

Bacillus phage endolysin, lys46, bactericidal properties against Gram-negative bacteria

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ABSTRACT

Background and Objectives: The great potential of bacteriophage for removing pathogen bacteria via targeting the cell wall is highly concerned. With a priority for overcoming drug-resistance, we screened against endolysins targeting Gram-negative bacteria to introduce a new antibacterial agent. This study was aimed to identify endolysins from the lysogenic phage of the *Siphoviridea* family in *Bacillus subtilis*.

Materials and Methods: The *Bacillus subtilis* strain DDBCC46 was isolated from a preliminary antibacterial screening program. The endolysin (s) was extracted, concentrated with ammonium sulfate saturation, and their activity evaluated against the indicator bacteria. The phage particles were extracted from the bacteria using the minimum inhibition concentration of mitomycin C, followed by testing the phage inhibitory effect on the growth of indicator bacteria. The NCBI, Virus-Host DB, and EXPASY databases were used to obtain and confirm the sequences of the genes encoding PG hydrolases in *Siphoviridea* phages hosted in *B. subtilis*.

Results: An 816 bp gene encoding an endolysin enzyme, was approved in the *B. subtilis* DDBCC 46, with specific primers of *Bacillus* phage SPP1. The purified-endolysin indicated antibacterial activity against *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Proteus* (sp), and *Escherichia coli*. SDS-PAGE profiling followed by silica gel purification, led to introduce Lys46₃₀ as a therapeutic product and food preservative.

Conclusion: lys46₃₀ showed antibacterial effects on the common Gram-negative pathogens in clinics and food industries; *E. coli*, *P. aeruginosa* and *Salmonella* (sp).

Keywords: Antibacterial activity; *Bacillus* phage; Endolysin; *Siphoviridea*

INTRODUCTION

Recently, the enzybiotics are concerned because of their specific antibacterial function and low level of drug-resistant in the clinical and agricultural micro-

biome (1). Enzybiotics have been mainly classified into bacteriocins and phage lysine (2). Endolysins, with cell wall lytic features, are known from phages. These enzymes eliminated pathogens through a non-toxic effect (3). Phage lytic proteins break down the cell wall of infected bacteria to release the phages. Additionally, endolysins are usually specific for a particular bacterium species and do not generally lyse commensal bacteria, as much as they exhibit more specific interaction than antibody and antigen. On the other hand, in a diverse view, the specificity of the cell-binding domain of endolysins is used for the identification; of the pathogen and or non-pathogen

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bacteria. This is a distinct advantage for endolysin in the accurate diagnosis in the clinical and food area (2). Moreover, structural and functional diversity of endolysins provides a powerful tool for protein engineering as well as designing new endolysins with desired properties. Today, endolysins have been developing with defined roles in the control of clinical antibiotic-resistant pathogens (4).

Phage endolysins, unlike bacterial autolysins, lack the signal peptide and require a phage protein; holin for their action. Holins create a hole in the cell membrane to release endolysins from the membrane, thus endolysins bind to the peptidoglycans and hydrolyze them. They precisely regulate the time for cell lysis and virion release, which is known as a biological clock (5).

In vivo and *in vitro* investigations show that lysins kill Gram-positive bacteria while the outer membrane in Gram-negatives works as an inhibitor, meaning most probably there are no endolysins targeting Gram-negatives. Nowadays, endolysins interact with the outer membrane of Gram-negative are introducing one by one. The documents show that these enzymes have only one catalytic domain in their structure. An alpha helix module in the endolysins with amphipathic properties allows the enzyme to pass through the outer membrane where the catalytic domain targets the peptidoglycans leading to cell wall penetrating. The proposed mode of action is that the acidic amino-acids bind to the negatively charged outer membrane components, such as lipopolysaccharide (6).

Various endolysins found that inhibit pathogens including *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, *S. aureus*, *Mycobacterium* spp., *P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae* (7). Gram-positive endolysins show a mass size of 25-40 kDa. On the contrary, the mass of endolysins targeting Gram-negative bacteria are in a range of 15-20 kDa with a single catalytic domain (8).

Based on the diversity of endolysins, a common mechanism of function has not been understood. Less than 1,000 types of these proteins are currently known. In spite of the diversity in the genome of phages in Earth's ecosystems, there is no any data bank for endolysins (9). Because of the importance and application of endolysins in industry and medicine, the antibacterial effects of the endolysin against Gram-positive and Gram-negative bacteria was studied in this project.

MATERIALS AND METHODS

Screening for lysogenic bacteria. Soils were sampled from Dehsufian's village, Semnan, and Ahmadabad village, Shahroud, under aseptic conditions. The samples were diluted in normal saline in a ratio of 1:10, and incubated at 37°C at 120 rpm for 1 h. 100 µl of each sample was inoculated on nutrient agar supplemented with the cell-wall of the indicator bacteria; *Streptococcus pyogenes* (ATCC19615), *Bacillus subtilis* (ATCC 12711), *Bacillus* sp., *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *S. typhimurium* DDBCC1001, *Proteus* sp. *K. pneumoniae* (ATCC 13883), and a clinical *K. pneumoniae* as previously described (10). To discover the possible role of endolysins and phages, the strains with clear zones were further examined.

Evaluation of antibacterial activity. The candidate strains were cultured in BHI (Brain Heart infusion broth) for 24 h at 37°C, 120 rpm. 100 µl of the cell-free supernatant; CFS (0.2 µm Millipore), was added in 1 mm well on LB medium supplemented with heat-killed indicator bacteria. The strains with the clear zone were selected for surveying their antibacterial activities. Same experiments were done for liquid bactericidal assay adding 3 ml of the dead indicators to 1 ml of CFS (11). Activity unit of the endolysin (AU) was calculated using this formula ($p < 0.05$) (12):

$$\text{Endolysin activity (mm}^2\text{/ml)} = Lz - Ls/V$$

$$Lz = \text{Clear zone area (mm)}^2$$

$$Ls = \text{Well area (mm)}^2$$

$$V = \text{Volume of sample (ml)}$$

Evaluation of enzyme activity. The CFS was partially-purified using ammonium sulfate saturation at 60%, dialyzed and stored at 4°C for enzyme assay. Using the Bradford method, the concentration of total protein was calculated (13). An autoclaved-culture of indicator bacteria with $OD_{600} = 2$ was subjected to a 1:10 serial dilution of the partially-purified endolysin for 30 min at 25°C. A dilution of the purified-supernatant that reduced turbidity of the heat-killed indicators to 50% was introduced as the activity of the endolysin ($p < 0.05$) (14).

Extraction of phage and FTIR spectra. Mitomycin (0.1, 0.2, 0.5 and 1 µg/ml) was used for 5 h at 37°C

to induce cell death in the candidate strains (15). The viable cells and cell debris were removed by 0.45 µm Millipore. The proteins (mainly phage capsids) were incubated for 16 h, at 4°C in 20% (v/v) NaCl and 10% (w/v) Polyethylene glycol (PEG) 8000, precipitated for 30 min at 4°C, 9000 rpm (16), and finally studied with soft agar assay (2, 17). The plaques were diluted in Saline-Magnesium buffer (SM). The lytic effects of the isolated phages were examined in the spot test (18). 100 µl of the indicator bacteria ($OD_{600}=0.4$) was spread on LB plates. 3 µl of each phage was placed on the plates followed by 16 h incubating at 37°C. Bacteriophage solution measured by FT-IR4800s, Shimadzu.

Identification of the endolysin producer. 16S rRNA amplicons were obtained by universal primers, 27F (5'-AGACTTT GGA TCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGT TACGACTT-3). The PCR reaction initiated with 96°C for 3 min, 35 cycles of 96°C for 40 s, 58°C for 40 s, and ended with a final extension of 72°C for 7 min. The PCR products were analyzed by Bioneer company services (19). The Basic Local Alignment Search Tool (BLAST) provided the data for the molecular identification of the isolates.

The functional analysis of the endolysin. Zymogram analysis is a procedure to test function of endolysin (10). Here, the endolysins were purified on 12% SDS PAGE as well as on a 12% SDS PAGE supplemented with 0.4% (w/v) of the autoclaved- indicators to detect the possible function of the enzyme. The loaded-samples were separated by zymogram gels and incubated in 50 Mm sodium phosphate buffer (pH 6.0) with 0.1% (v/v) Triton X-100 for 16 h at 37°C under gentle rocking. The SDS gel was methylene blue stained.

Purification of Lys46 using gel column. The partially-purified endolysin was purified by gel filtration chromatography (Sephadex G75 Superfine; Sigma) with phosphate washing buffer (20, 21).

Molecular identification lysogenic phages. Lysogenic phages from the *Syphoviridae* family were identified in the genome of *Bacillus subtilis*. The endolysins from *Syphoviridae* of *B. subtilis* were categorized in five types based on data from Virus-Host DB, NCBI, and EXPASY databases. The nucleotide sequence of the peptidoglycan hydrolase from each

phage was analyzed. Then primers were designed for fishing every endolysin. The PCR's were performed as 94°C for 3 min, 35 cycles of 94°C for 40 s, 50°C for 45 s), and ended with a final extension of 72°C for 5 min, the specific primer pairs for catalytic domains were used in PCR reaction (Table 1).

Statistical analysis. The data were tested for normality by SPSS (Statistical Package for the Social Sciences) version 22 and evaluated for the traits using analysis of variance and Duncan's multiple range test ($p < 0.05$).

RESULTS

Isolation of soil bacteria. The soil samples from Dehsufian's village, and Ahmadabad village were plated to isolate the culturable bacteria. The colonies with a halo zone around were characterized on their morphological features. The screening led to 82 bacteria those digested the cell wall of *S. pyogenes*, *S. faecalis*, and *B. subtilis*. Table 1 exhibits the number of isolates able to kill every indicator. In the liquid bactericidal assay, the isolates showed lytic activity against *E. coli*, *P. aeruginosa*, and *B. subtilis*. Based on the spectrum of effect, nine lysogenic strains were selected for further study.

The antibacterial activity of the supernatant. The ability of the isolates for cell wall lysis is demonstrated in Fig. 1. DDBCC46 lysed the cell wall of *Salmonella* (sp), *K. pneumoniae*, and *B. subtilis*. A stronger impact of the CFS from DDBCC46 was detected on *P. aeruginosa*, *E. coli*, and *Proteus* (sp).

Production of the enzyme and evaluation of its activity. The protein residue from ammonium sulfate-purification was dissolved in 10 mM Tris buffer. Approximately 70% in the activity of the lysin-purified from DDBCC46 was detected in comparison to the CFS. The partially-purified enzyme from DDBCC46, namely Lys46, lysed all of the Gram-negative plus one of the Gram-positive indicators. The activity for Lys46 was detected in a range of 22 to 30 mm corresponding to the cell wall of *P. aeruginosa* <*K. pneumoniae* <*E. coli* <*Salmonella* (sp) <*Proteus* (sp). The non-purified CFS digested the dead indicators as; *Proteus* (sp) 35 mm > *E. coli*, *Salmonella* (sp) and *K. pneumoniae* 18 mm.

Table 1. The oligonucleotide of peptidoglycan hydrolase from Syphoviridae

Oligonucleotides	Sequences	TM°C
FrSpo1	5'- ATGACTTATTCTTTAAACGATCTTTTAAGCAAAGTG -3'	57.6
RpSpo1	5'- TTAAAACATCATATTCCAAGTTTTTAAACCAACAAT-3'	
FRPPM	5'-TAAGGATCCCATGCATTTACCCTAGTTGTACAGGGATACA-3'	46.8
RPPPM	5'-TATCTCGAGCTACCTCCTTTTATATCTCGGTGCTGCCATC-3'	
FRSPP1	5'-GAAGGATCCTATGAGTAAATTAGTTTGGTTGGATGCAGG-3'	52
RPSPP1	5'-ATTCTCGAGTTACTTCTCCTTTACCGTTACATATTTACTTGCC-3'	
FR1051	5'-AAAGGATCCAATGGTGAAAATCACAAAGGACTTTATTCC-3'	64
RP1051	5'-CGTCTCGAGTTATTTTACTGTTGCCCTGTGGACTT-3'	
FRSPB2	5'-TTTGGATCCTATGTCAGTTTCACTAATAGCTACATTCCAGTC-3'	61.5
RPSPB2	5'-TAACTCGAGTTAGATTGTTGATCCAAACTGTCCCGAGATA-3'	

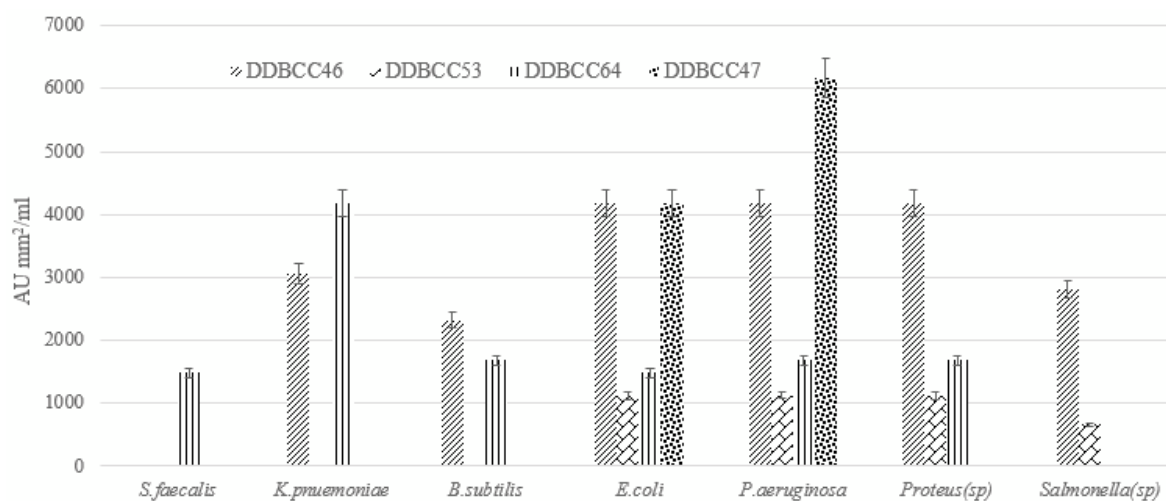


Fig. 1. The lytic activity of the strains on the cell wall of the indicators

SDS-PAGE and zymogram analyses. The proteins isolated from DDBCC46 plus the results from zymogram tests confirmed the enzymatic activity of the protein with a mass of 22 kDa (Fig. 2).

Evaluation of enzyme activity. The enzyme activity of CFS and partially-purified CFS, Lys46, was compared. The activity was calculated as 1 Unit/ml in CFS and 3 Unit/ml for Lys46. The enzyme activity assay showed a reduction in the turbidity of *Salmonella (sp)*, *K. pneumoniae* and *P. aeruginosa* in 30 min. No significant alteration was observed in the OD₆₀₀ *E. coli* and *Proteus (sp)* within the test.

Biochemical and molecular identification of the candidates. The phage-hosting bacterium was characterized as a Gram-positive, catalase, and oxidase-positive, rod-shaped and spore-forming bac-

terium. Analyzing of sequences of 16S rRNA gene showed 99% similarity to *B. subtilis* (accession number MW193081).

Gel purification of lys46₃₀. Two proteins in size of 11 kDa and 30 kDa were detected on the SDS-PAGE. Lys46 was purified by gel chromatography to separate the proteins followed by approving the fraction on SDS-PAGE (Fig. 3). The test for antibacterial activity was done with the eluted-11 kDa and-30 kDa proteins. The results revealed a lytic impact of 30 kDa protein (lys46₃₀) on *K. pneumoniae* and *P. aeruginosa* (40 mm), *E. coli* (35 mm), and *Salmonella (sp)* (22 mm). After 65 hours, the clear around was raised to 36 mm for *Salmonella (SP)* and 39 mm for *E. coli*. No significant changes were observed in the halo zone for the other indicators after 65 hours. The enzymatic activity of the lys46₃₀ was compared with

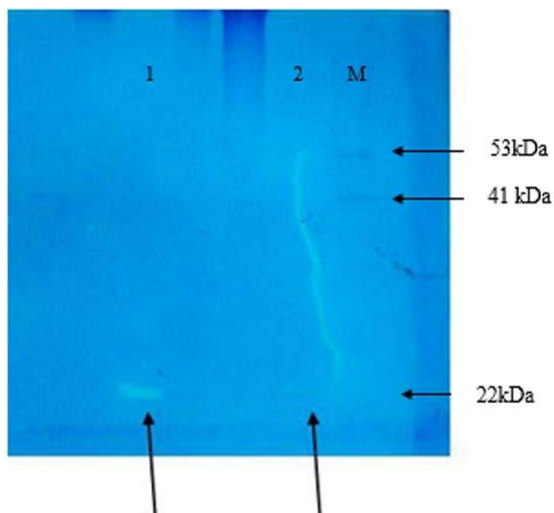


Fig. 2. The results from zymogram tests were confirmed the enzymatic activity of the protein with a mass of 22 kDa. line1. partially-purified CFS Lys46; line2. CFS Lys46; M. Size Marker proteins 10-170 kDa. The arrows are showing the digestion of cell wall.

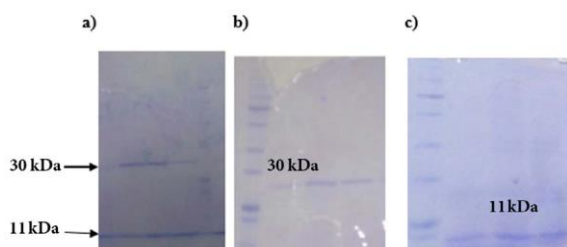


Fig. 3. Lys46 was purified by gel chromatography to separate two proteins in size of 11 kDa and 30 kDa those detected on the SDS-PAGE

purified-lys46 (Fig. 4). The bactericide activity of 0.16 mg/ml lys46₃₀ was 10 AU/ml against the clinical isolate of *K. pneumoniae*.

Phage extraction and FTIR spectrum of candidate phage. The phage DDPCC46 (Dasht Desert Bacteriophage Culture Collection) was extracted with 0.1 µg/ml mitomycin C. The formed-plaque in soft agar confirmed the extraction of phages. The lytic effect of DDPCC46 was shown by plaque formation on the indicator strains. The plaques formed against *Salmonella* (sp), *K. pneumoniae*, *Proteus* (sp) where, no plaque was observed on *E. coli* and *P. aeruginosa*. Three picks were detected in the results of FTIR at 568.96, 1635.52 and 3272.98 cm⁻¹ (Fig. 5).

Molecular identification of candidate phage.

Five specific lysogenic phage groups of the *Syphoviridae* family were identified in the genome of *B. subtilis* strains by Virus-Host DB, NCBI and EXPASY databases. The endolysin gene in the phi105 phage is 903 bp, in phage SPP1; 816 bp, in SPβ; 1104 bp and in PM1 258 bp.

PCR was performed using the pairs of specific primers for catalytic domains of *B. subtilis* endolysins. The expected 800 bp amplicon was detected by specific primer SPP1 (Fig. 6).

DISCUSSION

Phage endolysin as an antibacterial agent can be substituted to the antibiotic. Bacteria–phage coevolution limits resistance to endolysins in bacteria. Furthermore, conserved binding ability and activity, rare chances of mutation in binding sites, and binding to target extracellular peptidoglycan are the main advantages for endolysin encoded in the genome of the phages (22).

Despite ongoing endolysin clinical trials, the development of endolysins into over –the –counter medication is still in its infancy (22). Therefore, discovering new endolysin (s) with new, and distinct features was aimed in this study. Formerly we isolated with the same method DDPCC1 (Dasht Desert Bacteriophage Culture Collection) from a strain of *B. subtilis*, DDBCC10 (10). The soft agar supplemented with autoclaved-indicator bacteria *S. pyogenes* was used for isolating lysogenic bacteria by Rolflood et al. (23). Mishra, A. K (2014) documented the lytic effects of six phages and their lysins against 124 isolates of *S. aureus*. They showed that the host range of endolysins was increased in comparison to their related phages (24).

Gram-negative phage endolysins hydrolyze bacterial outer membrane protein. Recently endolysin from a Gram-negative bacteria; lys68, was described (25). Park et al. studied recombinant AP50-31 and LysB4, which displayed broad bacteriolytic activity against all the *Bacillus* (sp) (14). Oliveria et al. 2016, produced a recombinant endolysin (Abgp46) from *Acinetobacter* phage vb_AbaP_CEB1, which inhibited several multidrug-resistant *A. baumannii*. They use the endolysin in companion with a membrane-permeabilizing component, which resulted in the antibacterial activity against Gram-negative

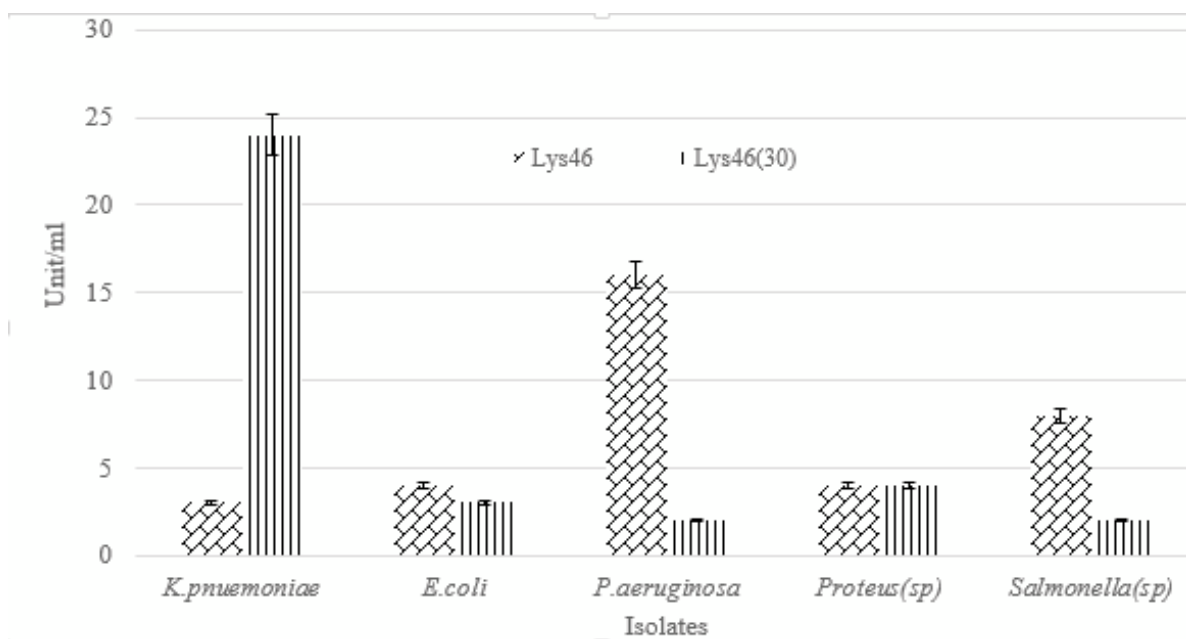


Fig. 4. Comparison of the enzyme activity, the purified lys46, and the lys46 on the indicator bacteria.

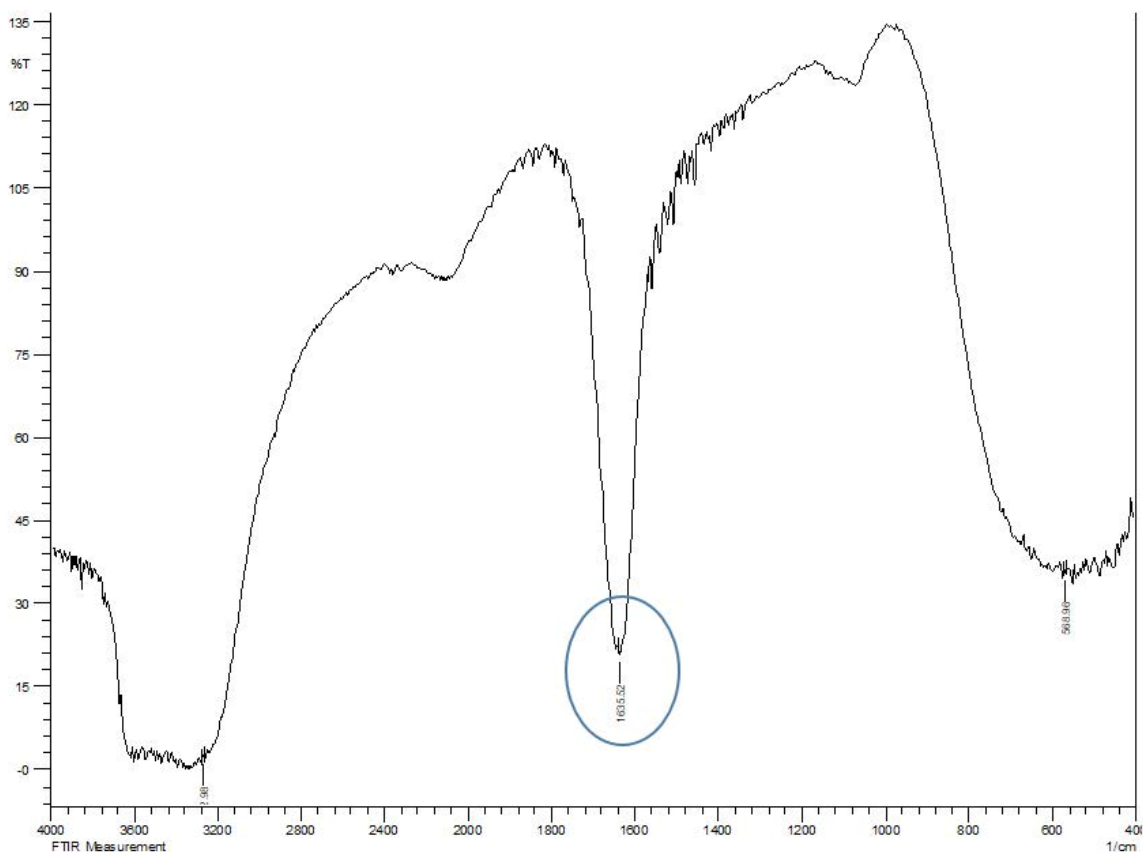


Fig. 5. FTIR spectrum of phage ssp1. The prominent peak indicating the syphoviridae is blue circled.

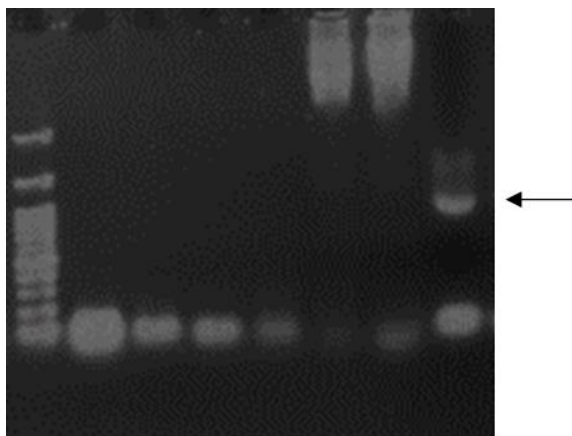


Fig. 6. PCR was performed using the pairs of specific primers for catalytic domains of *B. subtilis* endolysins. The expected 800 bp amplicon was detected by specific primer SPP1

bacteria such as *P. aeruginosa* and *Salmonella typhimurium* (26). It is mentioned that the bacteria in the log phase are more susceptible to the endolysins because of the post modifications in LPS in the stationary phase may reduce the antibacterial effects of the endolysins (27).

Salazar et al. 2017 used the silica gel column chromatography for purification of two bacteriocin-like molecules, displayed different physical properties (28). Yadav et al. (2017) isolated and purified the bioactive component from a halophilic *B. subtilis* subsp. *Spizizenii* by gel chromatography column. The component showed a higher antimicrobial activity to the impure- extract (29).

To approve the phage isolation, the host bacteria were subjected to the purified phage as described elsewhere (30). Siddiqui et al. introduced the rapid method for screening a wide variety of *S. aureus* in phage-typing system using 0.01 µg/ml to 1 µg/ml mitomycin C for the isolation of lysogenic bacteriophage (15). Doria et al. (2013) found the UV method more effective than mitomycin C for phage extracting from lysogenic *Oenococcus oeni*. 60% of the strains were entered to lytic phage with subjecting to UV, whereas only 32% of the strains were induced by mitomycin C. Although the mitomycin method is more expensive than UV radiation, most studies have suggested that mitomycin is preferred for induction of pro-phage (31).

The study of bacteriophage T5 in *Syphoviridae* and receptor binding pb5 showed the pick 1635 cm⁻¹, representative of this family (32, 33). Physicochemical

study of the proteins featured the pick 1635 for the Amid I which is characterized for tail of *Syphoviridae* family (34).

A reasonable numbers of studies have performed to identify and confirm lysogenesis by bioinformatic, molecular and electron microscopy (35). Bioinformatic analysis requires complete sequencing of the phage genome. Five specific lysogenic phage groups of the *Syphoviridae* family were identified in the genome of *B. subtilis* strains by Virus-Host DB, NCBI and EXPASY databases.

Electron microscopy analyzing also has high preparation cost; therefore, molecular techniques such as polymerase chain reaction from conserved gene can be a more cost-effective method for early detection and confirmation of the presence of a phage-specific. PCR was performed using the pairs of specific primers for catalytic domains of *B. subtilis* endolysins. The expected 800 bp amplicon was detected by specific primer SPP1. Lys46 was purified by gel chromatography to separate the proteins in the size of 11 kDa and 30 kDa. SDS-PAGE profiling followed by a silica gel purification led to introduce lys46₃₀ as a therapeutic product and food preservative. The antibacterial activity tests revealed the lytic impact of 30 kDa protein (lys46₃₀) against *K. pneumoniae*, *S. typhimurium*, *Proteus*, (sp) and *E. coli*.

The outer membrane in Gram negative bacteria is a strong protective barrier. The endolysins those target this barrier are useful molecular tools to omit the pathogens from food preservative in food spoilage and nosocomial infectious diseases (25).

CONCLUSION

Here, we introduce lys46₃₀ as an antibacterial agent with antibacterial effects on the much-known Gram-negative pathogens in clinic and food industries; *E. coli*, *P. aeruginosa*, and *Salmonella* (sp). The endolysin showed an activity of 3 U/ml on *Salmonella* (sp), *K. pneumoniae*, and *P. aeruginosa*. The enzyme is 30 kDa in mass. In a best-case scenario, Lys46₃₀ could be a candidate for biotechnological application.

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