Purification of amplified PCR products

After the amplification, the lysostaphin PCR products were run on 0.8% agarose gel and the specific bands corresponding to the expected gene fragment further were excised for purification. Removal of excess primers, nucleotides and enzymes ensured that the DNA fragments were suitable for downstream cloning experiments. After the purification, the concentrations of the samples were quantified using NanoQuant spectrophotometry. The purified PCR products showed concentrations ranging from 85.10 ng/µL to 163.63 ng/µL, with A260/A280 ratios between 1.87 and 1.90, confirming that the DNA fragments were sufficiently pure for insertion into the expression vector.

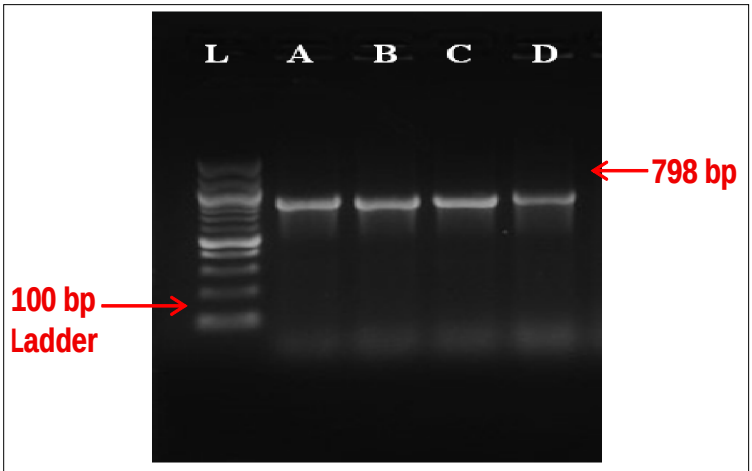
Cloning of the lysostaphin gene into the pcDNA3.1/CT-GFP-TOPO expression vector

The purified lysostaphin PCR products were ligated into the pcDNA3.1/CT-GFP-TOPO expression vector to generate recombinant constructs. The ligation mixture was subsequently introduced into competent TOP10 *E. coli* cells using the heat-shock transformation method. After transformation, the cells were plated onto LB agar plates containing ampicillin. Numerous colonies appeared on the plates after overnight incubation, indicating successful uptake of recombinant plasmids. These colonies, visible in Fig 5, represent bacterial clones potentially carrying the lysostaphin gene insert.

To obtain pure colonies for further screening, selected colonies were streaked onto fresh antibiotic-containing agar plates. The streaked colonies shown in Fig 6 demonstrate successful isolation of individual recombinant clones.

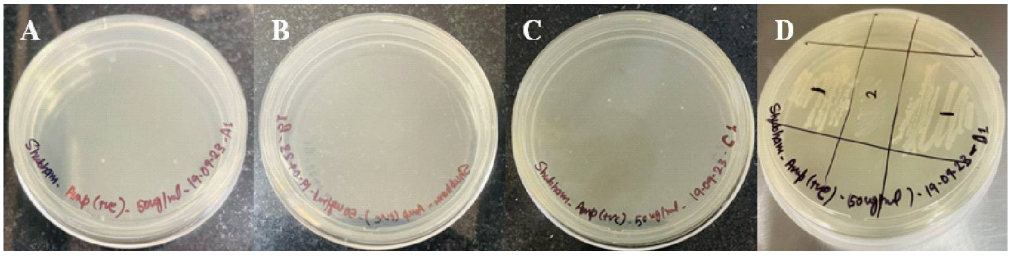
Screening of recombinant clones by colony PCR

To verify that the lysostaphin gene had been successfully inserted into the vector, bacterial colonies were screened



Lane: L-100 bp ladder; 1-A; 2-B; 3-C; 4-D represents amplified PCR products of lysostaphin using different sets of primers. (Product size-798 bp).

Fig 4: Gel image of modified lysostaphin amplified from the synthesized pUC57 vector.



A: Set-A; B: Set-B; C: Set-C; D: Set-D.

Fig 5: Agar plates of pcDNA3.1/CT-GFP-TOPO fusion vector having the different sets of amplified PCR products.

using colony PCR. Lysostaphin-specific primers were used to amplify the gene directly from bacterial lysates. Agarose gel electrophoresis of the colony PCR products revealed the presence of the expected gene fragment in several clones. The gel image shown in Fig 7 demonstrates clear amplification of the lysostaphin gene in multiple colonies. Among the screened clones, six clones from Set A, one clone from Set B, three clones from Set C and two clones from Set D were confirmed to contain the recombinant plasmid.

Isolation and quantification of recombinant plasmids

Colonies that tested positive in colony PCR were cultured overnight in LB broth containing ampicillin for plasmid isolation. Recombinant plasmids were extracted using the QIAprep Spin Miniprep Kit. Spectrophotometric analysis showed plasmid DNA concentrations ranging from 323.97 ng/µL to 547.67 ng/µL, with A260/A280 ratios between 1.73 and 1.85, indicating acceptable purity of the plasmid preparations for sequencing and further analysis.

Verification of gene orientation in recombinant constructs

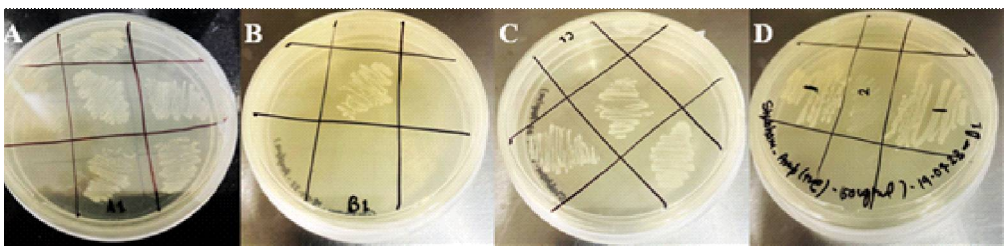
Correct orientation of the lysostaphin insert within the expression vector was verified by PCR amplification using the T7 forward primer together with a lysostaphin-specific reverse primer. Successful amplification of the expected DNA fragment confirmed that the lysostaphin gene had been inserted into the vector in the correct orientation. The PCR products confirming the orientation of the insert are clearly visible in Fig 8.

Sequence validation of recombinant plasmids

To further confirm the accuracy of the cloned constructs, selected recombinant plasmids were subjected to sanger sequencing. Sequencing was performed using vector-specific primers to read across the inserted lysostaphin gene. Sequence analysis confirmed that the lysostaphin gene had been inserted correctly into the pcDNA3.1/CT-GFP-TOPO vector without any mutations and that the reading frame was preserved. Representative sequencing chromatograms and alignment results are presented in Fig 9, illustrating the correct nucleotide sequence of the cloned gene.

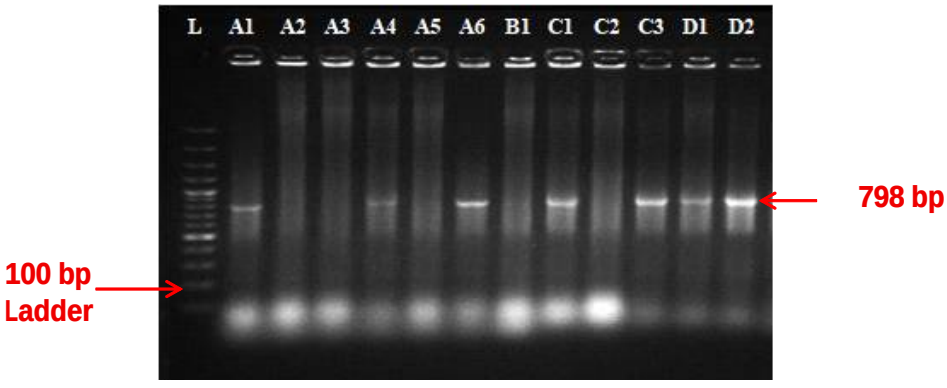
Transfection of recombinant plasmids into buffalo fibroblast cells

To evaluate expression of the lysostaphin gene in mammalian cells, the confirmed recombinant plasmids were transfected into buffalo fibroblast cells. The pcDNA3.1/CT-GFP-TOPO vector contains a GFP reporter gene, which allows visual detection of transfected cells. After transfection, clear green fluorescence signals were observed in the



A: Set-A; B: Set-B; C: Set-C; D: Set-D.

Fig 6: Streaked agar plates of pcDNA3.1/CT-GFP-TOPO fusion vector having the different sets of amplified PCR products.



Ladder: 100 bp.

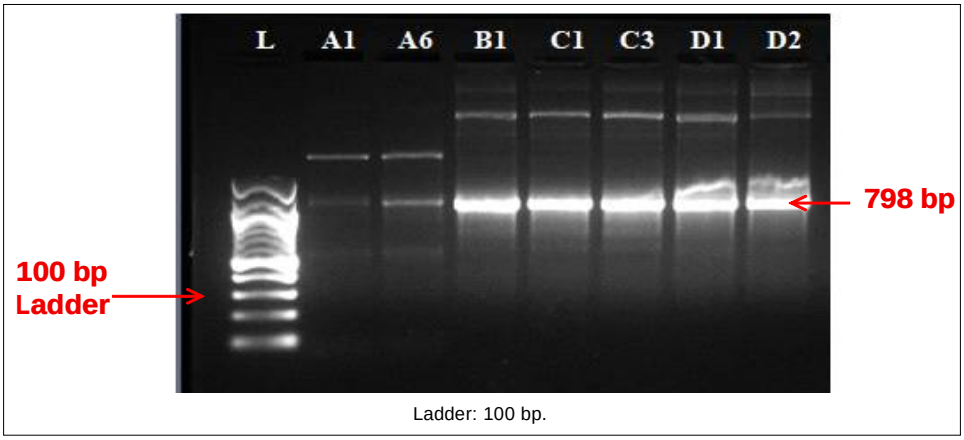
Fig 7: Colony PCR showing six isolated clones from set-A (A1-A6), one clone from set-B (B1), three clones from set-C (C1-C3) and two clones from set-D (D1 and D2) have recombinant plasmid containing lysostaphin.

fibroblast cells, indicating successful uptake and expression of the recombinant plasmids. The fluorescence observed in the transfected cells is shown in Fig 10.

Antibacterial activity of lysostaphin expressed in fibroblast cells

The antibacterial activity of lysostaphin expressed in transfected fibroblast cells was evaluated using a qualitative plate inhibition assay against *Staphylococcus aureus*. Lysates prepared from the transfected cells were applied onto agar plates containing a bacterial lawn of *S. aureus*, with purified lysostaphin used as a positive control for comparison. After incubation, visible zones of inhibition were observed around the lysate spots, indicating antibacterial activity of the expressed lysostaphin. The inhibition patterns observed are presented in Fig 11.

Mammary gland infections are a major economic burden in the dairy industry due to reduced milk production, treatment costs and compromised animal health. Among the pathogens involved, *Staphylococcus aureus* is a key contagious mastitis agent causing about 15-30% of infections and its intracellular survival, immune evasion and biofilm formation make treatment particularly challenging (Barkema *et al*., 2006; Bradley, 2002). The therapeutic potential of lysostaphin against *S. aureus*-associated infections has been demonstrated in several experimental models. Earlier studies showed that lysostaphin effectively reduced *S. aureus* burden in murine mastitis models, highlighting its promise as an alternative anti-staphylococcal therapeutic (Kerr *et al*., 2001). In addition, lysostaphin has demonstrated efficacy against methicillin-resistant *S. aureus* (MRSA), biofilm-associated infections,

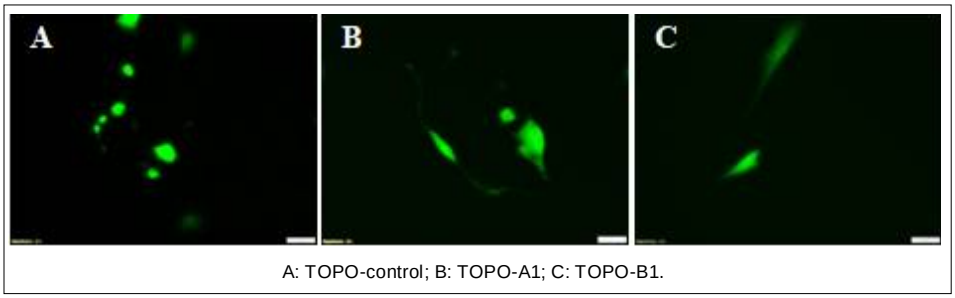


Ladder: 100 bp.

Fig 8: PCR amplification showing seven isolated clones (A1, A6, B1, C1, C3, D1, D2) of pcDNA3.1/CT-GFP-TOPO fusion vectors containing modified LST gene in correct orientation as amplified by T7 forward primer and LST reverse primer.



Fig 9: Sequence alignment of the plasmid Set A_Topo_Clone 1 (Tube 3) with bacterial lysostaphin gene carrying buffalo βLG signal sequence (sequencing done by T7 forward primer).



A: TOPO-control; B: TOPO-A1; C: TOPO-B1.

Fig 10: Expression of the pcDNA3.1/CT-GFP-TOPO fusion vector having GFP as screening marker in the transfected buffalo fibroblast cells.



A: TOPO-A1 and B: TOPO-B1.

Fig 11: Inhibition of staphylococcal strains by lysostaphin-producing cell lysate.

keratitis and experimental endocarditis, supporting its broader translational relevance (Kokai-Kun *et al*., 2003; Climo *et al*., 1998; Hole *et al*., 2026). Previous researchers have also explored recombinant expression of lysostaphin in eukaryotic systems by replacing the native bacterial translational elements with eukaryotic-compatible expression components. Successful expression in COS-7 cells and cell-free eukaryotic systems demonstrated that lysostaphin could be produced in a mammalian-compatible environment while retaining biological activity (Williamson *et al*., 1994). However, mammalian expression systems may introduce post-translational modifications such as glycosylation, which can potentially affect protein folding, secretion, stability and enzymatic activity. To overcome this limitation, the present study employed a modified lysostaphin construct in which two predicted N-linked glycosylation-associated asparagine residues (N125 and N232) were substituted with glutamine to improve compatibility with mammalian expression while preserving antibacterial functionality. Similar approaches have been explored previously, where control of glycosylation-associated modifications was shown to be important for maintaining lysostaphin activity (Huang *et al*., 2013). The modified lysostaphin gene was successfully cloned into the pcDNA3.1/CT-GFP-TOPO mammalian expression vector and following transfection into buffalo fibroblast cells, observable GFP fluorescence suggested successful uptake and expression of the recombinant construct.

The antibacterial assay further demonstrated visible inhibition of *S. aureus* growth by lysates obtained from transfected buffalo fibroblast cells, while purified lysostaphin served as a positive control for comparison. These findings suggest that the expressed recombinant lysostaphin retained antibacterial activity following mammalian expression. Compared with bacterial expression systems such as *Escherichia coli*, which are often associated with endotoxin contamination, inclusion body formation and complex downstream purification requirements (Rosano and Ceccarelli, 2014), mammalian systems may offer a more physiologically relevant environment for recombinant therapeutic protein production. Yeast-based expression systems such as *Pichia pastoris* provide an alternative eukaryotic platform, but issues related to hyperglycosylation and heterogeneous post-translational modifications remain potential concerns (Ahmad *et al*., 2014). From a translational perspective, recombinant lysostaphin expressed in mammalian systems may have potential applications in both veterinary and human medicine. In dairy animals, such an approach may contribute toward the development of alternative therapeutic interventions against *S. aureus*-associated mastitis, potentially reducing dependence on conventional antibiotics and helping address antimicrobial resistance. Previous studies involving transgenic expression of lysostaphin in mammary tissue have also demonstrated protective effects against staphylococcal mastitis, further supporting its veterinary relevance (Wall *et al*., 2005).

However, the findings of the present study should be interpreted within the scope of its design. This investigation was intended as a preliminary qualitative proof-of-concept assessment; therefore, quantitative transfection efficiency analysis, replicate-based statistical validation, recombinant protein purification and detailed enzymatic characterization were not performed. Further studies focusing on expression optimization, quantitative antimicrobial evaluation, protein purification and validation in relevant animal infection models will be necessary to establish reproducibility and therapeutic feasibility more robustly.

CONCLUSION

A preliminary qualitative proof-of-concept for the expression of modified lysostaphin in a mammalian expression system using buffalo fibroblast cells was demonstrated successfully. The modified lysostaphin construct, generated by substituting two predicted N-glycosylation-associated asparagine residues with glutamine, was successfully cloned into the pcDNA3.1/CT-GFP-TOPO expression vector and confirmed through PCR-based orientation analysis and Sanger sequencing. Following transfection into buffalo fibroblast cells, green fluorescence indicated successful uptake and expression of the recombinant construct. In addition, lysates obtained from the transfected cells showed antibacterial activity against *Staphylococcus aureus* in a qualitative plate inhibition assay, suggesting functional expression of the modified lysostaphin protein. These findings provide initial support for the feasibility of expressing biologically active lysostaphin in a mammalian host system and offer a basis for future quantitative optimization and further exploration of its therapeutic potential against staphylococcal infections, including mastitis-associated pathogens.

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Conflict of interest

The authors declare no conflict of interest.

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