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Study the effect of cloned pET-32a(+) plasmid by Lysostaphin gene against *Staphylococcus aureus*

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Abstract

Lysostaphin is a protein zinc metalloproteinase, extracted from *Staphylococcus simulans*, which disrupting peptide layer of S. aureus. In this study, Lysostaphin gene was detected in the *S. simulans* isolates. The molecular weight of the Lysostaphin gene is 750 bp. We were used the pET-32a(+) plasmid to cloning lysostaphin gene which transformed to competent rubidium chloride E. coli DH5α for producing the lysostaphin protein. The lysostaphin protein from this gene which isolated from *S. simulans*, then used the expression of used to killed S. aureus, which has the thick layer of wall that is the very difficult bacteria response to treatment. The result was reported succeeded pET-32a (+) plasmid to expressed lysostaphin gene and gave lysostaphin protein with high quality and quantity. As well as the result was appeared the high accuracy of his tag method in protein extraction and purification, and the quality and quantity more than other studies.

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Introduction

Bacteriocins are bacterial proteinaceous compounds, which have the activity of bactericidal against other bacterial spp., the bacteriocins have been proteolytic degradation (1). This bacterial proteinaceous compounds produced by gram positive bacteria. The bacterial proteinaceous can be divided into four classes, classes I and II bacteriocins are the most studied and have better clarified mode of action, since they possess prospect industrial and clinical applications (2). Treatment of staphylococcal infections has become difficult increasingly due to resistant strains colonization to some of the antibiotics (3). Staphylococci can be eliminated by lysostaphin from skin and nares of individuals increased risk infections *S. aureus* (4). It is binds to *S. aureus* and pentaglycine cross bridges cleaves within peptidoglycan, the cell wall envelope removing and osmotic rupture precipitating of staphylococci (5). Lysostaphin primarily active against coagulase positive *S. aureus* but some activity residual against coagulase negative *S. aureus*, it is requiring increased enzyme concentration and longer times incubation to be killed (6). *Lysostaphin* gene from the *S. simulans* isolates contain 1.5 kbp fragment of DNA (7). This gene was localized with in a *Hpa* II / *Hin*d III fragment. The gene coding for preproenzyme which consist of three regions distinct: signal peptide at the end of amino-terminal, a series of tandem repeats and the active *lysostaphin* gene (7,8). Promoter of the gene have (--35) and (--10) at nucleotides regions (89-95) and (110-119), respectively (7).

There are a number of the reports of the lysostaphin expression promoter dopeptidase. In many study, the r-lysostaphin produced from different pET vectors (9). One of the selective binding of the protein expressed is the His tags facilitate, it is affinity- nickel column. His tag sequences may be removed by protease optionally, another purification step requiring the tags which are no effect on the protein structure and function (10). Polyhistidines add by two ways. The first way by add repetitive histidine codons (CAT or CAC) to the PCR primers, as well as add

start or stop codon. The second technique by add a encoding His-tag sequences protein to the vector (11).

The aim of this study to expressed *lysostaphin* gene by pET-32a (+) plasmid and using the lysostaphin protein as bactericidal of *S. aureus*.

Materials and methods

Isolated genomic DNA from *S. simulans* according to Sambrook and Russel (12) with modification. This DNA used as a template for the amplification. The annealing temperature used in the PCR program was 53°C. The primer used in this study were Lys F1: (5' gct gca aca cat gaa cat tca 3'). Lys R1: (5' ctt tat agt tcc cca aag aac 3'). The total volume of PCR reaction was 50µl (5µl 10X buffer, 8µl 1.25 mM of dNTPs, 2µl forward primer, 2µl reverse primer, 0.5µl *Pfx*, 5µl enhancer, 0.5µl DNA template, 1µl $MgSO_4$, and 26µl ddH_2O. The program of PCR technique consist of several stages: the first initial denaturation PCR program, 6min at 95°C. followed by 35 cycles 95°C for 45sec, 58°C for 45sec, then 72°C for 45sec. The last stage as 72°C for 6min, and then down to 4°C for 30min.

Then used primers have two restricted enzymes by add two restricted sits (*Kpn* I and *Eco*R I), one of them to the forward primer (*Kpn* I), the restrict sit for this enzyme (ggt acc) as well as start codon (atg), and the second one to the reverse primer (*Eco*R I), the restrict sit for this enzyme (gaa ttc) as well as stop codon (tca), and finally we are add codon of six histidine to the reverse primer in order to extract and purify the lysostaphin protein from cloning bacteria which have His tag sequences, the codon of one histidine (atg) add to revers primer, know, the last primers with two restricted sits (*Kpn* I and *Eco*R I) with start and stop codons and codon of six histidine are: Lys F2: 5' GGT ACC ATG GCT GCA ACA CAT GAA CAT TCA 3'. Lys R2: 5'GAA TTC TCA ATG ATG ATG ATG ATG ATG CTT TAT AGT TCC CCA AAG AAC 3'.

Table 1 show the run of PET-32a(+) plasmid in the gel after cut by two restrict enzymes (*Kpn* I and *Eco*R I), then isolated and purified by gel extraction kit from the gel.

The PCR products from first PCR by Lys F1 and Lys R1 used as a template for sub PCR by using Lys F2 and Lys R2. The PCR product was run to electrophoresis, then the DNA from the gel was isolated and purified by the kit of DNA extraction. The extracted gene was cut by two restricted enzymes (*Kpn* I and *Eco*R I) as represented in the table 2, then the cut away gene cleaned from the few bp posts cut by these enzymes.

Clones were done to study the effect of PET-32a(+) plasmid, the cloning done between plasmid and PET-32a(+) cuts by *Eco*R I and *Kpn* I enzymes. Then ligated gene of *lysostaphin* digested by enzymes with PET-32a(+) digest by the same enzyme as described above, then transformed to rubidium chloride *E. coli* DH5α.

Colonies were replica-plated onto fresh (LB) Luria - Bertani solid medium, which containing 100µg/ml ampicillin, then incubated overnight at 37°C, then transformants bacteria confirmed by PCR, to detect successful cloning by colony PCR. This quick protocol which designed to screening the plasmids insert to the *E. coli* DH5α and this plasmid has the insert. which is called (constructive plasmid).

Table 1: Digestion of pET-32a(+) by *Kpn* I and *Eco*R I enzymes

No. of Samples	No. of Infection
pET-32A(+)	20µl
10 X buffer	5µl
BSA	5µL
ddH_2O	16µl
Kpn I	2µl
*Eco*R I	2µl
Total reaction	50µl

Table 2: Digestion of gene of *lysostaphin* by *Eco*R I and *Kpn* I enzymes

Substance	Quantity
Lysostaphin gene	20µl
10X buffer	5µl
BSA	5µl
ddH_2O	16µl
Kpn I	2µl
*Eco*R I	2µl
Total reaction	50µl

Determine the activity of cloning *E. coli* by *lysostaphin* gene to inhibit *S. aureus* by performed a standard disk diffusion assay. Prepare *S. aureus* and *E. coli* by culturing in the broth of nutrient to phase of exponential (OD_{620} 0.1). Then inoculated *S. aureus* were in the Muller - Hinton Agar (MHA), as well as prepared and placed in discs from *E. coli* in the plates centre. Overnight, under aerobic condition, incubated at 37°C, determined the inhibition zones of culture.

Post determined the activity against *S. aureus* by cloning bacteria, we are extract and purify by His-tag method lysostaphin protein from cloning bacteria. This processing done by used the FF crude columns of HisTrap (Sweden), then determined the concentration of protein by Bio-Rad Protein Assay. By SDS-PAGE analysis, limited the purity of protein (13).

Results

The result of this study in the figure 1 showed the result of electrophoresis of the total genomic DNA of *S. simulans*. In addition, the figure 2 showed the gel electrophoresis of pET-32a(+) plasmid and *lysostaphin* gene, this figure showed digested plasmid by *Kpn* I and *Eco*R I enzymes and digested gene of *lysostaphin* by same enzymes, which appeared the band of the gene about 750 bp. While figure 3 showing the product of *lysostaphin* gene from PCR cloned by pET-32a(+) plasmid by colony PCR, this figure showing band about 750 bp.

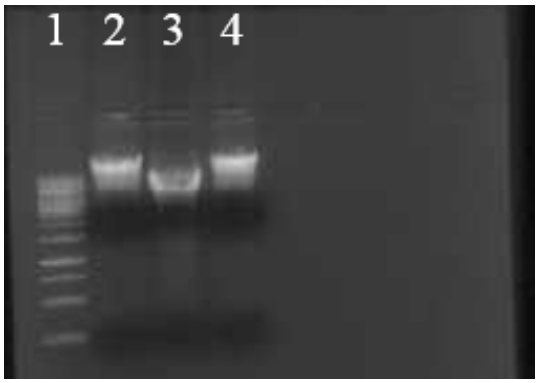

Figure 1: Electrophoresis of total genomic DNA of *S. simulans*, lane 1 is the ladder, lanes 2,3,4 are the total genomic DNA.

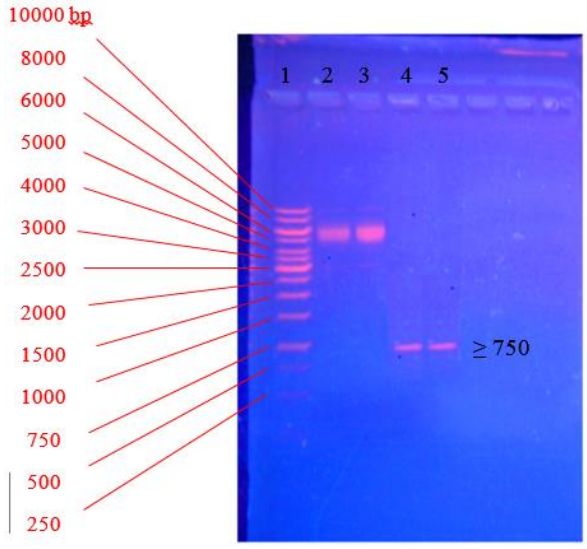

Figure 2: Gel electrophoresis of pET-32a(+) plasmid and *lysostaphin* gene, lane 1 is the ladder, lanes 2,3 digested plasmid by *Kpn* I and *Eco*R I enzymes and lanes 4,5 gene of *lysostaphin* digested by *Kpn* I and *Eco*R I enzymes.

The antibacterial activity of transformed *E. coli* have pET-32a(+) plasmid with gene of *lysostaphin* insert against *S. aureus* (Figure 4A), while figure 4B showed the effect of *E. coli* non-transformed by gene of *lysostaphin* against *S. aureus*. Table 3 showed the protein of lysostaphin concentration from cloned bacteria with pET-32a(+) plasmid after extraction by column of His-tag, also showing in this table first lysostaphin protein concentration 1.113 more than the third concentration of BSA 0.893 and less than second concentration of it 1.773. SDS-PAGE analysis of proteins showed in figure 5 with standard techniques, this figure showed only one protein band, this figure recoded lysostaphin molecular weight about 27,000 Dalton.

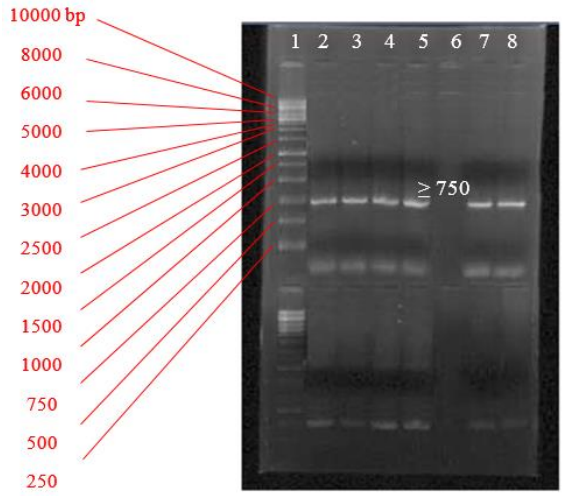

Figure 3: PCR product of *lysostaphin* gene from cloned bacteria by colony PCR, lane 1 is the ladder, lanes 2,3,4,5 are gene of *lysostaphin* from pET-32a(+) plasmid, lanes 7,8 are *lysostaphin* gene amplified by first detection about 750 bp, lane 6 is negative control.

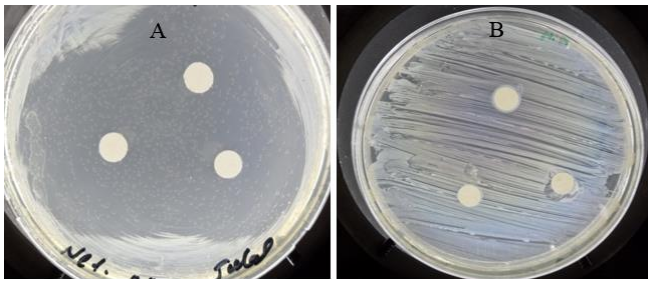

Figure 4: A. Effect of antibacterial activity of transformed *E. coli* by pET-32a(+) plasmid have *lysostaphin* gene against *S. aureus*, B. Effect of activity of antibacterial against *S. aureus* by non-transformed *E. coli*.

Table 3: Lysostaphin protein concentration at the absorbance 595 nm

Bio-Rad dye	BSA	Lysostaphin from pET-32a(+)
0.329	2.989	1.113
0.331	1.773	0.894
0.323	0.893	0.689
0.318	0.589	0.402
0.332	0.371	0.345

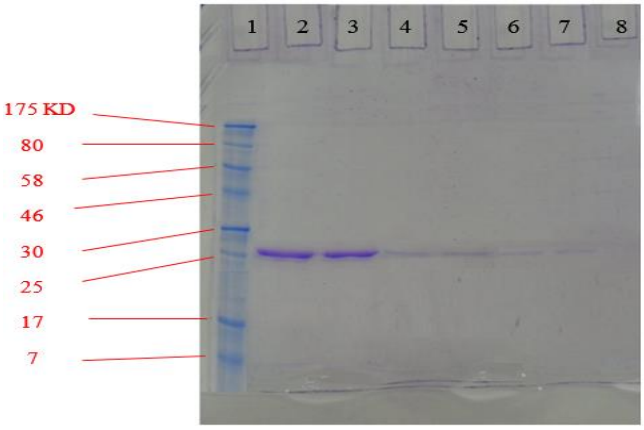

Figure 5: Lysostaphin protein in SDS-PAGE analysis, lane 1 is the marker, lanes 2,3 are lysostaphin protein extracted from cloning pET-32a(+) plasmid with first concentration, lines 4,5 second concentration, lines 6,7 the third concentration, and line 8 is negative control.

Discussion

Mastitis is one of the most important economic disease of dairy cattle, the effects of this disease come from resistance to treatment and costs losses (14). It is regarded as the first common disease, which causes losses of antibiotics by treatment in dairy cattle and responsible for antibiotics increasing resistance (15).

Mastitis treatment is very restricted and as a cause disease propagation, *S. aureus* is regarded a main cause of mastitis in the ruminants (16,17). One of the studies for detection of capsulated *S. aureus* strains from bovine and buffaloes mastitis is Shamoon (18). *S. aureus* appears as a one of the twelve most health-threatening pathogens in the resistant bacterial list compiled by WHO which promotes new antibiotics development (19,20). Staphylococcal species and especially *Staphylococcus aureus* have developed resistance multidrug lead to serious risks of health then treatments complex, by using the novel and effective strategies development to kill these bacteria (21). To the present study gave a new strategy to treat this bacterial problem of *S. aureus*.

The result of the current study showed the size of gene of *lysostaphin* was 750 bp which accepted by (7,22,23). The antibacterial activity of transformed *E. coli* have pET-32a(+), which showed the effect against *S. aureus* by *E. coli* non-transformed, this result agreement with the previously studies (11,23), which recorded of effectiveness against *S. aureus* by transformed *E. coli* have *lysostaphin* active gene 750 bp.

SDS-PAGE analysis of proteins recoded the molecular weight of lysostaphin protein about 27,000 Dalton, this result matching by Mustafa; Trayer and Buckley (11,24).

The result of the present study showed that limited increasing in concentration of lysostaphin protein when used pET-32a(+) plasmid compared with other study (25), which recorded less then concentration of lysostaphin protein with other plasmid, this study recorded lysostaphin concentration from pTrc99a plasmid 1.012mg/ml which less than our study 1.113 mg/ml, as well as 0.622 mg/ml concentration by used pBAD30 plasmid.

In many study, the r-lysostaphin were produced from different pET vectors, pET28a is one of them which yield 22 mg, the other one is pET 23b which yield 20 mg, and pET15b, yield 11 mg of protein purified from one later of *E. coli* (9,26,27). Our study gave successful of expressed lysostaphin gene by pET32a vector in *E. coli*, this protein production are mature r-lysostaphin because the activity against *S. aureus* and it is a large quantity of r-lysostaphin, this match and alignment with our data (9). It is found many different systems for expression have been developed, but the proteins recombinant production in E. coli remains the most one used (28).

The activity of lysostaphin protein against *S. aureus* is because capability to lysis peptidoglycan of cell walls bacteria (24,29). The site of action of lysostaphin protein is the cross-bridge pentaglycine of the peptidoglycan (30). Staphylococcal species in general and *S. aureus* is one of them composed of five glycine (Gly) residues (31). One of the antistaphylococcal agents is the lysostaphin, which is unique among in bacterial kills, it is have ability to killing a large number of organisms in the *Staphylococcus* genus because of the glycine-glycine bonds existence (32,33). Lysostaphin enzyme consists of two domains, those are: N-catalytic terminal domain and a cell wall binding domain (34).

Lysostaphin protein is a bacteriolytic metalloprotease secreted from *S. simulans* biovar *staphylolyticus*. It is function is degrading the staphylococci cell wall of multiple species by pentaglycine crosslinks hydrolysis within peptidoglycan (35).

Conclusion

We are conclude from succeeded pET-32a(+) plasmid to expressed *lysostaphin* gene and expressed protein lysostaphin in high quality and quantity. As well as conclude from our result, high accuracy of His tag technique in protein extraction and purification.

Aknowledgement

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Conflict of interest

Authors declares that they have no conflict of interest regarding publishing this review article.

References

1. Heng, NC, Wescombe PA, Burton JP, Jack RW, Tagg JR. In Riley MA, Chavan MA, editors. Bacteriocins: Ecology and evolution. New York: Springer; 2007. 45-92 p. Doi: 10.1007/s12602-008-9002-7
2. Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nat Rev Mic. 2005;3:777-788. Doi: 10.1038/nrmicro1273
3. Bradley JS. Newer antistaphylococcal agents. Cur Opi Ped. 2005;17(1):71-77. doi: 10.1097/01.mop.0000151555.17799.41
4. Kokai-Kun JF, Walsh SM, Chanturiya T, Mond JJ. Lysostaphin cream eradicates *Staphylococcus aureus* nasal colonization in a cotton rat model. Ant Age Che. 2003;47:1589-1597. Doi: 10.1128/aac.47.5.1589-1597.2003
5. Sloan GL, Smith EC, Lancaster JH. Lysostaphin endopeptidase-catalysed transpeptidation reactions of the amino-transfer type. J Bio. 1977;167:293-296. Doi: 10.1128/aac.47.5.1589-1597.2003
6. Zygmunt WA, Harrison EF, Browder HP, Tavormina PA. Comparative inhibition of methicillin-resistant strains of *Staphylococcus aureus* by lysostaphin and other antibiotics. App Mic. 1968;16(8):1174-1178. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC547615/pdf/applmicro00244-0072.pdf
7. Recsei PA, Gruss AD, Novick RP. Cloning, sequence, and expression of the *lysostaphin* gene from *Staphylococcus simulans*. Pro Nat Aca Sci. 1987;84:1127-1131. Doi: 10.1073/pnas.84.5.1127
8. Heinrich P, Rosenstein R, Bohmer M, Sonner P, Gotz F. The molecular organization of the lysostaphin gene and its sequences repeated in tandem. Mol Gen Gen. 1987; 209:563-569. Doi: 10.1007/bf00331163
9. Leila F, Ehsanollah GR, Neda M, Hamid A. Cloning, expression, and purification of recombinant lysostaphin from *Staphylococcus simulans*. Jundishapur J Mic. 2014;7(5):1-5. Doi: 10.5812/jjm.10009
10. Chant A, Kraemer-Pecore CM, Watkin R, Kneale GG. Attachment of a histidine tag to the minimal zinc finger protein of the *Aspergillus nidulans* gene regulatory protein AreA causes a conformational change at the DNA-binding site. Pro Exp Pur. 2005;39(2):152-159. Doi: 10.1016/j.pep.2004.10.017
11. Mustafa JY. Sequences and expression of the *lysostaphin* gene from *Staphylococcus simulans* isolated from bovine mastitis and its bactericidal effect on *Staphylococcus aureus* [PhD dissertation]. Bara: University of Basra, College of Science, Biology Department; 2011. 30-110 p.
12. Sambrook J, Russell DW. Molecular cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory Press; 2001. 59-78 p.
13. National Veterinary Institute (SVA). Department of Mastitis, Uppsala, Sweden. Accreditation Certificate, Methods File; 1998. 1-29 p.
14. Smith DL, Harris AD, Johnson JA, Silbergeld EK, Morris JJ. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. Pro Nat Aca Sci. 2002;99:6434-6439. Doi: 10.1073/pnas.082188899
15. Rajala-Schultz PJ, Smith KI, Hogan JS, Love BC. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. J Vet Mic. 2004;102(1-2):33-42. Doi: 10.1016/j.vetmic.2004.04.010
16. Sol J. Summer mastitis: pathogenesis, losses, incidence and prevention. 1983;108(11):443-452. https://pubmed.ncbi.nlm.nih.gov/6349011/
17. Hussein SA. Isolation and identification of bacterial causes of clinical mastitis in cattle in Sulaimania region. Iraqi J of Vet Sci. 2008;22(1): 35-41. Doi: 10.33899/ijvs.2008.5666.
18. Shamoon GN. Detection of *Staphylococcus aureus* capsules producer isolated from bovine and buffaloes mastitis. Iraqi J of Vet Sci. 2008;20(1).39-47. Doi: 10.33899/ijvs.2006.45782
19. WHO Mediacentre. WHO publishes list of bacteria for which new antibiotics are urgently needed. 2017. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed
20. Helena T, Vytas R, Linda K, Ulla P, Hannu M. Structural and functional insights into lysostaphin-substrate interaction. Fro Mol Bio. 2018;5(60):1-14. Doi: 10.3389/fmolb.2018.00060
21. Zeynep ED, Aişe Ü, Mehmet MÇ, Hayriye Ü, Barış B. Enhanced production of recombinant *Staphylococcus simulans* lysostaphin using medium engineering. J Pre Bio Bio. 2019;49(5): 521-528. Doi: 10.1080/10826068.2019.1599393
22. Bastos MC, Ceotto H, Coelho ML, Nascimento JS. Staphylococcal antimicrobial peptides: relevant properties and potential biotechnological applications. Cur Pha Bio. 2009;10(1):38-61. Doi: 10.2174/138920109787048580
23. Chong A. Characterization of the virulence-related roles of the *Legionella pneumophila* chaperonin, HtpB, in mammalian cells [PhD dissertation]. Nova Scotia: Dalhousie University Halifax; 2007. 79-81 p.
24. Trayer HR, Buckley III CE. Molecular properties of lysostaphin, a bacteriolytic agent specific for *Staphylococcus aureus*. J Bio Che. 1970;245(18):4842-4846. https://pubmed.ncbi.nlm.nih.gov/5456157/
25. Mustafa JY. Using two different promoters of *lysostaphin* gene to change protein production. Bas J Vet Res. 2014;1(2):141-150. https://www.iasj.net/iasj?func=article&aId=98806
26. Zhang B, Shangguan T, Ma H, Huang X, Zhang Y. Lysis of mastitis pathogens isolated from dairy cow milk samples by purified recombinant lysostaphin. Afr J Bio. 2012;11:4649-5922. DOI: 10.5897/AJB11.2430
27. Szweda P, Kotlowski R, Kur J. New effective sources of the *Staphylococcus simulans* lysostaphin. J Bio. 2005;117(2):203-213. Doi: 10.1016/j.jbiotec.2005.01.012
28. Rosano, GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: Advances and challenges. Fro in Mic. 2014; 5, 1-17. Doi: 10.3389/fmicb.2014.00172
29. Browder HP, Zygmunt WA, Young JR, Tavormina PA. Lysostaphin: Enzymatic mode of action. Bioc Biop Res Com. 1965;19:383-389. Doi: 10.1016/0006-291x(65)90473-0
30. Grudling A, Schneewind O. Cross-linked peptidoglycan mediates lysostaphin binding to the cell wall envelope of *Staphylococcus aureus*. J. Bact. 2006;188:2463-2472. Doi: 10.1128/JB.188.7.2463-2472.2006
31. Schleifer KH, Fisher U. Description of a new species of the genus *Staphylococcus*: *Staphylococcus carnosus*. Int J Sys Bac. 1982;32:153-156. Doi: 10.1099/00207713-32-2-153
32. Chen C, Huahao F, Yong H, Fan P, Hang F. Recombinant lysostaphin protects mice from methicillin-resistant *Staphylococcus aureus* pneumonia. Bio Med Res Int. 2014:1-10. Doi: 10.1155/2014/602185
33. Hanson MR Chung CL. Antibiotic selection for MRSA: case presentations and review of the literature. J Drug Der. 2009;8(3):281-286. https://pubmed.ncbi.nlm.nih.gov/19271378/
34. Paweł M, Elżbieta J, Elżbieta N, Janusz MB, Filip S, Dorota N, Matthias B, Izabela S. Structural bases of peptidoglycan recognition by lysostaphin SH3b domain. Sci Rep. 2019;9:5965. Doi: 10.1038/s41598-019-42435-z
35. Anna M, Agnieszka J, Grzegorz D, Benedykt W. Production of lysostaphin by nonproprietary method utilizing a promoter from toxin-antitoxin system. Mol Bio. 2019;61:774-782. Doi: 10.1007/s12033-019-00203-4

دراسة تأثير كلونة بلازميد pET-32a(+) بجين اللايسوستافين ضد بكتريا المكورات العنقودية الذهبية

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الخلاصة

اللايسوستافين هو بروتين من الخارصين الفلزي، ينتج من قبل بكتيريا المكورات العنقودية نوع سميلنس، يعمل على تحطيم الجدار الببتيدي لبكتريا المكورات العنقودية الذهبية. في هذه الدراسة تم عزل جين اللايسوستافين من المكورات العنقودية نوع سميلنس، وكان حجم الجين ما يقارب ٧٥٠ قاعدة نيتروجينية. استخدمنا بلازميد pET-32a(+) لكلونة جين اللايسوستافين الى بكتريا الايشيريشيا القولونية المستقبلة نوع (DH5α) وذلك لإنتاج بروتين اللايسوستافين من هذا الجين المكلون والمعزول من المكورات العنقودية نوع سميلنس الذي استخدم تعبيره الجيني في ما بعد كمضاد لقتل بكتريا المكورات العنقودية الذهبية أحد اصعب أنواع البكتريا بالعلاج لسمك جدارها الخارجي. أظهرت النتائج نجاح بلازميد pET32a-lys للتعبير عن جين اللايسوستافين وإنتاج بروتين اللايسوستافين وبكفائه عالية، كما أظهرت النتائج دقة تقنية الاستخلاص والتنقية باستخدام عمود الهستدين المتعدد، وكون كمية البروتين وكفاءته اعلى من دراسات سابقة على بلازميدات أخرى.