USING TWO DIFFERENT PROMOTERS *OF LYSOSTAPHIN* GENE TO CHANGE PROTEIN PRODUCTION

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ABSTRACT

Bacteriocins are bacterial antimicrobial peptides with bactericidal activity against other bacteria. Staphylococcins are bacteriocins produced by staphylococci, which are gram-positive bacteria with medical and veterinary importance. In this study we are using two promoters from two plasmids to *lysostaphin* gene to change production of lysostaphin protein then killed *Staphylococcus aureus* and compared with pBluescript plasmid as control, pTrc99a get increased the production of lysostaphin protein while the pBAD30 are decreased the production in compared with pBluescript plasmid as control, at the same time both plasmid reported succeeded to *lysostaphin* expression and gave the protein with different concentration.

INTRODUCTION

The lysostaphin endopeptidase gene (lss) and the gene involved in lysostaphin resistance (lif) reside on plasmid pACK1 [1, 2]. Lss and lif are flanked by insertion sequences, suggesting that *S. simulans* biovar staphylolytic us received these genes by horizontal gene transfer [1].

Class III bacteriocins include large peptides (Mr ≥ 25 kDa) which are generally heat-labile. This class of bacteriocins was further subdivided by Heng into two distinct groups: (i) the bacteriolytic enzymes (or bacteriolysins) and (ii) the non-lytic antimicrobial proteins [3]. Staphylococci have been shown to produce bacteriolysins, from which lysostaphin is considered to be the prototype. Lysostaphin is an extracellular enzyme secreted by *S. simulans* biovar staphylolyticus ATCC1362 [4]. The peptidoglycan of gram-positive microorganisms, an important component of the bacterial cell wall, is hydrolyzed at specific times and sites during physiological growth of the exoskeleton [5]. This is accomplished by murein hydrolases. Lysostaphin is one example of such enzymes, most of these enzymes display a domain structure [6]. In general, murein hydrolases harbor an N-terminal signal peptide followed by a second domain containing the enzymatic activity. In addition, these proteins harbor repeated sequences that flank either the N- or C-terminal side of the enzymatic domain [7].

MATERIALS AND METHODS

Genomic DNA was isolated from *S. simulans* according to Sambrook and Russel [8] with some modification. DNA isolated from *S. simulans* used as a template for PCR amplification (tables 1, 2). The annealing temperature for this PCR was 53 °C. Then the PCR products were subjected to agarose gel electrophoresis, isolated and purified the DNA from gel by QIAquick Gel Purification Kit, this PCR product as template for this study,the primers used:

- Forward: 5’ GGT ACC ATG GCT GCA ACA CAT GAA CAT TCA 3’.
- Reverse: 5’ GAA TTC TCA CTT TAT AGT TCC CCA AAG AAC 3’.

Table (1) Typical conditions of PCR amplification

Stage	Step	Temp.	Time	No. cycle
1	---	95 °C	3 min	1
2	1	95 °C	30 sec	35
---	2	Anne. Temp.	30 sec	
---	3	72 °C	1 min/1 Kbp	
3	---	72 °C	10 min	1
4	---	20 °C	Hole	---

Table (2) Amount of PCR reaction

Substance	1 reaction	2 reaction
10X buffer	5 µl	10 µl
1.25 mM dNTPs	8 µl	16 µl
Forward primer	2 µl	4 µl
Reverse primer	2 µl	4 µl
Pfx	0.5 µl	1 µl
Enhancer	5 µl	10 µl
Template DNA	0.5 µl	1 µl
$MgSO_4$	1 µl	2 µl
ddH_2O	26 µl	52 µl
Total reaction	50 µl	100 µl

L*ysostaphin* gene promoter:

To study the effect of promoter function in *lysostaphin* gene, many restrictions to gene and plasmid were done according to:

1. Amplifying *lysostaphin* gene by especial primers, from PCR above to *lysostaphin* gene (sub PCR) those primers:

- Forward: 5’ GGT ACC ATG GCT GCA ACA CAT GAA CAT TCA 3’.
- Reverse: 5’GAA TTC TCA ATG ATG ATG ATG ATG ATG CTT TAT AGT TCC CCA AAG AAC 3’.

2. Cutting the pTrc99a plasmid (figure 1) by *Kpn* I and *Eco*R I (table 3). Run the plasmid in the gel, isolated and purified from the gel by Gel Extraction Kit.

3. Cutting the pBAD30 plasmid (figure 2) by *Kpn* I and *Eco*R I (table 3). Run the plasmid in the gel, isolated and purified from the gel by Gel Extraction Kit.

The PCR product were subjected to electrophoresis, isolated and purified the DNA from gel by extraction of DNA from agarose gels. After that the *lysostaphin* gene was cut as shown in table (4). Then cleaned the DNA (*lysostaphin* gene) from the few bp which formed after the cut by PCR Purification Kit.

Clones were done to study the promoter effect, the cloning were done between cuter plasmid with pTrc99a plasmid cuter first times and with pBAD30 plasmid cuter other times, those happened by:

1. Ligated *lysostaphin* gene digested by *Kpn* I and *Eco*R with pTrc99a digest by *Kpn* I and *Eco*R I as described and transformed to rubidium chloride competent cells *E. coli* DH5α.
2. Ligated *lysostaphin* gene digested by *Kpn* I and *Eco*R with pBAD30 digest by *Kpn* I and *Eco*R I as described and transformed to competent cells *E. coli* DH5α.

For all cases, colonies were replica-plated onto fresh LB solid medium, containing Ampicillin 100 µg/ml, incubated overnight at 37 °C. Transformants were confirmed by PCR analysis, restriction of harboured plasmids then done colony PCR, this protocol is designed to quickly insert plasmids (constructive plasmid) screening *E. coli* DH5α colonies.

Table (3) The substances and quantity of digestion pTrc99a and pBAD30 by *Kpn* I and *Eco*R I

Substance	Quantity
pTrc99a or pBAD30	20 µl
10 X buffer	5 µl
BSA	5 µl
ddH_2O	16 µl
Kpn I	2 µl
*Eco*R I	2 µl
Total reaction	50 µl

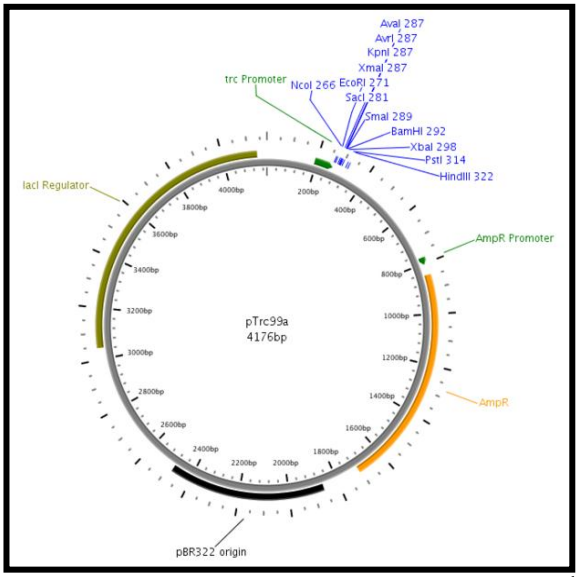

Figure (1) Map of the pTrc99a plasmid

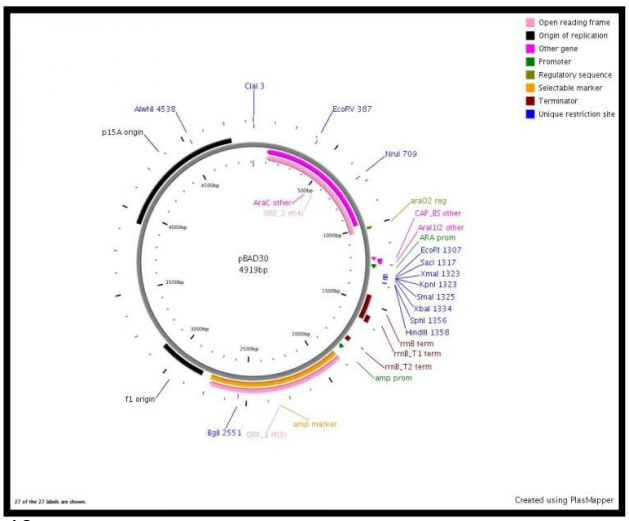

Figure (2) Map of the pBAD30 plasmid

Table (4) The substances and quantity of digestion lysostaphin gene by *Kpn* I and *Eco*R I

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

Determine the role of promoter in protein activity:
To determine if *E. coli* cloning (with *lysostaphin* gene) were able to inhibit the *S. aureus*, a standard disk diffusion assay was performed. Cultures of *E. coli* and *S. aureus* were incubated in nutrient broth for exponential phase (OD_{620} 0.1). *S. aureus* were inoculated in MHA while discs of *E. coli* were prepared and placed in the center of the plates. The cultures were then incubated at 37 °C overnight under aerobic conditions and the resulting zones of inhibition were determined.

Extraction and purification of lysostaphin protein from cloning bacteria by His-tag method:
By used HisTrap FF crude columns (GE Healthcare, SE-751 84 Uppsala, Sweden) are designed to direct purification of unclarified cell lysates without centrifugation or filtration in the sample preparation steps. The Bio-Rad Protein Assay from (Bio-Rad laboratories, Life Science Group, U.S. (800) 4BIORAD, California) can also be used with a microplate reader to determined protein concentration. SDS-PAGE analysis of proteins was performed by standard techniques (9).

RESULTS AND DISCUSSION

Figure (3) showing the PCR product result from *S. simulans* after running the product in the gel. This figure showed the band of product (*lysostaphin* gene) less then (750) bp. The result appeared the size of *lysostaphin* gene accepted to [10, 11, 12].

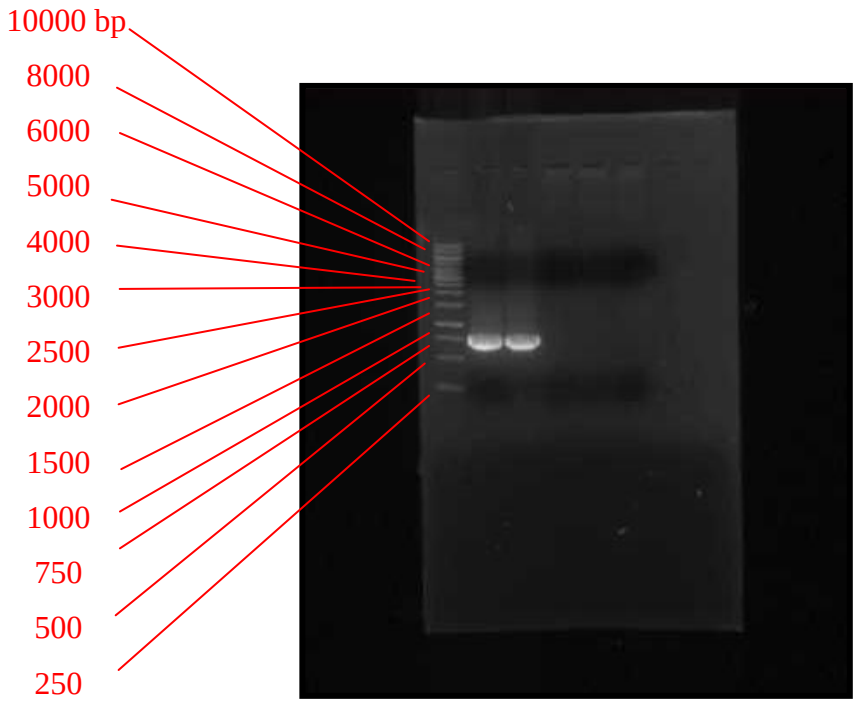

Figure (3) Band of PCR product less then (750) bp

Figure (4) showing PCR product from cloned bacteria, with *lysostaphin* gene from pTrc99a plasmid and *lysostaphin* gene from pBAD30 plasmid.

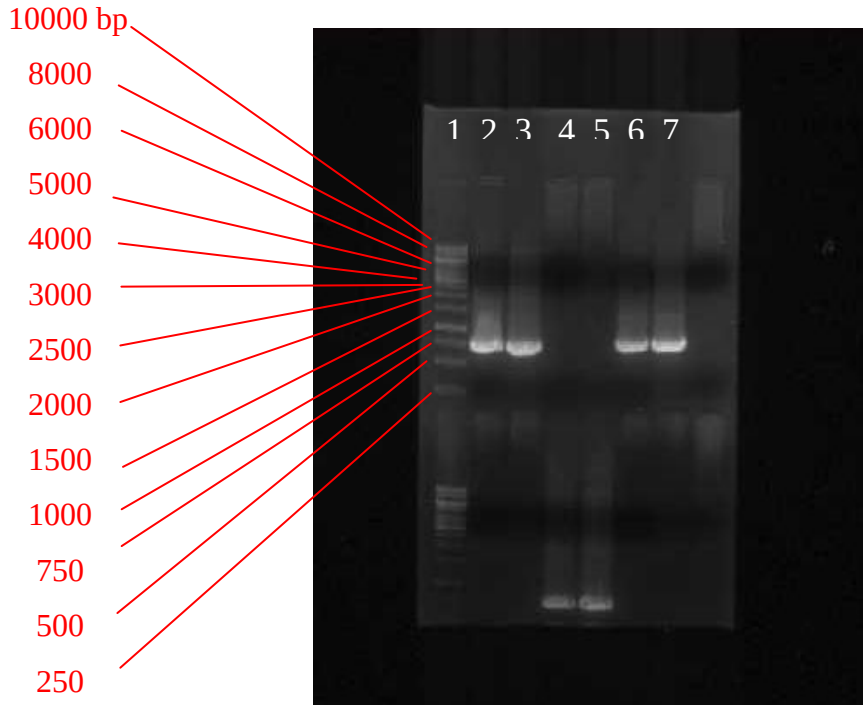

Figure (4) PCR product from cloned bacteria, lane (1) is the ladder, lane (2, 3) are a *lysostaphin* gene from pTrc99a plasmid, lane (4, 5) are negative control, lane (6, 7) are a *lysostaphin* gene from pBAD30 plasmid

The antibacterial activity of transformed *E. coli* by *lysostaphin* gene from pTrc99a plasmid first time, from pBAD30 plasmid second time and from pBluescript plasmid against *S. aureus* third time shown in figure (5), while non transformed *E. coli* by *lysostaphin* gene against *S. aureus* shown in figure (6). This result was in agreement with Chong and Mustafa in limited the good effect of transformed *E. coli* by *lysostaphin* gene against *S. aureus* by used the active *lysostaphin* gene less then (750 bp).

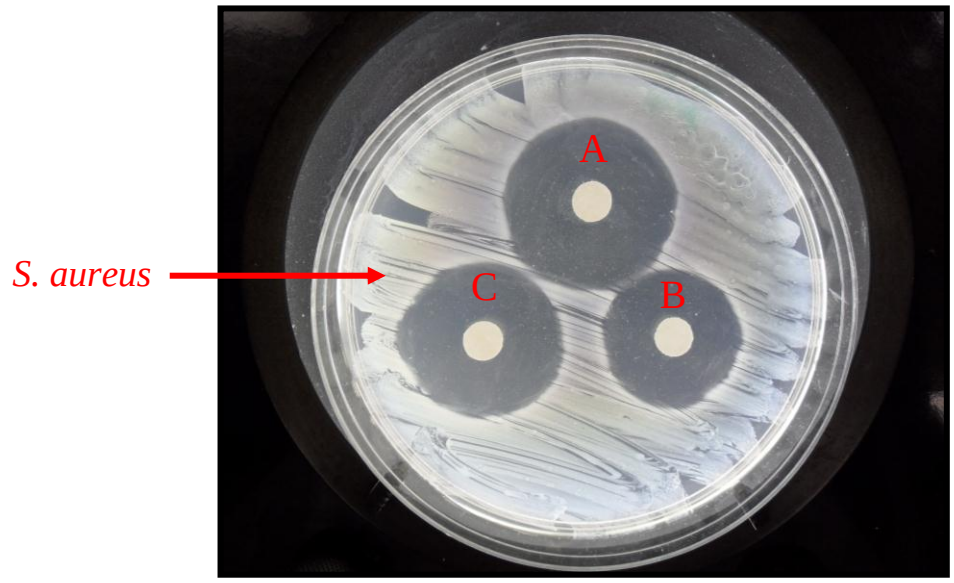

Figure (5) Transformed *E. coli* by *lysostaphin* gene. (A) *lysostaphin* gene from pTrc99a plasmid, (B) *lysostaphin* gene from pBAD30 plasmid, (C) *lysostaphin* gene from pBluescript plasmid against *S. aureus*

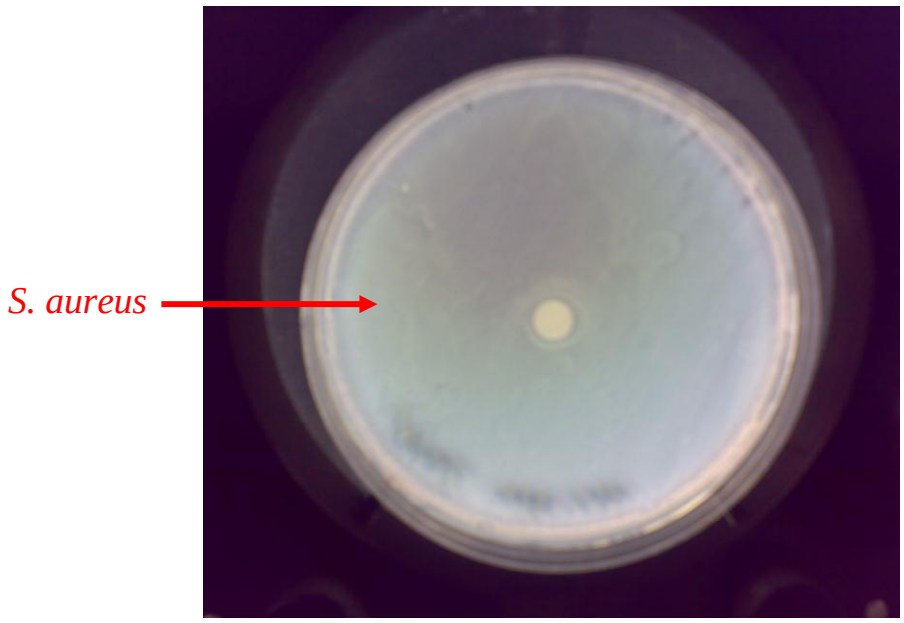

Figure (6) Not transformed *E. coli* while *S. aureus* spread onto plate

The concentration of lysostaphin protein from cloned bacteria by His-tag column was shown in table (5), also this table showed the lysostaphin from pTrc99a plasmid, and lysostaphin from pBAD30 plasmid. Figure (7) showed SDS-PAGE analysis of proteins by standard techniques, the figure showed one band of protein, the molecular weight of lysostaphin in all lanes were about 27,000 Dalton, it is agreed with [16, 12].

Our results was find very high variety of effect and concentration of lysostaphin protein according to plasmid types, that is mean we can change the effect and concentration if we change the promoter (plasmid) of gene.

Table (5) Protein concentration by measuring the absorbance at 595 nm

Bio-Rad dye	BSA	Lysostaphin from pTrc99a plasmid	Lysostaphin from pBAD30 plasmid
0.330	3.102	1012	0.622
0.335	1.617	0.835	0.398
0.325	0.971	0.662	0.316
0.310	0.581	0.390	0.311
0.312	0.301	0.310	0.307

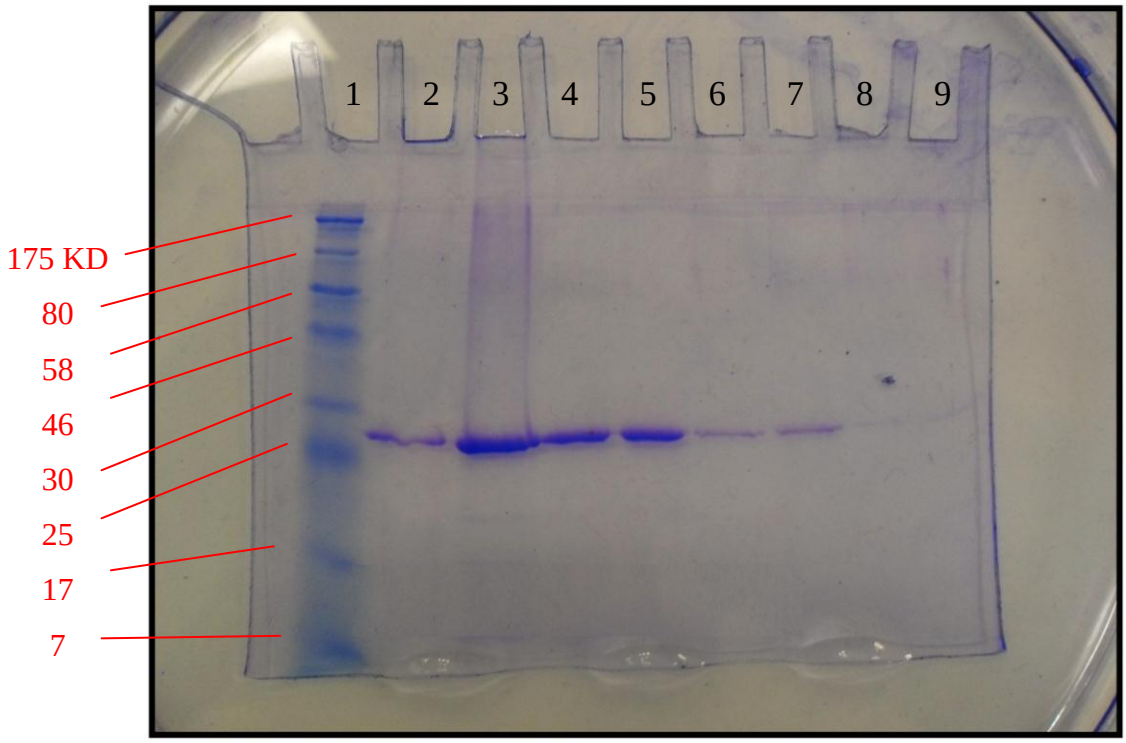

Figure (7) SDS-PAGE analysis of lysostaphin protein, the marker in lane (1), lysostaphin protein in lanes (2, 3) from *lysostaphin* gene from pTrc99a plasmid, (4, 5) *lysostaphin* gene from pBAD30 plasmid, (6, 7) *lysostaphin* gene from pBluescript plasmid while line (8, 9) are a control negative

Lysostaphin activity against staphylococci is due to its lysis capability of the peptidoglycan of bacterial cell walls [15, 16]. The target of the lysostaphin is the pentaglycine cross-bridge of the peptidoglycan [17]. *S. aureus* and other staphylococcal species are composed of five glycine (Gly) residues [18, 19]. Lysostaphin has the ability to cleave specifically between the third and the fourth Gly residues of the pentaglycine cross-bridge [20, 17]. The peptidoglycan of staphylococcal species is relatively resistant to lysostaphin containing higher amount of serine (Ser) than Gly [21].

Conclusions:

The research has arrived the conclusions, *E. coli* cloned with *lysostaphin* gene are very effective against *S. aureus*. pTrc99a get increased the production of lysostaphin protein while the pBAD30 are decreased the production in compared with pBluescript plasmid as control, at the same time both plasmid reported succeeded to *lysostaphin* expression and gave the protein with different concentration.

Recommendations:

The research recommends try to transport *lysostaphin* gene to eukaryotic system by transgenic method for a normal expression of this gene in second generation, and use lysostaphin in pharmaceutical in addition to antibiotics by to produce new successful antibiotics as a treatment of *S. aureus*.

استخدام نوعين مختلفين من المشغلات لجين اللايسوستافين لتغير كمية البروتين المنتج

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الخلاصة

البكترياسين هو ببتيد مضاد للبكتريا وله تأثير قاتل لنمو أنواع من البكتريا. الستافيلولايسين هو نوع من أنواع البكترياسين ينتج من المكورات العنقودية الموجبة لصبغة كرام والتي لها أهمية في المجال الطبي البشري والبيطري على حد سواء. وفي هذه الدراسة استخدمنا مشغلات (بروموتر) لبلازميدين مختلفين لجين اللايسوستافين لتغير كمية الإنتاج من هذا الجين لقتل بكتريا المكورات العنقودية الذهبية بالمقارنة مع بلازميد pBluescript كنقطة سيطرة. ووجد أن بلازميد pTrc99a أعطى زيادة ملحوظة في إنتاج بروتين اللايسوستافين بينما أعطى بلازميد pBAD30 نقصان بكمية إنتاج البروتين مقارنة مع بلازميد pBluescript كنقطة مقارنة. كما بينت الدراسة أن كلا البلازميدين نجحا في التعبير عن جين اللايسوستافين وإعطائه البروتين مع اختلاف في تركيز البروتين بالاعتماد على نوع البلازميد المستخدم.

REVERENCES

1. Thumm, G. and Götz, F. (1997). *Mol. Microbiol*., 23, 1251-1265.
2. Heath, H. E.; Heath, L. S.; Nitterauer, J. D.; Rose, K. E. and Sloan, G.L. (1989). *Biochem*. *Biophys. Res. Commun*., 160, 1106-1109.
3. Heng, N. C.; Wescombe, P. A.; Burton, J. P.; Jack, R. W. and Tagg, J. R. (2007). In *Bacteriocins: ecology and evolution*, (Riley, M.A. and Chavan, M.A., Eds.), Springer, New York, pp. 45-92.

4. Schindler, C. A. and Schuhardt, V. T. (1964). *Proc. Natl. Acad. Sci. USA.*, 51, 414-421.

5. Giesbrecht, P.; Wecke, J. and Recnicke, B. (1976). *Int. Rev. Cytol*., 44, 225-318.

6. Joris, B.; Englebert, S.; Chu, C. P.; Kariyama, R.; Daneo-Moore, L., Shockman, G. D. and Ghuysen, J. M. (1992). *FEMS Microbiol. Lett*., 70, 257-264.

7. Bastos, M. C.; Ceotto, H.; Coelho, M. L. and Nascimento, J. S. (2009). Staphylococcal Antimicrobial Peptides: Relevant Properties and Potential Biotechnological Applications, *Current Pharmaceutical Biotechnology*., 10, 38-61.

8. Sambrook, J. and Russell, D. W. (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., cited by Chong, A. (2007). Characterization of the Virulence-Related Roles of the *Legionella pneumophila* Chaperonin, HtpB, in Mammalian Cells. Ph D thesis. Dalhousie University Halifax, Nova Scotia., pp: 59-78.

9. *National Veterinary Institute (SVA)*. (1998). Department of Mastitis, Uppsala, Sweden. Accreditation Certificate, Methods File no., 3: 1-29.

10. Recsei, P. A.; Gruss, A. D. and Novick, R. P. (1987). Cloning, sequence, and expression of the *lysostaphin* gene from *Staphylococcus simulans*. *Proc*. *Natl*. *Acad*. *Sci*. USA., 84: 1127-1131.

11. Bastos, M. C. F.; Ceotto, H.; Coelho, M. L. V. and Nascimento, J. S. (2009). Staphylococcal antimicrobial peptides: relevant properties and potential biotechnological applications. *Curr*. *Pharm*. *Biotechnol*., 10 (1): 38-61.

12. Mustafa, J. Y. (2011). Sequences and expression of the *lysostaphin* gene from *Staphylococcus simulans* isolated from bovine mastitis and its bactericidal effect on *Staphylococcus aureus.* PhD thesis in Biology (Genetic Engineering), Basra University.

13. Chong, A. (2007). Characterization of the Virulence-Related Roles of the *Legionella pneumophila* Chaperonin, HtpB, in Mammalian Cells. Ph D thesis. Dalhousie University Halifax, Nova Scotia., pp: 79-81.

14. Heng, N. C. K.; Wescombe, P. A.; Burton, J. P.; Jack, R. W. and Tagg, J. R. (2007). The diversity of bacteriocins in Gram-positive bacteria. In Bacteriocins: Ecology and Evolution, *Springer*: New York, NY, USA, pp: 45-92.

15. Browder, H. P.; Zygmunt, W. A.; Young, J. R. and Tavormina, P. A. (1965). Lysostaphin: enzymatic mode of action. *Biochem*. *Biophys*. *Res*. *Commun*., 19: 383-389.

16. Trayer, H. R. and Buckley III, C. E. (1970). Molecular properties of lysostaphin, a bacteriolytic agent specific for *Staphylococcus aureus*. *J*. *Biol*. *Chem*., 245: 4842-4846.

17. Grudling, A. and Schneewind, O. (2006). Cross-linked peptidoglycan mediates lysostaphin binding to the cell wall envelope of *Staphylococcus aureus*. *J*. *Bacteriol*., 188: 2463-2472.

18. Schleifer, K. H. and Fisher, U. (1982). Description of a new species of the genus *Staphylococcus*: *Staphylococcus carnosus*. *Int*. *J*. *Syst*. *Bacteriol*., 32: 153-156.

19. Schleifer, K. H. and Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol*. *Rev*., 36: 407-477.

20. Schneewindt, O. and Fowler, A. (1995). Faull, K.F. Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. *Science*., 268: 103-106.

21. Kumar, J. K. (2008). Lysostaphin: an antistaphylococcal agent. *Appl*. *Microbiol. Biotechnol*., 80: 555-561.