EFFECT OF SIMULTANEOUS BETA-LACTAMASE INHIBITOR PROTEIN EXPRESSION ON CELLULAR BETA-LACTAMASE ACTIVITY

by

Kadriye Simay Yalaz

B.S., Chemical Engineering, Istanbul University, 2008

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Chemical Engineering

Boğaziçi University

2012

ACKNOWLEDGEMENTS

I would like to express my genuine gratitude to my thesis supervisor Assist. Prof. Elif Özkırımlı Ölmez, and my co-supervisor Assoc. Prof. Berna Sarıyar Akbulut for their guidance, understanding and support they have provided me during my research.

I would like to thank my thesis committee members Prof. Dilek Kazan, Prof. Zeynep İlsen Önsan, and Prof. Kutlu Ülgen for spending their time for reading and for their helpful comments.

I am indebted to my friends, in particular Aslıgül Doğan for her encouragements, accompany during my hardest times and for being such great friends.

I am grateful to Nilay Büdeyri and Gizem Buldum for their never-ending help and friendship. I would like to thank to my lab mate Celal Ceylan for his suggestions and help during the project. My special thanks go to KB-407 members for the friendly environment. I owe my deepest gratitude to my mother, to whom I dedicated this thesis, for her unending love, encouragement and support she have given me through my entire life.

This research would not have been possible without the financial assistance of TUBITAK Project No. 108M644, Project No. 109M229 and BAP Project No. 09HA504P.

ABSTRACT

EFFECT OF SIMULTANEOUS BETA-LACTAMASE INHIBITOR PROTEIN EXPRESSION ON CELLULAR BETA-LACTAMASE ACTIVITY

β-lactam antibiotics are commonly used in the treatment of bacterial infections. However, due to underuse and misuse of β-lactam antibiotics, bacteria have developed mechanisms that counteract β-lactam antibiotics. The most common mechanism is production of TEM-1 β-1actamase that hydrolyzes the β-lactam ring and enables bacteria to gain antibiotic resistantance. β-lactamase-inhibitory protein (BLIP), a 165 amino acid protein produced by *Streptomyces clavuligerus*, is a high affinity β-lactamase inhibitor. In this study, the inhibitory effect of BLIP was investigated by examining growth profiles, cell viability and *in-vivo* β-lactamase activity of recombinant cells simultaneously expressing β-lactamase and BLIP. β-lactamase activity per viable cell was determined in the absence and presence of BLIP. The presence of both BLIP and β-lactamase was verified by SDS-PAGE and Native-PAGE analysis. In the absence of BLIP, *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells showed comparable β-lactamase activity. The activity of β-lactamase in the periplasmic extract of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells decreased considerably when R-TEM-1 β-lactamase and BLIP were expressed simultaneously.

ÖZET

EŞZAMANLI BETA-LAKTAMAZ İNHİBİTÖR PROTEİN EKSPRESYONUNUN HÜCRESEL BETA-LAKTAMAZ AKTİVİTESİNE OLAN ETKİSİ

β-laktam antibiotikleri bakteriyel enfeksiyonların tedavisinde yaygın olarak kullanılmaktadır. Ancak, β-laktam antibiotiklerinin yetersiz ve yanlış kullanılmasına bağlı olarak, bakterileriler β-laktam antibiotiklerini etkisiz hale getirecek mekanizmalar geliştirmiştir. En sık görülen mekanizma, β-laktam halkasını hidroliz eden ve bakteriye antibiyotik direnci kazandıran TEM-1 β-laktamaz üretimidir. 165 amino asitten oluşan β-laktamaz inhibitör proteini (BLIP) *Streptomyces clavuligerus* tarafından üretilen, yüksek ilgiye sahip bir β-laktamaz inhibitörüdür. Bu çalışmada, BLIP'in inhibitor etkisi, hücrelerin büyüme profilleri, canlı hücre sayısı ve eşzamanlı β-laktamaz ile BLIP üreten rekombinant hücrelerdeki β-laktamaz aktivitesi incelenilerek araştırıldı. Canlı hücre başına β-laktamaz aktivitesi BLIP varlığında ve yokluğunda belirlendi. BLIP ile β-laktamaz varlığı SDS-PAGE ve Native-PAGE analizleri ile doğrulandı. BLIP yokluğunda, *E. coli* BL21(DE3) (pUC18 + pET-26EA) ve *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) hücreleri benzer düzeyde β-laktamaz aktivitesi göstermişlerdir. Eşzamanlı BLIP ve R-TEM-1 β-laktamaz ekspresyonu gerçekleştiğinde, periplasmadaki β-laktamaz aktivitesi önemli oranda düşmüştür.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

A	Ala, Alanine
D	Light path
D	Asp, Aspartic Acid
E	Glu, Glutamic Acid
F	Phe, Phenylalanine
G	Gly, Glycine
I	Iso, Isoleucine
K	Lys, Lysine
K_i	Inhibiton constant
L	Leu, Leucine
N	Asn, Asparagine
P	Pro, Proline
Q	Glu, Glutamine
R	Arg, Arginine
S	Ser, Serine
V	Val, Valine
V_s	Volume of enzyme
V_t	Total reaction volume
W	Trp, Tryptophan
Y	Tyr, Tyrosine
ε_λ	Extinction coefficient

LIST OF ACRONYMS/ABBREVIATIONS

APS	Ammonium Persulfate
BLIP	β-Lactamase Inhibitor Protein
CFU	Colony Forming Units
EDTA	Ethylenediaminetetraacetic Acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani Broth
MW	Molecular Weight
OD	Optical Density
rpm	Rotation Per Minute
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
T	Temperature
TEMED	N,N,N,N-Tetramethylethylendiamine
U	Unit

1. INTRODUCTION

The discovery of antibiotics in the 1930's, represented a turning point in the treatment of infectious diseases (Buynak, 2006). Among all antibiotics, β-lactam antibiotics utilization constitutes 50% of all used antimicrobials (Essack, 2001), owing to their comparatively high effectiveness, cost effectiveness, ease of delivery and minimal side effects (Wilke *et al*., 2005).

β-lactam antibiotics target the penicillin binding proteins (PBPs), therefore they are used in an effort to overwhelm bacterial infections by inhibiting bacterial cell wall synthesis (Mobashery *et al*., 2010, Zhu *et al*., 2010). Widespread use and misuse of β-lactam antibiotics led to progressively developed bacterial resistance against β-lactam antibiotics. Even though antibiotics have been effective in the treatment of infections, infectious diseases are still the outstanding cause of death globally as a result of both new and emerging diseases and also as a result of the increasing prevalence of antibiotic resistant pathogens (Essack, 2001).

β-lactamase production is the most common mechanism of bacterial resistance to β-lactam antibiotics. β-lactamases hydrolyze the amide bond of the four-membered β-lactam ring and therefore provide significant antibiotic resistance to their bacterial hosts (Wilke *et al*., 2005). The most common plasmid-mediated β-lactamase encountered in gram-negative bacteria is TEM-1 β-lactamase (Wiedemann *et al.,* 1989). β-lactamase-mediated antibiotic resistance has given rise to the development of β-lactamase inhibitors. An alternative way to fight with β-lactamase-mediated resistance has been the use of mechanism-based inhibitors such as clavulanic acid and sulbactam in combination with β-lactam antibiotics (Bush, 2002).

β-lactamase inhibitors do not possess antimicrobial activity, therefore they are used in conjunction with various β-lactams. The former protects the latter from the action of β-lactamase hence prevents the hydrolysis of the antibiotics (Rudgers and Palzkill, 1999). However, bacteria have also developed resistance to β-lactam - β-lactamase inhibitor combinations by introducing inhibitor resistant mutations in β-lactamases.

Streptomyces clavuligerus, the producer of the widely used small molecule inhibitor clavulanic acid, was also found to produce a proteinaceous β-lactamase inhibitor, BLIP (Doran *et al*., 1990). BLIP is a 165 amino acid protein that has been shown to bind to and inhibit the TEM-1 β-lactamase efficiently with a K_i of 0.1 - 0.6 nM (Petrosino *et al*., 1999, Rudgers and Palzkill, 1999, Strynadka *et al*., 1994). When compared to small molecule inhibitors, BLIP inhibits class A β-lactamases of gram-negative and gram-positive bacteria with relatively reduced affinities (Liu *et al*., 2004). Previous studies have indicated that peptides designed from key interacting residues of BLIP have inhibitory effects on β-lactamase activity (Strynadka *et al*., 2009, Rudgers *et al*., 2001). Thus, identification of small peptides that mimic the binding and inhibition activity of BLIP would facilitate the development of β-lactamase inhibitors and antibiotics (Rudgers and Palzkill, 2001).

This study aims to investigate the effect of the presence of intracellular BLIP on R-TEM-1 β-lactamase activity. pET-26b(+) vectors carrying the BLIP gene with either the *pelB* leader sequence or with its native leader sequence were used for periplasmic BLIP expression in *E. coli* BL21(DE3) cells. For simultaneous expression of BLIP and β-lactamase, pUC18 plasmid vector was also transformed into *E. coli* BL21(DE3) cells harboring the recombinant pET-26b(+) vector. First growth profiles and viabilities of the recombinant cells were investigated and then the proteins expressed were analyzed electrophoretically.

Biological background of β-lactamase, BLIP and a summary on previous studies about the interaction between β-lactamase and BLIP are given in Chapter 2. Materials and methods used in this study are given individually in Chapter 3 and Chapter 4. Results and Discussion of the experiments are given in Chapter 5. Conclusions and Recommendations for future studies are given in Chapter 6.

2. BIOLOGICAL BACKGROUND

2.1. β-Lactam Antibiotics and β-lactamase Mediated Resistance

Antibiotics constitute an important group in the pharmaceuticals market. In 2005, oral antibiotic market generated worldwide sales of $25.0 billion including only US sales of $8.5 billion (Christoffersen, 2006). β-lactam antibiotics (Figure 2.1), particularly penicillins and cephalosporins, represent the world's major biotechnology products with worldwide dosage form sales of approximately 65% of the total world market for antibiotics (Elander, 2003). Excessive and misuse of antibiotics have resulted in the emergence of antibiotic resistant bacteria (Zhu *et al*., 2010). The evolution of bacterial resistance to available antibiotics is a growing threat in the treatment of bacterial infections (Zhu *et al.*, 2010; Motamedi *et al*., 2010).

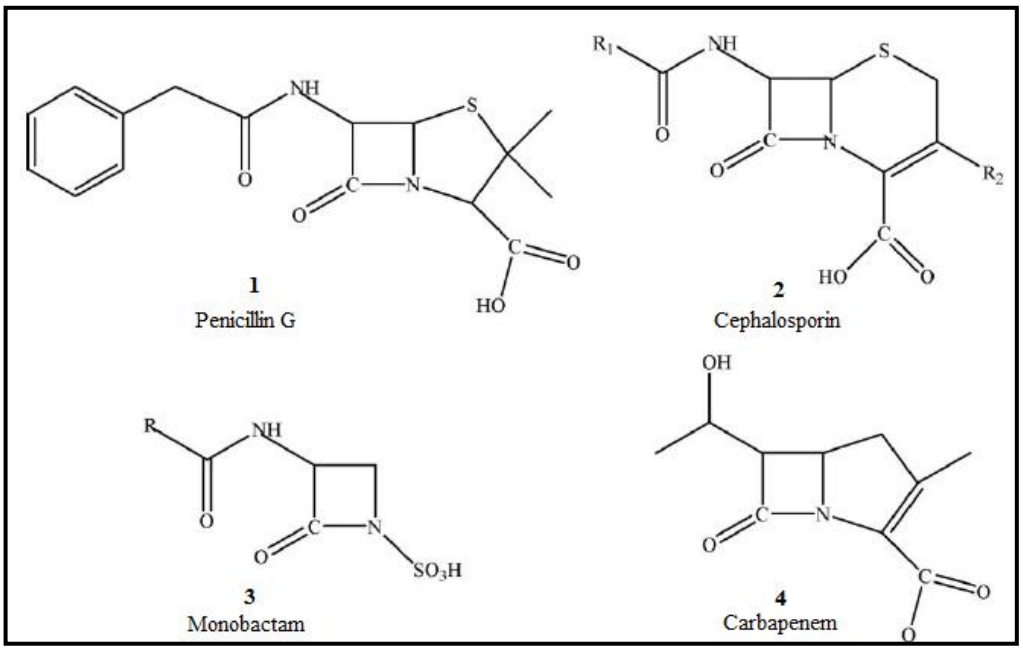

Figure 2.1. Chemical structure of β-lactam antibiotics (Babic *et al*., 2006).

Modern antibiotic therapy started with the discovery of a β-lactam antibiotic, penicillin, by Alexander Fleming in 1928 (Essack, 2001). The highly reactive four-member β-lactam ring is the distinctive structural feature of β-lactam antibiotics (Babic *et al*. 2006). Later, cephalosporin which is one of the β-lactam compound derivatives was

discovered (Abraham, 1990). To date, a large number of different β-lactam antibiotics, encompassing penicillins, cephalosporins, monobactams, and carbapenems are in use (Williams, 1999).

β-lactam antibiotics are capable of killing bacteria by repressing cell wall synthesis. Gram-positive bacterial cell walls may or may not be surrounded by a protein or polysaccharide envelope, while Gram-negative bacterial cell walls have an outer membrane (Figure 2.2). The critical attack site of anti-cell-wall agents is the peptidoglycan layer, which is essential for the survival of bacteria in hypotonic environments (Baron, 1996). β-lactam antibiotics inhibit the transpeptidases which are responsible for the construction of the peptidoglycan structure (Gagne and Savard, 2006). Cross-linked peptidoglycan units form the complex structure of the bacterial cell wall and also sustain rigidity of bacterial cell wall against osmotic pressure inside the cell (Babic *et al*., 2006). The glycan component is comprised of a basic repeating unit of an alternating disaccharide, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (Babic *et al*., 2006; Wilke *et al*., 2005). The individual peptidoglycan units are produced in the cytoplasm, subsequently final cross-linking reaction is catalyzed outside the cytoplasmic membrane by cell-wall transpeptidases (Babic *et al.*, 2006; Wilke *et al*., 2005).

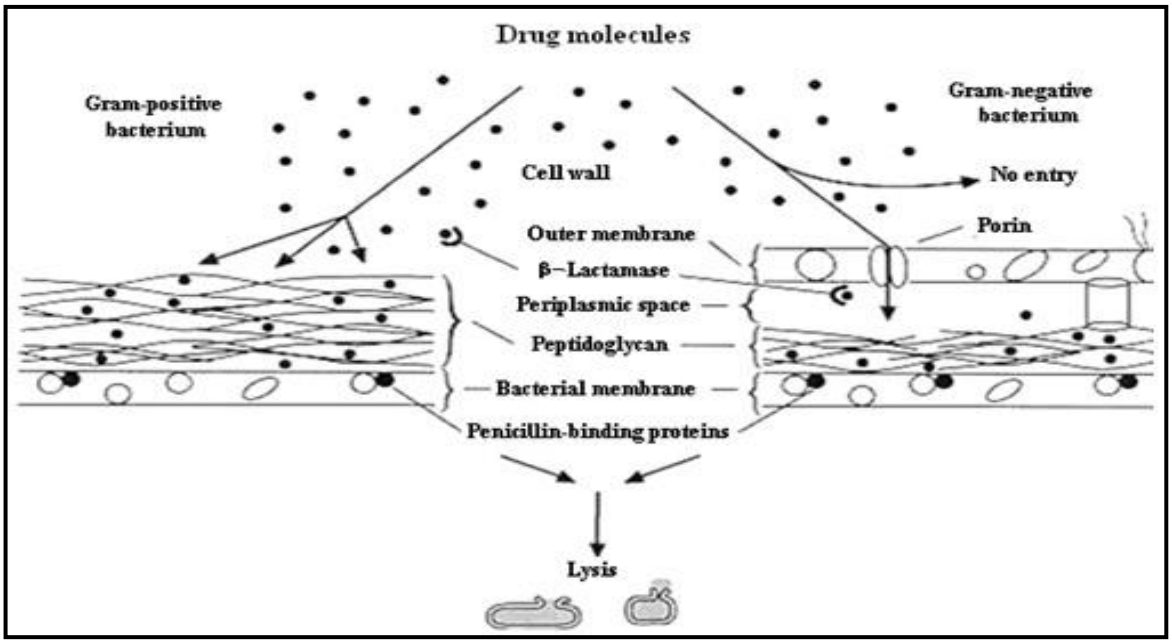

Figure 2.2. Outer wall of Gram-positive and Gram-negative bacteria (Baron, 1996).

Structural similarities between β-lactam antibiotics and the binding sites of transpeptidases, enable β-lactam antibiotics to attach and inactivate the transpeptidases that are involved in bacterial cell wall synthesis (Williams, 1999). Therefore, the growing bacteria become prone to cell lysis against loss or damage of this layer, which destroys the rigidity of the bacterial cell wall and resulting in death (Baron, 1996; Wilke *et al*., 2005).

Bacterial evolution towards antibiotic resistance depends on three different mechanisms. In the first case, bacteria produce enzyme called β-lactamase which hydrolyze the β-lactam ring thereby inactivating antibiotic before it reaches the penicillin binding protein target (PBP) (Wilke *et al*., 2005). The second mechanism is alteration of the antibiotic target site that has low affinity for β-lactam antibiotics (Babic *et al*., 2006; Wilke *et al*., 2005). In this case, cell wall transpeptidases that are insensitive to β-lactams, provide resistance to β-lactams (Babic *et al*., 2006; Wilke *et al*., 2005). The final mechanism is alteration of permeability or forced efflux, in order to fail entrance of the β-lactam antibiotic (Wilke *et al*., 2005). Missing or lessened expression of outer membrane proteins (OMPs) decreases entrance of β-lactams into the periplasmic space of gram-negative bacteria (Babic *et al*., 2006).

2.2. β-lactamase and TEM-1 β-lactamase

β-lactamase enzymes catalyze the hydrolysis of the β-lactam ring by cleaving the amide bond (Figure 2.3), consequently bacterial cell wall synthesis cannot be inhibited by the β-lactam antibiotics (Williams, 1999). β-Lactamases are grouped into four classes under Ambler Classification on the basis of amino acid sequence homology (Palzkill *et al*., 2009). Ambler classes A, C, and D contain a serine residue in their active sites, while class B enzymes have a Zn^{2+} ion in their active sites (Palzkill *et al*., 2009; Babic *et al*., 2006). Multidrug resistance has been increasing among Gram-negative bacteria, since it is strongly associated with the production of both chromosomal and plasmid-encoded β-lactamases. The number of the identified β-lactamases already exceeds 890 (Bush, 2010).

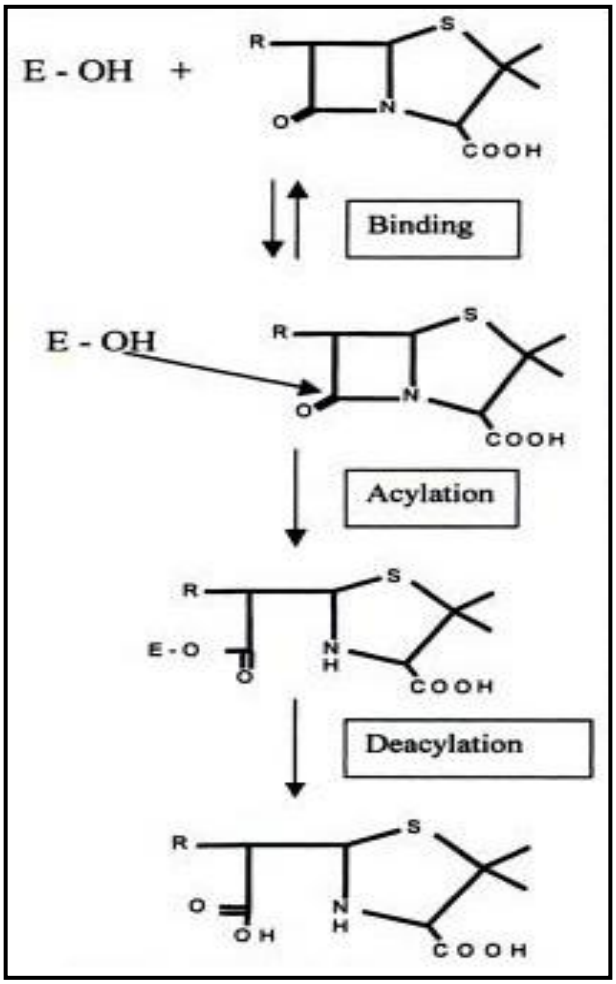

Figure 2.3. Hydrolysis of β-lactamase antibiotics (Robert *et al.*, 2007).

Once β-Lactamases are expressed in bacterial cell, they are secreted to periplasm in Gram-negative bacterial and to extracellular environment in Gram-positive bacteria.

TEM-1 β-1actamase was first isolated from *Escherichia coli* in Athens in 1963 (Datta and Kontomichalou, 1965). TEM-1 β-lactamase has 263 amino-acid residues with a molecular weight of 28.9 kDa (Gagne and Savard, 2006). The amino-acid sequence of TEM-1 β-lactamase is given in Figure 2.4. More than 130 TEM type and more than 50 SHV type β-lactamases (class A enzymes which share about 68% of its amino acids with TEM types and with similar overall structures) have been reported (Gupta, 2007).

HPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRID	60
AGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGP	120
KELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQ	180
QLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTG	240
SQATMDERNRQIAEIGASLIKHW	263

Figure 2.4. Amino acid sequence of TEM-1 β-lactamase (Reynolds *et al.,* 2006).

2.3. Plasmid-mediated β-lactamase Expression in Gram-negative Bacteria

Class A TEM-1 β-lactamase is the most common plasmid-mediated β-lactamase in gram-negative bacteria encoded by $bla_{\text{TEM-1}}$ gene (Petrosino *et al.*, 1999; Palzkill *et al*., 2009). pUC18 vector (Figure 2.5) carrying the $bla_{\text{RTEM-1}}$ gene expresses R-TEM-1 β-lactamase. The region containing the 861 bp TEM-1 β-lactamase gene on the plasmid confers resistance to ampicillin (Lim *et al*., 2001). The pUC18 derived R-TEM-1 β-lactamase has two amino acids mutations; I84V and V184A, when compared with TEM-1 β-lactamase. However, these substitutions do not have an effect on neither activity nor structure of the enzyme significantly (Avcı, 2011).

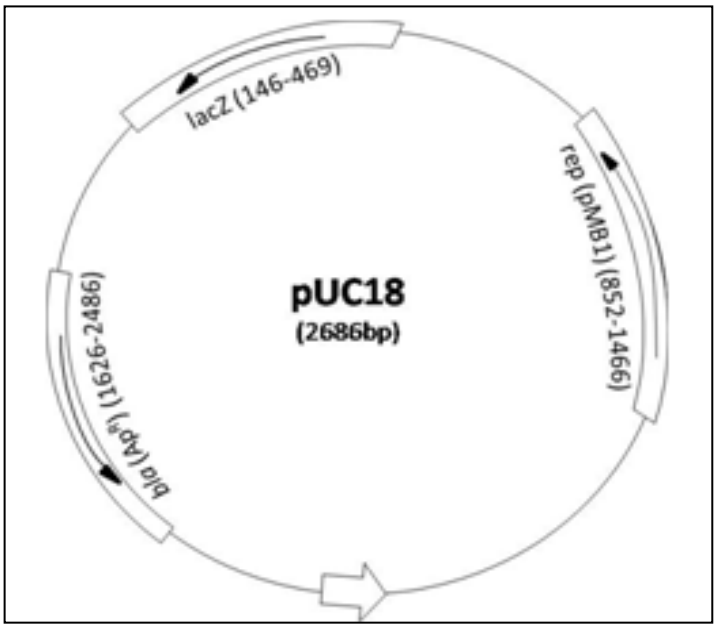

Figure 2.5. The pUC18 vector map (Novagen).

TEM-1 β-lactamase can hydrolyze both penicillins and cephalosporins. In order to overwhelm the drug resistance mediated by TEM-1 β-lactamases, extended-spectrum cephalosporins have been developed (Rudgers and Palzkill, 1999). Unfortunately, the use of extended spectrum antibiotics has led to TEM mutants capable of hydrolyzing these antibiotics. An alternative way to fight with β-lactamase-mediated resistance has been the use of mechanism-based inhibitors (Parker *et al.,* 1987).

2.4. β-lactamase Inhibitors

Inhibitors such as clavulanic acid, sulbactam and tazobactam (Figure 2.6) inactivate class A serine β-lactamases effectively by an irreversible mechanism (Buynak, 2006). The β-lactamase inhibitors are hydrolyzed very slowly or not hydrolyzed upon binding to the active site of β-lactamases or they yield 'permanently inactivated' species (Babic *et al*., 2006). Therefore, in clinical use, β-lactamase inhibitors are combined with a β-lactam antibiotics (Babic *et al*., 2006). These small molecule inhibitors protect the β-lactam drug from hydrolysis by β-lactamases and restore the therapeutic potential of the antibiotic (Rudgers and Palzkill, 2001).

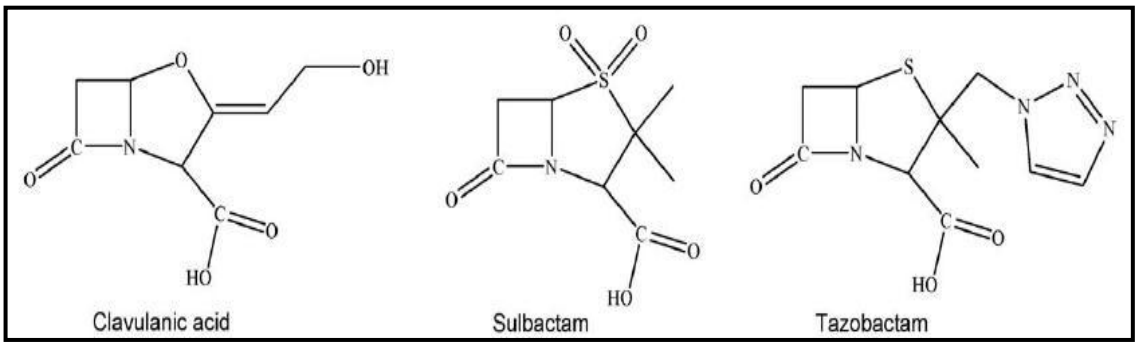

Figure 2.6. Chemical structures of β-lactamase inhibitors (Babic *et al*., 2006).

The small molecule clavulanic acid, which is capable of inhibiting various class A β-lactamases, was isolated from the gram-positive bacterium *S. clavuligerus*. Since clavulanic acid can pass through bacterial cell wall, it can effectively be used for the inactivation of both extracellular and intracellular β-lactamases (Essack, 2001).

2.5. β-lactamase Inhibitory Protein (BLIP)

The *bli* gene encoding BLIP was isolated on a 13.5 kilobase fragment of *S. clavuligerus* chromosomal DNA and is a protein responsible the inhibition of β-lactam antibiotics. BLIP has 165 amino-acid residues (Figure 2.7) and its molecular mass is 17.5 kDa. (Doran *et al*., 1990). The *bli* gene contains an N-terminal leader sequence of 36-amino-acids, which is important for translocation of the protein to the periplasm (Doran *et al.*, 1990). The amino acid sequence of the native leader sequence of the *bli* gene is underlined in Figure 2.7. Strynadka *et al.* identified the structure of BLIP revealing that it consists of two tandemly linked 76 amino acid domains (Strynadka *et al*., 1994). BLIP inhibits a number of class A β-lactamases including TEM-1 β-lactamases (Palzkill *et al*., 2009, Kotra and Mobashery, 1998).

BLIP	MRTVGIGAGVRRLGRAVVMAAAVGGLVLGSAGASNAAGVMTGAKFTQIQF	50
BLIP	GMTRQQVLDIAGAENCETGGSFGDSIHCRGHAAGDYYAYATFGFTSAAAD	100
BLIP	AKVDSKSQEKLLAPSAPTLTLAKFNQVTVGMTRAQVLATVGQGSCTTWSE	150
BLIP	YYPAYPSTAGVTLSLSCFDVDGYSSTGFYRGSAHLWFTDGVLQGKRQWDLV	201

Figure 2.7. DNA sequence of region encompassing *S.clavuligerus* BLIP gene (Doran *et al.*, 1990).

2.6. Expression of Recombinant Proteins and pET System

Approximately 20% of the proteins synthesized by bacteria are located partially or completely outside of the cytoplasm. Most of them reach their final destination by the general secretory pathway (GSP) (Pugsley, 1993). In gram-positive bacteria, proteins are released into the external milieu, whereas in gram-negative bacteria recombinant proteins are transported into the periplasmic space by secretory signal sequences (Pugsley, 1993).

The pET System is a powerful protein expression tool for tightly controlling target protein expression (Figure 2.8) using *E. coli* as the host (Leonhartsberger, 2006; Novagen,

2003). pET vectors allow target genes that are under control of the T7*lac* promoter and to be expressed in *E. coli* BL21(DE3) strain. This strain is the T7 host strain that is most widely used host for expression of cloned genes due to deficiency of proteases that can degrade target proteins in *E. coli* cells (Novagen, 2003).

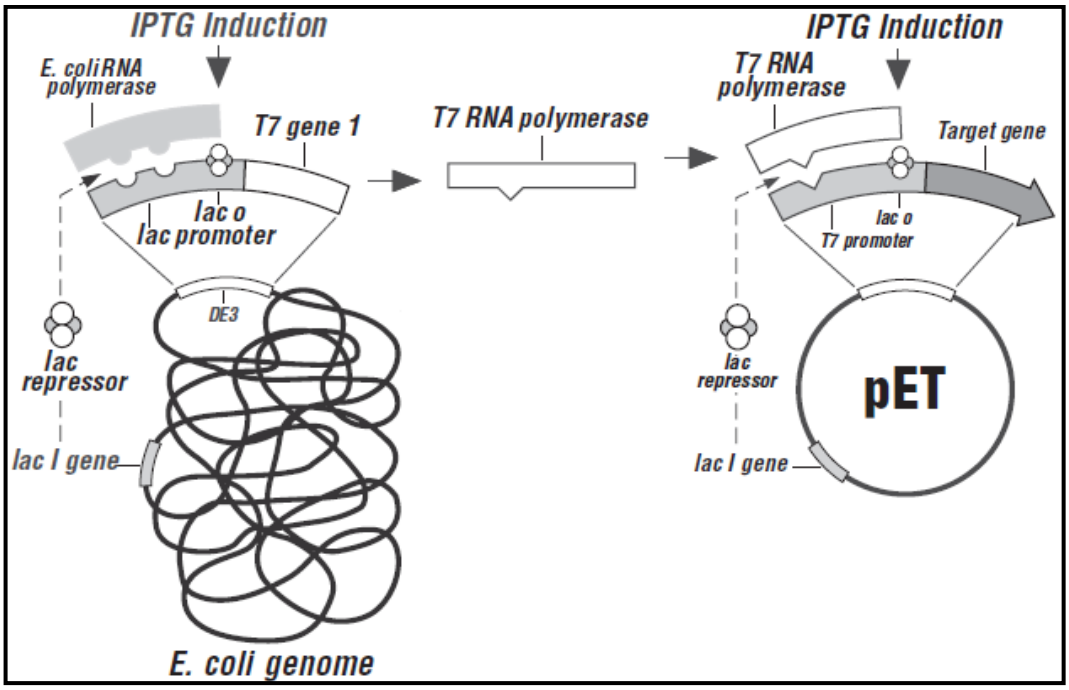

Figure 2.8. Control of T7*lac* Promoter on pET vector (Novagen, 2003).

Expression of target genes that are cloned in pET vectors are regulated indirectly (Novagen, 2003). Target genes are cloned by using BL21(DE3) host strain that carries a gene under the control of the inducible *lacUV5* promoter, for T7 RNA polymerase expression (Studier *et al*., 1990; Dubendorff and Studier, 1991). The T7 expression system of BL21(DE3) strain depends on an enzyme called T7 RNA polymerase, which is not endogenous to bacteria, however BL21(DE3) strain of *E. coli* has been engineered to carry the gene encoding T7 RNA Polymerase in a DNA fragment called the DE3 bacteriophage lambda lysogen (Novagen, 2003).

pET vectors contain a *lac* operator sequence just downstream of the T7 promoter and the natural promoter and *lacI* gene coding sequence for the *lac* repressor. When this type of vector is used in DE3 lysogens, the *lac* repressor acts both at the *lacUV5* promoter in the host chromosome to repress transcription of the T7 RNA polymerase gene by the host

polymerase and at the T7*lac* promoter in the vector to block transcription of the target gene (Studier *et al*., 1990; Dubendorff and Studier, 1991). Under normal circumstances in these *E. coli* cells, the *lac* operon prevents expression of T7 RNA polymerase (Novagen, 2003). The *lac* repressor binds to the *lac* operator region between the T7 promoter and the gene encoding T7 RNA polymerase. This effectively prevents transcription of the T7 RNA polymerase gene.

When IPTG is added to the cell culture, it binds to the *lac* repressor, changing its conformation in such a way that it is no longer able to bind the *lac* operator. This enables the cells to produce T7 RNA polymerase that is specific for the T7 promoter of the transformed plasmid (Novagen, 2003). Therefore, expression of target gene is induced. This system provides the ability to maintain target genes transcriptionally inactive under uninduced conditions (Novagen, 2003).

2.7. pET-26b(+) Vector

pET-26b(+) vector, which is used for expression of recombinant proteins in host cells, has 5360 base pairs. The premature proteins contain secretory signal sequences such as, *pelB, ompA,* and *phoA*. These signal sequences allow target proteins to be transported outside the cytoplasm. pET-26b(+) vector (Figure 2.9) carrying the *bli* gene with the native leader or the periplasmic secretion signal peptide sequences (*pelB*) was used for transporting BLIP to the periplasmic space of *E. coli* BL21(DE3) cells. Therefore, recombinant proteins can be secreted efficiently in *E. coli* (Leonhartsberger, 2006).

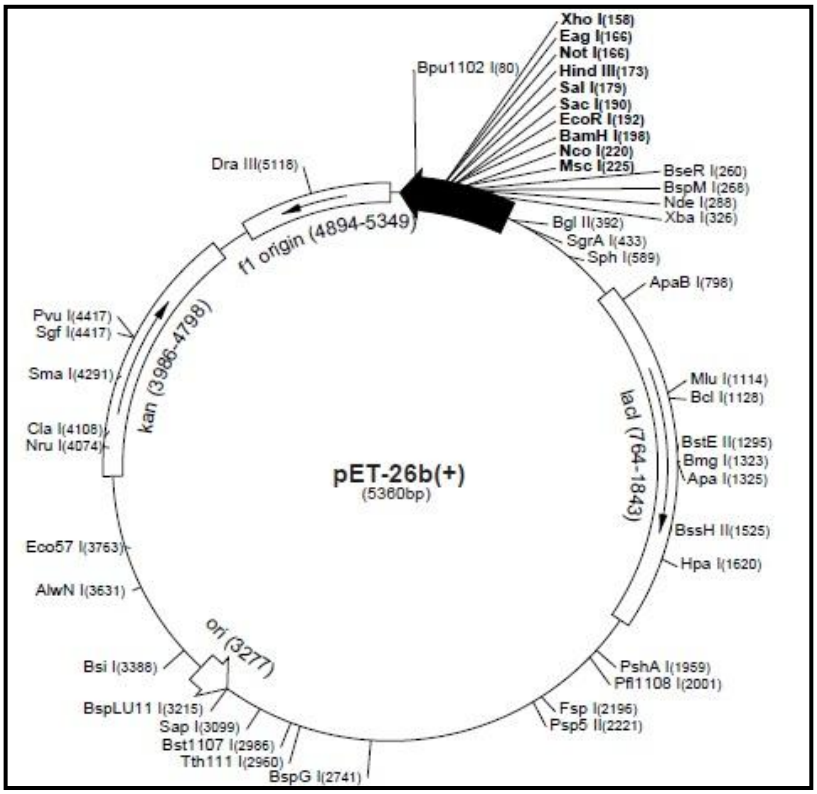

Figure 2.9. Vector map of pET26b(+).

pET-26b(+) plasmid carries the gene that enables kanamycin resistance. Amino acid sequence of native leader was given in Section 2.5. The N-terminal amino acid sequence of *pelB* leader and the expression region that is highlighted arrow in pET-26b(+) vector map are given in Figure 2.10.

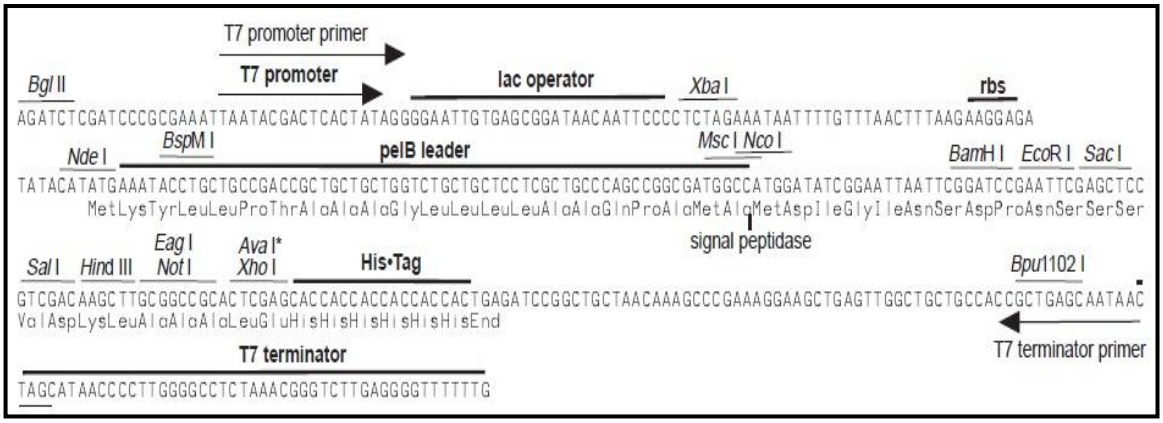

Figure 2.10. Expression region of pET-26b(+) vector.

2.8. *In-vitro* β-lactamase Inhibition

Kinetic characterization of β-lactamases can be detected by chromogenic substrates (Bebrone *et al*., 2001). CENTA (Figure 2.11) is a chromogenic substrate for β-lactamases and structurally resembles nitrocefin, cephaloridine, and cephalotin (Bebrone *et al*., 2001; Lim *et al*., 2001). CENTA displays a detectable color change from light yellow (λ maximum 340 nm) to chrome yellow (λ maximum 405 nm) concomitant with hydrolysis of the β-lactam ring (Jones *et al*., 1982).

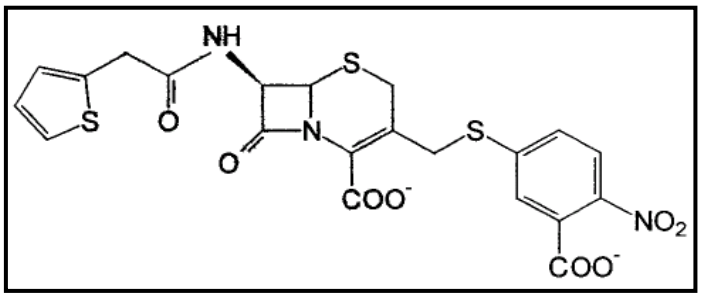

Figure 2.11. Structure of CENTA (Bebrone *et al.*, 2001).

CENTA, which can be prepared from the commercially available cephalothin, was shown to be susceptible to β-lactamase. The kinetic parameters obtained from the interactions between TEM-1 β-lactamase and CENTA are summarized in Table 2.1 (Bebrone *et al*., 2001).

Table 2.1. The kinetic parameters of TEM-1 β-lactamase-CENTA interaction (Bebrone *et al*., 2001).

Enzyme	CENTA (mM)	Enzyme (nM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
TEM-1 β-lactamase	0.025	11.5	110	70	1.6

TEM-1 β-lactamase efficiency can be linked to the k_{cat}/K_m value, which is given as 1.6 μM^{-1} s^{-1}, indicates that CENTA is a good substrate for TEM-1 β-lactamase. In addition to this, CENTA can be used in high-throughput screening tests for the selection of new β-lactamase inactivators (Bebrone *et al*., 2001).

2.9. β-lactamase - BLIP Interaction

BLIP inhibits class A β-lactamases from both gram-positive and gram-negative bacteria to varying degrees (Strynadka *et al*., 1994). The identification of the amino acid residues responsible for the binding and inhibition will facilitate the engineering of tighter, smaller inhibitors for β-lactamases (Petrosino *et al*., 1999).

Petrosino *et al*., (1999) reported that the wild type, His-tagged BLIP inhibited TEM-1 β-lactamase with a K_i of 0.11 nM, which was nearly 5 fold lower compared to K_i value of 0.6 nM for BLIP purified from *S. clavuligerus*.

The co-crystal structure of TEM-1 – BLIP complex suggested that Asp-49 and Phe-142 of BLIP mimic interactions made by penicillin G when bound in the active site of TEM-1. Petrosino *et al*. (1999) employed site-directed mutagenesis of Asp-49 and Phe-142 to alanine. Replacing Asp-49 with Ala and Phe-142 with Ala led 80 and to 300-fold increase in K_i, respectively. The D49A mutant inhibits TEM-1 with a K_i of 8.3 nM, whereas the F142A mutant inhibits with a K_i of 33 nM (Petrosino *et al*., 1999).

Rudgers and Palzkill (1999) have applied monovalent phage display method in order to identfy crucial residues for TEM-1 β-lactamase critical for BLIP binding. Functional mutants were selected amongst random mutants of TEM-1 β-lactamase, based on the ability to bind BLIP. Asp-101, Leu-102, Val-103, Ser-106, Pro-107, Thr-109, His-112, Ser-235, Gly-236, and Gly-238 were found to be critical for tight binding of BLIP. The selected β-lactamase mutants A113L/T114R and E240K had tighter binding to BLIP by over 6- and 11-fold, respectively. Combining A113L/T114R and E240K mutations resulted in 550-fold tighter binding between the enzyme and BLIP with a K_i of 0.40 pM.

Schroeder *et al*. (2002) performed inhibition assays by BLIP with R164S, R244S, and R164S/R244S mutant forms of TEM β-lactamase that were prepared by site-directed mutagenesis. Experiments demonstrated that inhibition constant of BLIP for wild-type TEM was K_i of 2.76 nM. The TEM R244S mutant and TEM R164S were shown to inhibit BLIP with K_i of 9.48 nM and K_i of 14.6 nM, respectively. Investigations also showed that K_i value of 6.86 nM was obtained in assays using double mutant TEM R164S/R244S

enzyme at a 100 nM concentration, while it was 13.3 nM at 200 nM concentration (Schroeder *et al.,* 2002). Zhang and Palzkill showed that the Y50A substitution in BLIP mutant resulted in TEM-1 β-lactamase 50-fold tighter than wild type BLIP. In addition, Y50A/E73A double mutant was shown to bind TEM-1 β-lactamase approximately 10-fold tighter than wild type BLIP (Zhang and Palzkill, 2003).

Fryszczyn and coworkers used Phage Display method that enables to capture tight-binding mutants by using a commercially available anti-His-tag antibody. The experiments concluded with the determination of BLIP Y50M mutant could inhibit TEM-1 β-lactamase with the affinity of 0.12 nM, which is 3 fold more than wild-type BLIP. This study also revealed that, inhibition constants for Y50A BLIP mutant was 0.07 nM, whereas it was 0.40 nM for wild-type BLIP (Fryszczyn *et al.*, 2011).

3. MATERIALS

3.1. Bacterial Strains and Plasmids

In this study, pET-26b(+) vector was used for periplasmic expression of BLIP. This vector is highly efficient for expression under the control of the strong T7 promoter (Novagen, 2003). The experiments were performed with the *E. coli* BL21(DE3) strain which is compatible with protein expression vectors that are under the control of the T7 promoter, such as pET vectors. pET-26b(+) plasmid vector was purchased from Novagen. pET-26b(+) vector carrying the BLIP gene with the pelB leader sequence (pET-26EA) was from a previous study by Ezgi Akkaya (Akkaya, 2010). pET-26b(+) vector carrying the BLIP gene with the native leader sequence (pET-26SJ) was a generous gift from Susan Jensen. *E. coli* BL21(DE3) cells were obtained from TÜBİTAK-GEBI. pUC18 plasmid vector was from our laboratory stock.

3.2. Chemicals and Enzymes

All the chemicals and the solutions used in this study were purchased from APPLICHEM (Germany), MERCK (Germany), MOLEKULA (Germany) or SIGMA (USA). DNA ladders and protein molecular weight markers were purchased from Fermentas (USA).

3.3. Laboratory Equipment

Table 3.1. List of laboratory equipments.

Purpose	Equipment
Absorbance Measurement	DU 640 Spectrophotometer (Beckman, USA) Specord 200 (Analytikjena, UK)
Centrifugation	16PK centrifuge (SIGMA, Germany)
Deepfreezer	Ultra Low Temperature Freezer U410 Premium (New Brunswick Scientific, USA)
Ice machine	FBOC Icematic
Incubation	FN500 Incubator (Nüve, Turkey)
Orbital Shaker	ZHWY-211B Shaker Incubator (ZHICHENG, China)
Sterilization	Steam Sterilizer OT40L (Nüve, TURKEY)
pH Measurement	pH meter (SCHOTT, Germany)
Pipetting	1-10, 10-100, 100-1000 µl pipettes (Thermo Electron Corporation, CANADA)
Polyacrylamide Gel Electrophoresis	Mini-PROTEAN® 3 Cell (Biorad, USA)
Power Supply	Power EC250-90 (Thermo Electron Corporation, CANADA)
Refrigerating	Refrigerator (RT59EBPN)
Sterile Environment	Microbiologic Safety Cabinet MN 120 (Nüve, Turkey)
Vortexing	Reax Top Vortex (Heidolph, Germany)
Water Purification Systems	MILLI-Q UF Plus (MILLIPORE, USA) MILLIPORE Elix® 5 UV (MILLIPORE, USA)
Weighting	Balance XB 220A (Precisa, Switzerland)

3.4. Growth Media

Table 3.2. LB medium.

Chemical	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Add dH_2O up to 1 liter	

Table 3.3. LB-agar medium.

Chemical	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Add dH_2O up to 1 liter	

3.5. Buffers and Solutions

3.5.1. SDS-PAGE Buffers and Solutions

Table 3.4. Electrophoresis separating gel buffer (1.5 M Tris, pH 8.8).

Chemical	Amount
Tris	18.15 g
Adjust pH to 8.8 with HCl and add dH_2O up to 100 ml	

Table 3.5. Electrophoresis stacking gel buffer (0.5 M Tris, pH 6.8).

Chemical	Amount
Tris	3 g
Adjust pH to 6.8 with HCl and add dH_2O up to 100 ml	

Table 3.6. Acrylamide-bisacrylamide solution (30:0.8).

Chemical	**Amount**
Acrylamide	29.2 g
Bisacrylamide	0.8 g
Add dH_2O up to 100 ml	

Table 3.7. 10% SDS.

Chemical	**Amount**
SDS	10 g
Add dH_2O up to 100 ml	

Table 3.8. 10% APS.

Chemical	**Amount**
APS	0.5 g
Add dH_2O up to 5 ml	

Table 3.9. SDS-PAGE separating gel (12% w/v, pH 8.8).

Chemical	**Amount**
Acrylamide bisacrylamide solution	4 ml
Electrophoresis separating gel buffer	2.5 ml
dH_2O	3.5 ml
10% SDS	100 µl
10% APS	50 µl
TEMED	5 µl

Table 3.10. SDS-PAGE stacking gel (5% w/v, pH 6.8).

Chemical	Amount
Acrylamide bisacrylamide solution	0.67 ml
Electrophoresis stacking gel buffer	1 ml
dH_2O	2.5 ml
10% SDS	42 µl
10% APS	30 µl
TEMED	5 µl

Table 3.11. 2X sample buffer.

Chemical	Amount
Electrophoresis separating gel buffer	2.5 ml
10% SDS	4 ml
Glycerol	2 ml
2-Mercaptoethanol	1 ml
Bromophenol blue	0.02 g
Add dH_2O up to 10 ml, store at -20°C	

Table 3.12. 10X electrophoresis running buffer.

Chemical	Amount
Tris	30.3 g
Glycine	144 g
SDS	10 g
Add dH_2O up to 1 liter	

Table 3.13. SDS-PAGE gel fixing solution.

Chemical	Amount
Ethanol	50%
Phosphoric acid	2%

Table 3.14. SDS-PAGE gel washing solution (Solution 2).

Chemical	Amount
Methanol	34%
Ammonium sulfate	17%
Phosphoric acid	2%

Table 3.15. SDS-PAGE gel staining solution.

Chemical	Amount
Methanol	34%
Ammonium sulfate	17%
Phosphoric acid	2%
Coomasie Brilliant Blue – G250	0.066%

3.5.2. Native-PAGE Buffers and Solutions

Table 3.16. 4X separating gel buffer (1.5 M Tris, pH 8.8).

Chemical	Amount
Tris	18.15 g
Adjust pH to 8.8 with HCl and add dH_2O up to 100 ml	

Table 3.17. 4X stacking gel buffer (pH 6.8).

Chemical	Amount
Tris	15.1 g
Adjust pH to 6.8 with HCl and add dH_2O up to 50 ml	

Table 3.18. 40% Acrylamide-bisacrylamide solution (37.5:1).

Chemical	**Amount**
Acrylamide	100 g
Bisacrylamide	2.65 g
Add dH_2O up to 250 ml	

Table 3.19. 10% APS.

Chemical	**Amount**
APS	0.5 g
Add dH_2O up to 5 ml	

Table 3.20. Native-PAGE Stacking gel mixture (pH 8.8).

Chemical	**Amount**
40% Acrylamide-Bisacrylamide Solution	0.4 ml
4X Stacking gel buffer	1.0 ml
dH_2O	2.6 ml
10% APS	50 µL
TEMED	5 µL

Table 3.21. Native-PAGE separating gel mixture (pH 8.8).

Chemical	**Amount**
Acrylamide bisacrylamide solution	2.5 ml
4X Separating gel buffer	2.5 ml
dH_2O	1 ml
10% APS	100 µl
TEMED	10 µl

Table 3.22. 2X sample buffer.

Chemical	Amount
4X Stacking Gel Buffer (pH 6.8)	2.5 ml
Glycerol	2 ml
Bromophenol blue	0.02 g
Add dH_2O up to 10 ml, store at -20°C	

Table 3.23. 1X running buffer (pH 8.3).

Chemical	Amount
Tris	6 g
Glycine	28.8 g
Add dH_2O up to 2 liter, pH should be 8.3 without adjustment	

Table 3.24. Native-PAGE destaining solution.

Chemical	Amount
Methanol	100 ml
Acetic acid	100 ml
Add dH_2O up to 1 liter	

Table 3.25. Native-PAGE Staining solution.

Chemical	Amount
Commassie Brilliant Blue–R250	0.25 g
Methanol	125 ml
Acetic acid	25 ml
Add dH_20 up to 100 ml	

3.5.3. Enzyme Activity Measurement Buffers and Solutions

Table 3.26. K^+PO_4 Buffer (1 M, pH 7.0).

Chemical	Amount
1M K_2HPO_4	450 ml
1M KH_2PO_4	550 ml

3.5.3.1. Preparation of CENTA Stock Solution. 25 mg CENTA was dissolved in 10 ml 50 mM K^+PO_4 buffer and aliquoted and stored at -20 °C. The concentration of the stock solution was 4.7 mM.

3.5.4. Other Stock Solutions

Table 3.21. Other stock solutions.

Chemical	Amount
Ampicillin	100 mg/ml
Kanamycin	50 mg/ml
1M IPTG	23.8302 g/10 ml

4. METHODS

4.1. Sterilization

All experiments were conducted with sterilized equipments. Tips, glassware, centrifuge tubes, solutions and culture media were sterilized at 1 atm and 121 °C for 15 min in an autoclave. Stock solutions of antibiotics and IPTG were filter sterilized through 45 μm pore sized membrane. Experiments that required sterile environment were carried out under laminar flow.

4.2. Preparation of Bacterial Stocks

5 ml of sterile LB medium was inoculated with a single colony of bacteria from a master plate. Inoculation was carried out by a sterile tip. Preculture was incubated overnight in an orbital shaker that was adjusted to 37 °C and 180 rpm. When needed, LB medium was supplemented with appropriate antibiotics. Grown cells were added at 1:1 ratio to sterile centrifuge tubes containing 50% sterile glycerol and stocks were stored at -80 °C.

4.3. Growth Conditions

Preculture was pipetted into sterile culture and cultures were grown in orbital shakers at 37 °C at 180 rpm. In order to provide efficient aeration, culture volume was adjusted to one fifth of the flask volume. All experiment were conducted with LB medium which was supplemented with 50 μg/ml kanamycin for the pET-26b(+) harboring cells and 100 μg/ml ampicillin for the pUC18 harboring cells. Growth was monitored by measuring the OD at 600 nm using a spectrophotometer. The induction of the expression of the recombinant protein was provided by adding IPTG to the growing culture to give a final concentration of 1 mM or 0.2 mM when OD_{600} nm reached the value of 0.5 per ml of culture.

4.4. Determination of the Growth Curve

100 ml sterile LB medium was inoculated with preculture at a final OD of 0.7 and placed in the orbital shaker at 37 °C. At desired time intervals, 0.25 - 1 ml of samples were taken from the culture and optical density of the sample was measured at 600 nm to monitor growth. LB medium was used for both the blank and diluting the samples to keep the spectrophotometric readings within reliable range of 0.2 to 0.6.

4.5. Viable Cell Count

Bacterial samples were plated to determine the number of colony forming units. For a ten-fold dilution, 100 µl of the bacterial sample taken at the desired time interval was transferred into a centrifuge tube containing 0.9 ml of fresh LB medium and vortexed to homogenize. This procedure was repeated for each ten-fold dilution. In order to obtain desired dilution, this procedure was repeated with samples taken from the previous centrifuge tube containing 1 ml diluted sample. Finally, 1 ml of the appropriate dilution was deposited in a sterile Petri dish, in triplicate, and agar containing appropriate antibiotic were poured into Petri dishes. The sample and the liquid agar were mixed gently by moving the plates through an eight-shaped path on the ground. The plates were left to cool to harden and then incubated overnight at 37 °C.

4.6. Extraction of Periplasmic Proteins

The periplasmic proteins of *E. coli* cells were extracted by using the osmotic shock protocol described by Nossal and Heppel (1966). Sterile LB medium was inoculated with a preculture at a 1:100 dilution and was incubated in the orbital shaker at 37 °C and 180 rpm. When the cells reached the optical density of 0.5 at 600 nm, they were induced with 0.5 or 0.2 mM IPTG. 3 hours after the induction time, cells were harvested by centrifugation at 7,000 rpm for 10 minutes at 4°C. Afterwards, the supernatant was discarded and the cells were resuspended in osmotic shock solution containing 20% w/v sucrose, 30 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The suspension was gently incubated at room temperature for 20 minutes and then centrifuged at 9,000 rpm for 20 minutes at 4 °C. Supernatant was resuspended in ice-cold 5 mM $MgCl_2$ and gently incubated on ice for 20 minutes. The cells

were removed from the periplasmic extract by centrifugation at 9,000 rpm for 20 minutes. The supernatant contained periplasmic proteins including TEM-1 β-lactamase and stored at -20 °C for further use.

4.7. Determination of Protein Concentration

Protein concentration in the periplasmic protein extract was determined using the method described by Bradford (1976). This assay is based on monitoring absorbance change at 595 nm, according to binding of the dye, Coomassie Brilliant Blue G-250 to protein. Bovine Serum Albumin (BSA) was used as the standard for the preparation of the calibration curve. Bradford samples with different BSA concentrations were prepared and the absorbances were measured at 595 nm. Monitored absorbance values were plotted using linear regression analysis. The equation obtained from the linear regression was used for determination of the protein concentration of cell extracts. The calibration curve obtained is given in Appendix A.

4.8. In-vivo Enzyme Activity Measurement

In-vivo β-lactamase - BLIP interaction in the periplasmic protein extract was detected by measuring β-lactamase activity. Periplasmic extract from *E. coli* BL21(DE3) cells harboring pUC18 plasmid was used as the source of β-lactamase and periplasmic protein extracts from *E. coli* BL21(DE3) cells harboring pET-26b(+) plasmid carrying the BLIP gene was used as the source of BLIP. Inhibition assays were performed in a total reaction volume 1 ml. 470 μM CENTA (Calbiochem) was used as the substrate and was mixed with the pre-determined amounts of osmotic shock fluid. Hydrolysis of CENTA was observed by monitoring the change in the optical density at 405 nm. One Unit (U) of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at 25 °C and pH 7.0 (Equation 4.1):

$$U = \frac{\frac{dA}{dt} \times V_t \times 10^6}{\lambda \times V_s \times d} \qquad (4.1)$$

where V_t is total reaction volume (ml); dA/dt is absorbance change per time (min^{-1}); ε_λ = 6,400 $M^{-1}cm^{-1}$ for CENTA; V_s is volume of enzyme (ml); d = 1 cm.

4.9. SDS-PAGE Analysis of Periplasmic Proteins

SDS-PAGE was performed to separate proteins based on their sizes. Osmotic shock samples were mixed with 2X sample buffer at 1:1 ratio and boiled for 5 minutes for denaturation of proteins. The marker and the samples were loaded into the SDS polyacrylamide gel wells and electrophoretic separation was performed at 110 V for approximately 75 minutes. Gels were gently removed from glass plates and placed in fixing solution overnight to fix the proteins. The next day, gels were washed with Solution 2 for an hour and were stained with Commassie G-250 at least overnight. Distilled water was used to remove unbound stain. The gels were dried.

4.10. Native PAGE Analysis of Periplasmic Proteins

Native PAGE uses the same discontinuous chloride and glycine ion fronts as SDS-PAGE to form moving boundaries that stack and then separate polypeptides by charge to mass ratio. Protein samples to be run on native gels should be avoided from denaturation therefore; samples were not heated and kept refrigerated in order to retain the activity of endogenous proteases to a minimum. Proteins were prepared in a nonreducing nondenaturing 2X sample buffer, which maintained secondary structure of the proteins and native charge density. After loading marker and samples, electrophoretic separation was performed at 120 V for nearly 75 min. When the electrophoretic separation was finished, the gels were gently removed from the glass plates and placed in staining solution for 2.5-3 hours. The gels were placed in destaining solution and left for an overnight in order to remove excess dye and fix the proteins.

4.11. Enzyme Activity Measurement by Ampicillin Hydrolysis

Kinetic hydrolysis of ampicillin by β-lactamase was examined in order to find out whether ampicillin can be used as a substrate for β-lactamase in place of CENTA. A curve for the rate of ampicillin hydrolysis as a function of ampicillin concentration was plotted.

For this purpose, seven stock solutions with varying ampicillin concentrations were prepared. Change in optical density was recorded during ampicillin hydrolysis. Initial rates were determined for these seven substrate concentrations and plotted as a function of ampicillin concentration. All rate measurements were conducted in duplicate.

In-vitro enzyme activity was determined by monitoring the hydrolysis of ampicillin by β-lactamase, at 240 nm ($\Delta\varepsilon = 538\ M^{-1}/cm^{-1}$) (Vakulenko *et al*., 2002). Enzyme assays were performed in 0.05 M KPO_4^+ buffer (pH 7.0). Periplasmic protein extracts obtained from *E. coli* BL21(DE3) cells were used to measure the kinetics of ampicillin hydrolysis. Varying concentrations of the substrate and the osmotic shock fluid were mixed and the final volume of the reaction was 1 ml. Total reaction was carried out in a 0.1 or 1.0-cm pathlength kuvartz cuvette. Values of enzyme activity were determined by using Equation 4.1 that is given in the Section 4.8. Recorded values of absorbance change at 240 nm per minute at each ampicillin concentration and extinction coefficient of the subtsrate were substituted into the equation, therefore activity of β-lactamase to hydrolyze varying concentrations of ampicillin were obtained as U/L. The results were expected to shed light on similarity between results that were obtained from kinetics of CENTA hydrolysis.

5. RESULTS AND DISCUSSION

In this study, growth profiles and viability of *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under induced and uninduced conditions were investigated. In order to gain insight about *in-vivo* β-lactamase - BLIP complex formation, total β-lactamase activity per viable *E. coli* BL21(DE3) (pUC18 + pET-26b(+)), *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under induced and uninduced conditions were calculated. In order to visualize the presence of BLIP - β-lactamase complex, periplasmic protein extracts were electrophoretically analyzed.

To investigate the effect of *pelB* and native leader sequence on BLIP expression and translocation, inhibition of β-lactamase by BLIP in *E. coli* BL21(DE3) (pUC18 + pET-26EA) and in *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under induced conditions were compared.

5.1. Bacterial Constructs

In this study, simultaneous expression of two different proteins encoded from pET-26 and pUC18 expression vectors, carrying *bli* and $bla_{\text{RTEM-1}}$ genes, respectively, was studied in *E. coli* BL21(DE3) cells. pUC18 plasmid was used for the expression of R-TEM-1 β-lactamase. pET-26b(+) vector possessing the BLIP gene with *pelB* signal sequence (pET-26EA) and native signal sequence (pET-26SJ) were used for periplasmic expression of BLIP. β-lactamase is produced constitutively whereas the expression of BLIP is efficiently achieved by inducing the T7 promoter with IPTG.

In an effort to investigate simultaneous expression of BLIP and β-lactamase and *in-vivo* inhibition of β-lactamase by BLIP, *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26EA or pET-26SJ plasmid was used. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) were used as control for comparative analysis.

5.2. Simultaneous Expression of BLIP and β-lactamase

β-lactamase is a periplasmic protein in Gram negative hosts such as *E. coli* hence it is translocated to the periplasm following expression. BLIP is also synthesized as a periplasmic protein in its host *S. clavuligerus*. When expressed in other hosts, natural periplasmic proteins such as BLIP may not be translocated from the cytoplasm. However, in order to achieve *in-vivo* β-lactamase inhibition by BLIP, BLIP should be translocated to the periplasm in *E. coli*. For this reason two different BLIP constructs have been used. The first BLIP construct possessed the native BLIP signal sequence and the second one possessed *pelB* leader sequence. In this manner, the purpose was to direct BLIP to the periplasm where β-lactamase resided. This would enable BLIP to recognize, bind and eventually inhibit β-lactamase. As a result of β-lactamase inhibition, decreased growth rate or cell death was expected. To test this hypothesis, growth profiles of *E. coli* BL21(DE3) cells harboring pUC18 in addition to pET-26EA or pET-26SJ were monitored and the number of viable cells was compared in the presence and absence of BLIP expression.

5.2.1. Growth Profiles

In order to investigate the effect of simultaneous BLIP and β-lactamase expression on cell growth, *E. coli* BL21(DE3) cells harboring two plasmids were induced with 0.2 mM IPTG when OD_{600} nm reached 0.5 to facilitate BLIP expression. Since β-lactamase was constitutively expressed, upon induction BLIP synthesis and hence growth inhibition due to BLIP and β-lactamase complex formation was expected. *E. coli* BL21(DE3) (pUC18 + pET26b(+)) cells grown under the same induced and uninduced conditions were used for comparison.

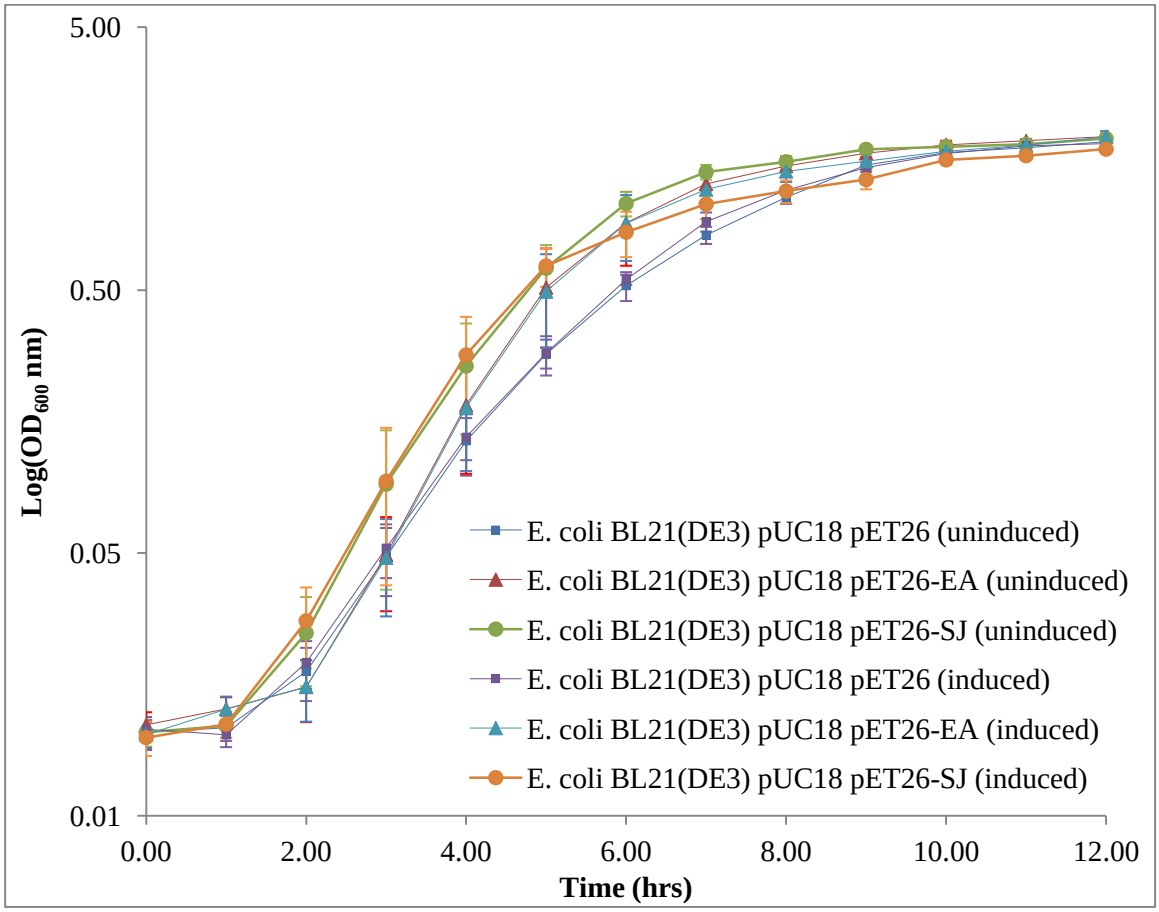

Figure 5.1. Growth profiles of uninduced and 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26b(+)), *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells.

Figure 5.1 showed that all cells harboring double plasmids had comparable growth profiles. When each cell type was considered separately, it was found that induction did not have any effect on the growth of *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells. This is not unexpected since these cells do not synthesize the recombinant protein BLIP. They could only synthesize β-lactamase. Similarly, the growth of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells were not affected by induction although BLIP expression was induced with IPTG. It was seen that there was no measurable β-lactamase inhibition. On the other hand, the growth of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were significantly reduced 1 hour after induction when compared to the uninduced cells. This suggested that there might be β-lactamase inhibition by BLIP since cell growth slowed down following BLIP expression. However since BLIP is a recombinant protein, retardation in cell growth might also be the cause of metabolic burden.

5.2.2. Growth and Viability Profiles

In order to examine the effect of BLIP expression on cell growth and viability, *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells grown under uninduced and induced conditions were compared. Final IPTG concentrations of 0.2 and 1 mM were tested. Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells were used as control. There were not expected to synthesize BLIP.

Optical density for uninduced and induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells were plotted in Figure 5.2 for the case when 1 mM IPTG was used for induction. These experiments have been performed in triplicate and error bars represent the standard error of the mean.

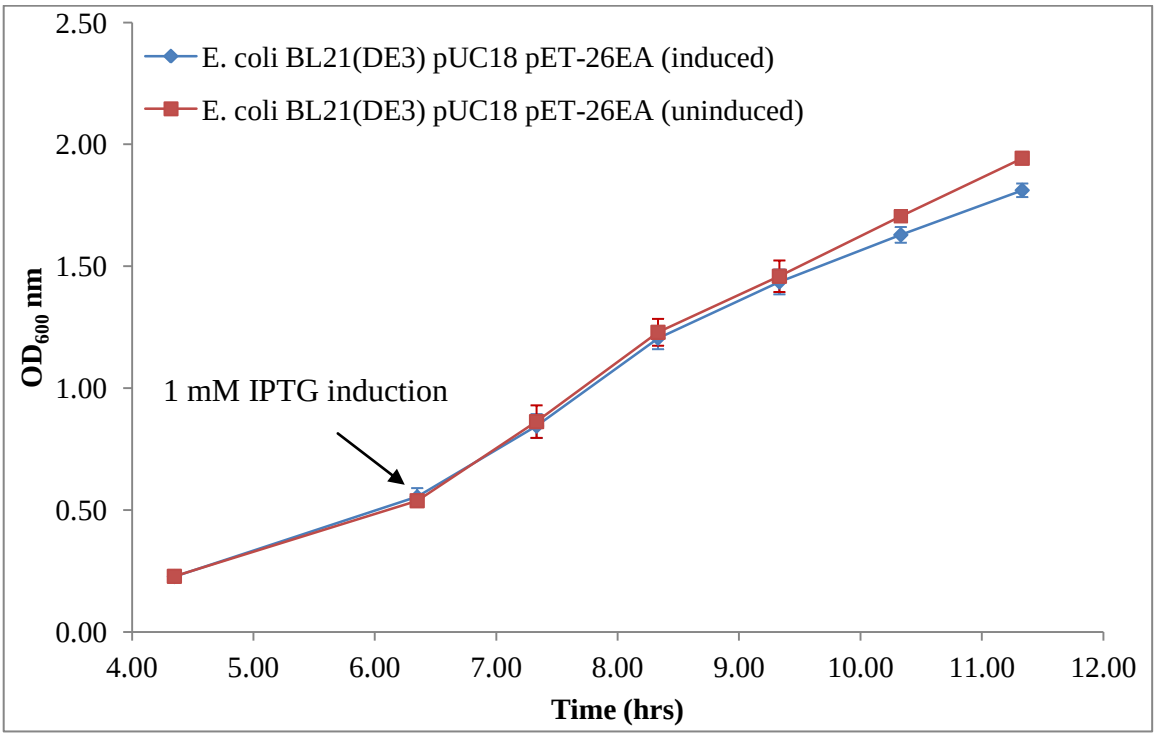

Figure 5.2. Growth curves of *E. coli* BL21(DE3) cells harboring pUC18 and pET-26EA vectors with 1 mM IPTG induction and without induction.

Based on the results in Figure 5.2, growth profile of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells measured as optical density did not vary significantly upon induction. Only about 5 hours after induction, the final optical density of the induced cells was

slightly lower. This suggested that 1 mM IPTG did not have a significant effect on cell growth measures as optical density.

As a second attempt, IPTG concentration used was reduced to observe its effect on cell growth. Figure 5.3 shows the growth profile of BL21(DE3) (pUC18 + pET-26EA) cells when induce with 0.2 mM IPTG. These cells were allowed to grow 3 hours after induction. These experiments have been performed in triplicate and error bars represent the standard error of the mean.

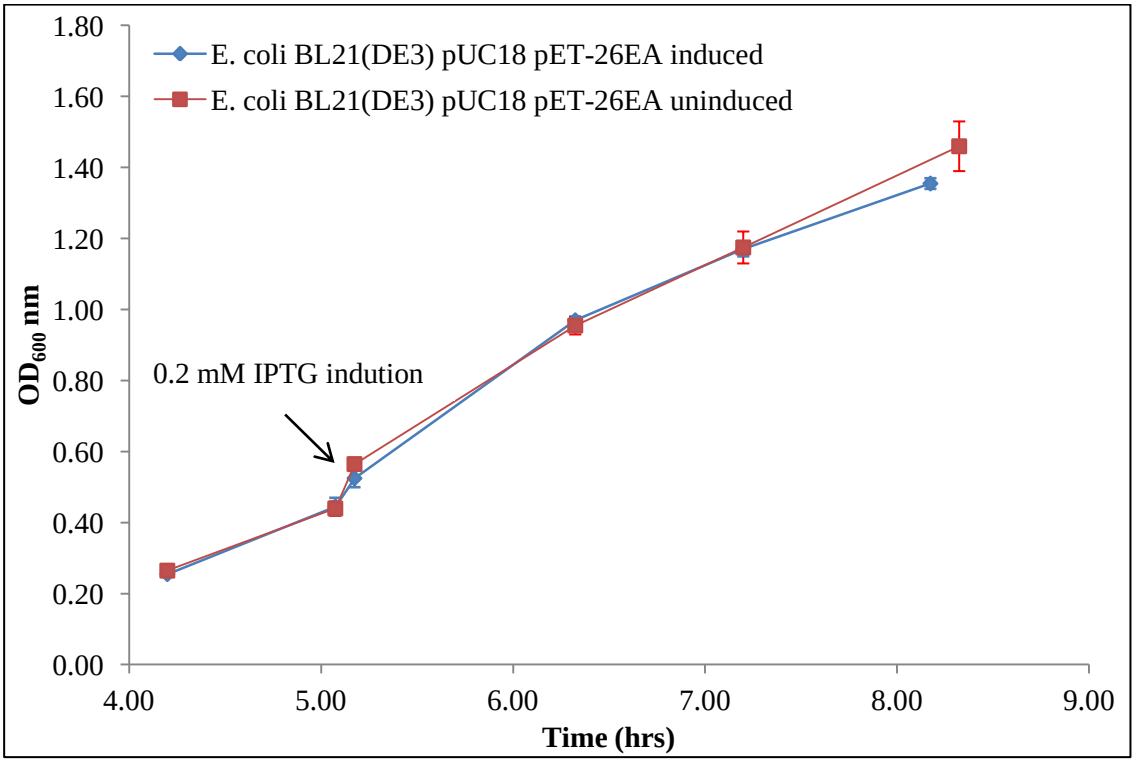

Figure 5.3. Growth curves of *E. coli* BL21(DE3) cells harboring pUC18 and pET-26EA vectors with 0.2 mM IPTG induction and without induction.

According to Figure 5.3, optical density of both *E. coli* BL21(DE3) (pUC18 + pET-26EA) cultures expressing only β-lactamase and both β-lactamase and BLIP are in the same range 2 hours after IPTG induction. However, after three hours of induction, optical density of the induced cells was slightly lower than that of the uninduced cells.

Since measurement of optical density relies on turbidity, it cannot provide reliable information on the number of viable cells in the cultures. For that reason, viable *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells were counted following induction, as described in Section 4.2. Number of viable cells may not be directly proportional to the optical density of growing cell cultures because the sample may include both dead and live cells. Since β-lactamase was expected to be inhibited by BLIP, a decline in the number of viable cells was expected following BLIP expression.

Upon induction with 0.2 mM IPTG, growth and viable cells of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cultures were measured. Results for both induced and uninduced cultures were plotted in Figure 5.4. Experiments were performed in triplicate and error bars indicated the range of error.

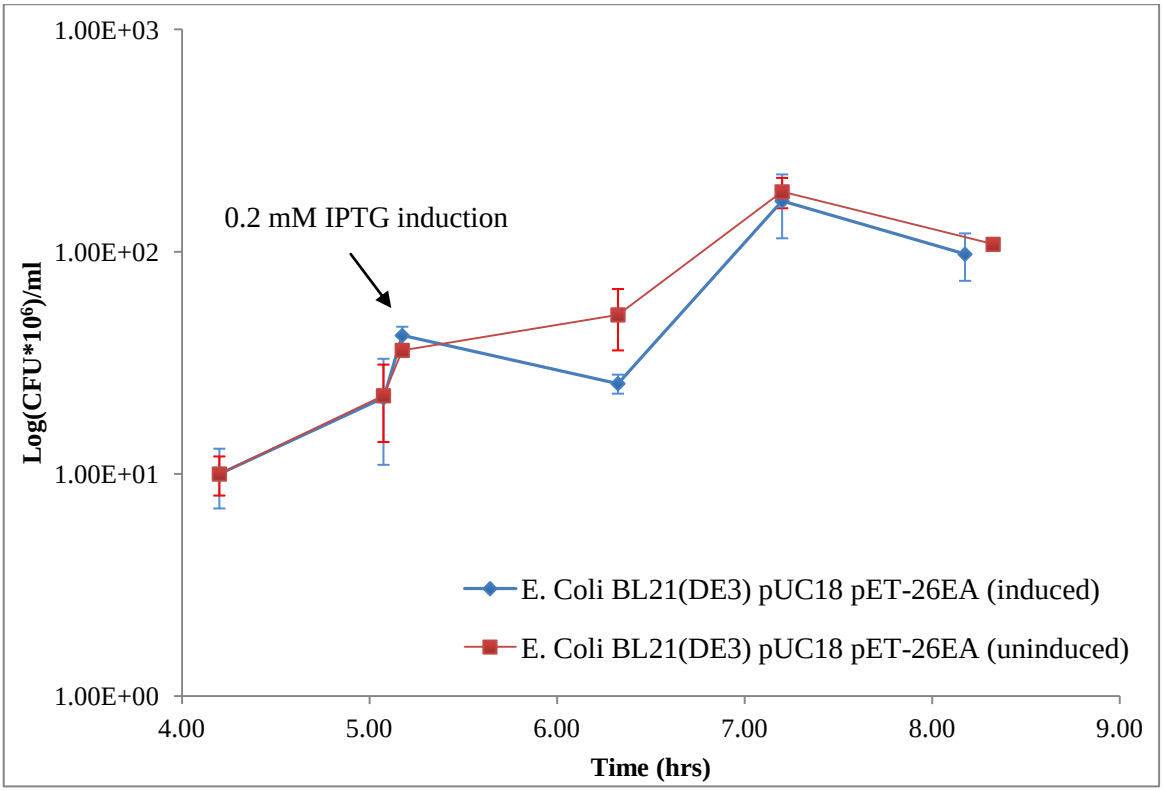

Figure 5.4. Cell viability profiles of 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells, uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells.

As it can be seen from Figure 5.4, before induction, the number of viable cells in both cultures was similar. Following induction, the number of viable cells in induced

cultures fell below the value obtained for uninduced cultures. Although the trend was similar for both cultures, the viable cell number in induced cultures always remained below the value of uninduced cultures.

Until the end of exponential phase cells multiply in number and there is no considerable cell death. Under this condition optical density and the number of viable cells should be linearly proportional. Cell death becomes significant as cells enter stationary phase of growth. Unfortunately spectrophotometers cannot differentiate between dead cells and live cells, since optical density measurements are based on turbidity. Therefore it might not be possible to directly relate optical density and live cell number.

In order to investigate the relationship between the number of live cells and optical density, viable cell number versus optical density at 600 nm was plotted (Figure 5.5) for *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells grown under induced and uninduced conditions. This experiment has been conducted in triplicate and error bars represent the error range from the average of the cells.

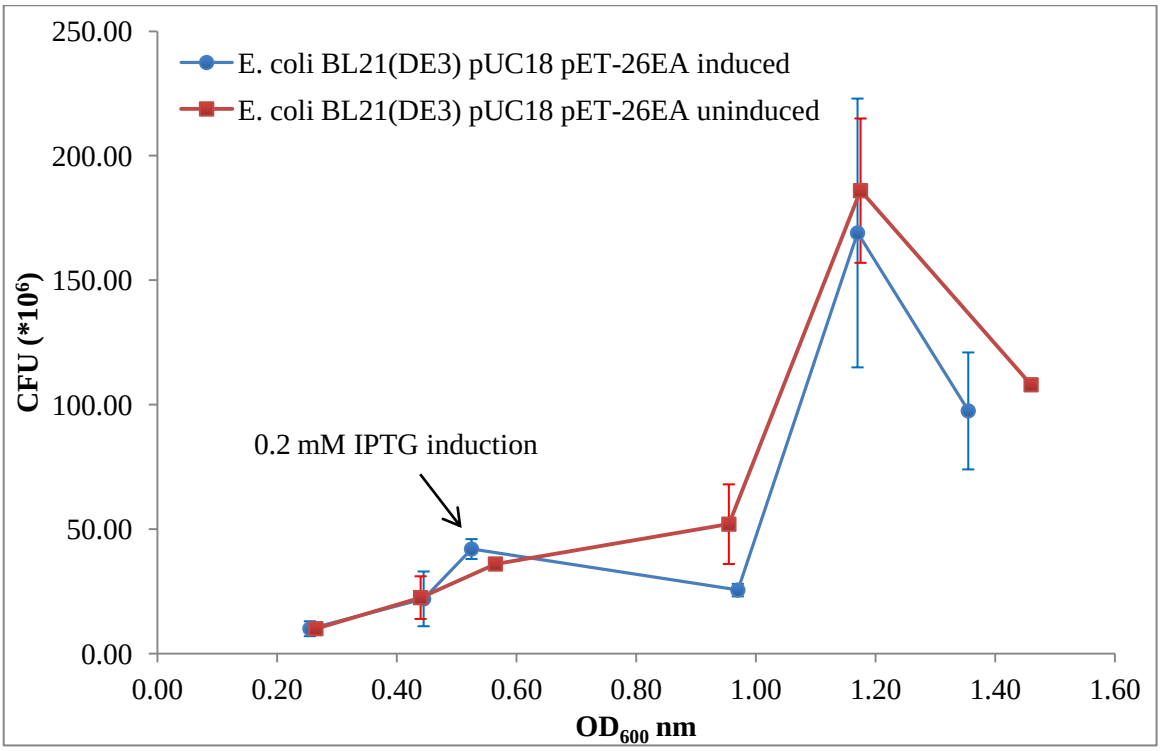

Figure 5.5. Cell viability versus OD_{600} nm; 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells, uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells.

In the light of Figure 5.5, it can be seen that the CFU increases with OD until the late exponential phase (OD_{600} = 1.2) after which the OD continues to increase while the CFU starts to decline. This result indicates that the cell cultures contain dead cells after the late exponential phase as expected. Unfortunately, there as no linear relation between optical density and viable cell number until late exponential phase. This should probably be due to error hence these experiments should be repeated. Overall, these results have shown that viable cell numbers are more reliable in terms of monitoring cell death and if optical density is to be monitored as a measure of cell growth, that should be only until the end of exponential phase.

5.2.3. Intracellular β-Lactamase Activity

Investigation of growth profiles, monitored as optical density, or measurement of viable cell number has not yielded sufficient information on β-lactamase – BLIP interaction in the periplasmic space of *E. coli*. With the initiation of BLIP expression, inhibition of β-lactamase was expected due to the formation of β-lactamase - BLIP complex. This should lead to a reduction in total β-lactamase activity. To test this hypothesis β-lactamase activity of the extract obtained from *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells grown under induced and uninduced conditions was compared. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were used as control.

Table 5.1 summarizes total β-lactamase activity (U/L) of the periplasmic protein extracts obtained from *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and total β-lactamase activity per total protein found in the periplasmic extract (U/mg).

Table 5.1. β-lactamase activity in the absence and presence of BLIP expression for 1.5 hours.

		1.5 hours after induction	
Expression System	**Proteins expressed**	**β-lactamase Activity (U/L periplasmic extract)**	**β-lactamase Activity (U/mg protein)**
E. coli BL21(DE3) (pUC18 + pET-26EA) **uninduced**	β-lactamase	642.19	1.81
E. coli BL21(DE3) (pUC18 + pET-26EA) **induced**	β-lactamase + BLIP	93.44	0.22
Inhibition		85%	88%

Table 5.2. β-lactamase activity in the absence and presence of BLIP expression for 3 hours.

		3 hours after induction	
Expression System	**Proteins expressed**	**β-lactamase Activity (U/L periplasmic extract)**	**β-lactamase Activity (U/mg protein)**
E. coli BL21(DE3) (pUC18 + pET-26EA) **uninduced**	β-lactamase	937.50	3.98
E. coli BL21(DE3) (pUC18 + pET-26EA) **induced**	β-lactamase + BLIP	742.41	1.77
Inhibition		21%	56%

Results in Table 5.1 and Table 5.2 indicate that total β-lactamase activity of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells was 642.19 and 937.50 U/L 1.5 and 3 hours after OD_{600} reached 0.5, respectively. β-lactamase activity was reduced by 85% in the presence of BLIP after 1.5 hours and by 21% in the presence of BLIP after 3 hours. When β-lactamase and BLIP were co-expressed, β-lactamase was inhibited by BLIP leading to a decrease in total intracellular β-lactamase activity. Although, β-lactamase activity of uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cell cultures increased 1.5 fold, β-lactamase activity per periplasmic extract (U/L) of induced *E. coli* BL21(DE3) (pUC18 +

pET-26EA) cell cultures increased 8 fold as induction period increased from 1.5 to 3 hours.

5.2.4. Investigation of Relation Between β-lactamase Expression and Number of Viable Cells

The amount of β-lactamase production was normalized based on number of viable cells. In an effort to compare the β-lactamase activity of each culture, total β-lactamase activity per CFU found in the culture was measured. *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells, were used for simultaneous expression of β-lactamase and BLIP and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were used as control. The number of viable cells in the culture was counted at different OD values. Under uninduced conditions, β-lactamase activity of both *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were examined. The cells were plated as described in Section 4.5 when OD_{600} reached 0.9 - 1.0. Experiments were conducted in duplicate and results are given in Table 5.3.

Table 5.3. Range of Activity, OD_{600}, and CFU values of uninduced cells when cells were harvested at OD_{600} = 0.9 - 1.0.

	Experiment 1		**Experiment 2**	
	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**
Activity (U/L periplasmic extract)	51.46	77.74	16.35	48.32
Harvest OD_{600} nm	0.93	0.96	0.91	0.89
Activity/Harvest OD_{600} nm	55.06	81.26	17.87	54.17
CFU*10^6	167.00	412.00	95.00	331.50
Activity/CFU*10^6	0.31	0.19	0.17	0.15
Protein concentration (mg/µl)	0.11	0.10	0.17	0.12

Based on the results presented in Table 5.3, in the absence of BLIP expression, β-lactamase activity per viable cell of *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) were in the same range. β-lactamase activity per colony forming units (CFU) of cells expressing both proteins varied between 0.17 - 0.31 and similarly β-lactamase activity per CFU values of control cells varied between 0.15 - 0.19. β-lactamase activity per viable cell of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and the control were comparable.

Although optical density at the time cell harvest were very close for both cultures, the number of viable cells in *E. coli* BL21(DE3) (pUC18 + pET-26EA) cultures was considerably lower than the control culture. Activity of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells was slightly lower than the control. Mainly, the normalized activities remained in the same range.

These experiments were repeated, but cells were harvested at optical density of 0.5 to 0.6. Results of the duplicate experiments are listed in Table 5.4.

Table 5.4. Range of Activity, OD_{600}, and CFU values of uninduced cells when cells were harvested at OD_{600} = 0.5.

	Experiment 1		**Experiment 2**	
	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**
Activity (U/L periplasmic extract)	159.50	46.48	405.33	45.85
Harvest OD_{600} nm	0.60	0.49	0.58	0.54
Activity/Harvest OD_{600} nm	265.52	94.89	693.22	85.47
CFU*10^6	164.00	100	260.00	160.50
Activity/CFU*10^6	0.97	0.47	1.56	0.29
Protein concentration (mg/µl)	0.14	0.08	0.06	0.11

According to Table 5.4, number of viable cells of E. coli BL21(DE3) (pUC18 + pET-26EA) cells ($164*10^6$ - $260*10^6$) was in the same range with the *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells ($100*10^6$ - $161*10^6$). β-lactamase activity per viable cell of *E. coli* BL21(DE3) (pUC18 + pET-26EA) was about 3 fold fold higher (1.00 - 1.56) than β-lactamase activity per viable cell of *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) (0.29 - 0.47).

5.2.5. Investigation of BLIP Inhibitory Activity on β-lactamase

The effect of simultaneous co-expression of BLIP and β-lactamase was investigated by measuring β-lactamase activity. *E. coli* BL21(DE3) (pUC18 + pET-26EA), *E. coli* BL21(DE3) (pUC18 + pET-26SJ) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cell cultures were induced with 0.2 Mm IPTG, when OD reached 0.5 at 600 nm. Induction OD was chosen as 0.5, which corresponds to the exponential growth phase of cell cultures. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were used as control due to absence of BLIP gene. pET-26EA and pET-26SJ vectors harbored the gene encoding BLIP with *pelB* and native leader sequence, respectively. Both *pelB* and native signal sequences were used for periplasmic localization of BLIP in *E. coli* BL21(DE3) cells.

All cultures were compared under induced conditions and effect of both *pelB* and native leader sequences and BLIP expression on β-lactamase actvity was investigated. Cell cultures were grown 3 hours after induction. Then optical density was measured and cells were plated to obtain CFU. Average values of experiments were summarized in Table 5.5. Results of experiments were given in APPENDIX C.

Table 5.5. CFU, Activity/Harvest OD, and Activity/CFU values of induced cells when cells were harvested 3 hour after induction.

	Average of Induced Experiments		
	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26SJ)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**
Activity (U/L periplasmic extract)	561.62	332.97	194.03
Harvest OD at 600 nm	1.4087	1.1570	1.5375
Induction OD at 600 nm	0.5184	0.5147	0.5089
Activity/Harvest OD at 600 nm	392.09	283.70	126.22
CFU*10^6	217.72	116.70	590.75
Activity/CFU*10^6	3.07	6.23	0.33
Protein concentration (mg/µl)	0.19	0.17	0.05

Table 5.5 shows that average values obtained for β-lactamase activity in *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were 561.62 and 332.97, respectively. While in the absence of BLIP, average β-lactamase activity was 194.03. *E. coli* BL21(DE3) (pUC18 + pET-26EA), *E. coli* BL21(DE3) (pUC18 + pET-26SJ) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cell cultures were induced with 0.2 mM IPTG at almost similar OD values (0.51). Harvest OD of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells was 1.4087, while that of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) was slightly lower (1.1570). The optical density of *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cell culture was 1.5375 just before harvest. The average number of viable *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells was 218 and 116, respectively. This was approximately 3-4 fold lower than average of viable cell number. However, in the presence of BLIP, β-lactamase activity per viable *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells was 10 fold higher, that of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells was 20 fold higher than that of *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells.

The 4 fold decrease in the number of viable cells number can be related to β-lactamase inhibition by BLIP. In contrast, higher β-lactamase activity and β-lactamase activity per viable cell number in the presence of BLIP can be due to the increase in expression level or the activity of β-lactamase in living cells.

In the light of these findings, it was found that comparing different cell cultures under induced conditions may led to misleading and inconsistent results, resulting from differences in copy numbers of plasmids. Therefore, to obtain reliable results about the effects of simultaneous BLIP expression on β-lactamase activity, induced and uninduced conditions of the same cell cultures were compared.

5.2.6. BLIP Inhibitory Activity on β-lactamase During 1.5 Hours After Induction

In Section 5.2.3, only total intracellular activity was examined. To further investigate relation between activity, harvesting OD and viable cell number, both uninduced and induced cell cultures were harvested 1.5 hours after induction. Uninduced cell cultures were used as control.

Experiments were conducted with *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cell cultures. In Table 5.1, it was shown that after 1.5 hours of induction period, β-lactamase inhibition was significantly lower when comparided with the inhibition value after 3 hours of induction period. For that reason, cell cultures were induced with 0.2 mM IPTG when OD_{600} reached 0.5 and grown 1.5 hours after induction. Experiments were performed once and results are given in Table 5.6.

Table 5.6. CFU, Activity/Harvest OD, and Activity/CFU values of induced cells after 1.5 hours of induction.

	Experiment 1		**Experiment 2**	
	Induced *E. coli* BL21(DE3) (pUC18 + pET-26EA)	**Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA)**	**Induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ)**	**Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ)**
Activity (U/L periplasmic extract)	43.25	239.73	302.97	1177.32
Harvest OD at 600 nm	1.1356	1.2196	1.0776	1.1326
Induction OD at 600 nm	0.4921	0.5851	0.5042	0.5231
Activity/Harvest OD at 600 nm	38.08	196.56	281.15	1039.48
CFU*10^6	5.35	108.00	60.00	370.00
Activity/CFU*10^6	8.08	2.22	5.05	3.18
Protein concentration (mg/µl)	0.1919	0.1341	0.1184	0.1157

According to Table 5.6, activity in uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells was approximately 6 fold higher than the activity in induced cells. OD values that were measured just before induction, are 0.4921 for data set and 0.5851 for control set. Harvest OD values were measured as 1.1356 and 1.2196 for induced and uninduced cell cultures, respectively. Although harvest OD values were close to each other, induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cell culture had a 20 fold lower CFU ($5.35*10^6$) when compared with uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cell culture ($108*10^6$). However, activity per CFU values of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells were 8.1 and 2.2 under induced and uninduced conditions, respectively. When *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were considered, activity in induced cells was 4 fold higher than found in uninduced cells. *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cell cultures had similar harvesting OD values 1.5 hours after induction. Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cell culture had a 6 fold higher CFU value than the induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cell culture. Similarly, activity per CFU of induced *E. coli* BL21(DE3) (pUC18 + pET-

26SJ) cell culture is slightly higher than uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cell culture.

In the light of these findings, it can be suggested that initiation of BLIP expression after IPTG induction resulted in cell death. Increased expression of β-lactamase helped induced cells to survive. This has led to an increase in β-lactamase activity per viable cell.

5.2.7. The Effect of Higher Ampicillin Concentration on β-lactamase Inhibition

In order to investigate the effect of higher ampicillin concentration on β-lactamase inhibition, *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cell cultures were grown in the presence of higher ampicillin concentration. Since β-lactamase is produced against ampicillin, the exposure to increasing ampicillin concentrations in the growth media could make a difference in the production level of β-lactamase. Therefore, ampicillin concentration was increased from 100 μg/ml to 200 μg/ml and 400 μg/ml in growth media. *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells were induced with 0.2 mM IPTG, when OD_{600} reached 0.5, in order to express both β-lactamase and BLIP. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were used as control. Findings were listed in Table 5.7.

Table 5.7. Activity values of the induced cells in the presence of high antibiotic concentration.

	β-lactamase Activity of Induced Experiments (U/L)	
Ampicillin Concentration (mg/ml)	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**
100	10.69	28.28
200	15.37	127.41
400	205.23	176.13

The results in Table 5.7 showed that, changing concentrations of ampicillin, caused variations in total β-lactamase activity. When both cell cultures were exposed to 100 mg/ml ampicillin, β-lactamase activity in *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells

were almost 3 fold higher than activity in *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells (28.28 - 10.69). When ampicillin concentration was increased to 200 mg/ml, activity of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells decreased 8.5 fold as compared to the control. Although, induction of BLIP production in 400 mg/ml ampicillin concentration did not cause a significant difference on β-lactamase activity between both cells, when compared with the results of 100 mg/ml and 200 mg/ml ampicillin concentrations, activity of *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells increased 19 and 14 fold, respectively. On the other hand, activity in *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells showed 6 and 1.4 fold increase when compared to activity measured in the presence of 100 mg/ml and 200 mg/ml ampicillin concentrations.

In the light of these observations, it can be argued that increasing ampicillin concentration from 100 mg/ml to 400 mg/ml had a considerable effect on activity in *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells showed the maximum difference in activity as ampicillin concentration increased from 100 mg/ml to 200 mg/ml.

5.3. Kinetics of Ampicillin Hydrolysis

β-lactamase activity measurements were performed with CENTA as the substrate. Since, β-lactamase is known to hydrolyze ampicillin, enzyme activity was also monitored examined by measuring the rate of ampicillin hydrolysis. Change in the absorbance during ampicillin hydrolysis by β-lactamase was used to determine whether ampicillin may be used instead of CENTA in kinetic assays or not. Periplasmic protein extracts of *E. coli* BL21(DE3) (pUC18 + pET-26EA), *E. coli* BL21(DE3) (pUC18 + pET-26SJ) and *E. coli* BL21(DE3) (pUC18 + pET-26) cells were used as the enzyme source. Changes in the values of OD_{240} nm with per time (min) for varying ampicillin concentrations was plotted (Figure 5.6).

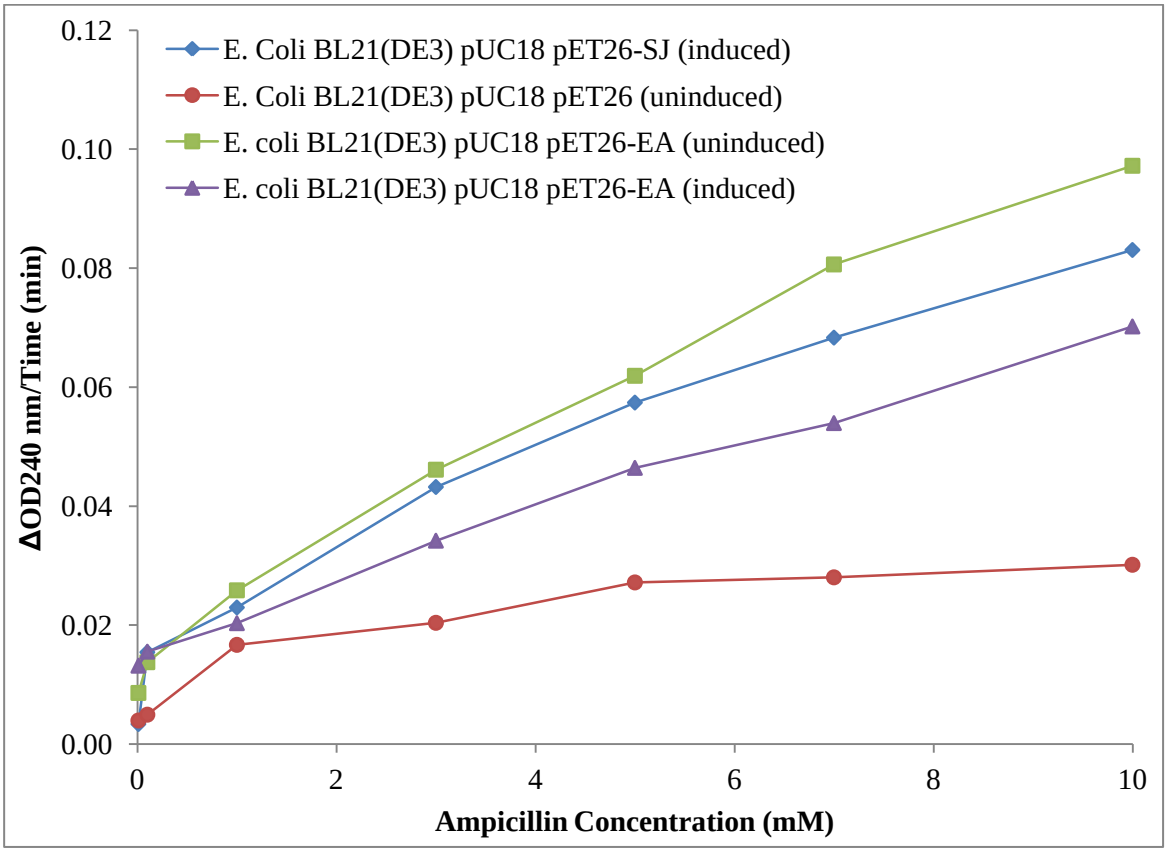

Figure 5.6. Ampicillin hydrolysis by induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26b(+)), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells.

According to the calibration curve obtained for ampicillin at 240 nm, up to a concentration of 7 mM, the absorbance readings were linearly correlated to concentration (APPENDIX B). Each periplasmic sample was mixed with 0.01, 0.1, 1, 3, 5, 7 or 10 mM ampicillin stocks, and optical densities were recorded. Absorbance values of CENTA (470 mM) hydrolysis at 405 nm using the same samples were also measured (Figure 5.7) for comparison.

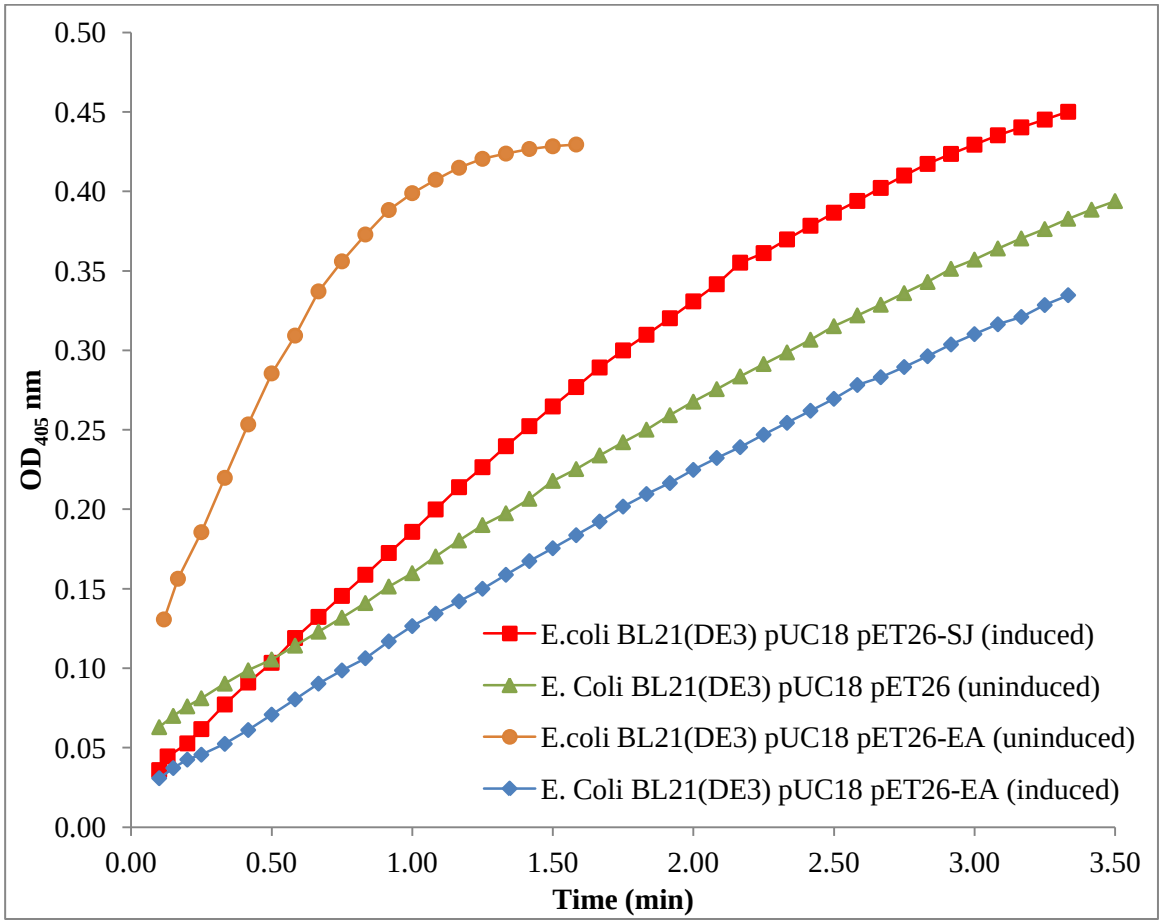

Figure 5.7. CENTA hydrolysis by induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26b(+)), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells.

Each line on Figure 5.6 represents the changes in OD_{240} nm per minute at seven different ampicillin concentrations while, Figure 5.7 was plotted for only hydrolysis of CENTA at 470 µM. These observations suggest that enzyme assays based on ampicillin hydrolysis may give as reliable results as CENTA hydrolysis. Results of OD_{240} nm change per minute per ampicillin concentration were used to calculate β-lactamase activity. Values are listed in Table 5.8 and results of CENTA hydrolysis by the periplasmic samples are given in Table 5.9.

Table 5.8. β-lactamase activity of induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26b(+)), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells during ampicillin hydrolysis.

	β-lactamase activity (U/L periplasmic extract)			
Ampicillin Concentration (mM)	**Induced *E. coli* BL21(DE3) (pUC18 + pET-26EA)**	**Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA)**	**Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	**Induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ)**
0.01	452.06	294.95	135.98	115.59
0.1	533.05	472.20	169.62	529.75
1	697.21	886.71	572.26	787.73
3	1172.91	1582.42	699.38	1482.59
5	1592.87	2123.81	932.32	1969.39
7	1851.05	2766.02	961.69	2343.99
10	2408.38	3334.94	1034.05	2849.28

Table 5.9. β-lactamase activity of induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26b(+)), uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells during CENTA hydrolysis.

	β-lactamase activity (U/L periplasmic extract)			
CENTA	**Induced *E. coli* BL21(DE3) (pUC18 + pET-26EA)**	**Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA)**	**Uninduced *E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	**Induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ)**
470 μM	157.89	405.33	45.85	272.66

5.4. Electrophoretic Analysis of Periplasmic Protein Extracts

In an effort to visualize presence of β-lactamase and BLIP, protein analysis was performed with periplasmic protein extracts of *E. coli* cells harboring both pUC18 and

pET-26EA or pET-26SJ plasmids. Protein concentrations were determined by Bradford Assay. The samples were prepared with the same amount of protein and were loaded to both SDS-polyacrylamide and Native-polyacrylamide gels.

5.4.1. SDS-PAGE Analysis of Periplasmic Protein Extracts

E. coli BL21(DE3) (pUC18 + pET-26EA) cells and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were grown under uninduced and induced conditions. *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were grown until OD reached 0.5 at 600 nm and were induced with 0.2 mM IPTG for 1.5 hours, while uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were used as control. Periplasmic protein extracts were prepared as described in Section 4.6 and the amount of periplasmic protein was measured as described in Section 4.7. Samples were loaded to SDS-polacrylamide gels with the same amount of protein and analyzed after Commassie G-250 staining (Figure 5.8).

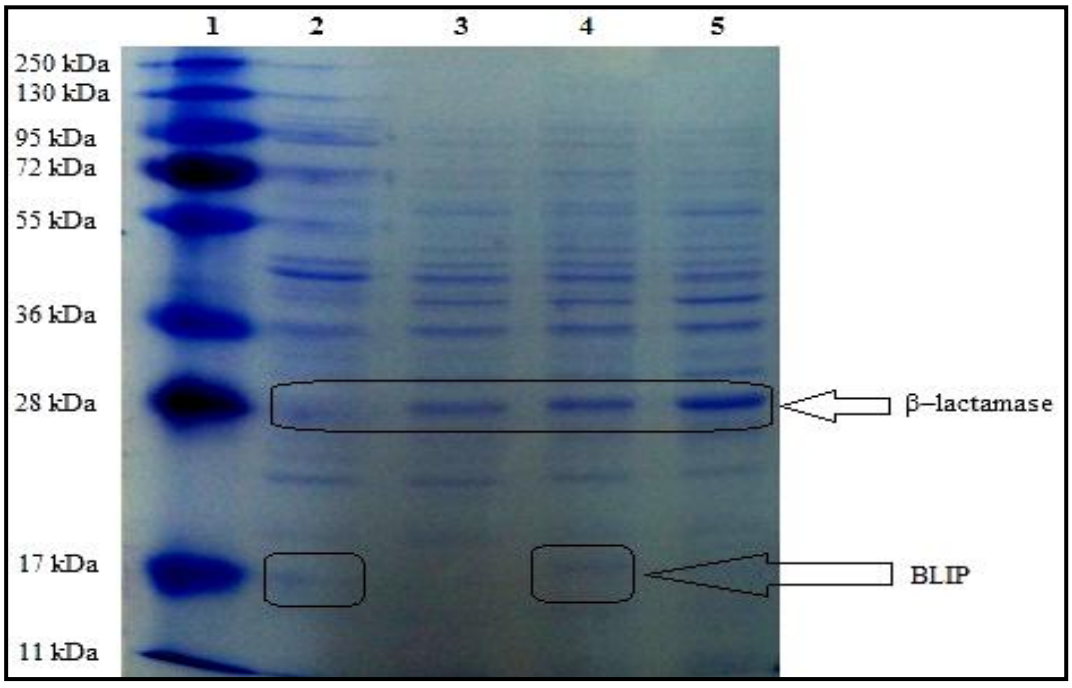

Figure 5.8. SDS-PAGE Electrophoresis. Lane 1: Weight Marker, Lane 2: 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26EA), Lane 3: uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA), Lane 4: 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ), Lane 5: uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ).

Bands corresponding to the size of β-lactamase (28.9 kDa) and BLIP (17.523 kDa) were observed in the protein extracts of the cells harboring both β-lactamase and BLIP, on Lane 2 and Lane 4. Bands corresponding to the size of β-lactamase (28.9 kDa) were observed in the protein extracts of uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells on Lane 3 and Lane 5, respectively.

5.4.2. Native-PAGE Analysis of Periplasmic Protein Extracts

In Native PAGE electrophoresis, protein migration depends upon the net charge, size and shape of the native structure, while proteins are separated according to their mass in SDS-PAGE. In contrast with SDS-PAGE electrophoresis, proteins can retain their native structure upon denaturant (SDS) absence in Native gel electrophoresis. Therefore, proteins in a complex are separated on a Native gel as one band. For that reason, periplasmic proteins extracts obtained from *E. coli* BL21(DE3) cells were run on Native gel in order to visualize BLIP - β-lactamase complex.

E. coli BL21(DE3) (pUC18 + pET-26EA), *E. coli* BL21(DE3) (pET-26EA), *E. coli* BL21(DE3) (pUC18 + pET-26SJ), *E. coli* BL21(DE3) (pET-26SJ) and *E. coli* BL21(DE3) (pUC18) cells were grown under induced conditions. Each *E. coli* BL21(DE3) cell was grown until OD reached 0.5 at 600 nm and were induced with 0.2 mM IPTG for 3 hours. Periplasmic protein extracts were prepared as described in Section 4.6 and the amount of periplasmic protein was measured as described in Section 4.7. The samples were loaded to Native-polacrylamide gels with the same amount of protein. Proteins were analyzed after Commassie G-250 staining Figure 5.9.

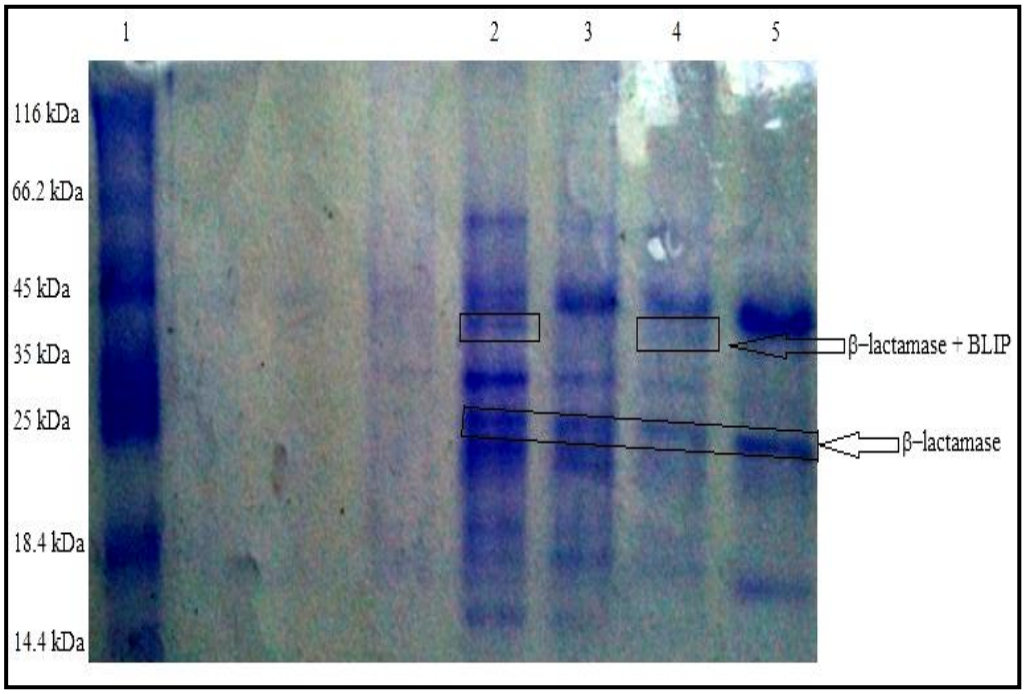

Figure 5.9. Native-PAGE Analysis. Lane 1: Weight Marker, Lane 2: 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26EA), Lane 3: uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA), Lane 4: 0.2 mM IPTG induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ), Lane 5: uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ).

According to Figure 5.9, a band corresponding to the size of β-lactamase - BLIP complex (46.423 kDa) was observed in the periplasmic protein extracts of *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells induced with 0.2 mM IPTG. Additionally, a band corresponding to the size of β-lactamase were observed in uninduced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Concluding Remarks

In this study, the effect of simultaneous BLIP and R-TEM-1 β-lactamase expression on the cell was examined. β-lactamase was expressed by the pUC18 plasmid and BLIP was expressed by the pET-26b(+) vector system. β-lactamase is a periplasmic protein. BLIP was also transported to the periplasm by either the pelB (pET-26EA) or the native leader (pET-26SJ) sequence. *E.coli* BL21(DE3) cells showed slower growth after induction of BLIP expression. Number of viable cells also showed a decrease under induced conditions.

In-vivo inhibiton of β-lactamase by BLIP was examined by measurement of β-lactamase activity in β-lactamase and BLIP expressing cells. BLIP expression significantly inhibited β-lactamase activity. In the presence of BLIP, β-lactamase was inhibited by 85% after 1.5 and by 21% after 3 hours of induction period. Expression of BLIP led to 4 fold reduction in viable cell number. On the other hand, results of β-lactamase activity per viable cell were not comparable for cells harboring different plasmid sets. Expression of BLIP led to a reduction in viable cell number and an increase in β-lactamase activity per viable cell, when compared for the same cells. The increase in β-lactamase activity may be due to an increase in either copy number or activity of β-lactamase, under induced conditions. Similar behaviour was observed when CENTA or ampicillin was used in the activity assays.

SDS-PAGE analysis confirmed the simultaneous expression of BLIP and β-lactamase in the periplasmic fraction of both *E. coli* BL21(DE3) (pUC18 + pET-26EA) cells and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells.

6.2. Recommendations for Future Studies

Enzyme kinetics were conducted with periplasmic extracts containing TEM-1 β-lactamase and BLIP. The effect of the signal sequences on BLIP - β-lactamase interaction can be determined by purifying BLIP, which has the *pel*B signal sequence (pET-26SJ) or the native signal sequence (pET-26EA), and using this pure BLIP in kinetic assays. Purification of BLIP can be achieved by appropriate methods such as column chromatography or pure BLIP can be provided commercially.

The presence of β-lactamase and BLIP was verified by SDS-Page analysis based on the expected molecular weight of the proteins. Western blot analysis can be used to confirm the presence of these proteins. Western blot can be performed on the native gel to check complex formation.

APPENDIX A: BSA CALIBRATION CURVE

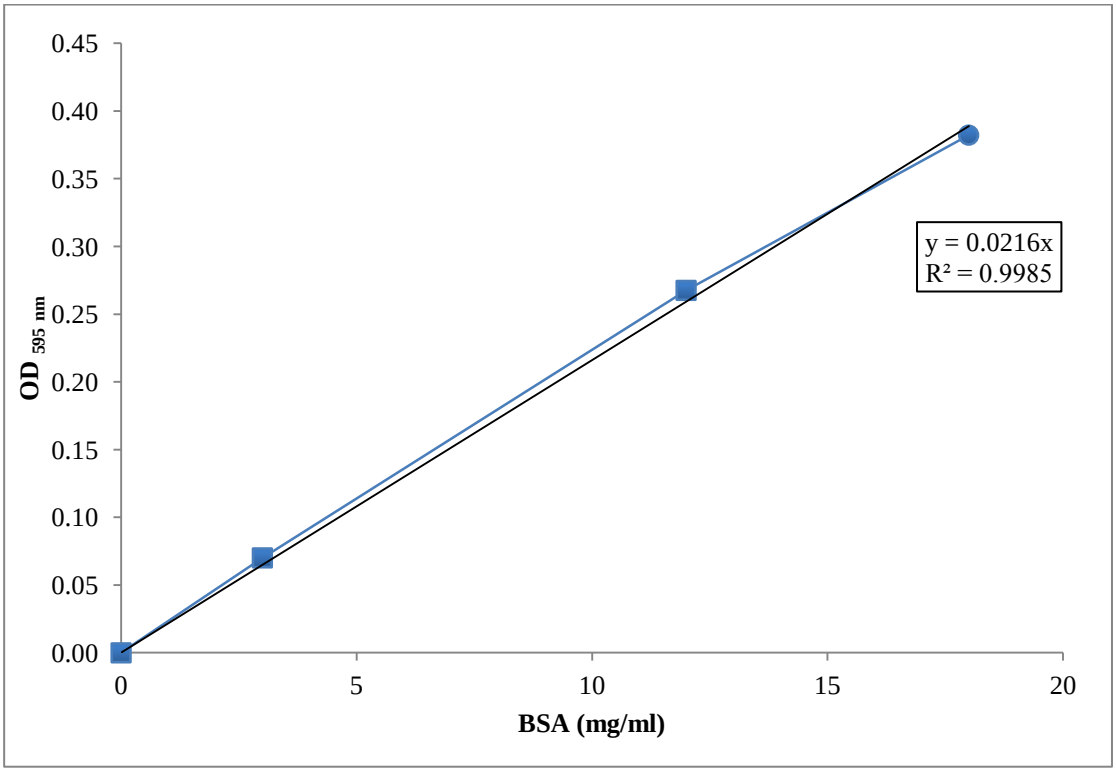

Figure A.1. BSA calibration curve.

APPENDIX B: AMPICILLIN CALIBRATION CURVE

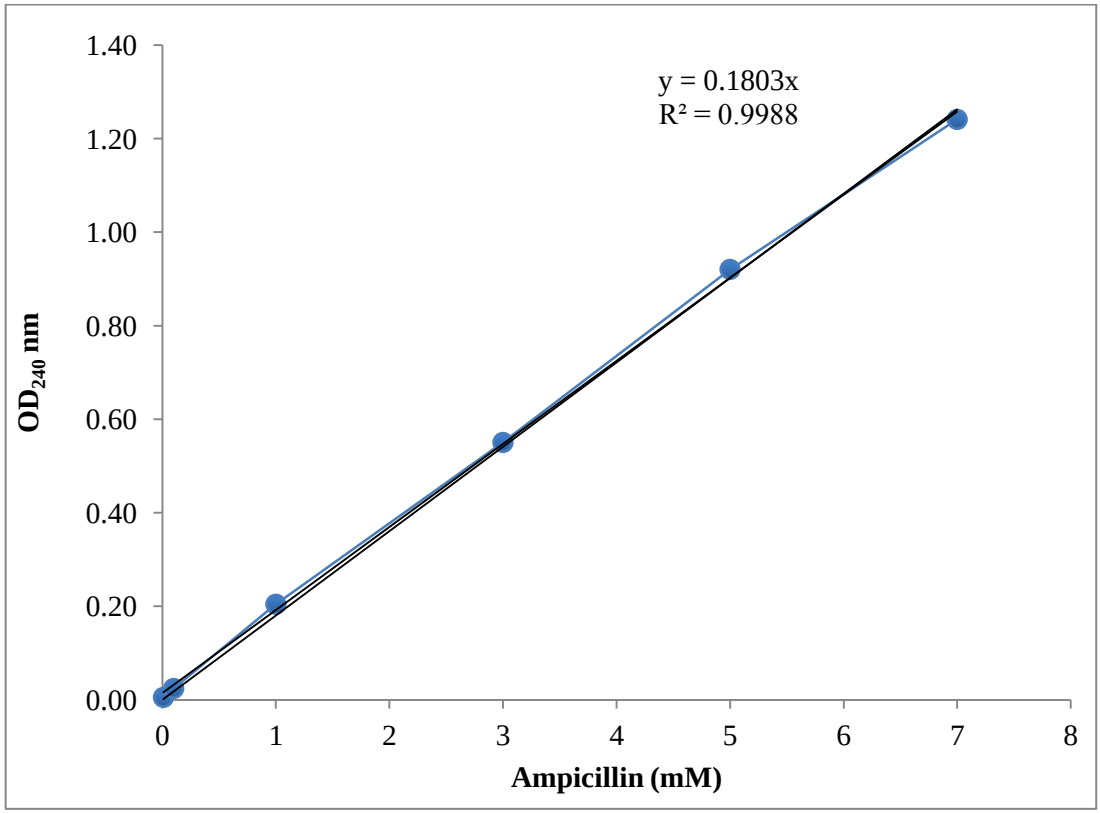

Figure B.1. Ampicillin calibration curve.

APPENDIX C: RESULTS OF INDUCED EXPERIMENTS

Table C.1. Results of induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells.

	Experiment 1		**Experiment 2**		**Experiment 3**		**Experiment 4**	
	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26b(+))**
Activity (U/L periplasmic extract)	798.16	218.01	680.26	146.12	557.34	176.53	301.91	235.48
Harvest OD at 600 nm	1.41	1.62	1.44	1.55	1.44	1.48	1.30	1.50
Induction OD at 600 nm	0.5335	0.5366	0.5045	0.5034	0.5063	0.4958	0.5077	0.4999
Activity / Harvest OD at 600 nm	566.07	134.58	471.36	94.27	387.15	119.15	232.60	156.90
CFU*10^6	141.00	620.00	183.50	595.00	188.50	543.00	130.00	605.00
Activity/CFU*10^6	5.66	0.35	3.71	0.25	2.96	0.33	2.32	0.39
Protein concentration (mg/µl)	0.24	0.02	0.12	0.03	0.18	0.07	0.20	0.08

Table C.2. Results of induced *E. coli* BL21(DE3) (pUC18 + pET-26EA) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells.

	Experiment 1		**Experiment 2**		**Experiment 3**		**Experiment 4**	
	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26SJ)**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26SJ)**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26SJ)**	***E. coli* BL21(DE3) (pUC18 + pET-26EA)**	***E. coli* BL21(DE3) (pUC18 + pET-26SJ)**
Activity (U/L periplasmic extract)	569.95	493.56	943.14	316.18	539.90	253.78	102.33	268.36
Harvest OD at 600 nm	1.35	1.32	1.49	1.09	1.57	1.108	1.27	1.11
Induction OD at 600 nm	0.5042	0.5089	0.5176	0.5096	0.5241	0.5150	0.5500	0.5256
Activity/Harvest OD at 600 nm	422.8	373.91	632.98	290.07	343.19	229.04	80.57	241.77
CFU*10^6	168.50	169.75	224.25	26.00	637.00	241.00	69.00	30.00
Activity/CFU*10^6	3.38	2.91	4.21	12.16	0.85	1.05	1.48	8.80
Protein concentration (mg/µl)	0.22	0.18	0.12	0.06	0.27	0.25	0.20	0.18

REFERENCES

Abraham, E., 1990, "Selective Reminiscences of Beta-lactam Antibiotics - Early Research on Penicillin and Cephalosporins", *BioEssays*, Vol. 12, No. 12, pp. 601-606.

Akkaya, E., 2010, "*Investigation of In-vivo Inhibiton of β-lactamase by β-lactamase Inhibitor Protein"*, M.S. Thesis, Boğaziçi University.

Avcı, N. G., 2011, "*Production and Purification RTEM-1 β-lactamase and its Inhibition by Peptides"*, M.S. Thesis, Marmara University.

Babic, M., A. M. Hujer, and R. A. Bonomo, 2006, "What's New in Antibiotic Resistance? Focus on Beta-lactamases", *Drug Resistance Updates*, Vol. 9, No.3, pp. 142–156.

Baron, S., 1996, "Medical Microbiology", University of Texas Medical Branch, 4th edition, Galveston, Texas.

Bebrone, C., C. Moali, F. Mahy, S. Rival, J. D. Docquiler, G. M. Rossolini, J. Fatstrez, R. F. Pratt, J. M. Frere, and M. Galleni, 2001, "CENTA as a Chromogenic Substrate for Studying Beta-lactamases", *Antimicrobial Agents and Chemotherapy*, Vol. 45, No. 6, pp. 1868-1871.

Bradford, M., 1976, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding", *Analytical Biochemistry*, Vol. 72, pp. 248-245.

Bush, K., 2002, "The Impact of β-lactamases on the Development of Novel Antimicrobial Agents", *Current Opinion in Investigational Drugs*, Vol. 3, pp. 1284-1290.

Bush, K., 2010, "Bench-to-bedside Review: The Role of β-lactamases in Antibiotic-Resistant Gram-negative Infections", *Critical Care*, Vol. 14, No. 224, pp. 1-8.

Bush, K., G. A. Jacoby, and A. A. Medeiros, 1995, "A Functional Classification Scheme for β-lactamases and its Correlation with Molecular Structure", *Antimicrobial Agents and Chemotherapy*, Vol. 39**,** pp. 1211-1233.

Buynak, J. D., 2006, "Understanding the Longevity of the Beta-lactam Antibiotics and of Antibiotic/Beta-lactamase Inhibitor Combinations", *Biochemical Pharmacology*, Vol. 71, No. 7, pp. 930-940.

Christoffersen, R. E., 2006, "Antibiotics - An Investment Worth Making?", *Nature Biotechnology*, Vol. 24, No. 12, pp. 199-207.

Datta, N. and P. Kontomichalou, 1965, "Penicillinase Synthesis Controlled by Infectious *R* Factors In Enterobacteriaceae", *Nature*, Vol. 208, pp. 239-241.

Doran, J. L., B. K. Leskiw, S. Aippersbach, and S. E. Jensen, 1990, "Isolation and Characterization of a Beta-Lactamase Inhibitory Protein from Streptomyces-Clavuligerus and Cloning and Analysis of the Corresponding Gene", *Journal of Bacteriology*, Vol. 172, No. 9, pp. 4909-4918.

Dubendorff, J. W. and F. W. Studier, 1991, "Controlling Basal Expression in an Inducible T7 Expression System by Blocking the Target T7 Promoter with *lac* Repressor", *Journal of Molecular Biology*, Vol. 219, No. 1, pp.45-59.

Elander, R. P., 2003, "Industrial Production of Beta-lactam Antibiotics", *Applied Microbiology and Biotechnology*, Vol. 61, No. 5, pp. 385-392.

Essack, S. Y., 2001, "The Development of Beta-lactam Antibiotics in Response to the Evolution of Beta-lactamases", *Pharmaceutical Research*, Vol. 18, No. 10, pp. 1391-1399.

Fryszczyn, B. G., N. G. Brown, W. Huang, M. A. Balderas, and T. Palzkill, 2011, "Use of Periplasmic Target Protein Capture for Phage Display Engineering of Tight-binding Protein-Protein Interactions", *Protein Engineering, Design & Selection*, Vol. 24, No. 11, pp. 819-828.

Gagne, S. M. and P. Y. Savard, 2006, "Backbone Dynamics of TEM-1 Determined by NMR: Evidence for a Highly Ordered Protein", *Biochemistry*, Vol. 45, No. 38, pp. 11414-11424.

Gupta, V., 2007, "An Update on Newer β-lactamases", *Indian Journal of Medical Research*, Vol. 126, pp. 417-427.

Jones, R. N., H. W. Wilson, J. William, J. R. Novick, A. L. Barry, and C. Thornsberry, 1982, "*In-vitro* Evaluation of CENTA, a New Beta-lactamase Susceptible Chromogenic Cephalosporin Reagent", *Journal of Clinical Microbiology*, Vol. 15, pp. 954-958.

Kotra, L. P. and S. Mobashery, 1998, "Beta-lactam Antibiotics, Beta-lactamases and Bacterial Resistance", *Bulletin De L Institut Pasteur*, Vol. 96, No. 3, pp. 139-150.

Leonhartsberger, S., 2006, *"E. coli* Expression System Efficiently Secretes Recombinant Proteins into Culture Broth", *BioProcess International*, Vol. 4, No. 4, pp. 64-66.

Lim, D., H. U. Park, L. D. Castro, S. G. Kang, H. S. Lee, S. Jensen, K. J. Lee, and N. C. J. Strynadka, 2001, "Crystal Structure and Kinetic Analysis of Beta-lactamase Inhibitor Protein-II in Complex with TEM-1 Beta-lactamase", *Nature Structural Biology*, Vol. 8, No. 10, pp. 848-852.

Liu, H. B., K. S. Chui, C. L. Chan, C. W. Tsang, and Y. C. Leung, 2004, "An Efficient Heat-Inducible Bacillus Subtilis Bacteriophage phi 105 Expression and Secretion System for the Production of the Streptomyces Clavuligerus Beta-lactamase Inhibitory Protein (BLIP)", *Journal of Biotechnology*, Vol. 108, No. 3, pp. 207-217.

Mobashery, S., L. I. Llarrull, S. A. Testero, and J. F. Fisher, 2010, "The Future of the Beta-lactams", *Current Opinion in Microbiology*, Vol. 13, No. 5, pp. 551-557.

Motamedi, H., H. Mirzabeigi, and T. Shirali, 2010, "Determining of Antibiotic Resistance Profile in Staphylococcus Aureus Isolates", *Asian Pacific Journal of Tropical Medicine*, Vol. 3, No. 9, pp. 734-737.

Nossal, N. G. and L. A. Heppel, 1966, "The Release of Enzymes by Osmotic Shock from *Escherichia coli* in Exponential Phase", *The Journal of Biological Chemistry*, Vol. 241, No. 13, pp. 3055-3062.

Novagen, 2003, *pET System Manual, 10th Edition*, http://lifeserv.bgu.ac.il/wb/zarivach/media/protocols/Novagen%20pET%20system%20manual.pdf, accessed at January 2012.

Palzkill, T., J. Yuan, W. Z. Huang, and D. C. Chow, 2009, "Fine Mapping of the Sequence Requirements for Binding of Beta-lactamase Inhibitory Protein (BLIP) to TEM-1 Beta-lactamase Using a Genetic Screen for BLIP Function", *Journal of Molecular Biology*, Vol. 389, No. 2, pp. 401-412.

Parker, R. H. and M. Eggleston, 1987, "Beta-lactamase Inhibitors: Another Approach To Overcoming Antimicrobial Resistance", *Infection Control*, Vol. 8, No. 1, pp. 36-40.

Petrosino, J., G. Rudgers, H. Gilbert, and T. Palzkill, 1999, "Contributions of Aspartate 49 and Phenylalanine 142 Residues of a Tight Binding Inhibitory Protein of Beta-lactamases", *Journal of Biological Chemistry*, Vol. 274, No. 4, pp. 2394-2400.

Pugsley, A. P., 1993, "The Complete General Secretory Pathway in Gram-Negative Bacteria", *Microbiological Reviews*, Vol. 57, No. 1, pp. 50-108.

Reynolds, K. A., J. M. Thomson, K. D. Corbett, C. R. Bethel, J. M. Berger, J. F. Kirsch, R. A. Bonomo, and T. M. Handel, 2006, "Structural and Computational Characterization of the SHV-1 β-Lactamase-β-Lactamase Inhibitor Protein Interfaces", *The Journal of Biological Chemistry*, Vol. 281, No. 36, pp. 26745-26753.

Robert, A., R. A. Bonomo, E. Marcelo, and M. E. Tolmasky, 2007, "Enzyme Mediated Resistance to Antibiotics Mechanisms, Dissemination, and Prospects for Inhibition", *American Society for Microbiology Press*, Washington D.C.

Rudgers, G. W. and T. Palzkill, 1999, "Identification of Residues in Beta-lactamase Critical for Binding Beta-lactamase Inhibitory Protein", *Journal of Biological Chemistry*, No. 274, No. 11, pp. 6963-6971.

Rudgers, G. W. and T. Palzkill, 2001, "Protein Minimization by Random Fragmentation and Selection", *Protein Engineering*, Vol. 14, No. 7, pp. 487-492.

Schroeder, W. A., T. R. Locke, and S. E. Jensen, 2002, "Resistance to β-Lactamase Inhibitor Protein Does Not Parallel Resistance to Clavulanic Acid in TEM β-Lactamase Mutants", Antimicrobial Agents Chemotherapy, Vol. 46, No. 11, pp. 3568-3573.

Strynadka, N. C. J., M. Gretes, D. C. Lim, L. D. Castro, S. E. Jensen, S. G. Kang, and K. J. Lee, 2009, "Insights into Positive and Negative Requirements for Protein-Protein Interactions by Crystallographic Analysis of the Beta-Lactamase Inhibitory Proteins BLIP, BLIP-1, and BLP", *Journal of Molecular Biology*, Vol. 389, No. 2, pp. 289-305.

Strynadka, N. C. J., S. E. Jensen, K. Johns, H. Blanchard, M. Page, A. Matagne, J. M. Frere, and M. N. G. James, 1994, "Structural and Kinetic Characterization of a Beta-Lactamase Inhibitor Protein", *Nature*, Vol. 368, No. 368, pp. 657-660.

Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff, 1990, "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes", *Methods in Enzymology*, Vol. 185, No. 1986, pp. 60-89.

Wilke, M. S., A. L. Lovering, and N. C. J. Strynadka, 2005, "Beta-lactam Antibiotic Resistance: A Current Structural Perspective", *Current Opinion in Microbiology*, Vol. 8, No. 5, pp. 525-533.

Williams, J. D., 1999, "Beta-lactamases and Beta-lactamase Inhibitors", *International Journal of Antimicrobial Agents*, Vol. 12, No. 1, pp. 3-7.

Zhang, Z. and T. Palzkill, 2003, "Determinants of Binding Affinity and Specificity for the Interaction of TEM-1 and SME-1 Beta-lactamase with Beta-lactamase Inhibitory Protein", *Journal of Biological Chemistry*, Vol. 278, No. 46, pp. 45706-45712.

Zhu, Y., S. Jovetic, G. L. Marcone, F. Marinelli, and J. Tramper, 2010, "Beta-lactam and Glycopeptide Antibiotics: First and Last Line of Defense?", *Trends in Biotechnology*, Vol . 28, No. 12, pp. 596-604.