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A THESIS SUBMITTED FOR THE DEGREE OF

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THESIS SUPERVISOR

ASSOC. PROF. DR. BARIŞ BİNAY

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2020

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

REKOMBİNANT *Staphylococcus simulans* LİZOSTAFİNİN BÜYÜK ÖLÇEKTE ÜRETİMİNİN İYİLEŞTİRİLMESİ VE KARAKTERİZASYONU

ZEYNEP EFSUN DUMAN
YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ANABİLİM DALI

DANIŞMANI
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GEBZE
2020

SUMMARY

Staphylococcus aureus (S. aureus), developed a multidrug resistance, leads to most of the healthcare-associated infections acquiring in healthcare settings such as hospitals, nursing homes, dialysis centers. These infections are required complex treatments and can cause significant medical and economic losses along with costs. Thereby, developing a new and effective strategy to rule these bacteria has gained significant importance.

Lysostaphin (EC 3.4.24.75) is a peptidoglycan hydrolase that has the ability to degrade the cell wall of almost all known staphylococcal species; in particular, *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. carnosus*, *S. saprophyticus*, *S. hominis*, *S. hyicus* ATCC 11249 and *S. intermedius* ATCC 29663. Although lysostaphin has considerable potential on biotechnological applications and ability as a bacteriocin, the high production cost limits its usage in healthcare settings (1 mg–134 €/ Sigma-Aldrich). Regarding this issue, optimization and the scale-up studies of lysostaphin by decreasing the production cost of the enzyme on larger scales become important.

In this study, increasing the expression yield of recombinant lysostaphin in *Escherichia coli* TOP10 (*E. coli* TOP10) has been aimed. Firstly, medium optimization was applied by testing the yield of arabinose induction and auto-induction mediums. The effect of temperature on expressed lysostaphin yield and activity were tested as a second task. The results revealed that the composition of auto-induction media increased the amount of lysostaphin production 5-times with the highest level of active lysostaphin at 30 °C. Following, optimized conditions were transferred to scale-up studies and recombinant lysostaphin was produced via bench-top culturing of *E. coli* cells. In conclusion, the tested conditions improve the protein yields up to 184.076 mg/L in a 3-L STR benchtop bioreactor and the production cost was decreased 4 folds when compared with the commercially available ones. Moreover, this study proved that lysostaphin is an effective bacteriocin on both commercially available and national *S. aureus* species. Outputs of the study will contribute to further larger-scale production of lysostaphin.

Keywords: Auto-induction; cost analysis; benchtop bioreactor; scale-up; *E. coli* expression system; lysostaphin; medium optimization; L- (+) arabinose.

ÖZET

Çoklu ilaca direnç geliştiren *Staphylococcus aureus* (*S. aureus*), hastaneler, bakım evleri, diyaliz merkezleri gibi sağlık ortamlarında görülen enfeksiyonların birçoğunun sebebidir. Bu enfeksiyonlar karmaşık ve yüksek maliyetli tedaviler gerektirir; önemli tıbbi ve ekonomik kayıplara neden olabilir. Bu nedenle, bu bakterilerle mücadele etmek için yeni ve etkili stratejiler geliştirmek önem kazanmaktadır.

Lizostafin (EC 3.4.24.75), bilinen tüm stafilokok türlerinin hücre duvarını parçalama yeteneğine sahip olan bir peptidoglikan hidrolazdır; özellikle şu türler üzerinde etkilidir, *S. aureus, S. epidermidis, S. haemolyticus, S. carnosus, S. saprophyticus, S. hominis, S. hyicus* ATCC 11249 ve *S. medius* ATCC 29663. Lizostafin biyoteknolojik uygulamalar ve bakteriyosin olarak önemli bir potansiyele sahip olsa bile, yüksek üretim maliyeti tedavi ve araştırma amaçlı kullanımını sınırlandırmaktadır (1 mg-134 € / Sigma-Aldrich). Bu bağlamda, enzimin üretim maliyetini azaltmak üzere daha büyük ölçeklerde yapılacak optimizasyon çalışmaları önem kazanmaktadır. Bu çalışmada *Escherichia coli* TOP 10' da (*E. coli* TOP10) rekombinant lizostafin ekspresyon veriminin arttırılması amaçlanmıştır. İlk olarak, arabinoz indüksiyonu ve oto indüksiyonlu besi yerlerinin verimi test edilerek besi yeri optimizasyonu uygulandı. Bununla birlikte, sıcaklığın ifade edilen lizostafin verimi ve aktivitesi üzerindeki etkisi, test edildi. Sonuçlar, otoindüksiyon ortamının bileşiminin, 30 ° C'de en yüksek seviyede aktif lizostafin üretim miktarını 5 kat arttırdığını ortaya koydu. Ardından, optimize edilmiş koşullar scale-up çalışmalarında kullanıldı ve *E. coli* hücrelerinin tezgâh üstü kültürlenmesi yoluyla rekombinant lizostafin üretildi. Sonuç olarak, test edilen koşullar, 3 L tezgâh üstü biyoreaktörde 184.076 mg/L'ye kadar protein verimlerini iyileştirdi ve enzimin üretim maaliyeti ticari olarak satılana göre 4 kat düşürüldü. Ayrıca, elde edilen sonçlar, lizostafinin hem ticari olarak hem de izole edilen *S. aureus* türleri üzerinde etkili bir bakteriyosin olduğunu kanıtlamıştır. Çalışmanın çıktıları daha büyük ölçekli lizostafin üretimine katkıda bulunacaktır.

Anahtar kelimeler: Oto-indüksiyon; maliyet analizi, tezgâh üstü biyoreaktör; scale-up; *E. coli* ekspresyon sistemi; lizostafin, besi yeri optimizasyonu; L- (+) arabinoz.

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TABLE OF CONTENTS

LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		Explanations
µ	:	Micro
°C	:	Celsius Degree
BCA	:	Bicinchoninic Acid
His	:	Histidine
HCl	:	Hydrochloric Acid
NaOH	:	Sodium Hydroxide
L	:	Liter
mL	:	Milliliter
kDa	:	Kilodalton
M	:	Molar
mM	:	Millimolar
OD	:	Optical Density
min	:	Minute
sec	:	Second
g	:	Gram
mg	:	Milligram
SDS-PAGE	:	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOC	:	Super Optimal Broth with Catabolite Repression
LB	:	Luria-Bertani
ENLB	:	Enriched Luria-Bertani
PLB	:	Peptone Luria-Bertani
TB	:	Terrific Broth
SB	:	Super Broth
FM	:	For Medium
Std	:	Studier Medium
dH_2O	:	Distilled water

Amp	:	Ampicillin
dcw	:	Dry Cell Weight
DO	:	Dissolved Oxygen
vvm	:	Vessel Volume Per Minute
Lys	:	Lysostaphin
rpm	:	Revolutions per Minute
IC_{50}	:	The Half Maximal Inhibitory Concentration
Conc.	:	Concentration

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1. INTRODUCTION

1.1. The Aim, Contribution and Content of The Thesis

This thesis is focusing on mainly three topics which are, improving the expression yield of recombinant lysostaphin on a small scale, introducing a more feasible production process for lysostaphin on a benchtop bioreactor and testing the bactericidal properties of produced lysostaphin.

Lysostaphin (EC 3.4.24.75) is a glycylglycine endopeptidase produced by *Staphylococcus simulans* biovar *staphylolyticus* (ATCC 1362, NRRL B-2628) exclusively. Even though lysostaphin is able to degrade the cell wall of almost all known staphylococcal species and has been demonstrated as a promising agent for the treatment of biofilm-associated and blood-borne staphylococcal infections, and biotechnological applications have limited due to its high cost. This study reveals an inducible system and auto-induced medium for the expression of lysostaphin that is newly developed. Moreover, this study provides a cost-effective methodology through the implementation of optimized parameters to stirred tank bioreactor system. The outputs of the bioreactor studies will contribute to the future larger-scale production studies of lysostaphin.

Within the scope of this thesis, the aim, contributions, and content of the thesis are mentioned in the first chapter. In the second part, *Staphylococcus aureus* and related diseases, basic information about enzymes, the properties of lysostaphin enzyme and its bacteriocin potential and basic steps for bioprocess development from recombinant protein production and protein purification were briefly explained. Then, in the third part, the information about the optimization studies both on shake-flask and stirred tank bioreactor, detailed economic analysis and antimicrobial characterization tests were shared. In the fourth section, the results were reported. In the fifth and final section, comments on these results were explained.

2. BACKGROUND INFORMATION

2.1. *Staphylococcus aureus* and It's Infections

Staphylococcus aureus (*S. aureus*) is a sphere-shaped (coccal) gram-positive bacteria due to containing highly thick peptidoglycan and teichoic acid. *S. aureus* strains are mostly resistant to high temperatures and high salt concentrations [1], [2]. It has been reported that staphylococcal species is one of the main reasons for nosocomial and community-acquired infections that are related to numerous diseases containing endocarditis, osteomyelitis, pneumonia, toxic shock syndrome, food poisoning, and various skin infections like folliculitis, furuncles, carbuncles, abscess, and mastitis [3], [4].

Robert Koch first described staphylococci in 1878, and in 1880 Pasteur produced them on broth. In 1881, Alexander Ogston emphasized that it is a pathogen for mice and guinea pigs. The term staphylococcus is derived from the Greek staphyle and is chosen by Alexander Ogston for their characteristic clusters. In 1884, Rosenbach called white colonies *Staphylococcus albus* and yellow-orange colonies *Staphylococcus aureus.* This distinction has continued until recent times. They are catalase-positive cocci within the *Micrococcaceae* family and in nature; are commonly found in dust, soil, animal skin, mucosal tissues, and extracts and are proliferating in human skin, nasal cavity and lesions. Staphylococcal infections in humans primarily emerging due to *S. auresus.* It has been reported as one of the most dangerous staphylococcal species. [5], [6].

The scientist has been trying to develop novel and effective antimicrobial agents since they identified them as a reason for serious infections. The treatment of *Staphylococcus aureus* infections became available after the discovery of penicillin, in the 1940s. However, in the 1950s the developing resistance to penicillin by *S. aureus* leads to difficulties. In the following decade, 1961, methicillin-resistant *S. aureus* (MRSA) was firstly isolated in the UK and it spread into a community in the 1990s named Community-Acquired Methicillin-Resistant *S. aureus* (CA-MRSA) [7]–[9]. Since the onset of the pandemic of MRSA over the past decades, it has become the most common cause of both hospital- and community-acquired infections worldwide. The historical diagram of

developing drug resistance for bacteria and the discovery of antimicrobial agents is provided in Figure 2.1.

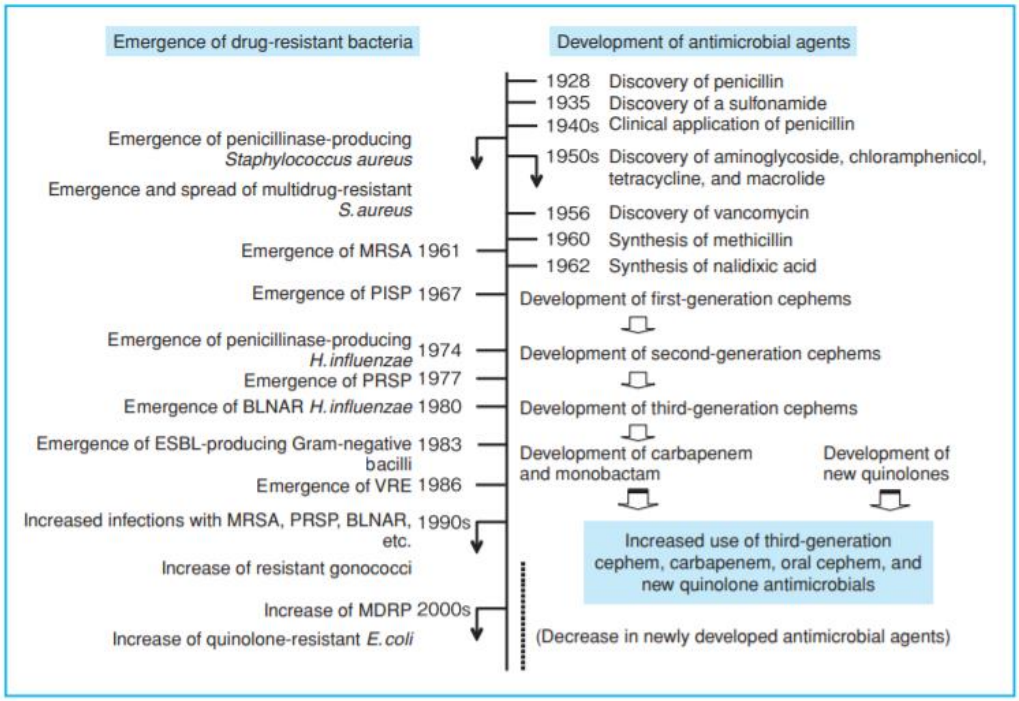


Figure 2.1: Timeline for the emergence of drug-resistant bacteria and their development of resistance against antimicrobial agents [2].

The drug-resistant bacteria which leads to the emergence of infections is a serious and growing global health concern. Therefore, significant efforts are being made in the development of new antimicrobial compounds with improved efficacy. World Health Organization (WHO) is estimating that the infections caused by antibiotic-resistant bacteria would be a major cause of death in 2050 where the expected number of deaths will be around 10 million every year. Moreover WHO has listed MRSA and recently emerged vancomycin-intermediate and resistant *S. aureus* (VRSA) as "high-priority" deadly bacterial pathogens owing to the emerging problem of resistance [10]. Therefore, alternative therapeutic strategies to combat the challenging situations in the management of multi-drug-resistant *S. aureus* infections, are of utmost importance.

2.2. Enzymes

Enzymes are biological catalysts in protein structure that accelerate biochemical reactions. Enzymes can be obtained from vegetable, animal or microbial sources. They are not consumed during the reaction and do not change at the end of the reaction. The production and destruction of organic substances in cells, digestion, muscle contraction, cell respiration are the result of various metabolic reactions and these reactions are made possible by the catalytic effect of enzymes. There are many factors that increase or decrease the activity of enzymes and thus the rate of reactions they catalyze. These factors are; substrate concentration, enzyme concentration, pH, temperature, reaction time, reaction products, enzyme inhibitors and activators, radiation, pressure and light.

There is a large number of known enzymes and in order to regulate the nomenclature of enzymes, the International Association of Biochemistry and Molecular Biology (IUBMB) classified the enzymes into six main groups containing subgroups according to the reactions they catalyze. Enzyme code numbers consist of four numbers separated by dots and the first number indicates the enzyme's six main enzyme groups; The names and tasks of the six main enzyme groups are shown in Table 2.1 [11].

Since the early ages, enzymes that have been used in the production of cheese, vinegar and alcoholic beverages and similar products have become a focal point for scientific studies and industrial applications with the advances in the field of recombinant DNA technology [12], [13]. Today, enzymes are used in many areas, from the food industry to the medical field for diagnosis and treatment. In 2016, the global market for the industrial enzymes was found to be approximately US $ 4.61 billion and is expected to reach US $ 6.30 billion by 2022. Currently, 4000 enzymes from about 200 microbial sources are identified and 75 % of these enzymes are hydrolytic enzymes such as proteases, carbohydrases and lipases [14]. It has great importance that makes suitable enzymes for industrial applications and reduces the production cost of the enzymes using for industrial purposes.

Table 2.1: Classification of Enzymes

Class	Name	Catalyzed Reaction and Mechanism	Enzyme Code
1	Oxidoreductases	Catalyze oxidation/reduction reactions	EC 1.X.X.X
2	Transferases	Transfer of a functional group from one substrate to another	EC 2.X.X.X
3	Hydrolases	Formation of two products from a substrate by hydrolysis	EC 3.X.X.X
4	Lyases	Non-hydrolytic addition or removal of groups from substrates	EC 4.X.X.X
5	Isomerases	Intramolecular rearrangement	EC 5.X.X.X
6	Ligases	Join two molecules by synthesizing of new C-O, C-S, C-N or C-C bonds with a simultaneous breakdown of ATP	EC 6.X.X.X

2.2.1. Hydrolases

Hydrolases belong to the third class of enzymes that catalyze hydrolysis reactions involving in the transfer of functional groups to water which leads to dividing a larger molecule to smaller molecules. Lipases, phosphatases, glycosidases and peptidases are some examples of hydrolases [15].

2.2.2. Lysostaphin and It's Potential as a Bacteriocin

Lysostaphin [EC 3.4.24.75] belongs to the metalloendopeptidase class of hydrolase enzymes which is a zinc-dependent glycylglycine endopeptidase. It is encoded natively by the *Staphylococcus simulans* biovar *staphylolyticus* [NRRLB- 2628 (gi|126496)] and a natural competitor of *S. aureus* strains [16], [17]. The 3D structure of lysostaphin supplied by protein data bank is represented in Figure 2.2.

Figure 2.2: The structure of *Staphylococcus simulans* lysostaphin extracted from the protein data bank (PDB No. 4qp5)

The polyglycine interpeptide bridges on the cell walls of *S. aureus*, *S. epidermidis* and *S. carnosus* in all metabolic states (growing, resting, or heat-killed) is hydrolyzed by the Lysostaphin activity [18], [19]. A schematic representation of the enzymatic activity of lysostaphin on the peptidoglycan cell-wall of *S. aureus* can be seen in Figure 2.3. This property of lysostaphin makes this enzyme a promising antimicrobial protein against the widespread hospital and community-acquired infections, particularly the multidrug-resistant *S. aureus* [20]–[22].

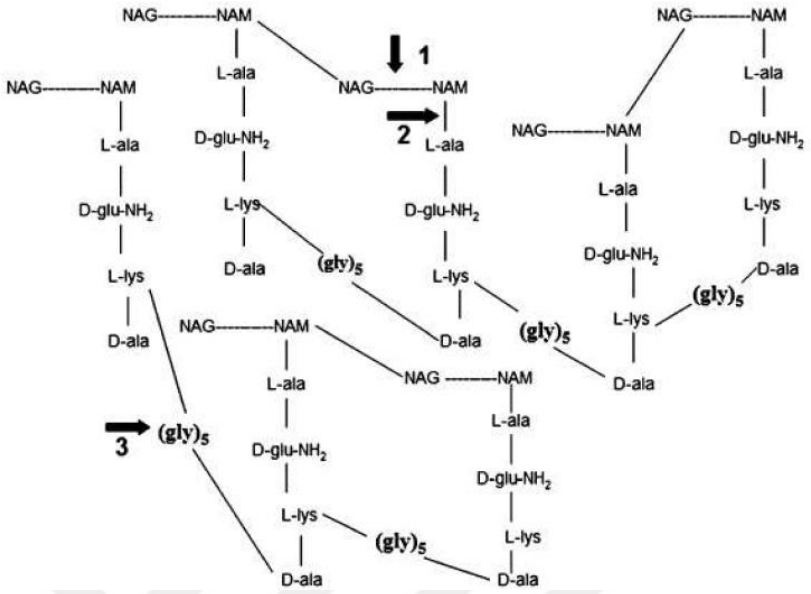


Figure 2.3: A schematic representation of the enzymatic activities of lysostaphin peptidoglycan cell-wall of *S. aureus*. Lysostaphin has the ability of multiple enzyme activities on 1) endo-β-*N*-acetyl glycosaminidase, 2) *N*-acetyl-muramyl-L-alanine amidase and 3) glycylglycine endopeptidase. The glycylglycine endopeptidase activity results in the solubilization of the pentaglycine bridges, Kumar et al., 2008.

Lysostaphin has been widely used in research laboratories for the identification of the staphylococcal species and also to hydrolyze the cell wall of staphylococcal for releasing of the nucleic acids, intracellular enzymes, surface components and cell membrane [23].

Additionally, lysostaphin is able to disrupt staphylococcal biofilms in vitro not only by killing the staphylococci that are normally tough to treat with antibiotics but also by the disintegration of the extracellular biofilm matrix from the artificial surface [19], [24]–[26]. According to scanning electron microscopy studies, lysostaphin eradicates both the sessile cells and the extracellular matrix of biofilms that provides a new treatment option for patients to treat staphylococcal infections of indwelling devices such as artificial heart valves and other prosthetic devices as well [22], [25], [27].

Improved production of lysostaphin was widely studied thanks to specific biotechnological applications and potential for research. Up to now, lysostaphin has been cloned and produced in various expression host cells [28]–[31] and expression systems [18], [30], [32], [33] (Table 2.2). Although some achievements have been obtained using

recombinant DNA technologies to produce lysostaphin, it is still not widely used in clinical applications that require high amounts of the enzyme [34]. Therefore, developing novel and feasible production process for lysostaphin through recombinant DNA technology has been gaining importance.

Table. 2.2: Recombinant lysostaphin expression through different expression systems.

Vector	Host Expression System	References
-	Rabbit reticulocyte cell-free translation system	Williamson C. M. Et al., 1993 [35]
-	COS-7 cells	
-	Transgenic mice	Kerr D. E. et al., 2001 [36]
*p*TYB12-*Lys*	*Escherichia coli* ER2566	Szweda P. et al., 2001 [37]
*p*BAD*Lys*	*Escherichia coli* TOP10F'	Szweda P. et al., 2005 [23]
-	*Lactobacillus plantarum* WCFS1	Liu H. et al., 2011 [38]
*p*ET32-*Lys*	*Escherichia coli* BL21	Farhangnia L. et al., 2014 [39]
*p*PIC9- *Lys*	*Pichia pastoris* GS115	Zhao H. et al., 2014 [31]
*p*UC18-*Lys*	*Escherichia coli* DH5α	Mądry A. et al., 2019 [40]

2.3. Recombinant DNA Technology

Recombinant DNA technology (rDNA) is a technology that involves the cutting of different DNA molecules from different biological species and combining them together by genetic engineering technology. DNA molecules formed by combining molecules from different species are called recombinant DNA. Genetic analysis and manipulations such as endonuclease assays, DNA sequencing, and directed evolution studies can be performed on recombinant DNA. The scientific basis of this technology is called recombination which is one of the important reasons for the diversity seen in nature.

rDNA makes it possible to isolate a gene or sequence DNA, define the nucleotide sequence, study its transcripts, mutate it in highly specific ways and reinsert the modified DNA into a living organism.

2.4. Bioprocess Development

Bioprocessing is a solid part of most of the pharmaceutical, food and chemical industries. Throughout the bioprocess operations, microbial, animal, and plant cells, and components of cells such as enzymes are utilized for manufacturing new value-added products and destroying the harmful wastes. Several steps involving different types of scientific expertise are necessary to bring the product into a commercial level (Figure 2.4) [41].

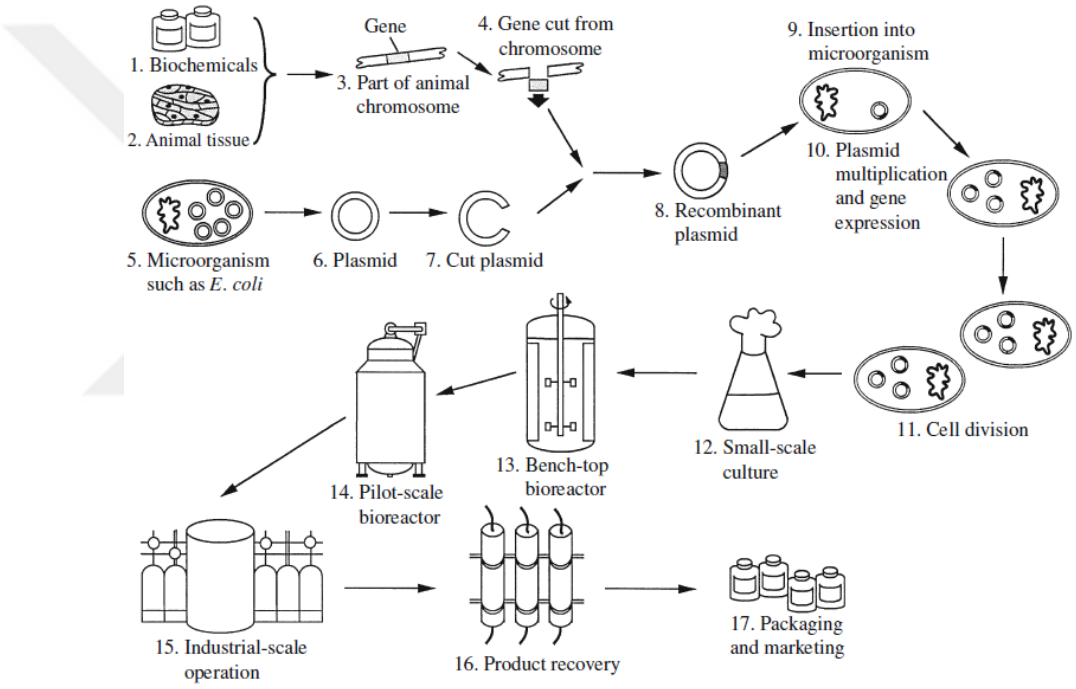


Figure 2.4: Development of bioprocess steps for commercial manufacture of a recombinant DNA-derived product [41].

Microbial fermentation remains a central feature of most industrial bioprocesses. Production of value-added products with bioprocess is carried out by numerous intracellular reactions with a suitable microorganism using mainly carbon sources such as glucose, glycerol, molasses, soy, etc. In the production of biotechnological products through high-efficiency bioprocesses, microorganisms with increased production capacity by with genetic manipulation of the host organism are developed [42].

Optimization studies of fermentation are ideally performed at a small scale (250 mL to 1 L capacity) to reduce time, cost and resource requirements. These are generally performed in vessels in which the engineering environment is not well characterized or controlled. A wide range of variables are also known to affect heterologous protein expression and numerous experiments are required to adequately characterize the total complement of interactions and effects. Consequently, the results obtained from these screening studies are used to reproduce at conventional bench-top bioreactors from 1 L to 2 L capacity. Following, those outputs are evaluated, processed and implemented to pilot scales with a vessel of capacity from 100 to 1000 L is designed according to conditions determined from the bench-scale prototype by aiming the examine the response of cells to scale-up [41], [43].

In bioprocesses, the designed bioreactor operating conditions are only physically feasible under sterile conditions, and the same bioreactor systems can be used to produce different biomolecules. However, besides the desired product, the formation of a large number of by-products, primarily amino acids and organic acids, and extracellular enzymes that need to be separated from fermentation medium should suggest that separation processes in bioprocess production should be achieved. Therefore, product recovery (Figure 2.4, Step 16) or downstream processing is considered as one of the most important parts of the total process. At the end of fermentation, the raw broth is treated in a series of steps to produce the final product. The product recovery step is difficult to manage and implement and generally expensive especially for some recombinant-DNA-derived products, purification process takes 80 to 90 % of the total processing cost. The selected method for downstream processing depends on the nature of the targeted product and the broth. On an industrial scale, many standard operations in the laboratory become unfeasible or impractical. For the separation of cells from the liquid part; filtration, centrifugation, and flotation are used in commercial procedures. After that, if the product is produced intracellularly, mechanical disruption of the cells is achieved via chemically or physically such as sonication. Following solvent extraction, chromatography, membrane filtration, adsorption etc. are used for purification of the product. The procedures applied industrially for downstream processing are first developed and tested using in small-scale [42], [44].

2.4.1. Protein Purification

Protein purification is a process that aims to isolate one or more proteins from a mixture of other protein and non-protein portions. usually. Protein purification is crucial for the characterization of a protein's function, to understand the structure and interactions with other molecules. Protein separation frequently based on the differences of size, binding affinity and physicochemical properties of the protein. Strategies for protein purification are as follows:

- Chromatography techniques: size exclusion, immunoaffinity, hydrophobic interaction, affinity and ion exchange; HPLC
- Metal binding-Polyhistidine-tag

The use of affinity tags has a benefit on the purification of recombinant proteins by simplifying the purification process. This method relies on affinity chromatography. Nickel-nitrilotriacetic acid (Ni-NTA) purification system has an extraordinary selectivity and affinity to the proteins tagged with 6 sequential histidines. Imidazole is a part of histidine's side chain. For protein purification, imidazole is responsible for binding to Ni^{2+} atoms. Using free imidazole at high concentrations outcompetes the binding of the histidine imidazole ring. The interaction between the 6xHis tag and Ni^{2+} can be seen in Figure 2.5.

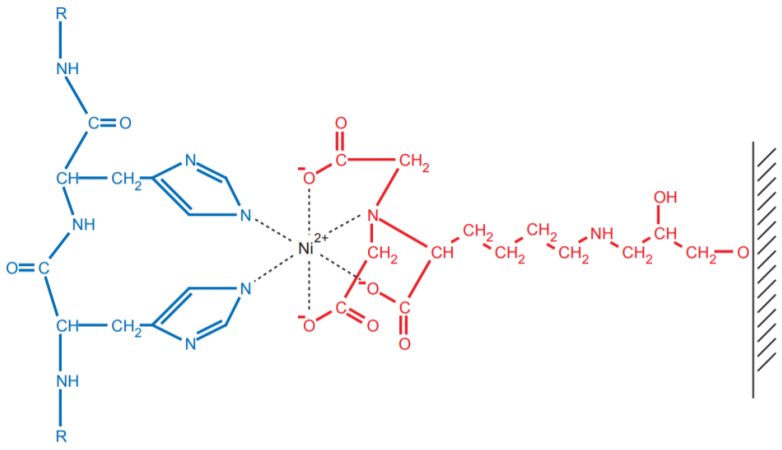


Figure 2.5: Mechanism of interaction between Ni-NTA and a 6xHis-tagged protein [45].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Devices

Laboratory tools and devices that were used during the study are indicated in Table 3.1.

Table 3.1: Laboratory equipment list.

Autoclave	BioTek
Centrifuge	Beckman Coulter
Deep freezers (-80 °C)	Eppendorf
Electrophoresis Power Supply	Bio-Rad
Magnetic Stirrer	WiseStir
Micropipettes	Axygen
Microplate Reader	Tecan
Microplate Shaker Incubator	BioTek- ELx808
pH Meter	Hanna
SDS-PAGE Electrophoresis System	Bio-Rad
Shaking Incubator	N-BioTek
Sonicator	(Bandelin Sonoplus HD2200
Thermal Cycler	Techne
Vortex	BioTek
Water-Bath	Stuart
Bench-Top Bioreactor (3L)	Applikon

3.1.2. Strains, Chemicals and Kits

All chemicals have been supplied from Sigma-Aldrich (St. Louis, Missouri, USA) unless indicated otherwise. BCA Protein Assay Kit has been supplied via Thermo Scientific (Waltham, Massachusetts, USA), Vivaspin® Centrifugal Concentrator (30 kDa) and Syringe Filters (0.45 nm) have been supplied from Sartorius (Göttingen, Germany) and HisTrap and PD-10 columns have been supplied from GE Healthcare (Pittsburgh, Pennsylvania, USA).

Chemically competent *E. coli* TOP10 strain (Invitrogen) was used for the purpose of preparing plasmids and also an expression of lysostaphin. The *p*BAD*Lys* vector has been supplied by Szweda et al. [23] based on an identified lysostaphin gene sequence (GenBank access no. M15686) with the *araBAD* promoter. Recombinant lysostaphin activity was detected by means of the strain *S. aureus* ATCC 29213 (American Type Culture Collection, Manassas, VA). The susceptibility of produced Lysostaphin to *S. aureus* species were tested via *S. aureus* strains (Sa3, Sa16, Sa35) obtained from milk samples with subclinical mastitis from the milk production centers of Kocaeli and neighboring villages between December 2006 and May 2007 were isolated from Gebze Technical University Molecular Biology and Genetics Department Microbiology Laboratory and included in the culture stocks of Gebze Technical University Chemistry Department Biochemistry Laboratory. All the measurements were conducted in triplicates.

3.1.3. Growth Mediums

Arabinose induction auto-induced mediums and their composition ratios are represented in Table 3.2 and Table 3.3. All mediums were prepared by dissolving all indicated components in dH_2O and sterilized via autoclave at 121 °C for 15 mins.

Table 3.2: Composition of arabinose induction growth mediums.

	Name of Growth Medium				
Components (g/L)	**LB**	**ENLB**	**PLB**	**TB***	**2xYT**
Peptone	-	16	10	-	16
Tryptone	10	-	-	12	-
Yeast extract	5	10	5	24	10
NaCl	10	5	10	-	5
KH_2PO_4	-	4	-	-	-
Glycerol (mL)	-	3	-	-	-
*900 mL TB medium was completed to 1L with 10X TB salt solution (0.17 M KH_2PO_4, 0.72 M K_2HPO_4)					

Table 3.3: Composition of auto-induced growth mediums.

	Name of Growth Medium				
Components (g/L)	**SB**	**FM**	**TB**	**LB**	**Std**
Yeast extract	20	5	24	5	5
Tryptone	35	10	12	10	20
NaCl	5	-	-	-	5
Na_2HPO_4	10	7.1	7.1	7.1	6.67
KH_2PO_4	6.8	6.8	6.5	6.5	3
$MgSO_4$	0.15	0.15	0.15	0.14	-
$(NH_4)_2SO_4$	3.3	3.3	3.3	3.3	-
Glucose	0.5	0.5	0.5	0.5	0.5
Glycerol (mL)	-	-	-	-	6

3.1.4. Buffers and Solutions

Buffer A and Buffer B were used for purification of 6xHis-tagged lysostaphin and 20 mM Tris-HCl with 150 mM NaCl buffer was used for the determination of the activity of purified lysostaphin.

3.1.4.1 HisTrap Column Regeneration Buffers and Solutions

Buffers for the regeneration of HisTrap column are listed below:

- EDTA buffer: 500 mM Na_2-EDTA (168.11 g/L) dissolved in dH_2O and the pH value was adjusted to 8.0.
- NaOH solution: dH_2O was added dropwise onto 100 mM NaOH (4 g/L).
- $NiSO_4.6H_2O$ solution: 50 mM $NiSO_4.6H_2O$ (7.74 g/L) was dissolved in dH_2O.
- 20 % (v/v) ethanol solution: 50 mL absolute ethanol was mixed with 950 mL dH_2O.

3.1.4.2. Protein Purification Buffers

Purification of proteins from bacterial lysate was performed by using HisTrap protein purification columns. Buffers used in protein purification are given below:

- Buffer A: 20 mM Tris-HCl (3.64 g/L), 500 mM NaCl (29.22 g/L) and 30 mM Imidazole (2.04 g/L) were dissolved in dH_2O and the pH value was set to 7.4 by using HCl.
- Buffer B: 20 mM Tris-HCl (3.64 g/L), 500 mM NaCl (29.22 g/L) and 30 mM Imidazole (34.04 g/L) were dissolved in dH_2O and the pH value was set to 7.4 by using HCl.

Buffer A with 100 mM Imidazole (50 mL): 42.50 mL Buffer A and 7.50 mL Buffer B were mixed. Buffer A with 200 mM Imidazole (50 mL): 32 mL Buffer A and 18 mL Buffer B were mixed. Buffer A with 400 mM Imidazole (50 mL): 10.50 mL Buffer A and 39.50 mL Buffer B were mixed.

3.1.4.3. Enzyme Activity Assay Buffer

20 mM Tris-HCl (3.64 g/L) and 150 mM NaCl (8.77 g/L) were dissolved in dH_2O and the pH value was adjusted to 7.4.

3.2. Methods

3.2.1. Transformation of *p*BAD*Lys* vector into *E. coli* TOP10 competent cells

The *p*BAD plasmid containing the *lysostaphin* gene was transformed into chemically competent *E. coli* TOP10 cells via heat shock methodology [46]. Competent cell tubes stored at -80 °C and plasmid store at -20 °C were thawed on the ice for 10 mins. 1-5 µL containing 1-100 ng of *p*BAD*Lys* was added into the cell mixture and flicked 4 to 5 times to mix plasmid and cells. The mixture was placed on the ice for 30 mins then heat shocked at 42 °C for 30 secs. After that, it was placed on ice for 5 mins. 300 µL of SOC medium was introduced to the mixture and incubated at 37 °C at 230 rpm for 1 hour. 100 µL of the mixture was spread onto the LB agar plate (agar 20 g/L, yeast extract 5 g/L, peptone 10 g/L, NaCl 10 g/L) which contains ampicillin (Amp 100 µg/L) and incubated for 14-16 hours at 37 °C. Individual colonies were picked and inoculated into overnight cultures for optimization of the expression conditions.

3.2.2. Preparation of glycerol stock

A single colony of transformed *E. coli* TOP 10 containing *p*BAD*Lys* was inoculated into 5 mL LB broth containing Amp (100 µg/L) and incubated at 37 °C, at 180 rpm overnight. 250 µL of the overnight culture mixed with 50 % (v/v) sterilized glycerol solution and then stored at -80 °C as a stock.

3.2.3. Optimization of Recombinant Lysostaphin Expression Level on a Shake Flask

High-cell-density fermentations were performed on various growth medium at different temperatures for the purpose of obtaining larger quantities of active and soluble lysostaphin in *E. coli* TOP 10. Hence, in the content of this study two different induction systems, auto-induction and arabinose induction were applied under the control of the *araBAD* promoter.

3.2.3.1. Preparation of pre-culture

LB (5 mL) broth containing 100 µg/mL Amp was inoculated with a single transformed *E. coli* TOP 10 colony containing *p*BAD*Lys* and incubated for 14-16 hours at 180 rpm and at a temperature of 37 °C.

3.2.3.2. Expression of Lysostaphin via Arabinose Induction Medium

250 µL of the overnight transformed *E. coli* TOP10 culture was pipetted into the 250 mL baffled flasks including 50 mL five different mediums with 100 µg/mL Amp at pH 7.0. The mediums used in the arabinose induction strategy (LB, PLB, TB, 2xYT and ENLB) were given in Table.3.2. All cultures were incubated at a temperature of 37 °C, a 230 rpm till the OD_{600} figures reached to 0.2-0.4. When OD_{600} was reached to the desired level, the filter-sterilized arabinose solution was introduced to a concentration of 0.2 % (v/v). After arabinose induction, the cultures were incubated at varying temperatures at 37 °C, at 30 °C and at 25 °C for 4 hours. Samples (1 mL) were collected before and after induction (2 h, 4 h) in order to follow cell growth profile and lysostaphin expression level via detecting OD_{600}, performing SDS-PAGE analysis respectively. Product yield on biomass (Y_{PX} (mg/g), mg produced yield on produced biomass) was calculated based on equation (3.1). After completion of the incubation process, the cells were separated from the fermentation medium via centrifugation at 4 °C, 4500 rpm for 15 mins. The pH value of the supernatant part was measured after centrifugation and pH changes were determined

after the incubation period. The pellet parts were kept at -20 °C for further experiments. All experiments were carried out in triplicate and *E. coli* TOP10 cells with lysostaphin-free *p*BAD plasmid were used as a control.

3.2.3.3. Expression of Lysostaphin via Auto-induced Medium

The induction time optimization was performed for auto-induced mediums by using FM auto-induced medium up to 48 h. After detecting the optimum expression level through time, the pH of all auto-induced mediums that are LB-AI, TB-AI, SB, FM and Std. represented in Table 3.3 was adjusted to 7.0 before inoculation. Following, auto-induced mediums (50 mL on a 250 mL baffled flasks) consisting of 100 µg/mL Amp and 0.1 % (v/v) filter-sterilized arabinose solution were inoculated with 250 µL of the transformed *E. coli* TOP10 pre-culture and incubated at 37 °C, 30 °C and 25 °C, 230 rpm for 24h seperately. Samples (1 mL) were collected at 16 h and 24 h of the fermentation in order to follow cell growth profile through OD_{600} values, detect lysostaphin expression level via performing SDS-PAGE analysis and calculate (Y_{PX}) through Equation (3.1). Once the incubation has been completed, the cells were separated from the medium by means of centrifugation step at 4 °C, 4500 rpm through 15 mins. The pH change was detected by measuring the final pH of the supernatant part after centrifugation. The pellet parts were kept at -20 °C for further analysis. All of the experiments were carried out in triplicate and *E. coli* TOP10 cells with lysostaphin-free *p*BAD plasmid were used as a control.

3.2.4. Expression of Recombinant Lysostaphin on a Bench-Top Bioreactor

Recombinant lysostaphin was expressed on a 3 L Stirred Tank Bioreactor that was equipped with a temperature probe, a sparger, a DO probe and a pH probe. The optimum expression temperature and fermentation medium were identified as 30 °C, FM on the shake flask studies and implemented into the scale-up studies on 3 L jacked Bench-Top Bioreactor. The inoculum for the fermenter was initiated by transferring the 5 mL overnight *E. coli* TOP10 pre-cultures into 25 mL LB broth with 100 µg/mL Ampicillin containing shake flasks and incubating them overnight at 37 °C for 230 rpm. 10 % of 2 L auto-induced FM consisting of 100 µg/mL Amp and 0.1 % (v/v) filter-sterilized arabinose solution was inoculated with inoculum and incubated for 48 h. Glucose and glycerol composition of the auto-induced FM and the agitation speed were varied between low (400 rpm) and high (800 pm) stirring speed to observe the effect of carbon source and stirring speed on enzyme activity and yield. Three different batch operation was performed; the composition of the fermentation medium and the fermentation parameters were shown at Table.3.4 and Table.3.5 respectively. The pH was maintained through the addition of 3 M KOH and 3 M H_3PO_4. 1 mL samples were collected at 0, 2, 6, 16, 24 and 48 hours of the fermentation to measure dcw and OD_{600}, thereby calculate Y_{PX} with Equation (3.1) and to perform SDS-PAGE analysis. The pellet part of the fermentation broth was separated by centrifugation at 4500 rpm (30 mins) and kept at -20 °C for future experiments.

$$\mathrm{YPX} = \frac{\textbf{mg produced lysostaphin (mg/L)}}{\textbf{g cells produced (g dcw/L)}} \tag{3.1}$$

Table 3.4: Composition of FM used in different batch operations.

Composition of the Fermentation Medium (g/L)			
Component	**Batch 1**	**Batch 2**	**Batch 3**
Yeast extract	5	5	5
Tryptone	10	10	10
$Na_2HPO_4.7H_20$	10	10	10
KH_2PO_4	6.80	6.80	6.80
$MgSO_4$	0.15	0.15	0.15
$(NH_4)_2SO_4$	3.30	3.30	3.30
Glycerol	-	6	6
Glucose	1.50	1.50	1.50

Table 3.5: Fermentation parameters of the different batch operations.

Fermentation parameters	**Batch 1**	**Batch 2**	**Batch 3**
DO (%)	-	-	-
pH	7.0	7.0	7.0
Stirring speed (rpm)	800	800	400
Air Flow (vvm)	1	1	1
Temperature (°C)	30	30	30

3.2.5. Purification of Recombinant Lysostaphin

6xHis-tagged Lys enzyme was purified via 5 mL His-Trap HP column immobilized metal affinity chromatography (IMAC) and 30 to 500 mM imidazole gradient was applied during purification.

3.2.5.1. Preparation of the His-Trap Column

The 5 mL HisTrap HP column was regenerated through the following washing steps:

1) 50 mL of dH_2O
2) 50 mL of EDTA buffer (500 mM Na_2-EDTA, pH 8.0)
3) 50 mL of NaOH (100 mM)
4) 50 mL of dH_2O
5) 20 mL of $NiSO_4.6H_2O$ (50 mM)
6) 10 mL of dH_2O

Lastly, the column was equilibrated with 50 mL of Buffer A and stored with 20 % (v/v) ethanol after each purification.

3.2.5.2. Purification via His-Trap Column

The cell pellets (~1 g) were resuspended on lysis buffer (30 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.40, 1.0 mg/mL lysozyme) and incubated on the ice at 45° for 30 mins. The suspension was placed on ice and it was sonicated with a 10-seconds blasting followed by a 10-seconds cooling through 10 cycles via ultrasonication. Following the ultrasonication process, the cells were separated in centrifuge (at 11,000 rpm, for 1 h, at 4 °C) in order to the crude extract and the lysate was filtered through the 0.45 nm filter, then the 6xHis-tagged lysostaphin was purified with optimized TAGZyme purification system (QIAGEN) by eluting with buffer A which embodies varying concentrations of imidazole (100 mM, 200 mM, 400mM, and 500 mM). The obtained fractions were analyzed by SDS-PAGE to determine pure lysostaphin fractions which were then pooled and diluted to 50 mL by addition of 20 mM of Tris-HCl pH 7.40. Protein samples were concentrated with ultracentrifuge tubes (Vivaspin® centrifugal concentrator, 30 kDa). The buffer was exchanged to Tris-HCl (20 mM, pH 7.0) and imidazole removed using a PD-10 desalting column at 4 °C and the sample was kept at a temperature of 4 °C until the next analysis [47].

3.2.6. Determination of Recombinant Lysostaphin Concentration

The protein concentration of each sample was determined by following the instructions of the Thermo Scientific BCA Protein Assay Kit which containing bovine serum albumin as a standard [48].

3.2.7. Lysostaphin Activity Assay

For the purpose of analyzing the bacteriolytic effectiveness of lysostaphin via spectrophotometric measurement, slight changes were applied to a previously reported method [49]. Briefly, overnight *S. aureus* ATCC 29213 cell culture was centrifuged and the cell pellets were provided being resuspended in the activity measurement buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.40) to adjust OD_{600} to 0.5. An aliquot of the appropriate dilution of recombinant lysostaphin solution was added to *S. aureus* ATCC 29213 suspension and decrease in the OD of cells at 600 nm after 10 mins incubation at 37 °C was recorded. Cell suspension incubated with 20 mM Tris-HCl pH 7.40 and 150 mM NaCl buffer was used as a negative control. One unit of lysostaphin activity was defined as the amount of enzyme causing a 50 % reduction in turbidity of the 200 mL reaction volume within 10 mins at 37 °C in a 96-well microtiter plate.

3.2.8. Determination of a Storage Stability

The effects of storage time and temperature on purified enzyme stability were determined by incubating the aliquots of purified lysostaphin in 20 mM Tris-HCl pH 7.40 at four different temperatures of -80 °C, -20 °C, 4 °C, and 25 °C. Measurements were carried out at the 1^{st},15^{th},30^{th},45^{th} and 70^{th} days of the incubation using the method of standard assay which was previously explained. The relative activities were calculated with 100 % acceptance on the lysostaphin's activity on the first day.

3.2.9. Cost Analysis of Lysostaphin

The production cost for one batch of recombinant lysostaphin was analyzed with the Equation (3.3-3.6) which includes the cost for components contained by the medium ($Cost_{cm}$), reagents ($Cost_{rea}$), operational expenses ($Cost_{op}$) and the costs for labor ($Cost_{lab}$) with minor changes to the study described by Osma et al [50].

$$Cost\ cm = \sum(\text{cost of medium components}) \quad (3.3)$$

$$Cost\ rea = \sum(\text{cost of medium reagents}) \quad (3.4)$$

$$Cost\ op = \frac{\text{Ei x Dmax}}{\text{Capi}} \quad (3.5)$$

E_i is the energy consumption of incubator, where D_{max} stands for maximum incubation duration and Cap_i is the incubator capacity.

$$Cost\ lab = \frac{\text{Sm x tb}}{160} \quad (3.6)$$

The labor expenses were estimated based on 500 EUR/month (160 hours per month). Where S_m=monthly salary, t_b= maximum time required for one batch.

The cost of lysostaphin production ($Cost_{Lys}$) was calculated on the basis of EURO per 1000 units of respective enzyme activity with maximum enzyme activity ($A_{lysostaphin}$) and volume of enzyme production ($V_{lysostaphin}$) as indicated in Equation (3.7) [51].

$$Cost\ Lys = \frac{Costcm + Costrea + Costop + Costlab}{\text{Alysostaphin x Vlysostaphin}} \quad (3.7)$$

3.2.9. Determination of Antimicrobial Properties of Lysostaphin

The antimicrobial properties of recombinant lysostaphin were tested against commercially available *S. aureus* species (*S. aureus* ATCC 29213 and *S. aureus* ATCC 25233) and national isolates *S. aureus* strains (Sa3, Sa3-1, Sa16, Sa35) obtained from milk samples with subclinical mastitis and included in the culture stocks of Gebze Technical University Chemistry Department Biochemistry Laboratory.

The *S. aureus* species from the culture stocks of Gebze Technical University Chemistry Department Biochemistry Laboratory were selected qualitatively by using Mannitol Salt Agar (MSA) containing 5.0 g/L of Pancreatic Digest of Casein, 5 g/L of Peptic Digest of Animal Tissue, 1.0 g/L of Beef Extract, 75 g/L of Sodium Chloride, 10 g/L of D-Mannitol, 0.25 of g/L Phenol Red and 15 g/L of agar as described earlier by Freeman et al., 1989 [52]. The plates were incubated at 37 ºC for 24 h and *S. aureus* species were detected by following the color change from red to yellow. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25233) were used as a control.

3.2.9.1. Determination of IC_{50}

The half-maximal inhibitory concentration (IC_{50}) of lysostaphin was identified for each strain. The procedure employed by Schindler and Schuhardt [53] was applied with slight changes. Overnight cultures (18 h, 37 ºC, 180 rpm) of *S. aureus* ATCC 29213, *S. aureus* ATCC 25233 and isolated *S. aureus* species (Sa3, Sa16, Sa35) grown on tryptic soy broth (TSB, 17.0 g/L Tryptone (Pancreatic Digest of Casein), 3.0 g/L Soytone (Peptic Digest of Soybean), 2.5 g/L Glucose, 5.0 g/L NaCl, 2.5 g/L K_2HPO_4) culture were centrifuged and cells were washed with activity measurement buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.40) once. Washed cells were resuspended with activity measurement buffer and the OD_{600} values were set to ~0.85. IC_{50} concentrations were assayed by adding 2-fold dilutions of 0.9 µg/mL lysostaphin to each well. OD_{600} was followed at 20-sec intervals during 10 mins duration. The linear regression method was used in order to calculate IC_{50} concentrations for each tested strain [54].

3.2.9.2. Disk Diffusion Method

Different concentrations of recombinant lysostaphin susceptibility were tested against various *S. aureus* strains through a procedure of NCCLS with slight modifications [55]. *S. aureus* ATCC 29213, *S. aureus* ATCC 25233 and *S. aureus* strains (Sa3, Sa16, Sa35) obtained from Biochemistry Laboratory stocks of Gebze Technical University were grown on Muller-Hinton broth (2.0 g/L beef infusion solids, 17.5 g/L casein hydrolysate, 1.5 g/L starch) at 37 ºC, 180 rpm overnight. After incubation, the cells were diluted with Tris-HCl (20 mM, pH 7.5) buffer solution containing NaCl (150 mM) to ~10^7 CFU. 100 µl of diluted *S. aureus* strains were plated on Mueller-Hinton agar plates. Sterilized wells were opened to plates and 50 µl of serially diluted lysostaphin enzyme (0.34, 0.17, 0.085, 0.425, 0.2125, 0.010625 µg/mL), 10 µg Ampicillin as a control agent was dropped to the wells. The plates were incubated for 16 hours at 37 ºC and the diameters of the zone around the discs were measured. The % yield of degradation for lysostaphin dilutions in comparison to the Amp were calculated based on equation (3.8).

$$\%\ \text{Yield of Degradation} = \frac{\text{Zone dimater due to lysostaphin (mm)}}{\text{Zone diamter due to 10 µg Amp (mm)}} \text{x}100 \qquad (3.8)$$

4. RESULTS AND DISCUSSIONS

4.1. Transformation of a *p*BAD*Lys* vector into *E. coli* TOP10 competent cells

Transformation of *p*BAD*Lys* vector to *E. coli* TOP10 competent cells was successfully done. Bacterial colonies on the selection plates containing ampicillin (100 µg/mL) were seen in Figure 4.1.

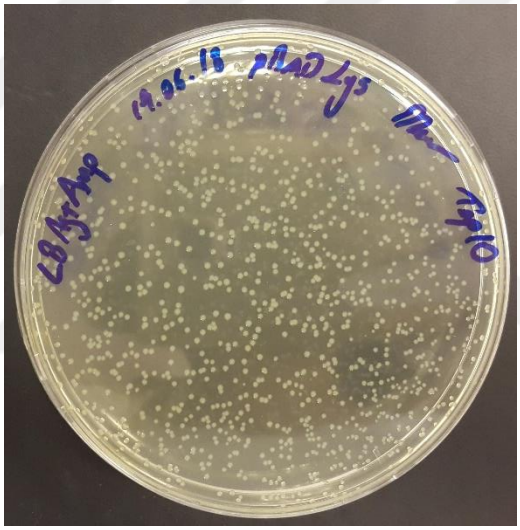


Figure 4.1: Colonies of transformed *E. coli* with *p*BAD*Lys* vector after transformation.

4.2. Expression and Purification of Lysostaphin

The recombinant lysostaphin expression was conducted under the control of an *araBAD* promoter with the addition of a filter-sterilized arabinose solution. The production of the recombinant protein with arabinose induction resulted in an amount of approximately 42 % of total cell proteins in *E. coli* TOP10 using LB medium. The C-terminal 6xHis-tag allowed purification of the protein using immobilized metal affinity chromatography (IMAC) and the expected size of lysostaphin protein (~25 kDa) was detected on a Coomassie-stained gel (Figure 4.2). The imidazole gradient from 10 to 500 mM was applied to optimize the purification method. As confirmed by SDS-PAGE, the purity of the lysostaphin is adequate for further analysis (Figure 4.2, Lane E3–5). The fractions containing the pure protein were pooled, concentrated and obtained lysostaphin yield was 9 mg/L culture (LB) with over 95 % purity based on SDS- PAGE analysis. The purity and the quantity of recombinant lysostaphin from this expression were enough to allow further characterization.

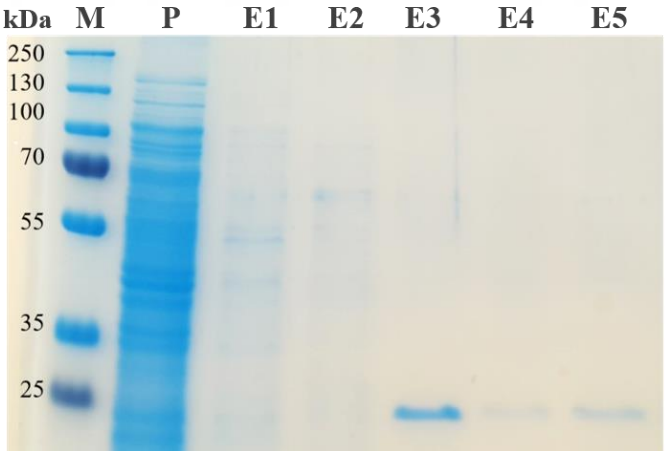


Figure 4.2: SDS-PAGE Analysis of Purification of Lysostaphin; M: Marker, P: Pellet after centrifugation, E1: Buffer A with 10 mM imidazole, E2: Buffer A with 100 mM imidazole, E3: Buffer A with 200 mM imidazole, E4: Buffer A with 400 mM imidazole, E5: Buffer A with 500 mM imidazole.

4.3. Optimization of Recombinant Lysostaphin Expression Level on a Shake Flask

On this step of the experiment, the time required for optimum fermentation was identified for the auto-induction first. The protein that was expressed in the FM auto-induced medium has reached the optimum value at the 24th hour of reaction (Figure 4.3). That being the case, the incubation time was determined 24 h for further experiments.

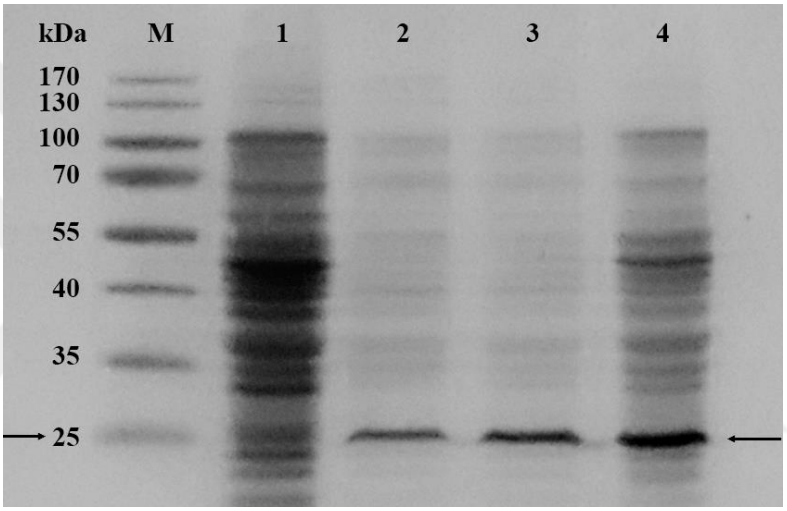
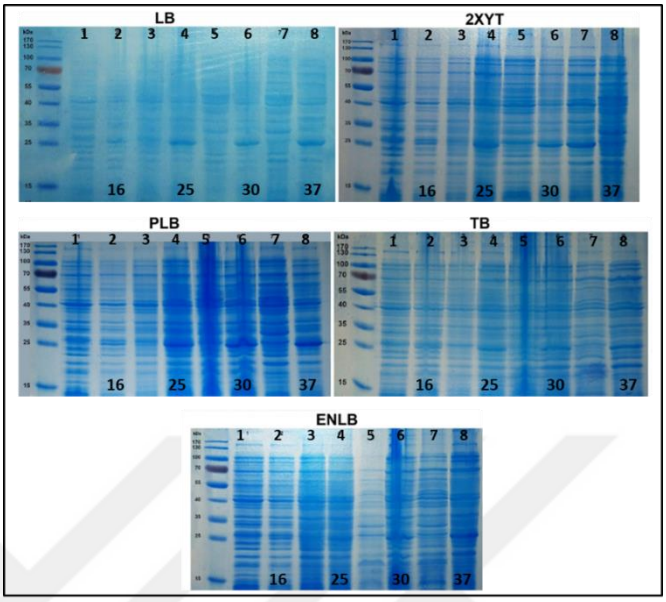


Figure 4.3: SDS-PAGE analysis of auto induced FM at different time points of fermentation. M: Marker; 1: Cell lysate without arabinose induction, 24 h; 2: Cell lysate by inducing with arabinose, 16 h; 3: Cell lysate by inducing with arabinose, 24 h; 4: Cell lysate by inducing with arabinose, 48 h.

The optimum temperature values settled for auto-induction and arabinose induction methods. Detected lysostaphin amount, dry cell weights, activity and specific activity of purified lysostaphin incubated in arabinose induction and auto-induced mediums were given in Table 4.1 and Table 4.2 respectively. The total amount of produced lysostaphin was observed to upon the temperature was increased from 25 °C to 37 °C (Figure 4.4).

a)



b)

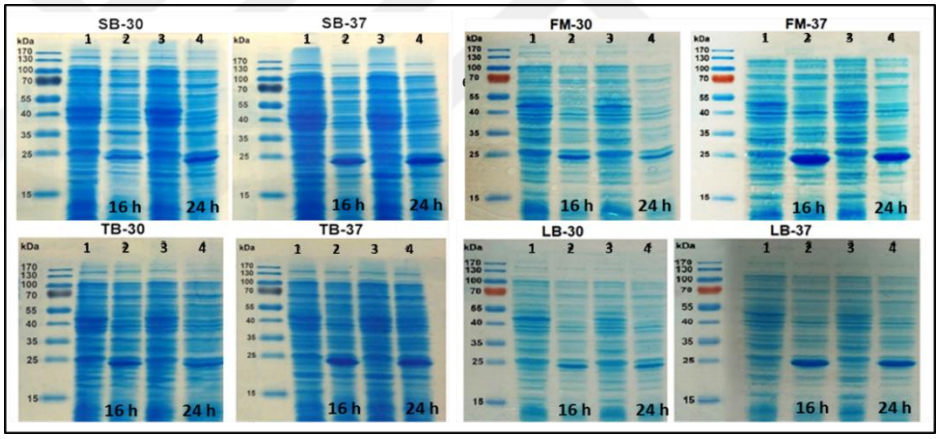


Figure 4.4: SDS PAGE Analysis a) 0.2 % Arabinose Induction Mediums: 1) 16 °C, before induction 2) 16 °C, 4h arabinose induction, 3) 25 °C, before induction, 4) 25 °C, 4h arabinose induction, 5) 30 °C, before induction, 6) 30 °C, 4h arabinose induction, 7) 37 °C, before induction, 8) 37 °C, 4h arabinose induction. b) Auto Induced Mediums: 1) Control (Without arabinose), 16 h 2) *p*BAD*Lys E. coli* TOP10, 16 h 3) Control (Without arabinose), 24 h 4) *p*BAD*Lys E. coli* TOP10, 24 h.

Table 4.1: Dry cell weight (g/L), Y_{PX} (mg/g), Concentration of Lysostaphin (mg/L), Activity (U/L) and Specific Activity (U/mg) of lysostaphin produced by using induction with 0.2 % (v/v) arabinose for 4 hours at 37, 30, 25 °C.

Temperatures (°C)	**Arabinose induction mediums**	**Dry Cell Weight (g/L)**	**Y_{PX} (mg/g)**	**Lys Concentration (mg/L)**	**Activity (U/L)**	**Specific Activity (U/mg)**
37	LB	0.2 ± 0.0	51.9 ± 0.9	9.6 ± 1.6	284.1 ± 0.3	29.6 ± 2.2
	TB	0.3 ± 0.0	128.8 ± 1.3	316.3 ± 20.26	676.4 ± 0.6	5.3 ± 1.9
	PLB	0.3 ± 0.0	657.6 ± 4.6	182.8 ± 12.48	1005.4 ± 1.1	5.5 ± 2.9
	ENLB	0.7 ± 0.1	244.6 ± 2.4	172.9 ± 7.1	1521.5 ± 1.9	8.8 ± 1.9
	2xYT	0.1 ± 0.0	136.8 ± 1.8	171.1 ± 2.2	1762.3 ± 2.4	10.3 ± 3.6
30	LB	0.3 ± 0.0	7.1 ± 0.1	2.2 ± 0.9	88.4 ± 1.2	40.2 ± 2.6
	TB	1.1 ± 0.0	20.7 ± 0.3	24.3 ± 2.6	396.1 ± 0.5	16.3 ± 0.9
	PLB	0.3 ± 0.0	18.6 ± 0.6	6.5 ± 4.1	525.8 ±0.8	80.9 ± 4.8
	ENLB	0.6 ± 0.0	7.2 ± 0.7	4.5 ± 0.7	477 ± 1.1	106.0 ± 7.2
	2xYT	0.5 ± 0.01	11.6 ± 0.6	5.4 ± 1.7	392.6 ± 0.1	72.7 ± 4.5
25	LB	0.2 ± 0.1	215.4 ± 1.5	34.9 ± 1.1	876.1 ± 1.0	25.1 ± 0.7
	TB	0.3 ± 0.0	12.7 ± 1.0	3.6 ± 0.2	303.8 ± 0.2	84.4 ± 5.8
	PLB	0.2 ± 0.0	10.4 ± 0.4	2.6 ± 0.8	141.7 ± 0.2	54.5 ± 4.6
	ENLB	0.2 ± 0.0	33.7 ± 0.6	8.2 ± 0.0	866.7 ± 0.3	105.7 ± 6.6
	2xYT	0.3 ± 0.0	24.5 ± 0.7	7.9 ± 0.0	966.2 ± 0.7	122.3 ± 8.2

Table 4.2: Dry cell weight (g/L), Y_{PX} (mg/g), Concentration of Lysostaphin (mg/L), Activity (U/L) and Specific Activity (U/mg) of lysostaphin produced by using auto-induction with 0.1 % (v/v) arabinose for 24 hours at 37, 30, 25 °C.

Temperatures (°C)	Auto-induction mediums	Dry Cell Weight (g/L)	Y_{PX} (mg/g)	Lys Concentration (mg/L)	Activity (U/L)	Specific Activity (U/mg)
37	LB-AI	0.6 ± 0.0	116.4 ± 2.6	65.9 ± 2.5	388.8 ± 8.8	5.9 ± 0.4
	TB-AI	1.1 ± 0.1	31.4 ± 2.4	35.7 ± 8.2	207.1 ± 6.2	5.8 ± 0.3
	SB	0.7 ± 0.0	7.2 ± 0.4	5.3 ± 0.6	14.3 ± 0.6	2.7 ± 0.1
	FM	0.7.± 0.0	137.0 ± 0.3	90.4 ± 0.1	307.4 ± 3.2	3.4 ± 0.1
	Std	1.4 ± 0.1	39.9 ± 1.6	56.9 ± 3.9	187.8 ± 1.4	3.3 ± 0.0
30	LB-AI	0.7 ± 0.0	54.5 ± 1.7	35.2 ± 8.6	109.1 ± 3.0	3.1 ± 0.1
	TB-AI	0.9 ± 0.0	18.8 ± 0.6	17.4 ± 0.1	116.6 ± 2.4	6.7 ± 0.1
	SB	0.7 ± 0.0	33.7 ± 2.4	22.5 ± 2.6	76.5 ± 0.9	3.4 ± 0.2
	FM	0.5 ± 0.1	401.8 ± 3.5	200.1 ± 3.1	1140.6 ± 3.7	5.7 ± 0.2
	Std	2.2 ± 0.1	21.0 ± 1.7	46.5 ± 2.1	88.4 ± 1.9	1.9 ± 0.0
25	LB-AI	0.5 ± 0.1	90.7 ± 1.3	43.9 ± 6.2	127.3 ± 2.9	2.9 ± 0.1
	TB-AI	0.9 ± 0.0	24.8 ± 1.3	21.7 ± 0.1	56.4 ± 1.2	2.6 ± 0.1
	SB	0.6 ± 0.0	38.8 ± 0.9	23.2 ± 2.6	234.3 ± 1.6	10.1 ± 0.4
	FM	0.6 ± 0.0	13.7 ± 1.0	8.7 ± 1.1	52.2 ± 2.1	6.0 ± 0.2
	Std	1.8 ± 0.1	21.3 ± 0.9	39.0 ± 3.3	101.4 ± 1.4	2.6 ± 0.1

With respect to the induction of arabinose, varying combinations of yeast extract and peptones have been tested because of the easy preparation and availability preparation of essential growth factors and vitamins. Therefore, five different types of growth medium were used in the arabinose induction system to be able to achieve higher efficiency of enzyme production. The amount of glycerol and yeast extract and also types of the peptone source were varied between the five different mediums where the effect of different carbon and nitrogen components on the yield of the lysostaphin production was tested. Mineral salts K_2HPO_4 and KH_2PO_4 were shown to be outstanding elements of the medium. Mineral salts had a significant contribution to the increasing enzyme yield. With respect to the TB medium, the expressed enzyme yield was much higher compared to the LB medium. This result can be explained as the TB medium contained a variety of mineral salts and carbon sources. As a conclusion, the most productive enzyme expression was

achieved as 316.3 mg/L with 676.4 U/L activity on TB Medium induced with 0.2 % (v/v) arabinose at 37 °C for 4 h (Table 4.1).

The auto-induction system is based on the function of *araBAD* promoter that has been used for the expression of soluble and active recombinant proteins in *E. coli* [56]–[58]. The auto-induction medium consisted of well-proportioned carbon sources such as glycerol and glucose, and other necessary nutrients for the host's metabolism. This facilitated the higher cell densities and higher yields of a target protein in comparison to procedures conducted through the manual addition of an inducer in the mid to late log phase [59], [60]. Additionally, the desired protein was produced automatically without any need for monitoring the density value of the cell or adding an inducer at specific time intervals. The auto-induction system is known as being an alternative way to achieve high-throughput growth of bacterial cells and the higher expression of recombinant proteins. However, there are no reported studies where the auto-induction method was used to enhance the production of lysostaphin. Outcomes of the experiments has indicated that the use of FM auto-induction medium determined the most effective level of soluble and active lysostaphin at a temperature of 30 °C and the highest Y_{PX} compared to the other auto-induction medium (200.1 mg/L lysostaphin after 24 h incubation with a higher enzyme activity of 1140.6 U/L) (Table 4.2).

More cells and less protein yield than other cultures were obtained via LB and TB-AI medium containing rich carbon sources like glycerol and yeast extract at 25 °C (Table 4.1, Table 4.2). In addition, the trace element-rich composition of the auto-induction medium has significantly changed the amount of soluble lysostaphin production in the cytoplasm. Due to this reason, the activity of lysostaphin expressed in the auto-induced system was higher than the arabinose induced system. On the other hand, enriched trace elements in the auto-induction medium (FM), caused a reduction in the specific activity. Whereas the yield of purified protein in small-scale production was demonstrated to be only between 10 and 20 mg/L in previous studies, [18], [23], [33] this study shows that the arabinose induction system can provide over 10-fold increase in protein yield than the best-reported result. The results presented that the increased temperature values have evidently changed the production level of active lysostaphin in both systems. The most probable reason for observing less activity in higher protein yields is due to the fact that

enzyme folding is restricted by the rate of protein expression [32]. As understood from the Y_{PX} values, particularly for those which were incubated at 30°C and 37 °C, increased biomass production did not lead to higher lysostaphin expressions.

The pH values of all cultures were adjusted to 7.0 at the beginning of the reaction and the final pH values were measured from supernatant parts of the cultures after harvesting the cells. The recorded pH values were represented in Figure 4.5.

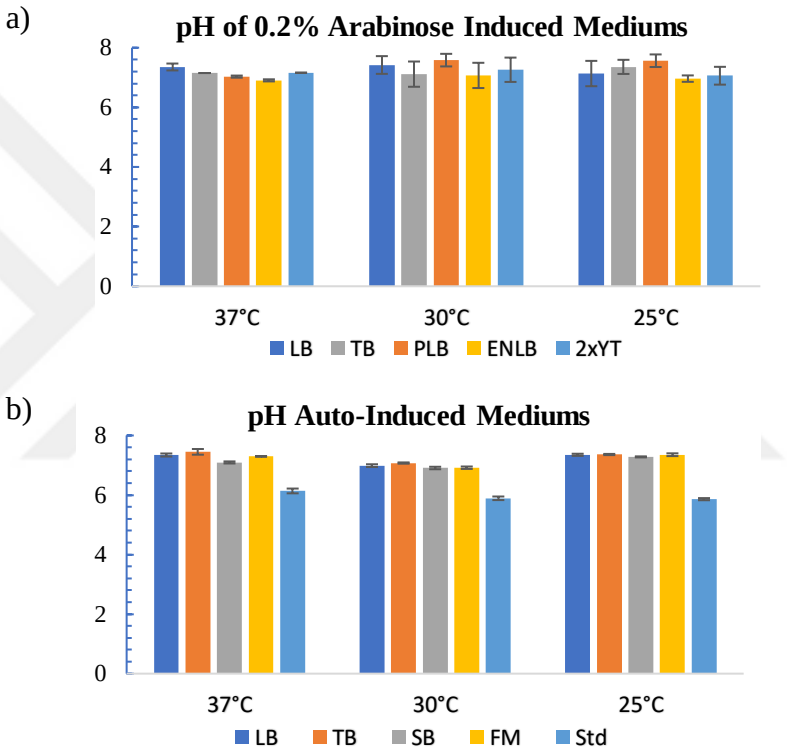


Figure 4.5: Final pH values of cultures: a) Represents the final pH of the 0.2 % (v/v) arabinose induced cultures. b) Represents the final pH of the auto-induced with 0.1 % (v/v) arabinose cultures.

According to the measurements, while the pH of arabinose induced cultures not significantly changed, especially the pH of Studier's medium was decreased. Since the enzyme activity was conserved in neutral pH values, the pH of the fermentation medium was set to 7.0 in the bioreactor studies [32].

4.4. Expression of Recombinant Lysostaphin on a Bench-Top Bioreactor

The scale-up studies were performed on 3-L Bench Top STR and the recombinant lysostaphin was produced through *E. coli* TOP10 culturing successfully. In the optimization of shake flask studies, the temperature and the type of induction system medium where the highest amount of recombinant lysostaphin was identified as 30 °C and auto-induced FM medium, respectively. The cell growth profiles containing OD_{600} and g dcw/L of different batch operations represented in Figure 4.6. The highest cell density of transformed *E. coli* TOP10 culture was detected in Batch 2 run in auto-induced FM medium containing 6 mL/L glycerol and 1.5 g/L glucose at high stirring speed (800 rpm). On the other hand, the obtained cell density was lower in Batch 3 with FM medium containing the same amount of glycerol and glucose but incubated at a low stirring speed (400 rpm). The lowest cell concentration was measured in Batch 1 at a high mixing speed and FM medium without glycerol. In all batch operations, the cell concentrations were maintained constant after approximately 24 hours due to the depletion of carbon source in the fermentation medium.

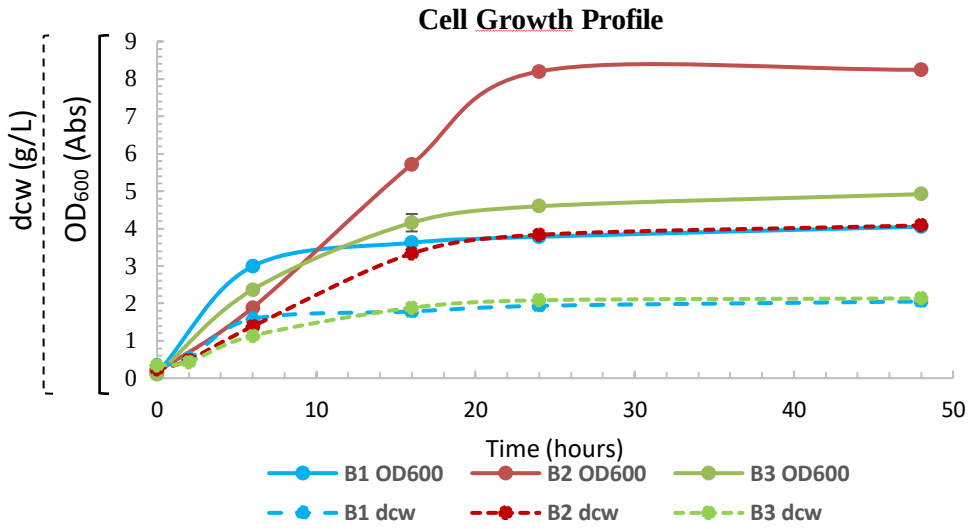


Figure 4.6: Cell growth profile of Batch 1 (blue line OD_{600}, blue dashed line dcw), Batch 2 (red line OD_{600}, red dashed line dcw) and Batch 3 (green line OD_{600}, green dashed line dcw).

Expression levels of lysostaphin enzyme through different batch operations were analyzed by SDS-PAGE and represented in Figure 4.7. The amount of protein expression was increased from Batch 1 to Batch 3. When all operations are examined, it is observed that lysostaphin expression began after the second hour of the fermentation.

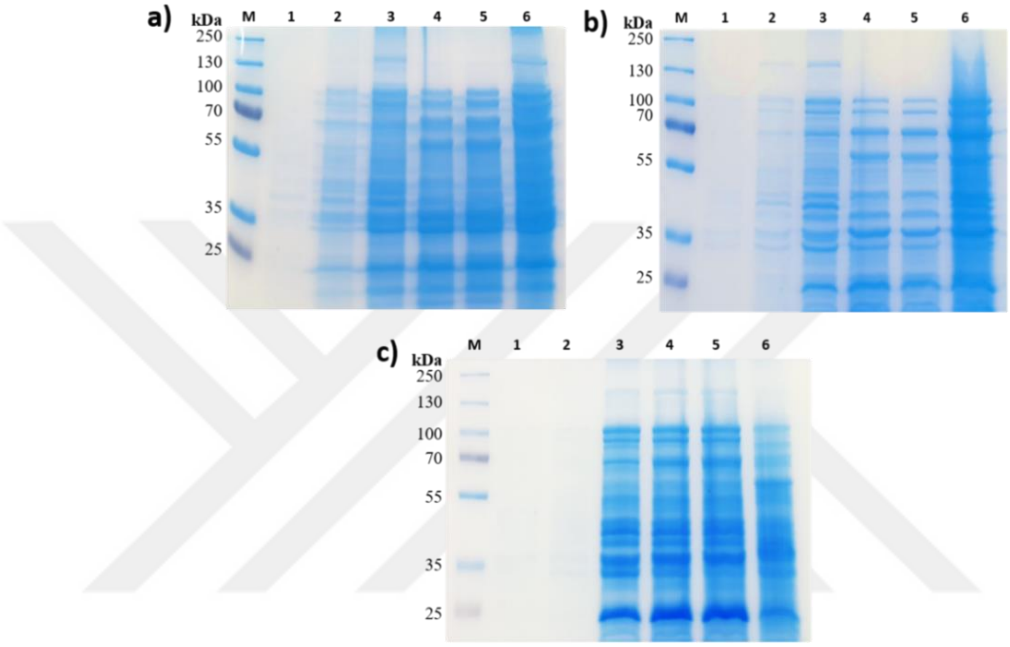


Figure 4.7: SDS PAGE analysis of a) Batch 1, b) Batch 2 and c) Batch 3 during expression at different time points; M: Marker, 1: pellet at 0 h, 2: pellet at 2 h, 3: pellet at 6 h, 4: pellet at 16 h, 5: pellet at 24 h, 6: pellet at 48 h.

The calculated activity (U/L), specific activity (U/mg), enzyme concentration (mg/L) and $Y_{P/X}$ (mg/g) of recombinant lysostaphin were given in Table 4.3. When the highest enzyme amount (~184 mg/L) and in Batch 3 in the presence of FM containing both glycerol and glucose as a carbon source and run at low stirring speed (400 rpm), it decreased thereby applying the high agitation speed (Batch 2). Additionally, the 2-folds decreasing the mechanical stirrer speed enhanced 2-folds the product yield on biomass. On the other hand, the specific activity of protein reached the highest value via Batch 1 (22.4 U/mg) that composed of glucose as a carbon source and at 800 rpm. With this respect, the specific activity of lysostaphin reduced in the supplementation of both glucose and glycerol as a carbon source as reported by Chan et al., 2002 [61].

Table 4.3: Activity, specific activity, lysostaphin concentration and product yield on the biomass of Batch 1, Batch 2, Batch 3.

	Batch 1	Batch 2	Batch 3
Activity (U/L)	2211.5 ± 1.2	2149.9 ± 0.5	3385.3 ± 0.7
Specific Activity (U/mg)	22.4 ± 0.3	12.8 ± 0.0	18.4 ± 0.4
Lys Concentration (mg/L)	98.6 ± 0.3	168.3 ±0.1	184.1 ± 0.4
$Y_{P/X}$ (mg/g)	48.1 ± 0.3	41.2 ± 0.4	86.3 ± 0.2

4.5. Determination of Storage Stability of Lysostaphin

The effect of storage time and temperature on the activity of produced recombinant lysostaphin was tested and represented in Figure 4.8. The relative activity of the purified enzyme was accepted as 100 % for each storage temperature on the first day. While the calculated relative activity of lysostaphin stored at -80 °C, on the 15th day was the highest, 4 °C and 25 °C storage temperatures caused the loss of approximately half of the activity of an enzyme. Although there was no significant activity loss for the lysostaphin stored at -20 °C and 4 °C on the 30th day, the activity loss of the enzyme was about 15 % at -80 °C. According to the final measurement, recombinant lysostaphin possessed barely 5 % of its activity at the -20 °C, 4 °C, and 25 °C. On the other hand, it maintained almost 20 % of its activity for -80 °C (Figure 4.8). The best storage temperature for recombinant lysostaphin was demonstrated to be –80 °C where 75 % of the enzyme activity was retained for 30 days.

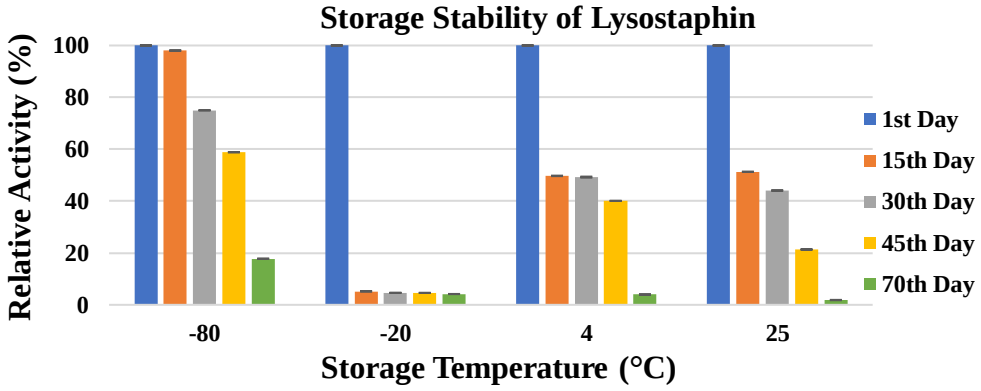


Figure 4.8: Relative Activity of lysostaphin at different storage temperatures (-80, -20, 25, 4 °C) for 70 days.

4.6. Cost Analysis of Lysostaphin

Despite lysostaphin is being readily reachable at the market, the preliminary studies have indicated that lysostaphin has remarkable potential in biotechnology practices such as the treatment of staphylococcal infections. Its applications are not very common in healthcare settings yet. High production cost is one of the reasons preventing the enzyme from being used in healthcare (1 mg and 15 mg 134 EUR and 1160 EUR, respectively/bestseller Sigma-Aldrich). The calculated production cost of 1000 U of lysostaphin produced by proposed conditions in this study (FM medium with 6 g/L glycerol and 1.5 g/L glucose, 30 °C, 800 rpm, pH 7.0) was only about 1.274 EUR which is 4-folds lower than previously reported minimum amount (Figure 4.9) [32]. The ease of the auto-induction system facilitates the adaptation of proposed system in any laboratory for the production of low-cost and large amounts of active lysostaphin for research, which could be rather difficult in the case of the technology developed by other groups [30], [32], [62].

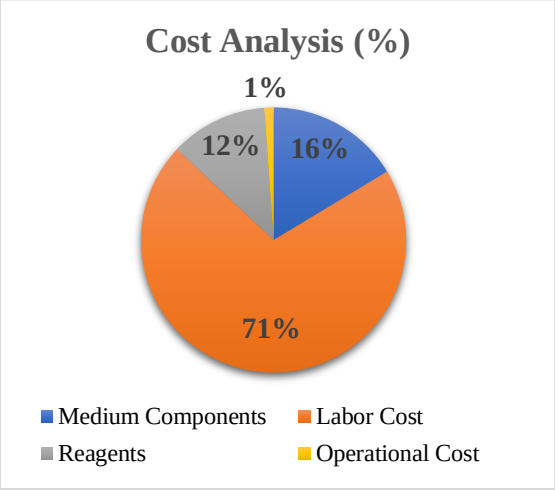


Figure 4.9: Cost analysis for a single batch run in the indicated optimum conditions and medium. The analysis included inoculation, fermentation, purification, and enzyme activity assay stages. Costs of reagents and medium components were based on Sigma-Aldrich catalog. Operational costs involved all electricity consumptions during fermentation, the other electricity-related costs were negligible. The labor cost was assumed at 500 EUR/month (160 h/month; 24 h/single batch).

4.7. Determination of Antimicrobial Properties of Lysostaphin

S. aureus stocks available in the Biochemistry Laboratory at the Department of Chemistry, Gebze Technical University was screened in order to differentiate *S. aureus* from the other staphylococcal species. Most of the staphylococcal species are able to grow on MSA containing high salt concentration. MSA contains mannitol as the only carbohydrate source in the medium and uses phenol red as a pH indicator to select the bacteria that can ferment the mannitol. The *S. aureus* colonies grew on MSA with yellow zones, while *Staphylococcus epidermidis* and other salt-tolerant organisms will produce small red or pink colonies resulting in no color change on the medium [63]. As observed in Figure 4.10, it is clear that when strain 3, 3-1, 16 and 35 are *S. aureus*, strain number 10 is a strain another staphylococcal species. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were tested as a control. Identified *S. aureus* strains were used for further antimicrobial studies.

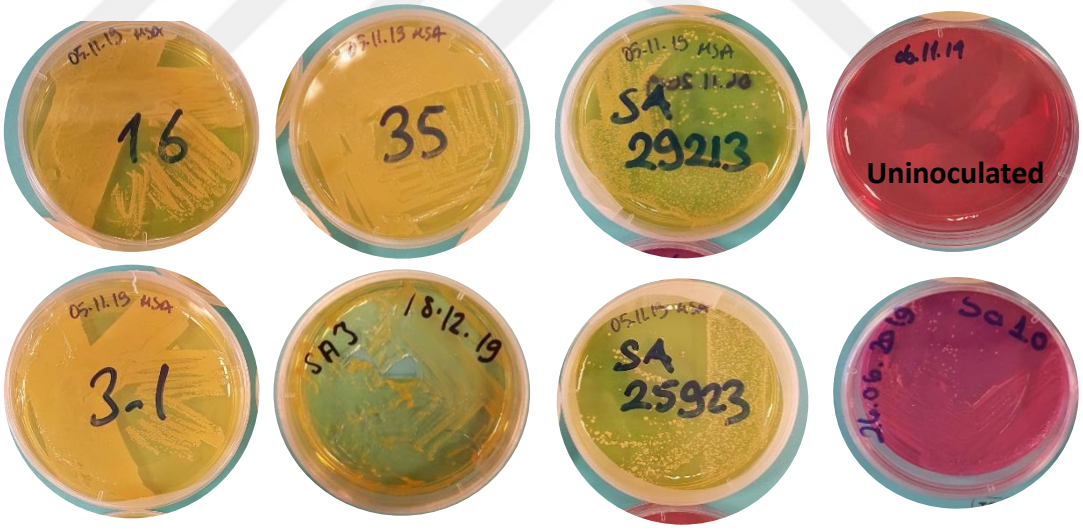


Figure 4.10: Results of MSA selection for *S. aureus* isolates (Sa3, Sa16, Sa35, Sa3-1, Sa10) and the *S. aureus* ATCC29213, *S. aureus* ATCC25923 as a control.

Bacteriocin capacity of purified lysostaphin was characterized via the calculation of IC_{50} concentrations and disk diffusion assay.

4.7.1. Determination of IC_{50}

The maximum half inhibition concentrations of produced lysostaphin were calculated and the maximum % lysis for each strain was represented in Table 4.4. The minimum IC_{50} concentrations were detected as 0.09 μg/mL and 0.06 μg/mL for Sa3 and *S. aureus* ATCC 29213 respectively. On the other hand, the IC_{50} concentration was quite similar for Sa16 and Sa35 strains and calculated as ~0.2 μg/mL. As represented in Figure 4.11, the IC_{50} value for *S. aureus* ATCC 25233 cannot be observed through the used lysostaphin concentration boundaries. However, it was estimated by using the linear regression method as 1.41 μg/mL. Thereby, recombinant lysostaphin has higher bacteriocin capacity for *S. aureus* ATCC 25233, Sa3 and Sa3-1 in comparison to the Sa16, Sa35 and *S. aureus* ATCC 25233.

When IC_{50} concentrations of Ceftaroline, Cefotaxime, Ceftriaxone and Penicillin G were reported as 0.5 μg/mL, 0.5 μg/mL, 0.25 μg/mL and 4 μg/mL respectively against commercially available *S. aureus* ATCC 29213 [64], the expressed recombinant lysostaphin in this study was 0.06 μg/mL. So, recombinant lysostaphin is about 10-folds more effective than β-lactams.

Table 4.4: Maximum lysis percentage and IC_{50} concentration for *S. aureus* species.

Name of strain	**% Lysis**	**IC_{50} (μg/mL)**
Sa 3	74.47 ± 0.05	0.09 ± 0.00
Sa 3-1	73.54 ± 0.36	0.08 ± 0.00
Sa 16	67.02 ± 0.16	0.20 ± 0.01
Sa 35	67.69 ± 0.43	0.21 ± 0.01
***S. aureus* ATCC 25233**	45.78 ± 1.12	1.41 ± 0.06
***S. aureus* ATCC 29213**	78.70 ± 0.46	0.06 ± 0.00

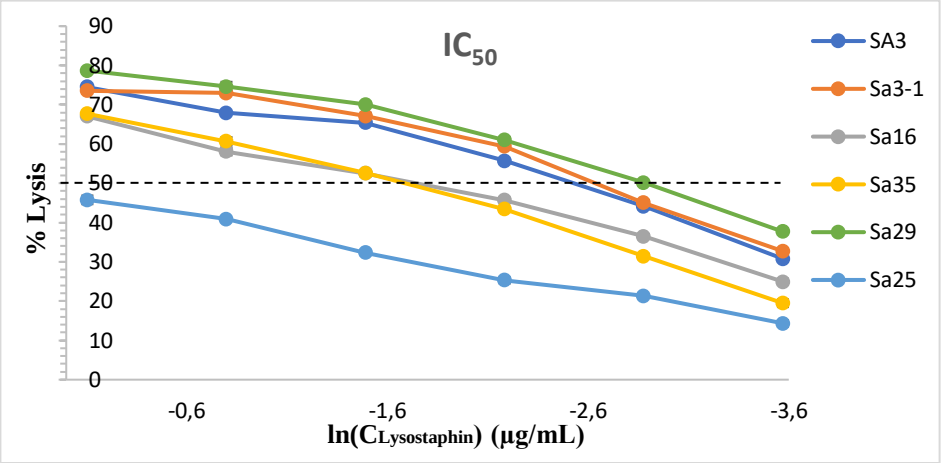


Figure 4.11: Representation of IC_{50} concentration with respect to the half lysis percentage.

4.7.2. Disk Diffusion Assay

Petri images of the disc diffusion method performed with *S. aureus* isolates (Sa35, Sa16, Sa3) of GTÜ Biochemistry laboratories and commercial strains (*S. aureus* ATCC 29213, *S. aureus* 25233) by pure lysostaphin enzyme were represented in Figure 4.12. It has been observed that zone diameters obtained by using pure enzyme in different concentrations increased and formed different zones in different *S.aureus* strains.

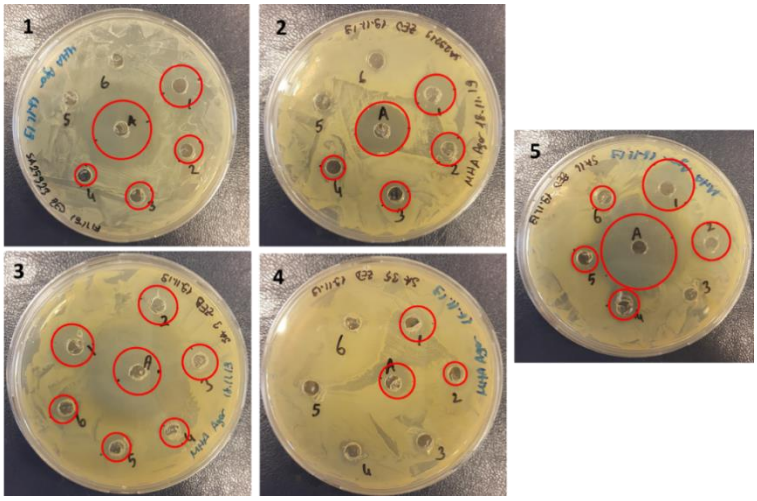


Figure 4.12: Disc diffusion analysis of the purified enzyme with *S. aurueus* strains. 1: *S. aureus* ATCC 25933; 2: *S. aureus* ATCC 29213; 3: *S. aureus* 3 isolate; 4: *S. aureus* 35 isolate; 5: *S. aureus* 16 isolate.

According to Table 4.5, even the lowest concentration of lysostaphin is able to eradicate the Sa 3 and Sa 16. On the other hand, with mid and lower concentrations the inhibition of the growth of Sa 35, *S. aureus* 29213, *S. aureus* 25923 were not measurable. Through the 0.34 µg/mL 0.17 µg/mL and 0.085 µg/mL concentrations of lysostaphin, the antimicrobial agent prevented the growth of all tested *S. aureus* strains.

Table 4.5: Measured zone diameters on disk diffusion assay with different lysostaphin concentrations.

Concentration of stock (µg/mL)	0.34	0.17	0.085	0.0425	0.02125	0.010625	Ampicillin (10 µg)
Name of Strain	**Zone diameter (mm)**						
Sa 3	15 ± 0.0	14 ± 0.1	12 ± 0.1	10 ± 0.0	9 ± 0.0	8 ± 0.0	15 ± 0.3
Sa 35	11 ± 0.0	9 ± 0.1	8 ± 0.1	-	-	-	11.5 ± 0.2
Sa 16	18 ± 0.1	16 ± 0.0	14 ± 0.1	13 ± 0.0	10± 0.0	8 ± 0.0	28.5 ± 0.5
Sa 29213	15 ± 0.1	12 ± 0.1	10 ± 0.1	8 ± 0.0	-	-	20 ± 0.2
Sa 25923	14 ± 0.1	11 ± 0.0	9 ± 0.1	8 ± 0.1	-	-	20 ± 0.4

The percent yields for degrading different strains of *S. aureus* by lysostaphin dilutions by taking 10 µg Amp. as reference were plotted and represented in Figure 4.13. The zone diameters for each strain because of the Amp were accepted as 100 %. As observed from calculated results, 0.34 µg/mL lysostaphin has about 70 % bacteriocin ability for all tested *S. aureus* strains in comparison to the 10 µg Amp. Moreover, even ~0.04 µg/mL lysostaphin was as approximately 45 % effective as Amp for Sa3, Sa16, *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923.

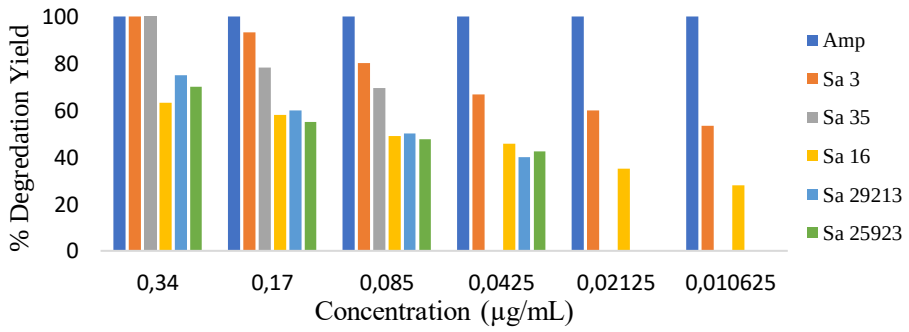


Figure 4.13: The percent yield of degradation for lysostaphin dilutions in comparison to the ampicillin.

5. CONCLUSION

Enhanced easy induction and low-cost of lysostaphin are crucial for both its implementation as an antibacterial agent and for further tests of its properties during preclinical and clinical research. In this thesis, high-density *E. coli* production for the purpose of high yielding and low-cost lysostaphin from shake flask scale and benchtop bioreactor scale was performed and optimum conditions were detected. Moreover, the antimicrobial efficiency of the produced enzyme was promising for further applications. The currently proposed auto-induction medium and fermentation parameters in bench-scale for low-cost production of lysostaphin will utilize larger-scale production of lysostaphin.

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BIOGRAPHY

Efsun completed her high school degree at E.C.A. Elginkan Anatolian High School. After that, she started her undergraduate studies at the Department of Bioengineering, Marmara University and graduated in second place. She involved in two compulsory internships during BSc years which are at Aziz Sancar Institute of Experimental Medicine, Istanbul University and Institute of Polymer Science and Technology, Spanish National Research Council (CSIC), Madrid. After graduating from a bachelor's degree, Efsun started a Biochemistry MSc. program at the University of Bonn, Germany. After completing one semester she transfers her MSc studies to the Institute of Biotechnology, Gebze Technical University under the supervision of Assoc. Prof. Barış Binay. She conducted a part of her MSc studies at the Process and Systems Engineering Centre (PROSYS), the Technical University of Denmark under the supervision of Prof. John M. Woodley.

APPENDICES

Appendix A: Publications related to the thesis

Duman Z. E., Unlu A., Cakar M. M., Unal H., Binay B., (2019) 'Enhanced production of recombinant S*taphylococcus simulans* lysostaphin using medium engineering', Preparative Biochemistry and Biotechnology, 49(5), p.521-528.

Duman Z. E., Woodley J. M., Unlu A., Unal H., Binay B., 'Enhanced Large-Scale Production of Recombinant *Staphylococcus simulans* Lysostaphin on Benchtop Bioreactor', 2nd Eurasia Biotechnological Approaches and Technologies Congress, Antalya, Turkey (26-29 October 2019).

Appendix B: Vector Map of *p*BAD_Lysostaphin

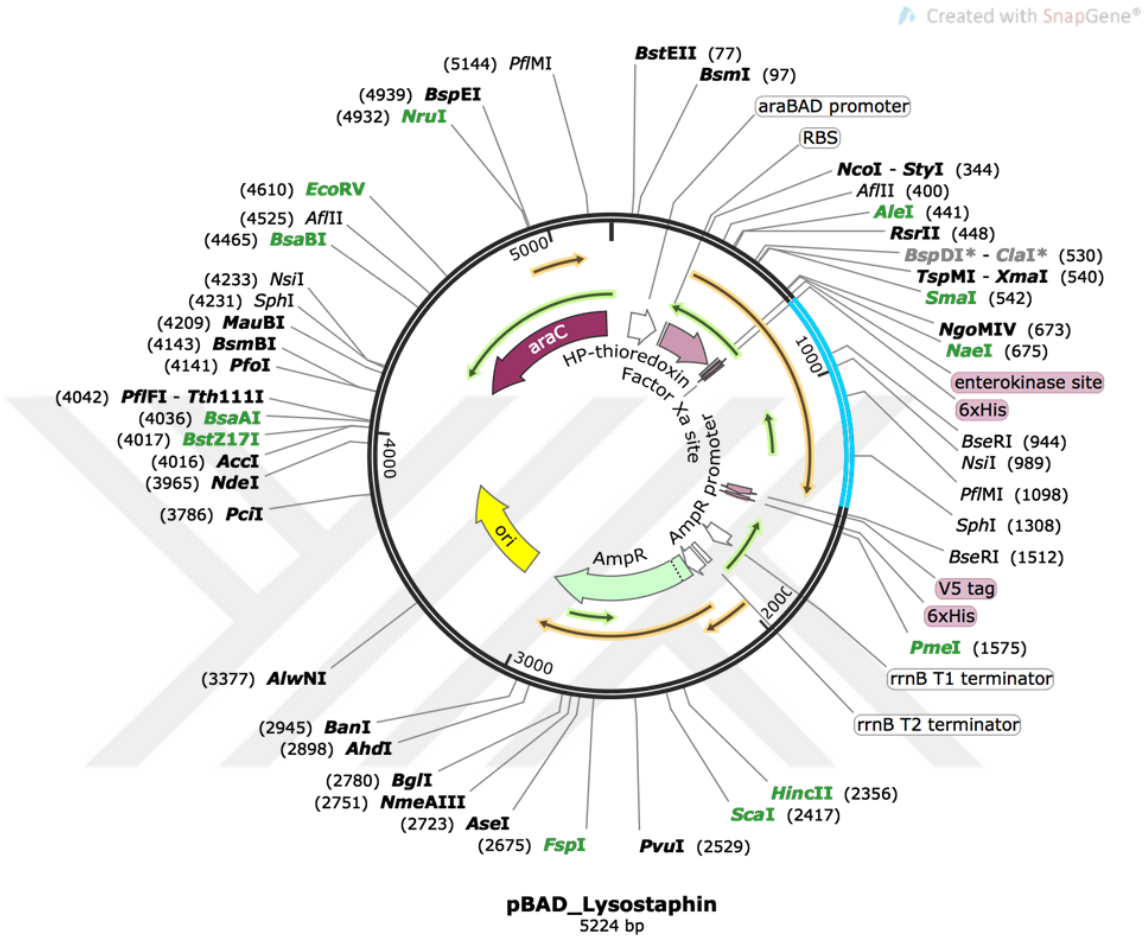

Figure B1.1: Cloned *lysostaphin* gene (blue DNA fragment) into *p*BAD vector.