BACTERIAL UPTAKE OF ANTIMICROBIAL PEPTIDES

by

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ABSTRACT

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The discovery of beta lactam antibiotics has been a groundbreaking improvement in the treatment of bacterial diseases. However, most bacteria have gained resistance against beta lactam antibiotics which rendered them ineffective. Beta lactamase mediated resistance is the most frequently encountered defence mechanism. To overcome this problem, several beta lactamase inhibitors have been developed such as clavulanic acid, sulbactam and tazobactam. They are generally small molecules that pose high affinity towards beta lactamase enzyme, thus protect beta lactam from being hydrolyzed. Unfortunately, bacteria started to produce different beta lactamases which are not sensitive to conventional inhibitors that led to the emergence of designing effective beta lactamase inhibitors. These inhibitors must have high affinity as well as good cell penetration capability to inhibit beta lactamase in the periplasmic space. In this study, the effect of adding five hydrophobic residues to beta lactamase inhibitory proteins was examined. The chimeric peptides P2,P4,P5, which included a hydrophobic part and an inhibitory part, and the inhibitory peptide P3 were examined for their inhibition properties and for their cell penetration capabilities ısing in vivo and in vitro techniques. P2 showed uncompetitive inhibition with K_i of 36μM and P3 indicated competitive inhibition with K_i of 412.44μM. P4 decreased K_m and V_{max} around 85%. The cell growth experiments showed that P4 was the most effective peptide that inhibited cell growth in resistant cells after 250μM.

ÖZET

ANTİMİKROBİYAL PEPTİDLERİN HÜCRE İÇİNE ALIMI

Beta laktam antibiyotiklerinin keşfi, bakteri kaynaklı hastalıkların tedavisinde çok önemli gelişmeler sağlamıştır. Zaman geçtikçe bazı bakteri tipleri beta laktam antibiyotiklerine karşı direnç geliştirmiş, bu durum da antibiyotiklerin etkisini önemli ölçüde azaltmıştır. En önemli direnç mekanizması bakteriler tarafından üretilen beta laktamaz enzimleridir. Antibiyotikleri beta laktamazın hidrolize edici etkilerinden korumak için sulbaktam, klavulanik asit ve tazobaktam gibi beta laktamaz inhibitörleri geliştirilmiş ve dünya çapında kullanılmaya başlanmıştır. Ne yazık ki, dirençli bakteriler farklı tipte bata laktamaz enzimleri üretmeye başlamış, bu durum da acil bir şekilde yeni inhibitörler geliştirme ihtiyacını doğurmuştur. Geliştirilecek inhibitörlerin beta laktamaz enzimlerine karşı yüksek bağlanma eğilimi göstermesi ve hücre içie etkili bir şekilde geçebilme özelliğine sahip olması gerekmektedir. Yaptığımız bu çalışmada, beta laktamaz inhibitörü olma özelliği taşıyan peptidlere, hidrofobik LLIIL kalıntısı eklenmiş, bu durumun, peptidlerin hücre içine taşınımı ve inhibite etme yetisi üzerine etkileri incelenmiştir. İnhibitör özelliği kanıtlanmış P3, ve hidrofobik kalıntılar içeren P2, P4 ve P5 kullanılarak in vitro ve in vivo deneyler yapılmış, hücre içine geçişleri, bakteri hücreleri üzerindeki etkileri ve beta laktamaz inhibite etme özellikleri incelenmiştir. Sonuçlar P2 peptidinin rekabetsiz inhibisyona neden olduğu ve K_i değerinin 36μM olduğunu, P3 peptidinin ise rekabetçi inhibisyona sahip olduğunu ve K_i değerinin 412.44μM olduğunu göstermiştir. P4 hem K_m hem de V_{max} değerinde kayda değer düşüşlere neden olmuş inhibisyon tipi belirlenememiştir. Hücre büyüme deneyleri de en etkili peptidein P4 olduğunu göstermiştir.

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d	Path length
K_m	Michaelis constant
K_i	Inhibitor Constant
V_0	Enzyme activity
V_s	Enzyme volume
V_t	Total reaction volume
λ	Absorptivity constant

LIST OF ACRONYMS/ABBREVIATIONS

3D	Three Dimensional
A	Alanine
atm	Atmospheric Pressure
BLIP	Beta Lactamase Inhibitory Protein
C	Carbon
CPP	Cell Penetrating Peptide
Cl	Chlorine
D	Aspartic Acid
DMSO	Dimethyl sulfoxide
E1	Enzyme set 1 used in the experiments with P2 and c. acid
E2	Enzyme set 2 used in the experiments with P3 and P4
EDTA	Ethyenediaminetetraacetic Acid
ESBL	Extended Spectrum Beta-Lactamase
G	Glycine
g	Gram
H	Hydrogen
H	Histidine
h	Hours
I	Isoleucine
IPTG	Isopropyl Beta-D-1-Thiogalactopyranoside
G	Glycine
K	Potassium
K	Lysine
L	Leucine
LB	Lysogeny Broth
Mg	Magnessium
ml	Milliliters
MIC	Minimum Inhibitory Concentration

N	Asparagine
O	Oxygen
OD	Optical Density
P	Potassium
P	Proline
P2	NH_2 – LLIILHAAGDYYAY – $CONH_2$
P3	NH_2 – RRGHYY – $CONH_2$
P3A	NH_2 – RRGHYYA – $CONH_2$
P4	NH_2 – LLIILRRGHYY – $CONH_2$
P5	NH_2 – LLIILYHFLWGP – $CONH_2$
PBP	Penicillin Binding Protein
pVEC	Vascular Endothelial-cadherin Protein
R	Arginine
rpm	Rate per Minutes
S	Sulphur
S	Serine
Tat	Trans Activating Transcriptional Activator
μM	micromolars
Y	Tyrosine
W	Tryptophan

1. INTRODUCTION

The discovery of beta lactam antibiotics have been a major success in the treatment of infectious diseases. Among all antibiotics, beta lactam antibiotics represent approximately 65% of the global market share [1]. The main mechanism of action is to prevent cell wall formation by targeting penicillin binding proteins (PBPs). They are highly effective against various types of gram positive and gram negative bacteria [2].

Due to misuse and mutations, most bacteria have gained resistance against beta lactam antibiotics. The most significant resistance mechanism is the production of beta lactamase enzymes which render the antibiotics ineffective by hydrolyzing the beta lactam ring [3]. In gram negative bacteria, TEM-1 is the most commonly encountered beta lactamase.

In order to solve the worldwide antibiotic resistance problem, beta lactamase inhibitors have been developed. They are small molecules which show high affinity towards beta lactamases and prevent the beta lactam ring from being hydrolyzed. These inhibitors are commonly used together with beta lactam antibiotics and increase the efficiency of their partner antibiotics. Clavulanic acid, tazobactam and sulbactam are the three well known inhibitors which are currently in commercial use [4]. Unfortunately, new types of beta lactamases have been produced by bacteria that show insensitivity towards these inhibitors. This problem led to the emergence of discovering new beta lactamase inhibitors.

Antimicrobial and inhibitory peptides are commonly used alone or combined together with antibiotics. They contain various amino acids which differ in terms of hydrophobicity and electrical charges. These peptides are small molecules which interact with the cell wall, thus becomes effective on different microorganisms. When designing antimicrobial peptides, cell penetration properties should also be taken into consideration. An effective inhibitor must have high affinity towards beta lactamase and brilliant penetration properties to reach beta lactamase. To obtain better cell

penetration, hydrophobic residues can be used which drags the peptide through the cell membrane.

In this study, the effect of adding five hydrophobic residues (LLIIL) from cell penetrating peptide pVEC to three beta lactamase inhibitory peptides were investigated. The aim was to reach enhanced cell penetration levels and consequently obtain increased beta lactamase inhibition. For that purpose, in vivo cell growth experiments and viable colony forming units (CFU)s were counted for both resistant and wild type cells in the presence of five peptides at different times. In addition, kinetic characterization of beta lactamase enzyme in the presence of selected peptide concentrations were conducted. The results were compared in Section 5 to understand the role of hydrophobic residues (LLIIL).

During the project *E.coli* K12 cells were used in the cell growth experiments as control. They do not posses resistance against TEM-1 beta lactamase, as a result indifferent to beta lactamase inhibitors. To obtain antibiotic resistance, pUC18 plasmid was transferred to wild type *E.coli* K12 cells as pUC18 was a plasmid that has plasmid been genetically engineered to include a gene for antibiotic resistance to ampicillin by producing TEM-1 beta lactamase.

2. BIOLOGICAL BACKGROUND

2.1. Beta Lactam Antibiotics

Antibiotics are chemicals which destroy the ability to produce and survive by sticking to crucial parts of the bacterial cell. Bacteria susceptible to antibiotics, will stops growing or simply die. Antibiotics are only effective against bacteria not on viruses, parasites or fungi [5].

Beta lactam antibiotics represent an important part of antibiotic family. Their discovery first started with Alexander Fleming's discovery of penicillin in 1928. In the light of this discovery, many types of antibiotics have been discovered or synthesized [5]. Since then, the potency, breadth of spectrum, activity against resistant pathogens, stability and pharmacokinetic properties have improved as a result of the developments on structural and regulatory biosynthetic genes and metabolic engineering [6]. With all these advances in biotechnology ,the production mechanisms have developed significantly which increased the global market share of beta lactam antibiotics to 65% of overall antibiotic consumption [1].

Beta lactam antibiotics contain a three carbon one nitrogen beta lactam ring which constitute the efficiency of the antibiotic by attacking to the active sites of bacterial enzymes [7]. They are subject to different classifications depending on their structure and function; and are commonly named by their origin microorganism. The beta lactam antibiotics include penicillins such as penicillin G, penicillin V, ampicillin, cloxacillin, and piperacillin; cephalosporins such as cephalothin, cephaloridine, cephalexin, and cefaclor; and cephamycins such as cefoxitin. Also, more recently developed non classical structures such as monobactams, and thienamycin are widely used beta lactam antibiotics [6]. The molecular structure of the four main beta lactam antibiotics are shown in Figure 2.1.

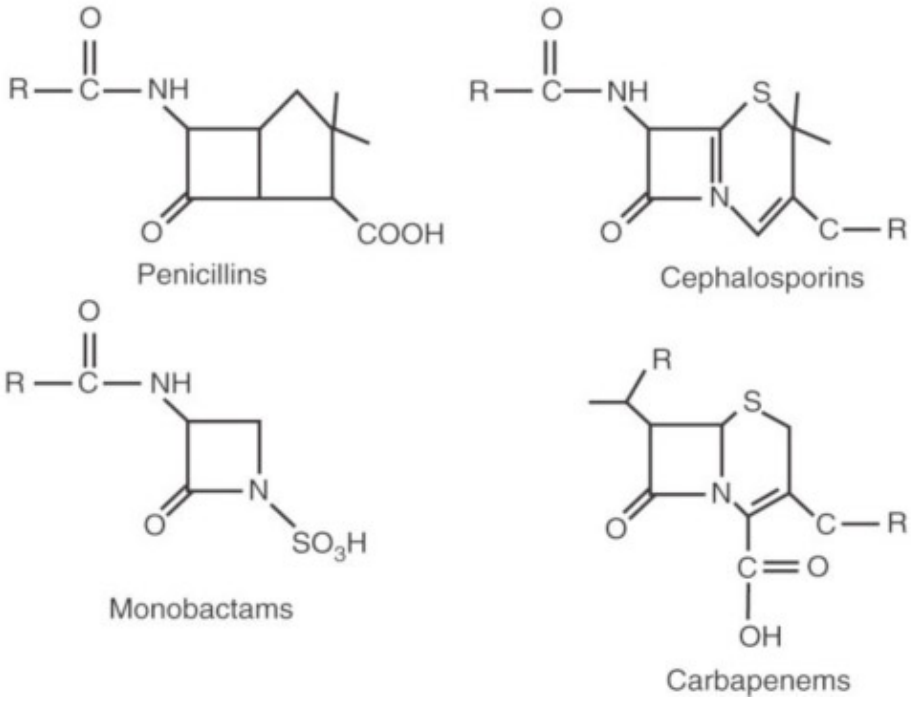

Figure 2.1. Chemical structures of penicillin, caphalosphorin, monobactam and carbapenem [8]

Beta lactams are effective both on gram negative and gram positive bacteria. Their activity against gram negative bacteria is related to the ability to resist beta lactamases , which are produced by the bacteria as a resistance mechanism, and to bind penicillin binding proteins (PBPs) [9]. PBPs are mainly responsible for catalyzing D-ala D-ala cross linkages of the peptidoglycan wall and consequently maintaining osmotic stability of the cell by preserving the integrity of the cell wall. Beta lactams show structural mimicry to D-Ala-D-Ala, bind PBPs instead and prevent the formation of cross linkages which eventually results in cell death due to lysis and osmotic imbalances [9].

Beta lactam antimicrobials are used as the most common treatment of bacterial infections. However they are also the main reason for the worldwide resistance of gram negative bacteria. Resistance to beta-lactams among the target bacteria for survival has been developed early in their history of use which affected the potency of the antibiotics to critical levels. A proper understanding of antibiotic resistance mechanisms is crucial

for the discovery of efficient drugs. Some of the identified resistance systems are the breakdown of the beta lactam ring through hydrolysis or redox process, inactivation by group transfer which modifies the beta lactam structure, and the modification of antibiotic target sites [10] .

2.2. Beta Lactamase Mediated Resistance

Beta-lactams constitute the most common treatment for bacterial infections and continue to be the major reason for resistance to beta lactam antibiotics among Gram-negative bacteria worldwide. The continuous exposure of bacterial strains to antibiotics has induced dynamic and continuous production and mutation of -lactamases among bacteria, expanding their activity even against the newly developed beta lactam antibiotics [10].

The first beta lactam antibiotics were biosynthesized directly by microorganisms to get leverage against the non-producing bacteria for food sources. Consequently the non-producing strains gained the ability to produce beta lactamases in order to overcome the threat. This skill provided them with a significant advantage for survival. Today, this natural selection process still proceeds with acceleration due to the heavy use of antibiotics and antibiotic resistance becomes even a more serious problem [11].

The origin of the beta lactamase enzymes is the penicillin-binding proteins (PBPs) which were responsible for cell wall synthesis. Both two were diverged from a common ancestor; however beta lactamases gained the ability to hydrolyze beta lactam ring [12]. Beta lactamases act by covalently binding the carbonyl portion of the beta lactam ring and breaking the amide bonds. Without excessive amounts, their contribution to antibiotic is insignificant; however especially in gram negative bacteria their presence poses a serious threat to the efficacy of the antibiotic [13].

Currently the beta lactamase superfamily has more than 255 members, many of which differ only by a single amino acid [14]. Beta lactamase enzymes are categorized by two different systems; Ambler classification system that uses sequence similarities

and catalytic mechanism and the other is the Bush Jacoby system which is based on substrate preferences [15].

The Ambler system recognizes three families of serine beta lactamases (Class A, C and D) a serine moiety in the active centre that catalyzes hydrolysis of the beta lactam ring through an acyl-intermediate of serine, and a 4^{th} family (Class B) which requires a metal cofactor (e.g. zinc in the natural form) to function, and for this reason, they are also referred to as metallo- beta-lactamases (MBLs) [16].The active-site serine -lactamases belong to a larger family of penicillin-recognizing enzymes that includes the penicillin-binding proteins (PBPs), which cross-link bacterial cell walls. They acylate -lactam antibiotics, like PBPs, and then use strategically positioned water molecules in order to hydrolyze the acylated -lactam [4]. Class C beta-lactamases share common structural features with D,D-carboxypeptidases and class A beta-lactamases, and class A and class C beta-lactamases have the same evolutionary origin as other beta-lactam target enzymes [17].

Table 2.1. Amber classification of beta lactamases.

Ambler Class	**Active Site**	**Enzyme Type**
A	Serine	Broad spectrums (TEM, SHV) ESBL (TEM,SHV,CTX-M) Carbapenemases(VIM,IMP)
B	Zinc binding thiol group	Carbapenemases (VIM,IMP)
C	Serine	AmpC cephamycinases (AmpC)
D	Serine	Broad spectrums (OXA) ESBL

Table 2.2. Bush Jacoby Medeiros classification of beta lactamases

B.J.M Group	**Subgroup**	**Substrate**	**Characteristics of Beta Lactamase Members**
1	1	all beta lactams except	Chromosome and plasmid encoded AmpC beta lactamases
2	2a	penicillins	Penicillinases produced by gram positive bacteria
	2b	cephalosphorins	Broad spectrum beta lactamases (TEM-1,TEM-2, SHV-1)
	2be	Penicillins , cephalosphorins, monobactams	Extended spectrum beta lactamases (ESBL)
	2br	penicillins	TEM ns SHV type inhibitor resistant beta lactamases
	2c	penicillins, carbenicillin	Carbenicillin hydrolyzing PSE type beta lactamases
	2e	cephalosphorins	Inducible cephalosphorins from Proteus spp.
	2f	Penicillins, cephalosphorins, carbapenems	Serine carbapenems
	2d	Penicillins, oxacillin	OXA beta lactamases that hydrolyze oxacillin
3	3a,3b,3c	Most beta lactams including carbapenems	metallo beta lactamases
4	undetermined	penicillins	Penicillinases which do not belong to other groups

TEM-1 is the most frequently encountered plasmid-encoded beta lactamase in gram negative pathogens and used as an antibiotic resistance determinant. Like other class A beta lactamases, it contains serine active site which makes is susceptible to inhibitors such as clavulanic acid. It was first discovered in the 1960s and so far, more than 170 variants with different resistance mechanisms and amino acid sequences have

been isolated [18]. TEM-1 is used as an antibiotic resistance determinant because of its effectiveness against penicillins and different types of cephalosphorins which makes it a significant cause of beta lactamase mediated resistance [19].

To overcome TEM-1 resistance, ampicillin or amoxicillin is used together with a beta lactamase inhibitor. TEM-1 shows 68% structural similarity to SHV-1 in terms of amino acid identities and substrate similarity. These two beta lactamases frequently occur together in pathogens. The approaches to overcome TEM-1 resistance thus also apply to SHV-1 [19].

2.3. Beta Lactamase Inhibitors

Beta lactamase mediated resistance poses a serious clinical threat against beta lactam antibiotics. To solve this problem, different strategies have been developed. The first strategy is to design new beta lactam antibiotics , and the second one is to inhibit beta lactamases in order to stop the breakdown of the antibiotic [4]. The inhibition is achieved through beta lactamase inhibitors. Beta lactam antibiotics are combined with beta lactamase inhibitors and the hydrolysis of the beta lactam ring is avoided which preserves the activity of the antibiotic [16] .

The studies on beta lactamase inhibitors first started 30 years ago. All inhibitors are small compounds that show structural similarity to penicillin, each containing a modified side chain enabling them to behave like suicide inhibitors which compete with antibiotic for the active site and inactivate beta lactamase by binding it irreversibly.Figure 2.2 shows sulbactam, tazobactam and clavulanic acid which are the three inhibitors currently in use [20]. Clavulanate, tazobactam and sulbactam are effective against class A beta lactamase enzymes as they target serine active sites. The inhibitory constant (K_i) of clavulanic acid for class A TEM-1 beta lactamase is reported as around 0.1μM, tazobactam as 0.01μM and sulbactam as 0.9 μM [22]. These three inhibitors contain a beta lactam ring which makes them vulnerable against the resistance due to the upregulation of beta lactamase expression and newly developed beta lactamases. In addition, multidrug resistant gram negative pathogens have evolved in

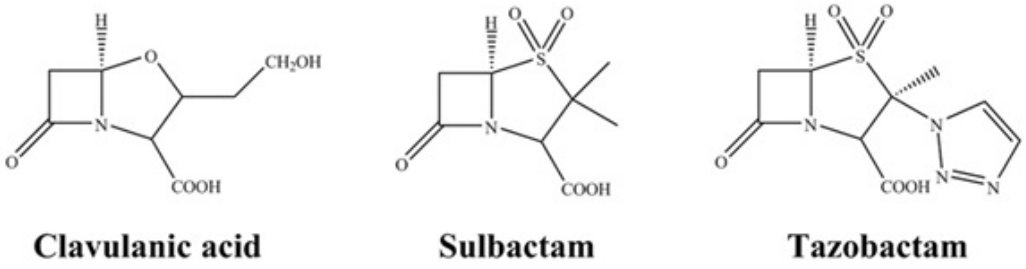

Figure 2.2. The chemical structures of three main beta lactamase inhibitors [21]

which these inhibitors lost most of its efficiency. As a result, an urgent need to develop new beta lactamase inhibitors has emerged [15].

In order to detect the right concentration of a drug or the inhibitor, minimum inhibitory concentrations (MICs) are used. It is defined as the minimum concentration of an antimicrobial that will totally inhibit the visible growth of a microorganism after overnight incubation. MICs are used by diagnostic laboratories to confirm resistance, and as a research tool to determine the in vitro activity of antimicrobials [23]. MICs can be determined on plates of solid growth medium and broth dilution methods. To be able to detect the MIC value through broth dilution, identical doses of bacteria are cultured in wells of liquid media containing progressively lower concentrations of the drug. The minimum inhibitory concentration of the substance is considered as between the concentrations of the last well in which no bacteria grew and the next lower dose, which allowed bacterial growth.

In the project, clavulanic acid, beta lactamase inhibitory protein (BLIP), potent TEM-1 beta lactamase inhibitors RRGHYY and CYHFLWGPC were used. All four are competitive suicide inhibitors of class A beta lactamases and bind to serine active sites of these beta lactamases.

2.3.1. Clavulanic Acid

Clavulanic acid is a competitive, irreversible suicide inhibitor obtained from *Streptomyces clavuligerus* [24]. It acts on beta lactamase enzyme found in periplasmic space and shows concentration dependent competitive inhibition properties, especially on class A TEM-1 [25]. It is used as a broad-spectrum beta-lactamase inhibitor and found effective against chromosomally and plasmid-mediated beta-lactamases of Gram-negative and Gram-positive bacteria as it binds to their penicillin-binding proteins (PBPs) [25].

Clavulanic acid, in its sodium salt form, is a bicyclic beta lactam, however it does not contain penicillin and cephalosphorin nucleus. Its antimicrobial properties are very limited, consequently it is used together with antibacterial agents and antibiotics [25]. It was proven that clavulanic acid highly increases the antibiotic susceptibility of amoxicillin resistant strains of bacteria to antibiotics [26]. As a result, they are affected more severely by antibiotics and get vulnerable. It restores the beta lactamase containing isolates, thus increases the spectrum of amoxicillin to larger extents. It is used together with ampicillin at a concentration around 5μg/ml, the MIC value of anpicillin was redsignificantly from 1000 μg/ml to 1.0 pg/ml [24].

The K_i value of clavulanic acid was detected as 0.11μM for beta lactamases obtained from *Streptomyces cacaoi* [27] and 0.1 μM for TEM-1 beta lactamase [22] which makes it a highly effective beta lactamase inhibitor.

2.3.2. Beta Lactamase Inhibitory Protein (BLIP)

Beta lactamase inhibitory protein (BLIP) is a potent TEM-1 beta lactamase inhibitor with a K_i value of 0.3nM. It is a 165 amino acid long protein produced by *S. clavuligerus* that contains a 76 amino acid long tandem repeat. It is commonly used as a model of beta lactamase inhibition [28].

TEM-1 beta lactamase contains two major domains where the substrate-binding site located at the interface. The Ala46–Tyr51 loop blocks the active site and the Asp49 residue of BLIP mimics the beta-lactam carboxyl group [29].

BLIP is the first inhibitor that shows inhibition to a single target enzyme with two similarly folded domains. In the TEM-1 BLIP co-crystal structure, two loops are inserted to the active site which results in complete inhibition [30]. Hydrogen bonding interaction between Tyr 105 and Ser 106 on TEM-1 and Glu 73 are encircled by the nonpolar interface with direct contact of BLIP with the active residues of TEM-1 [31]. This interaction was the first characterized and analyzed BLIP-beta lactamase interface and used as a model for protein-protein interactions to understand binding specificity, structure and thermodynamic properties [32].

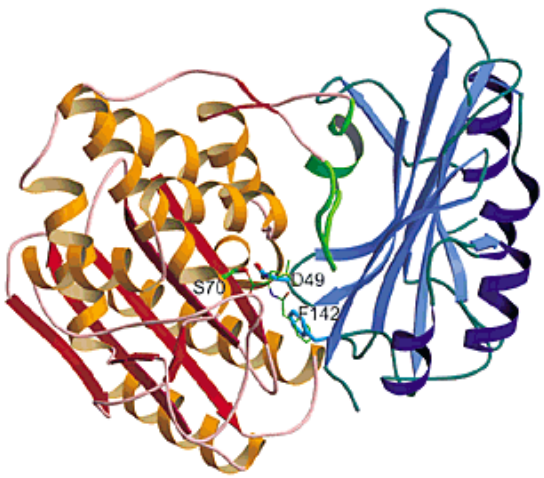

Figure 2.3. Interface between BLIP (blue) and TEM-1(orange)

The cocrystal structures showed critical interactions between the active site of TEM-1 and residues of BLIP between 46 to 51 (N-Cys-Ala-Ala-Gly-Asp-Tyr-Tyr-Cys-COOH). In addition to TEM-1, these 6 residue long peptide was found effective on class A beta lactamases from *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus licheniformis* with K_i values between 1 to 3 μM. BLIP does not show efficacy against class B, C, or D -lactamases [30]. BLIP did not have any inhibitory effects on these two beta lactamases which indicated that using the 6 residues, new novel small-molecule inhibitors of different beta lactamase classes can be designed [28].

The 45-53 loop of BLIP indicated competitive inhibition. When the hydrophobic residues were added, the viable cell counts of resistant cells were decreased where no effect was observed on wild type cells that shown beta lactamase inhibition. The SMD results showed improved interactions with the lipid bilayer. Also, the translocation of the peptide though the membrane caused more disordering effects than the one without hydrophobic residues on the lipid bilayer like many other antimicrobial peptides [29].

2.3.3. Peptide Based Inhibitor RRGHYY

RRGHYY-NH2 peptide was a peptide obtained with phage display method and its binding affinity was optimized by the synthesis of peptide arrays with SPOT synthesis technology. It is 50% identical with a type II beta turn sequence found in the beta lactamase inhibitory protein (BLIP) [33].

From the SPOT synthesis results, it was understood that the arginine residues were highly significant in beta lactamase binding. The beta lactamase inhibition assays showed that it was an inhibitor of TEM-1 beta lactamase with a K_i value of 136 μM, class A *Bacillus anthracis* Bla1 beta lactamase with a K_i of 42 μM and the class C beta lactamase, P99, with a K_i of 140 μM even though it was optimized only for TEM-1 beta lactamase [33].

The kinetic characteristics of RRGHYY for TEM-1, Bla1 and P99 enzymes inhibition were determined using kinetic experiments by Huang et al. For each tested beta lactamase enzyme, competitive inhibition was observed for RRGHYY. It was seen that the peptide's behavior towards all targets was the same and it binded at or near the active site of the beta lactamases to block entry of the beta lactam. It was concluded that it was possible that the RRGHYY peptide to bind in a similar manner to the BLIP beta turn with the arginine residues providing increased affinity [33].

2.3.4. Novel TEM-1 inhibior YHFLWGP

YHFLWGP is a cyclic heptapeptide where tryptophan and proline residues are responsible for the binding and inhibition of beta lactamases. It is rpoven to be a competitive inhibitor which is effective on TEM-1 beta lactamase with a K_i value of 333μM. This inhibitor contains a loop structure highly similar to that of beta lactamase inhibitory protein (BLIP) [34].

The inhibitor has the ability to both inhibit TEM-1 and penicillin binding proteins. Its K_i is lower than most beta lactamase inhibitors, however as it also shows inhibition towards PBPs, which makes it highly unique and promissing [34].

2.4. Inhibition Mechanisms

Enzymes are protein molecules which behave as catalysts in bioreactions .The inhibition mechanism depends on the covalent and noncovalent interactions between the enzyme and and its substrate [35]. It can be either reversible or irreversible. Reversible inhibition is identified by fast dissociation of the enzyme inhibitor complex where irreversible one shows slow dissociation due to tight bounds resulting from covalent or non covalent bonding [36].

Enzymes bind to the substrate from the active site using 3D lock-key arrangement which depends on various external conditions such as temperature, reactant concentration, reaction time and pH [35]. Inhibition schemes are generally the same and categorized in 3 classes which are classified as competitive, uncompetitive and noncompetitive inhibition depending on the binding modes to substrates. Figure 2.4 provides a schematic representation of these three inhibition types All three inhibition types show characteristic behaviours which can be analyzed through Lineweaver-Burk (double reciprocal) and Hanes-Woolf plots which provide a precise way to determine kinetic parameters such as V_{max} and K_m. Figure 2.5 shows the characteristic Lineweaver-Burk plots of three main inhibition types together. As seen in the figure, competitive inhibition is identified by the slope change, uncompetitive inhibition by the change in

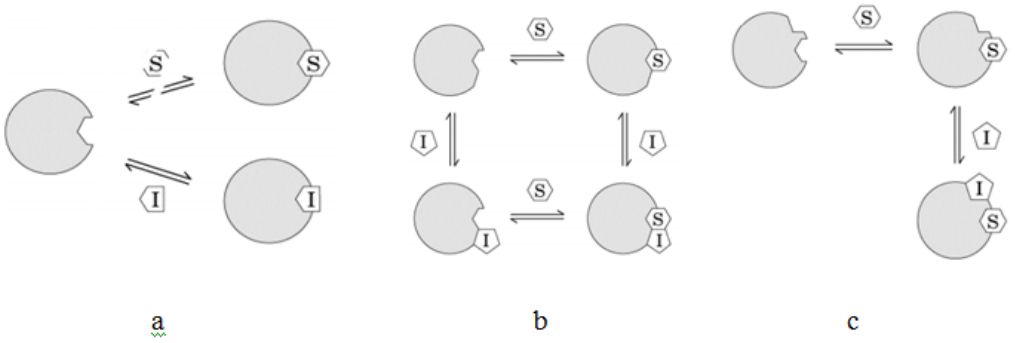

Figure 2.4. The molecular representations of (a) competitive, (b) uncompetitive and (c) noncompetitive inhibition [37]

the intercept and noncompetitive inhibition by the changes in both the slope and the intercept. For competitive inhibition, enzyme binds to the substrate or the inhibitor

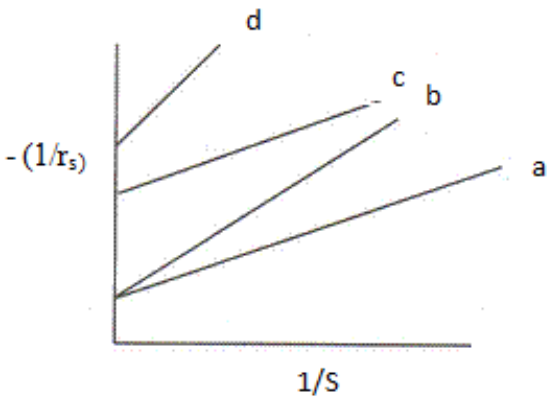

Figure 2.5. Lineweaver-Burk plots for three types of inhibition (a) no inhibition (b) competitive inhibition (c) uncompetitive inhibition (d) noncompetitive inhibition [37]

to form a complex. The molecular structure resembles to the substrate, and competes with the substrate for the active site. The active site is occupied together with the inhibitor and the substrate and the substrate binding is prevented to some extent which leads to the reduction of the reaction rate. As the availability of the free enzyme is reduced, the Michaels Menten constant K_m is increased but the maximum velocity V_{max} stays the same as the presence of the inhibitor can be overcome by high substrate

concentrations [35]. The inhibition constant K_i is calculated using equation 2.1.

$$\mathbf{K}_i = \mathbf{K}_m \left(1 + \frac{[I]}{(\mathbf{K}_m)app}\right) \tag{2.1}$$

In the case of uncompetitive inhibition, the inhibitor is structurally different from the substrate. The inhibitor binds to the free enzyme or the enzyme substrate complex which results in structural distortion of the active site and the allosteric sites of the enzyme. K_m and V_{max} values decrease at the same ratio. Consequently, K_i can be calculated using Equation 2.2 and Equation 2.3 [35].

$$\mathbf{K}_m(app) = \left(\frac{\mathbf{K}_m}{(1 + \frac{[I]}{\mathbf{K}_i})}\right) \tag{2.2}$$

$$\mathbf{V}_{max}(app) = \left(\frac{\mathbf{V}_{max}}{(1 + \frac{[I]}{\mathbf{K}_i})}\right) \tag{2.3}$$

The noncompetitive inhibition is distinguished by the simultaneous binding of the inhibitor and the substrate to the enzyme at different binding sites. The inhibitor lacks of structural similarity to the free enzyme and the enzyme substrate complex. The proportion of the enzyme molecules which bind to the enzyme stays the same as the substrate does not have an any effect on the binding ability of the noncompetitive inhibitor.

The inhibition cannot be overcome by increasing substrate concentration [36]. K_m stays the same but V_{max} is reduced significantly. These types of inhibitors are usually considered as [35] . K_i calculation is achieved through Equation 2.3.

The characterization of beta lactamases is achieved through chromagenic substrates. Together with nitrocefin and PADAC, CENTA is a widely used chromogenic substrate which is sensitive to different types of beta lactamase enzymes. Its hydrolysis

can be monitored by the detectable color change from light yellow (λ maximum ca. 340nm) to chrome yellow (λ maximum ca.405nm) [38] [39].

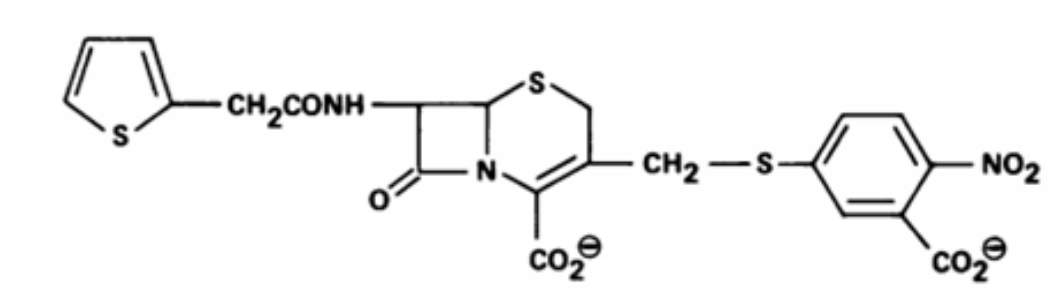

Figure 2.6. Molecular structure of CENTA

CENTA is a commercially available substrate prepared using cephalothin. Its structure is highly similar to cephalosphorin and it was shown to be sensible to many beta lactamase types. Because of its commercial availability and safety, it is conveniently used in kinetic studies.

2.5. Cell Penetrating Peptides (CPPs)

Most of the newly developed therapeutics fail to reach the clinic due to their low cellular uptake and poor delivery mechanisms [40]. The main reason is their hydrophobic nature [41]. In order to improve cellular uptake, different technologies including cell penetrating peptides (CPPs) have been proposed. CPPs are small molecules that enable the transportation of various bioactive molecules such as nucleic acids, peptides, proteins, liposomes and particles into the cell. Since their discovery 20 years ago, numerous CPPs have been described and analyzed [40]. Today, more than 100 CPPs are identified and divided into three classes: cationic, amphipathic and hydrophobic. 83% of these peptides have a net positive charge. Amphipathic CPPs contain both cationic and anionic peptides and form the biggest CPP class (44%) Hydrophobics constitute a total of 15% where anionic CPPs are not a part of any class and assigned to different classes [42]. Positively charged residues on CPPs enable them to make electrostatic interactions with anionic membrane parts, translocate the plasma membrane and ease

delivery to the cytoplasm or nucleus [43].

The first known CPP was cationic and was derived from the HIV-1 protein Tat (RKKRRQRRR). Cell-penetrating peptides (CPPs) are mostly short peptides that contain at most 35 amino acid residues. They are generally water-soluble, partly hydrophobic or polybasic and possess a net positive charge at physiological pH values [44]. Specific groups of CPPs are classified by their high sequence identity and common structural characteristics. However, generally CPPs show no sequence homology. The structural diversity leads to various uptake mechanisms, and numerous uptake levels depending on the cell line, and other conditions. Furthermore, the type of cargo carried by a CPP, which can be covalently or non-covalently attached to the CPP, can also affect profoundly mode and levels of uptake [42]. The process generally does not require energy and far from being toxic to the cell [43].

2.5.1. pVEC

pVEC is a well known amphiphatic cell-penetrating peptide (CPP) that contains 13 cytosolic and 5 trans membrane residues derived from murine vascular endothelial-cadherin (VE-caderin) protein. Like all CPPs, it is a short peptide that only contains 18 amino acids and has a sequence of LLIILRRRIRKQAHAHSK. It is mainly responsible for mediating physical contact between adjacent cells through dimerisation, In addition to this, it transfers information into the cell, controls vascular permeability and angiogenesis. [45]. Research showed that pVEC can be efficiently taken up by the cells and works as a potent carrier peptide [46].

The N terminus of pVEC peptide sequence is hydrophobic where the C-terminus is highly hydrophilic. The middle part contains four arginine and two lysine amino acids which makes it positively charged. Elmquist et al showed that, through N-terminus, pVEC interacts with plasma membrane which makes the N-terminus hydrophobic stretch highly important for cellular uptake and cell permeability [45].

In Akdağ et al, it was shown that the uptake potential decreased when a mutation occurred in the LLIIL residues to one by one transformation to alanine. Also it was detected that the deletion of the first three residues or replacement of the first five residues by L-alanine resulted in the destruction of cellular uptake [41].

Alaybeyoglu et al showed the results of adding LLIIL residues of pVEC to the N terminus of BLIP peptide on cellular uptake. It was proposed that adding hydrophobic residues to the N terminus would lead to promising results in terms of designing novel antimicrobial peptides [29].

3. MATERIALS

3.1. Bacterial Strains and Plasmids

The cell growth experiments were performed using *E.coli* K12 strain and the pUC18 plasmid carrying antibiotic resistant *E.coli* K12pUC18 cells. The pUC18 plasmid enables the bacteria cells to produce beta lactamase enzyme, thus makes them resistant to beta lactam antibiotics. They were both provided from our laboratory stock.

3.2. Chemicals

The chemicals used during the experiments are purchased from APPLICHEM (Germany), MERCK (Germany), MOLECULA (Germany) and SIGMA (USA).

3.3. Solutions

3.3.1. Potassium Phosphate Buffer

Potassium phosphate buffer is prepared for aliquoting CENTA substrate. Also it was used in the control sets during the cell growth experiments and also used as buffer in enzyme activity experiments. It was prepared at 1M concentration, and then diluted to the target concentration of 50μM. The chemicals used are shown in Table 3.1.

Table 3.1. KPO_4 buffer

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

3.3.2. CENTA

CENTA is a commercially produced chromagenic substrate used for beta lactamase enzyme and used in kinetic measurements. It was obtained from Calbiochem Chemicals and prepared at 4.7mM stock concentration and aliquoted in 50ul eppendorf tubes using 50μM potassium phosphate buffer. The aliquot was stored at -20°C.

3.3.3. Ampicillin

Ampicillin is added to the growth media of resistant *E.coli* K12pUC18 cells. It was aliquoted using sterile distilled water at 100mg/ml concentration. As ampicillin cannot be sterilized with steam sterilizer, it was filtered through 45μM pore sized membranes. The aliquot was stored in small volumes at -20°C.

3.4. Growth Media

All cell cultures are grown using LB medium and plated for Colony Forming Unit (CFU) experiments using LB-agar medium. These media are prepared using sterile distilled water. For *E.coli* K12pUC18 cells, ampicillin at a final concentration of 100ug/ml is added to the growth media. The contents of the mediums are given below.

Table 3.2. LB Medium

Chemical	**Concentration**
Tryptone	10g/l
Yeast Extract	5g/l
NaCl	10g/l

Table 3.3. LB-agar Medium

Chemical	**Concentration**
Tryptone	10g/l
Yeast Extract	5g/l
NaCl	10g/l
Agar	15g/l

3.5. The List of Used Peptides

In the scope of the project, the inhibitory effects of 5 peptides are investigated. In the table 3.4, the list of the peptides are given.

Table 3.4. The list of used peptides and their sequences

Name	**Sequence**	**Composition**	**Amount**
P2	NH_2–LLIILHAAGDYYAY–$CONH_2$	Obtained by combining antimicrobial BLIP based peptide and the first 5 amino acids of cell penetrating peptide pVEC	10.5mg
P3	NH_2–RRGHYY–$CONH_2$	Patented beta lactamase inhibitor	10.0mg
P3A	NH_2–RRGHYYA–$CONH_2$	P3 peptide with an additional alanin amino acid at the end	10.0mg
P4	NH_2–LLIILRRGHYY–$CONH_2$	Combination of P3 peptide and the first 5 hydrophobic residues (LLIIL)	10.0mg
P5	NH_2–LLIILCYHFLWGPC–$CONH_2$	Combination of TEM-1 beta lactamase inhibitor [34] and 5 hydrophobic residues of cell penetrating peptide pVEC	10.0mg

3.6. Laboratory Equipments

Table 3.5. The list of laboratory equipments

Aim	**Equipment**
Absorbance Measurement	Specord 200 (Analytikjenna, UK) Precision Cells (Hellma, Germany)
Microplate Incubation	Synergy HTX multi-mode reader (BioTek, USA) Tissue Culture Test Plate 96F (TPP,Switzerland)
Orbital Shaker	ZHWY-211B Shaker Incubator (ZHICHENG, China)
Incubation	FN5008(Nüve, Turkey)
Vortexing	Reax Top Vortex (Heidolph, Germany)
Sterilizer	Steam Sterilizer OT40L (Nüve, Turkey)
Water Purification	MILLI-Q UF Plus (MILLIPORE, USA) MILLIPORE Elix® 5UV (MILLIPORE, USA)
Pipetting	1ul-10ul, 10ul-100ul, 100ul-1ml pipettes (Thermo Electron Corporation, Canada)
Refrigeration	RT59EBPN(Samsung, South Korea)
Water Bath	nb5 bath (Nüve, Turkey)
Deep Freezer (-80°C)	Ultra Low Temperature Freezer U410 Premium (New Brunswick Scientific, USA)
Weighing	Balance XB 220A (Precisa, Switzerland)
pH Measurement	pHmeter (SCHOTT,Germany)
Magnetic Stirrer	CAT M6.1(Germany)
Working Area Sterilization	Microbiologic Safety Cabinet MN 120(Nüve, Turkey)

4. METHODS

4.1. Sterilization

The equipments used during in vivo experiments were always sterilized beforehand to avoid contamination. For the sterilization of pipette tips, glassware equipments, growth medium, distilled water and buffer solutions, a steam sterilizer was operated at 121°C for 15 minutes at 1 atm pressure. As ampicillin was degraded at high temperatures, it was filtered through 45μM pore sized membranes.

4.2. Preculture Preparation

100 ml fresh LB medium was inoculated with 1 ml cell stock which is kept at -80°C. Ampicillin was added to the preculture of the resistant *E.coli* cells at a concentration of 100μg/ml. The prepared preculture was incubated overnight using incubator shaker at 37°C and 180 rpm. After overnight incubation in the orbital shaker, 1ml of the culture was pipeted to 100 ml fresh LB medium. OD_{600} was waited to reach 0.9, then fresh LB medium was inoculated again using the same method.

4.3. Cell Growth Conditions

Precultures were grown using orbital shaker at 37°and 180 rpm. The growth was monitored by measuring optical density at 600 nm using a spectrometer. After two inoculations, they are transferred to microplates and peptides with selected concentrations were added. The microplate reader which was operated at 37°C kept optical density data for 20 hours per 5 minutes. For antibiotic resistant cells, ampicillin is added to the medium. 500 ml flask is used to keep the growth volume at one fifth of the flask for proper aeration.

4.4. Determination of Growth Curves

Cell growth was monitored using microplate reader. For the incubation in the microplate reader, first, 100 μl buffer was added to the target wells of 96 well plate. When the final OD of the culture reached 0.2, 100 μl samples were transferred to the 96 well-plate. The total volume of each used well reached to 200 μl and the plate was placed into the microplate reader. OD at 600 nm was measured at 37°C and 180 rpm for 20 hours by taking data per 5 minutes.

4.5. In Vivo Growth Inhibition

100 μl of buffer solution was added to all wells except for the one with the highest target concentration. 200 μl of the solution that contains peptide at twice the final concentration was added to the empty well. Two-fold dilutions were performed by taking 100 μl of the peptide-containing solution and transferring to the well on the left. The same dilution procedure was applied until the target concentrations were obtained. For each peptide concentration control wells that contain the same chemicals in the peptide aliquot were prepared using the same method. When optical density reached to 0.2, 100 μl of the cell culture was added to each well. 96-well plate was placed in the microplate reader. OD data at 600nm was taken for 20 hours at 37°C and 180rpm orbital shaking as advised in Andrews et al [23].

4.6. Colony Forming Units

To determine the differences in the colony forming units of the peptide containing wells and the control sets, bacterial samples were plated. Before plating ten-fold dilutions were performed. To obtain ten-fold dilution, 100 μl from all the samples were taken and transferred to centrifuge tubes each contained 900 μl LB medium. The mixtures were vortexed for homogenization. Ten-fold dilution was repeated until the target dilutions were obtained. The final 1 ml samples were transferred to sterile Petri dishes individually. Freshly prepared LB-agar was poured into the dishes and mixed carefully by moving through a circle path. When the dishes were cooled and thus hard-

ened, they were placed into the incubator overnight at 37°C. 16 to 20 hours later, the colonies were counted. The colony numbers below 30 and above 300 were not counted.

4.7. Extraction of Periplasmic Proteins

Osmotic shock method described by Nossal and Heppel was used to obtain periplasmic proteins. Sterile fresh LB medium was used for inoculation with *E.coli* BL21(DE3) preculture at a 1:100 dilution to keep optical density below 0.05. This inoculate was incubated at 37°C at 180 rpm. When the optical density reached 0.9 at 600 nm it was induced with 0.1 mm IPTG and incubated for 3 hours at 30°C and and 180 rpm. After the incubation, the samples were centrifuged at 14500 rpm for 20 min at 40°C. The pellets were collected and kept on ice for overnight. The cells were resuspended in 2 ml osmotic shock liquid which contained 20% sucrose in 30 mm Tris-HCl with 1mm ethylenediaminetetraacetic acid (EDTA) and incubated for 20 minutes at room temperature. After incubation the samples were centrifuged at 9000 rpm for 20 minutes, than pellets obtained were resuspended in ice cold 4 ml 5mm $MgSO_4$ to release the periplasmic proteins. The pellets were incubated for 20 minutes on ice, then rapidly centrifuged at 9000 rpm for 20 minutes at 40°C to remove the cells. The supernatant contained TEM-1 beta lactamase.

4.8. Protein Concentration Determination

To determine the protein concentration in the periplasmic extract, Bradford method (1976) was used. Samples from the beta lactamase enzyme was taken and subjected to 1:10 dilution. Bradford dye was added which binded to the proteins. The absorbance chance at 595 nm was monitored. To determine the calibration curve, Bradford samples with different Bovine Serum Albumin concentrations were prepared, the absorbance values were measured and plotted using linear regression analysis. The protein concentration in the sample was calculated using the equation obtained from the linear regression analysis that is shown in Figure A.1.

4.9. Beta Lactamase Activity Determination

Periplasmic extract from *E.coli* BL21 cells were used as the TEM-1 beta lactamase source. Two sets of enzymes (E1 and E2) which were obtained in different times were used in the experiments. The activities in the presence of selected CENTA concentrations were determined through the hydrolysis of CENTA (Calbiochem). CENTA was added to the 1ml precision cells that contain 50μM potassium phopsphate buffer and enzyme. At each run, 5μl enzyme was used. From Bradford Assay, it was found that enzyme solution 2 contained 0.4μg/μl pure enzyme, so 5μl enzyme solution contained 2μg TEM-1 beta lactamase. The molecular weight of TEM-1 was found as 165 kDa from Santa Cruz Biochemicals, so the final molarity of the enzyme in 1 ml precision cells used in the activity measurements was calculated as 12.12 nM. During the kinetic assays on RRGHYY, Huang et al used 45nM TEM-1 [33], and Alaybeyoglu et al used 36.33nM TEM-1 beta lactamase on the kinetic experiments on 45-53 loop of BLIP [29] where Huang et al used 11.2nM enzyme for 46-51 loops of BLIP [28]. It was decided not to use enzyme volumes higher than 5μl as it was seen that, 12.12nM gave satisfying results, so the kinetic experiments were proceeded with this enzyme concentration.

The color change with time due to hydrolysis was monitored at 405 nm by spectrometer. The maximum activies were calculated using equation. 4.1.

$$U = \frac{\frac{dA}{dT} \times V_t \times 10^6}{\lambda \times d \times V_s} \tag{4.1}$$

In equation 4.1 dA/dt represents the absorbance change, Vt total reaction volume (1ml), λ absorptivity constant 6400cm2 mole-1 for CENTA), d path length (1cm) and Vs volume of the enzyme(5μl). When the activities at different CENTA concentrations were obtained, Lineweaver-Burk and Hanes-Woolf plots were drawn and used to calculate V_{max} and K_m values of the enzyme.

4.10. In Vitro Enzyme Inhibition

To investigate the inhibitory effects of analyzed peptides on beta lactamase enzyme, they were added to the reaction volume in 25μM concentrations individually. The same procedure for pure enzyme activity measurement was repeated. The activities at different CENTA substrate concentrations were calculated. Lineweaver-Burk and Hanes-Woolf plots were drawn to obtain K_m and V_{max}. These values were compared with the pure enzyme and the inhibition types were determined. Finally the inhibition constant Ki, which shows the potency of the inhibitor, was calculated.

5. RESULTS AND DISCUSSION

The aim of the project was to analyze the effect of adding five hydrophobic residues (LLIIL) to beta lactamase inhibitory peptides to see whether the addition of LLIIL led to any improvements on inhibition properties and cell penetration capabilities. P2, P4 and P5 were designed using known class A beta lactamase inhibitory peptides and the hydrophobic LLIIL residues. Their inhibition capacities and cell penetration properties were investigated and compared with the inhibitory peptides P3 and P3A.

P2 is based on BLIP 45-53 residues with the addition of 5 hydrophobic residues (LLIIL) from the cell penetrating peptide pVEC. With the help of the additional residues, P2 was anticipated to reach beta lactamase more efficiently, thus be more effective as an antimicrobial agent. P3 is a known potent TEM-1 beta lactamase inhibitor [33]. P3A was constructed by adding alanine to its C terminus. P4 was designed by adding LLIIL on the N terminus of P3 peptide to potentially enhance penetration. P5 was designed by using a novel beta lactamase inhibitor [34] and the 5 LLIIL residues from pVEC.

The effects of the peptides were analyzed in two parts. In the first part, growth experiments were performed using wild type *E.coli* K12 and antibiotic resistant *E.coli* K12pUC18 cells. pUC18 plasmid is responsible for TEM-1 beta lactamase formation which resulted in antibiotic hydrolysis and thus antibiotic resistance. Wild type *E.coli* K12 lacked of TEM-1 beta lactamase enzyme, and used as control. Growth inhibition was detected by monitoring the changes in optical density at 600nm for 20 hours using the microplate reader Also, viable cell colonies were counted after 4 and 9 hours of microplate incubation. After 4 hours, the bacterial growth was in mid-exponential phase and after 9 hours, it was at the beginning of stationary phase. Together, they were thought to give a good idea on the decreases in cell colonies. The decreases in colony counts were reported in percentage changes by comparing with the peptide free control sample.

In the second part, kinetic characterization of TEM-1 beta lactamase obtained from *E.coli* BL21(DE3) cells was performed in the presence of peptides by monitoring CENTA substrate hydrolysis. 2 enzyme sets were used . Enzyme 1 (E1) was used in the experiments performed on P2 and clavulanic acid. Its concentration was not known exactly as it was obtained before the experiment and the results of Bradford Assay was not found. At each run, 5μl enzyme solution was added. For the in vitro experiments on P3 and P4, Enzyme 2 (E2) was used. To determine the protein concentration in the beta lactamase enzyme, Bradford assay was performed. As a result, the protein concentration was found as 0.4μg/μl. As a result, it contained 2μg enzyme in 5μl volume and the final enzyme volume in 1 ml precision cells was 12.12μM. Kinetic parameters (V_{max}, K_m and Ki) were calculated. The inhibition types and levels were determined.

5.1. Experimental Results In The Presence of Clavulanic Acid

Clavulanic acid is a potent, commercially used beta lactamase inhibitor. It is mainly combined with amoxicillin and ticarcillin to prevent the antibiotics from the destructive effects of beta lactamases. Potassium clavulanate used in the experiments was obtained in powdered form and aliquoted using potassium phosphate buffer in 1mM concentrations.

As its inhibition properties are well known, it was used as a control to understand the reaction of resistant *E.coli* cells to beta lactamase inhibitors. Enzyme kinetics and cell growth experiments were performed and repeated twice. During each run, 1μg of enzyme in 5μl volume was used.

5.1.1. Kinetic Characterization of Beta Lactamase In The Presence of Clavulanic Acid

In order to determine the inhibitory effect, the maximum activities of beta lactamase were measured using 47 μM CENTA substrate in the presence of 50μM, 100 μM and 200 μM potassium clavulanate. The CENTA hydrolysis with time was detected

by measuring the OD values at 405nm using spectrometer. The results were shown in Figure 5.1, Figure 5.2 and Figure 5.3. The activities were calculated using Equation 4.1.

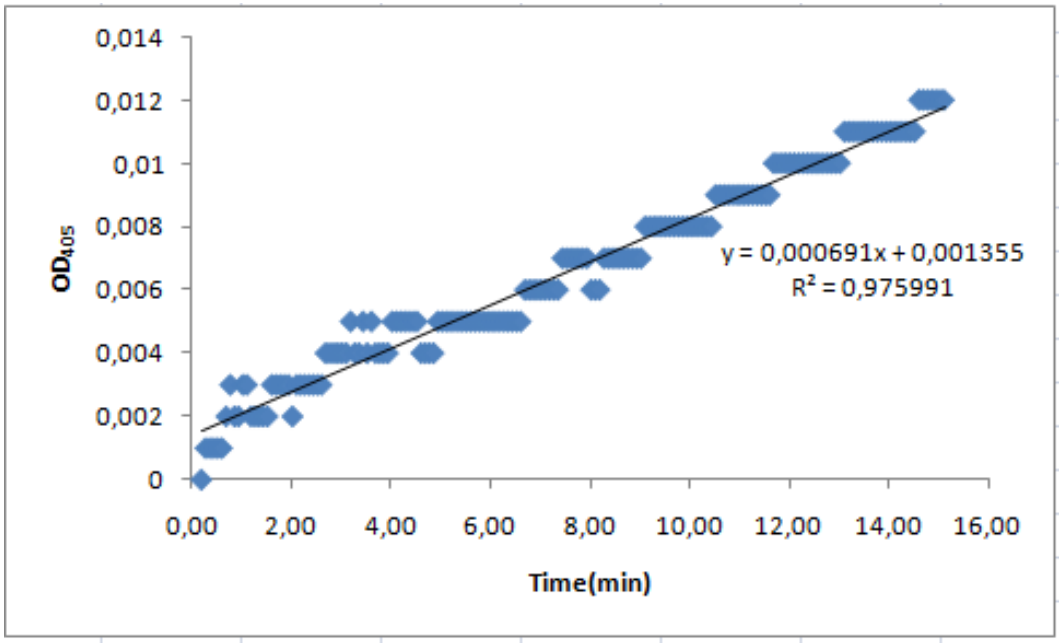

Figure 5.1. Beta lactamase mediated hydrolysis of 47μM CENTA in the presence of 50 μM clavulanic acid

The maximum activity of beta lactamase in the presence of 47μM CENTA substrate and 50μM clavulanic acid was found as 21.6 U/L where it was around 9000U/L without clavulanic acid. The maximum activity detected when 100μM clavulanic acid was present was very similar to the previous case and calculated as 25.87 U/L.

The maximum activity decreased to 10 U/L in the presence of 200μM inhibitor. The CENTA hydrolysis plots clearly showed high beta lactamase inhibition in the presence of clavulanic acid. No other experiments were performed for clavulanic acid and thus kinetic parameters were not calculated. For future studies, 50μM, 100 μM or lower concentrations can be tried to obtain Lineweaver-Burk and Hnes-Woolf plots. 200μM is not suggested as it showed more than 50% decrease in the activities.

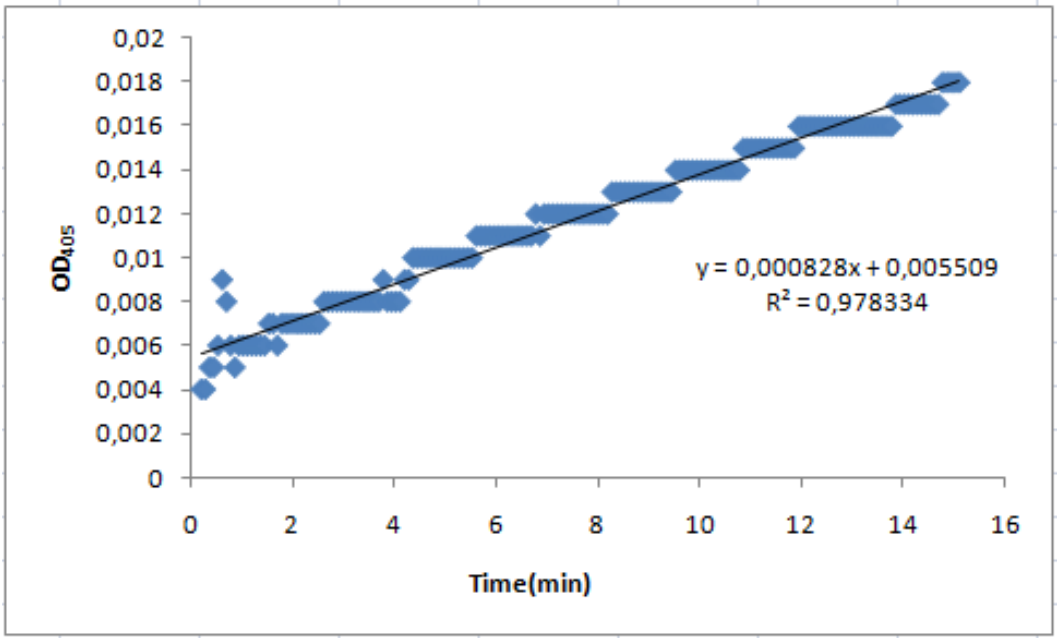

Figure 5.2. Beta lactamase mediated hydrolysis of 47μM CENTA in the presence of 100 μM clavulanic acid

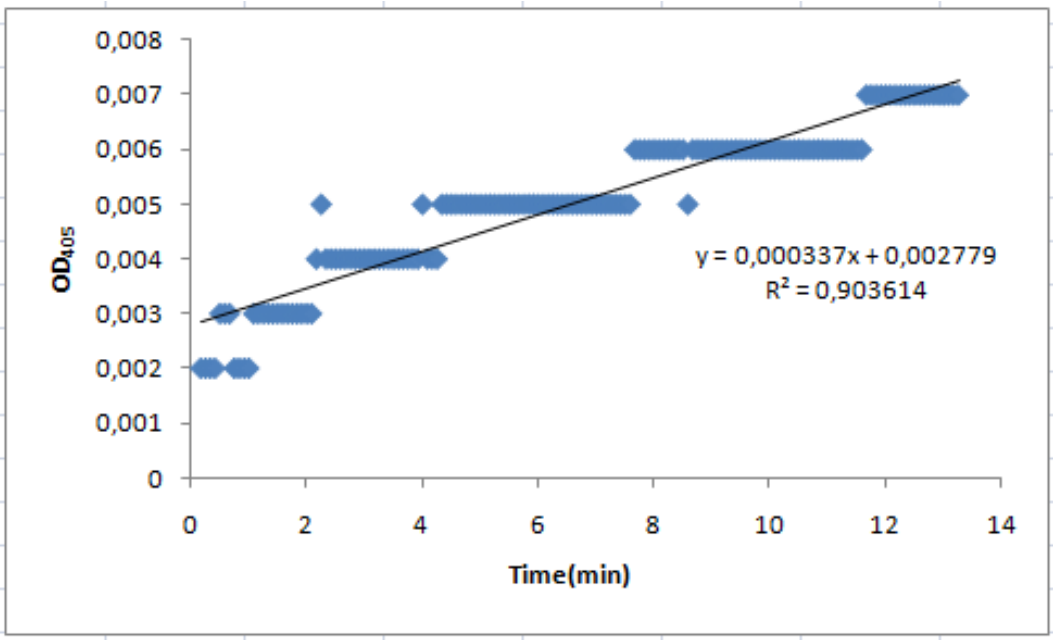

Figure 5.3. Beta lactamase mediated hydrolysis of 47μM CENTA in the presence of 200 μM clavulanic acid

5.1.2. Effect of Clavulanic Acid on Cell Growth

Growth experiments were performed on antibiotic resistant cells and wild type *E.coli* cells were used as control. 1mM ampicillin was added only to the growth medium of resistant *E.coli* cells. The aim was to detect beta lactamase enzyme inhibition by monitoring cell death.

Precultures were grown in an orbital shaker at 180 rpm and 37°C. The cell growth was monitored using microplate reader for 20 hours at OD_{600}. The results were shown in Figure 5.4 and Figure 5.5 It was seen from figure 5.4 that the highest growth inhibition

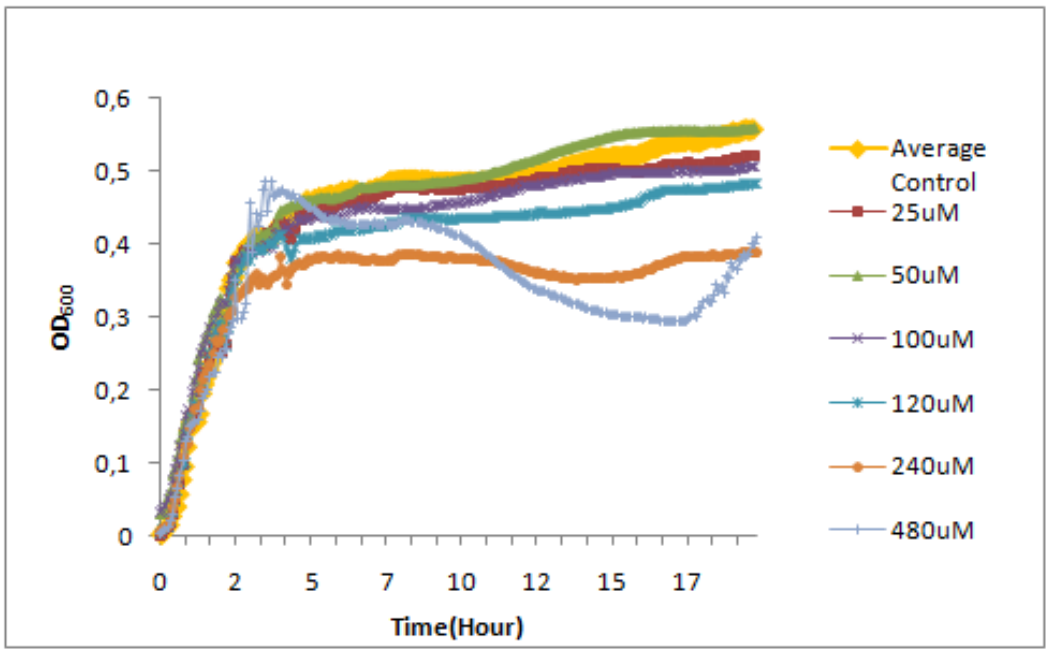

Figure 5.4. The growth profiles of wild type *E.coli* cells in the presence of 25μM, 50μM, 100μM, 120μM, 240μM and 480μM clavulanic acid and the average control sample

of wild type cells was observed at 240 μM and 480 μM clavulanic concentrations which was reflected as 33% decrease in the optical density. The cell growth in the presence of 480 μM decreased until 17^{th} hour, but started to increase afterwards. In general, a decrease around 15% was observed below 240 μM clavulanic acid concentration.

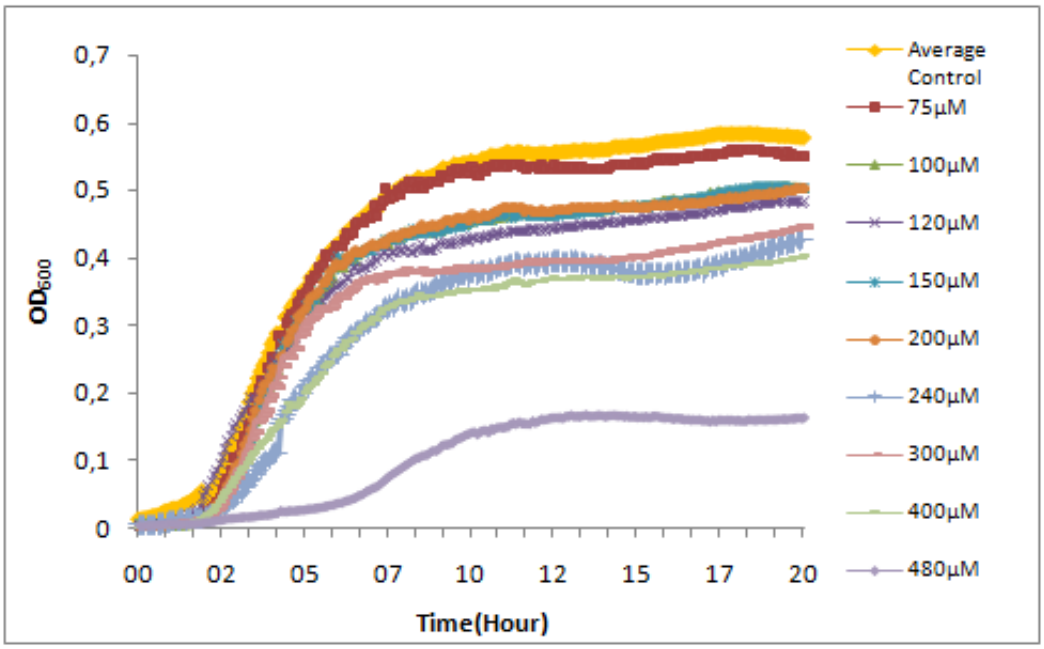

Figure 5.5. The growth profiles of resistant *E.coli* cells in the presence of 75μM, 100μM, 120μM, 150μM, 200μM, 240 μM, 300μM, 400μM and 480μM clavulanic acid and the average control sample.

For antibiotic resistant *E.coli* cells, not much change was observed for 75 μM. 15% decrease in OD_{600} values was detected for the concentrations between 100 μM and 200 μM. The decrease reached to 35% in the presence of 200 μM to 400 μM clavulanic acid concentrations.The growth rate was notably reduced compared to the control well after 400 μM. The maximum optical density reached only to 0.17 where the average control was 0.63 and resulted in 73% decrease . These results indicated that clavulanic acid was most effective after 400 μM for 1mM ampicillin concentration.

5.1.3. Viable Cell Count in the Presence of Clavulanic Acid

Viable cell counts of wild type *E.coli* and antibiotic resistant *E.coli* cells were counted after 4 hour of incubation in the microplate reader at 37°C and 180 rpm. 1mM ampicillin was added to the LB-agar growth medium of resistant cells. Serial dilutions were performed ranging from 10^6 to 10^9 before spreading the cells onto the petri dishes.

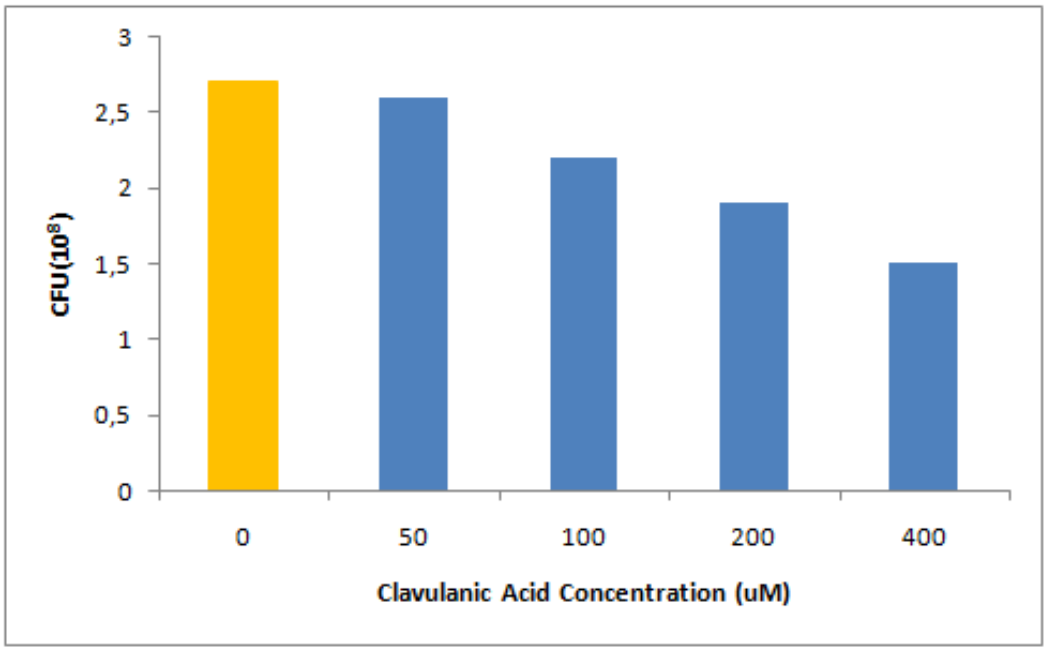

Figure 5.6. Viable cell counts of wild type *E.coli* K12 cells in the presence of 50 μM, 100μM, 200μM and 480μM clavulanic acid and the average control sample at the end of 4^{th} hour

The viable cell counts of wild type *E.coli* K12 cells after 4 hours of incubation showed a gradual decrease with increasing clavulanic acid concentrations. Not much difference was observed in the presence of 50 μM clavulanic acid. However, the cell counts decreased by 18% in the 100 μM and 30% in the 200 μM clavulanic acid containing wells. The most significant decrease was observed in the presence of 400μM c.acid which resulted in 40% decrease in cell growth. The results matched the growth curve results that indicated a maximum of 33% decrease.

Clavulanic acid ranging from 50 μM to 480 μM concentrations was added to the growth medium of resistant *E.coli* cells before microplate incubation. For each concentration, viable cells counts decreased from 8% to at most 96% were detected. This decrease became more and more significant with increasing concentrations. When the results of 400 μM and 480 μM were compared, a huge gap between cell counts were observed which was 0.9x10^7 for 400 μM and 0.11 x 10^7 for 480 μM clavulanic acid containing sample. It was concluded that when the 400 μM barrier was exceeded.

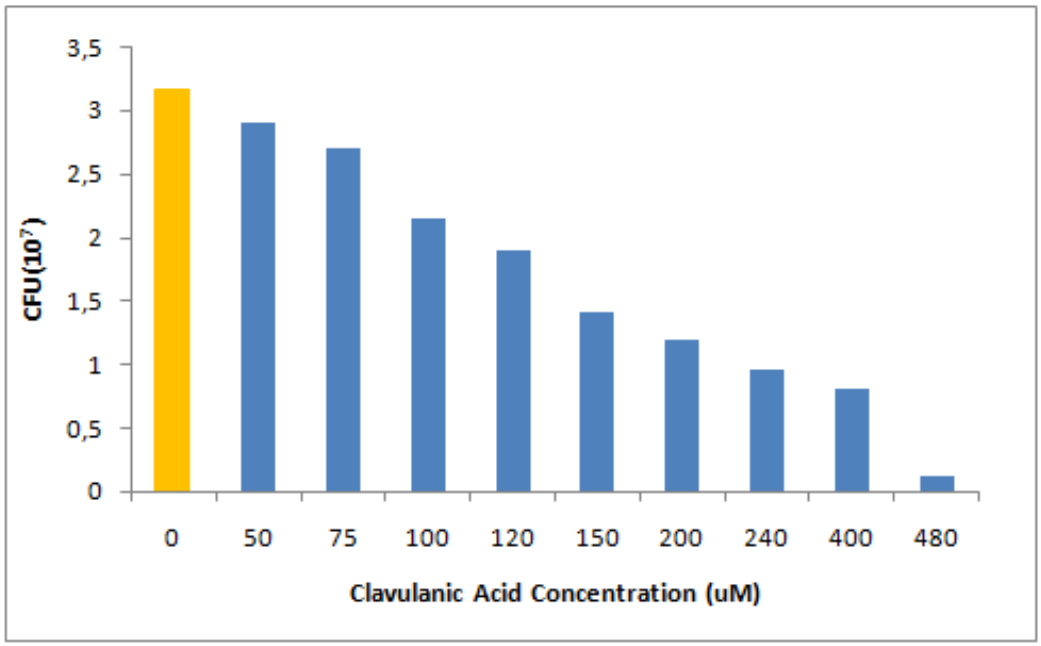

Figure 5.7. Viable cell counts of resistant *E.coli* cells in the presence of 50 μM, 75μM, 100μM, 120 μM, 150 μM, 200 μM, 240μM, 400 μM and 480μM clavulanic acid and the average control sample at the end of 4^{th} hour

After 400 μM concentration the growth inhibition increased notably. The results of cell growth and viable cell count experiments matched as they both showed 400 μM was an important barrier in growth inhibition.

5.2. Experimental Results In The Presence of P2

P2 was designed with BLIP 45-53 residues and the 5 hydrophobic (LLIIL) residues. To analyze and understand the effect of LLIIL on the cell capacity and antimicrobial properties of P2, both cell growth and in vitro enzyme kinetics experiments were performed. For the growth of resistant *E.coli* cells, ampicillin was added to the growth medium to stabilize the pUC18 plasmid.

The effect of P2 peptide was investigated in two parts. In the first part, cell growth of wild type and resistant *E.coli* cells in the presence of selected P2 concentra-

tions was monitored to construct growth curves. In the second part, beta lactamase enzyme activities in the presence of 25μM, 50μM and 100μM P2 were investigated for 47μM CENTA. 25μM P2 concentration was chosen to proceed with the kinetic characterization.

5.2.1. Effect of P2 on Cell Growth

Bacterial growth experiments were performed both on wild type *E.coli* K12 and resistant *E.coli* cells. Wild types were used as control and no ampicillin was added to the growth medium. P2 aliquots were prepared using 50% sterile water and 50% DMSO at 5mM concentrations. When added to the wells, DMSO percentage decreased to 2.5% which was below the concentration which could affect the growth curves. For each peptide concentration, control wells were prepared using the same type of *E.coli* cells with buffer and 2.5% DMSO. The experiments were repeated twice to increase the reliability of the results.

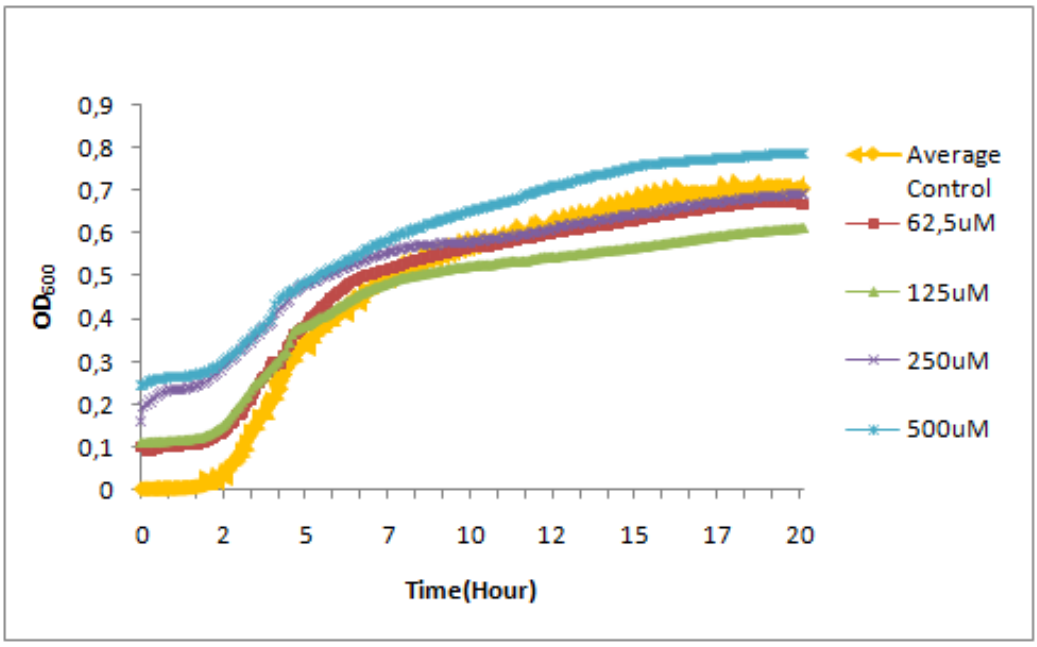

Figure 5.8. The growth profiles of wild type *E.coli* K12 cells in the presence of 75μM, 100μM, 120μM, 150μM, 200μM, 240 μM, 300μM, 400μM and 480μM P2 and the average control sample.

From Figure 5.8 it was seen that as the P2 concentration increased the starting OD_{600} values increased accordingly. P2 peptide had solubility problems. When added to the wells, precipitates were observed especially at high concentrations. This problem became more apparent at 250 μM and 500 μM concentrations which affected the reliability of the results.

Growth inhibition was observed for all concentrations, but this inhibition did not increase with increased peptide concentrations. When compared to the average control sample 15% decrease in cell growth was detected in all P2 containing wells.

The same experiments were repeated for resistant *E.coli*. The results were shown in Figure 5.9.

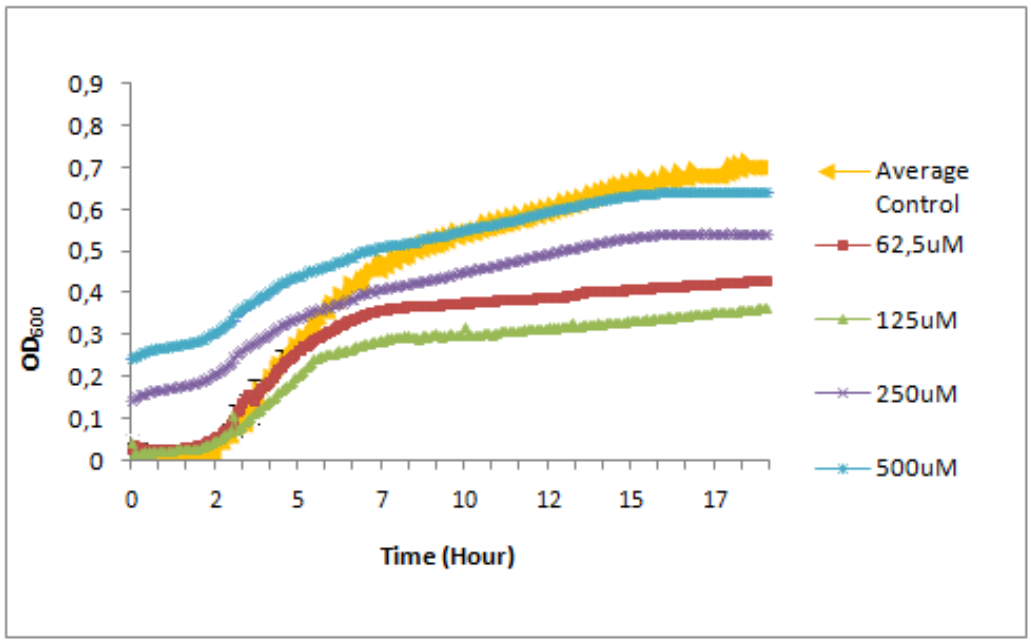

Figure 5.9. The growth profiles of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250 μM and 500μM P2 and the average control sample.

As P2 peptide was not dissolved homogeneously in 50% water-50%DMSO solution and resulted in visible particles when transferred to the 96 well-plates. Therefore the starting OD_{600} values were significantly higher compared to the control well which

was also observed in Figure 5.8.

When the growth curves of resistant cells in the presence of P2 peptide were compared with the control well, 50% decrease in cell growth was observed when 125μM P2 was added and 40% decrease in resistant cells was seen for 62.5μM P2. Significantly more growth inhibition was detected on resistant *E.coli* cells than wild type *E.coli* K12 cells. The results with higher concentrations were not evaluated as the wells showed insolubility which may have affected the results.

5.2.2. Viable Cell Count In the Presence of P2

To determine the viable cell counts of wild type and resistant *E.coli* cells, P2 containing and P2 free control samples of wild type cells were plated after 4 hours of incubation and the resistant cells were plated after 4 and 9 hours of microplate incubation which showed the changes in cell counts at mid exponential and stationary growth phases compared to the control samples. Addition of P2 was expected to decrease the cell numbers in resistant *E.coli* cells, due to beta lactamase inhibition. The experiments were repeated twice and the average CFU numbers are shown in Figure 5.10 figure 5.11 and 5.12.

In order to determine the cell colony counts, first CFU experiment on wild type *E.coli* K12 cells were conducted. As these *E.coli* cells lacked of beta lactamase enzyme and thus antibiotic resistance, the inhibitory effect of P2 on this enzyme was irrelevant. Therefore, it was used as control. From Figure 5.10, it was clear that up until 250μM P2 concentration, no difference was observed compared to the peptide free control sample. In the presence of 250μM P2, 20% decrease was observed and at 500 μM no decrease was observed. As not much decrease was observed in the presence of 62.5μM , 125μM and more importantly 500μM, the decrease in the presence of 250μM P2 concentration was thought to be human error.

The Figure 5.11 shows the viable colony counts of resistant *E.coli* cells in the presence of selected P2 concentrations. 62.5μM peptide concentration resulted in 25%

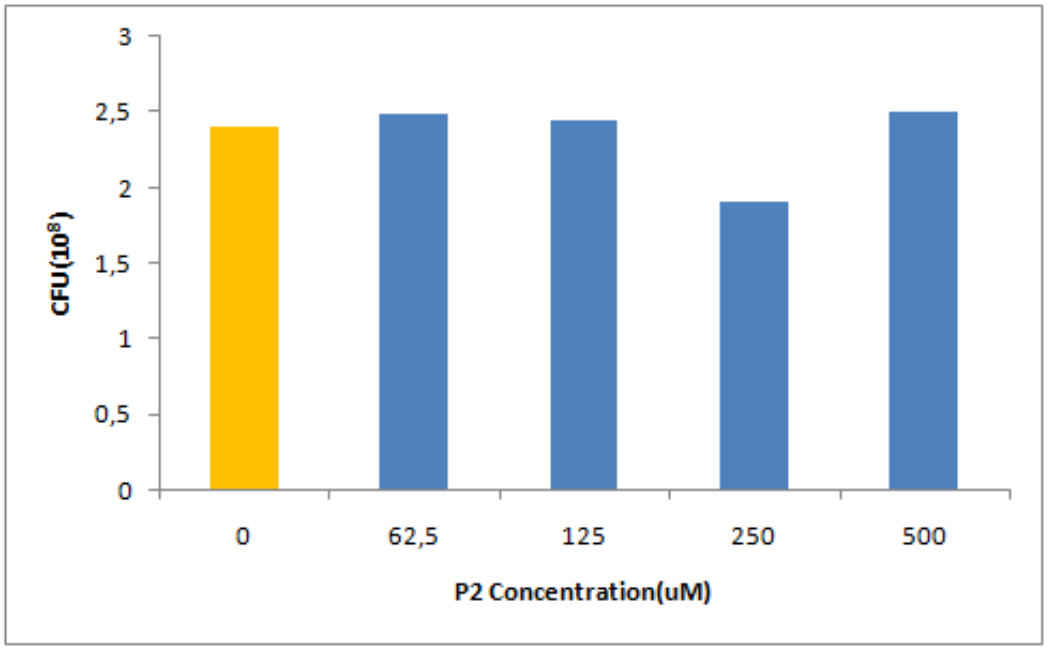

Figure 5.10. Viable cell count of wild type *E.coli* K12 cells in the presence of 62.5μM, 125μM, 250μM, 500μM P2 and the average control sample at the end of 4^{th} hour

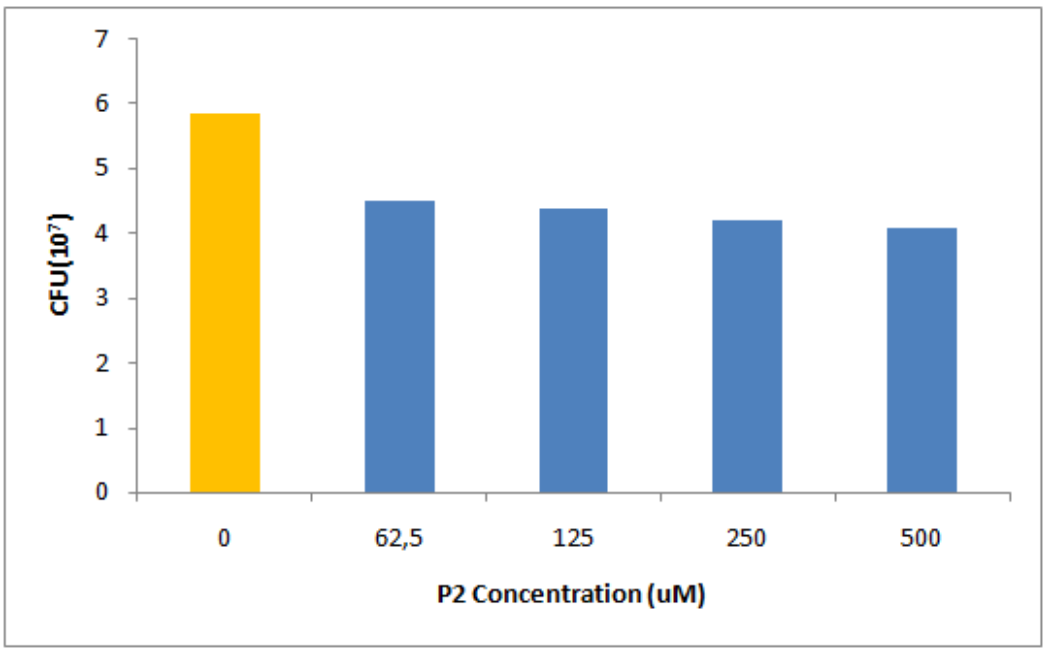

Figure 5.11. Viable cell count of resistant*E.coli* K12 cells in the presence of 62.5μM, 125μM, 250μM, 500μM P2 and the average control sample at the end of 4^{th} hour

decrease, 125 μM 27% decrease, 250 μM 30% and 500 μM 31% decrease. It was clear that increasing the concentration led to bigger decreases in the viable cell counts compared to the peptide free control sample.

When the growth curves and the CFU results were analyzed together it was understood that counting viable cells after 8th hour might lead to more realistic results as the inhibition was more visible after 8 hours of incubation for the growth curves. In order to reflect these differences in the CFU counts, another experiment was conducted by taking samples after 9 hours of microplate incubation.

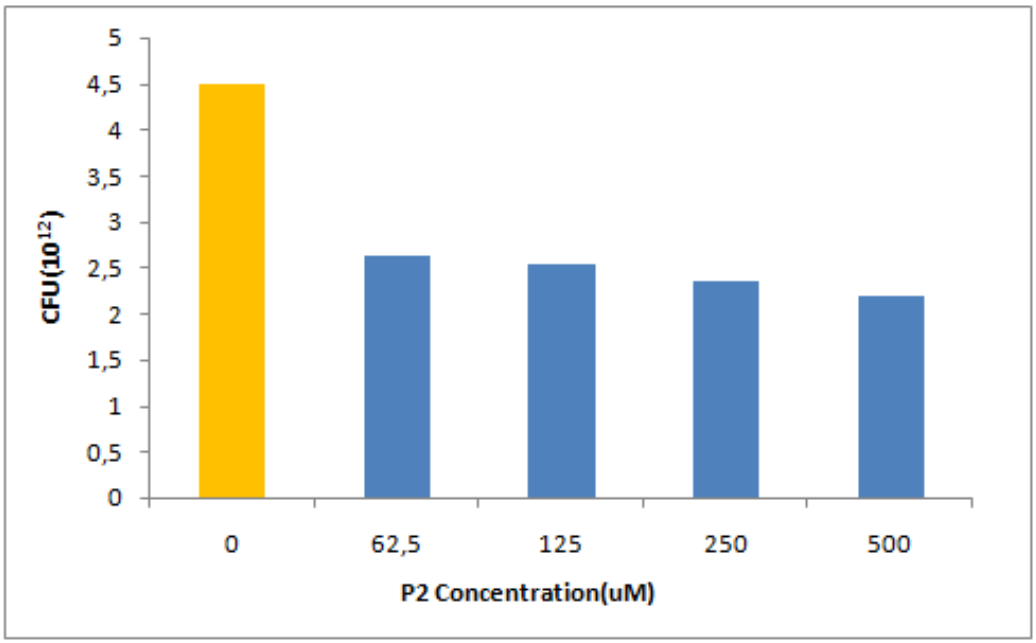

Figure 5.12. Cell colony numbers of resistant *E.coli* cells in the presence of 62.5μM 125μM, 250μM, 500μM P2 and the average control sample at the end of 9^{th} hour

Figure 5.12 shows the viable colony forming units of *E.coli* cells in the presence of four selected P2 concentrations after 9 hours of microplate incubation. At each concentration , a decrease was clear. This decrease was 42% for 62.5 μM, 44% for 125 μM, 47% for 250 μM and 53% for 500 μM P2. The cell growth experiments resulted in an average of 49% decrease in growth rate. Increasing peptide concentrations led to no considerable differences.

The results of CFU after 9 hours matched the results of the cell growth experiments. Increasing peptide concentration did not lead to significant decreases in cell counts and the decrease was between 42% to 54%.

5.2.3. Kinetic Characterization of Beta Lactamase In The Presence of P2

5.2.3.1. Activity Results of Beta Lactamase Enzyme. Kinetic characterization of the enzyme was achieved by monitoring CENTA hydrolysis. CENTA is a well known chromogenic substrate of beta lactamase enzyme. It is hydrolyzed by beta lactamase and the color change from light yellow to chrome yellow can be monitored by detecting OD405 change using spectrometer. In the experimental procedure, CENTA hydrolysis was followed by taking OD_{405} data for 10 minutes. At each run, 5μl enzyme was used. The experiments were repeated twice for each CENTA concentration.

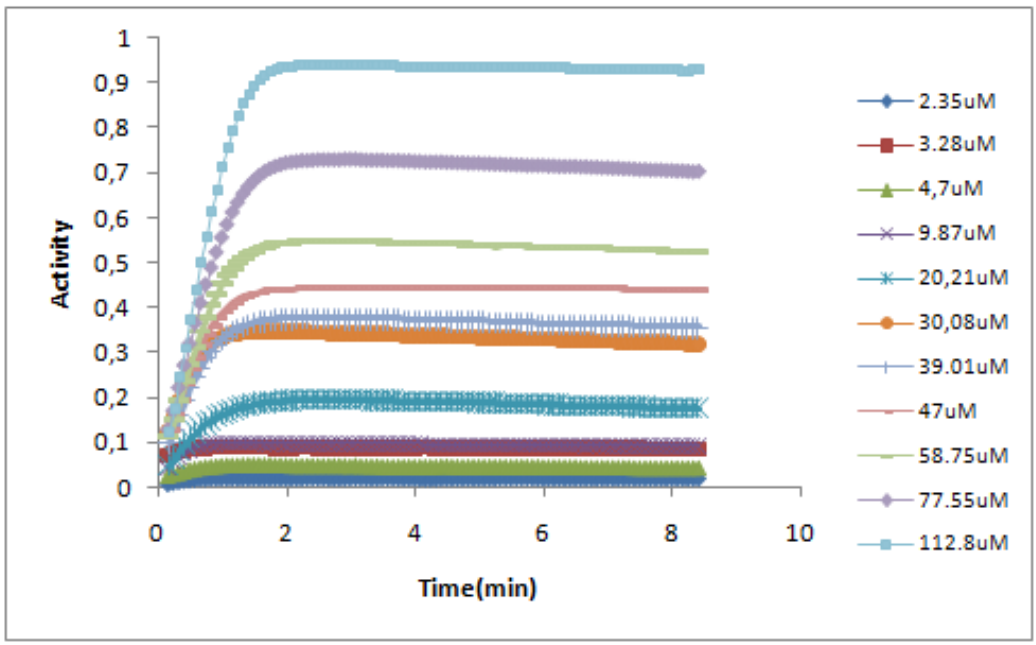

Figure 5.13. Activity graphs of enzyme 1 using different CENTA concentrations

Enzyme activity graphs were shown together in Figure 5.13. It was expected to see increased activities with a higher slope as CENTA concentration was increased. When the curves were investigated, it was seen that as the concentration was increased, the maximum activities increased with a higher slopes.

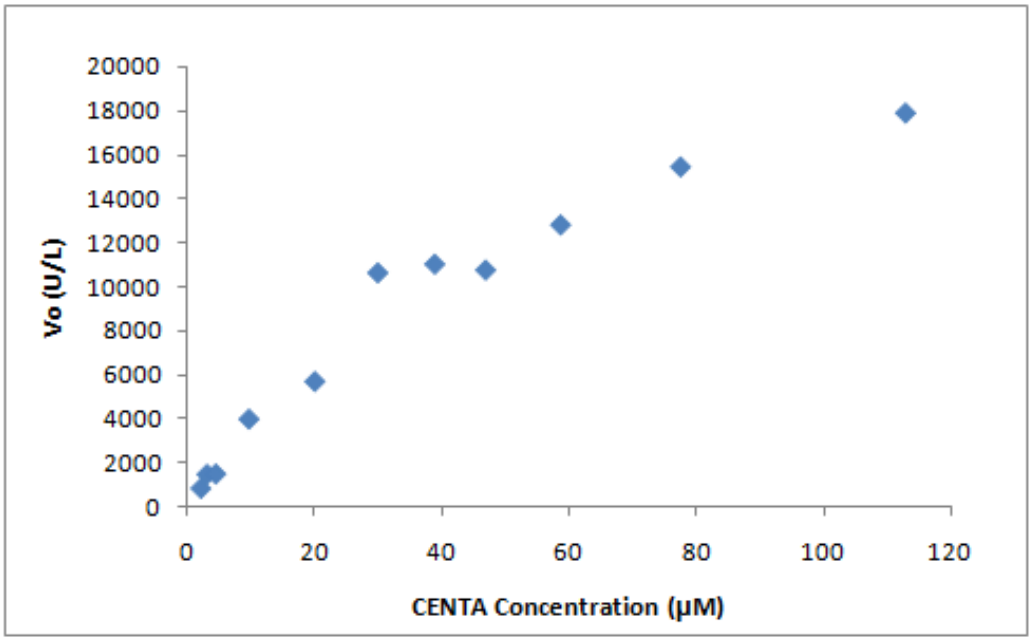

Figure 5.14. Activities of Enzyme 1 (Vo) for 11 selected CENTA concentrations

The maximum activities of enzyme 1 for different CENTA substrate concentrations were calculated using Equation 4.1 and shown in Figure 5.14. As expected the activities were positively correlated with CENTA concentrations.

While constructing the Lineweaver-Burk plots, the measurements of 2.35μM, 3.28 μM and 4.7 μM were not used as they were found unreliable. To detect the maximum activities in the presence of small CENTA substrate concentrations, CENTA in low volumes were used. While dealing with small volumes, the chance of making mistakes increased which could result in highly misleading outcomes. For each repeat, considerably high differences in maximum activities for same small CENTA concentrations were detected. Also, it was seen that the dominating part of the Lineweaver-Burk plot was the measurements obtained with low CENTA concentrations. Therefore to avoid mistakes, concentrations higher than 4.7μM were used. Kinetic parameters were calculated using Figure 5.15. Using Equation 4.1, K_m was found as 75μM and V_{max} as 25000 U/L.

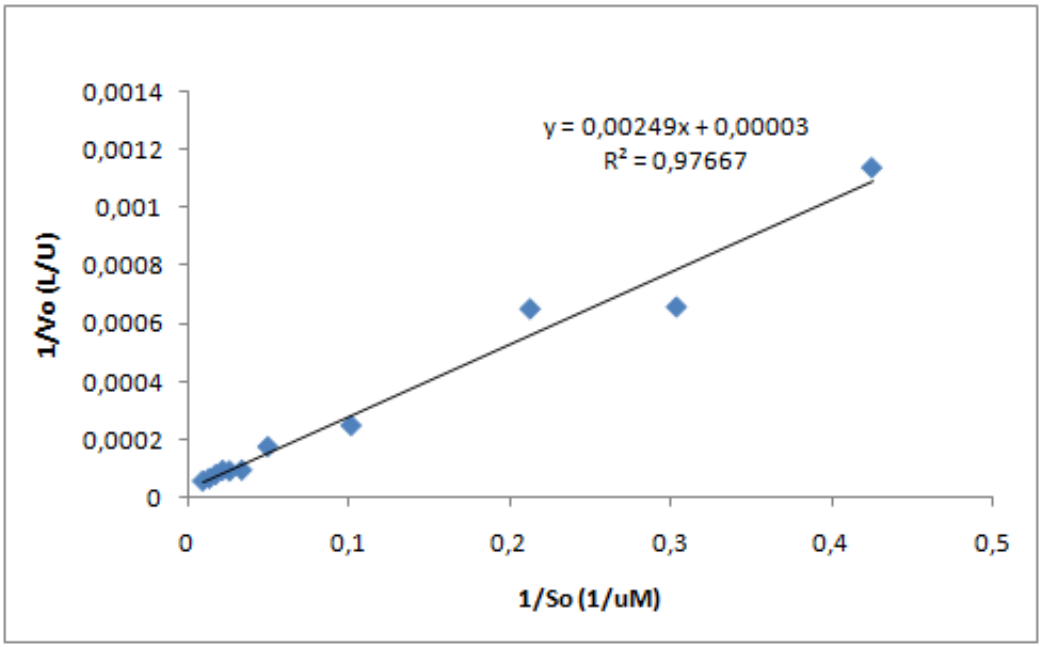

Figure 5.15. Lineweaver-Burk plot for enzyme 1

5.2.3.2. Beta Lactamase Activity In the Presence of P2. Using 47μM CENTA substrate, maximum activities were detected for 25μM, 50μM, 100μM and 200μM P2. The results were shown in Figure 5.16.

The Figure 5.16 was analyzed and 25μM P2 was chosen to proceed with the kinetic characterization experiments. The activity measurements were performed using 25μM P2 CENTA was known as a chromagenic substrate of beta lactamase enzyme, so it was used in different concentrations to determine activity values. Lineweaver-Burk and Hanes-Woolf plots were constructed. Kinetic parameters were calculated using these two plots.

The activities of enzyme 1 for 13 selected CENTA substrates in the presence of 25μM were shown together in Figure 5.17. The maximum activities and the slopes increased as the CENTA concentration was increased. Consequently it was concluded that the enzyme plots were reliable. The calculated maximum activities for different CENTA substrate concentrations for enzyme 1 with and without 25μM P2 were shown

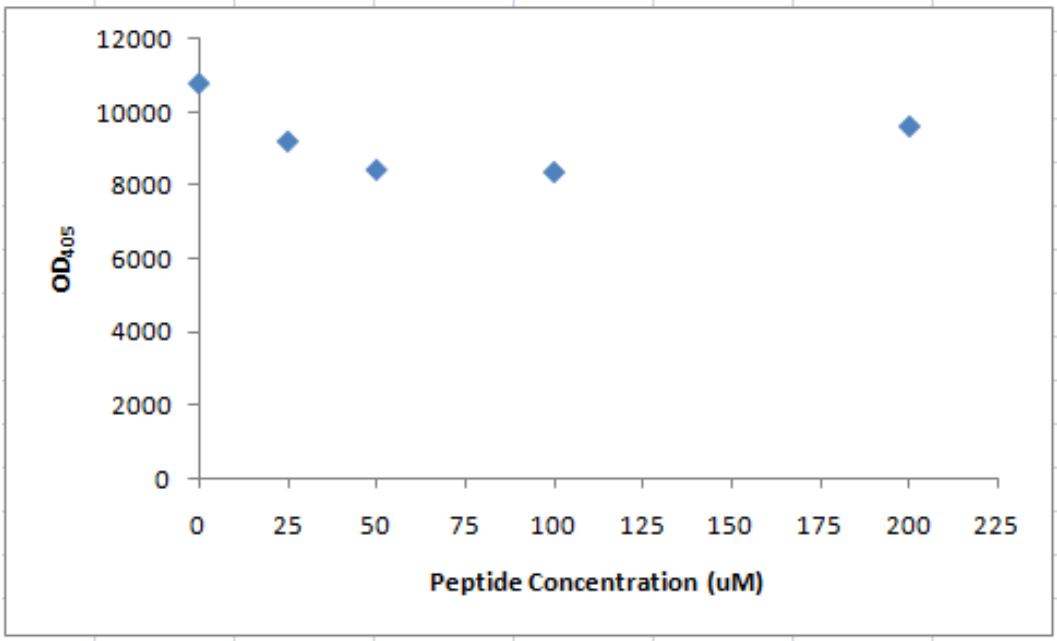

Figure 5.16. Activity Graphs of enzyme 2 for 25μM, 50μM, 100μM and 200μM P2 in the presence of 47μM CENTA

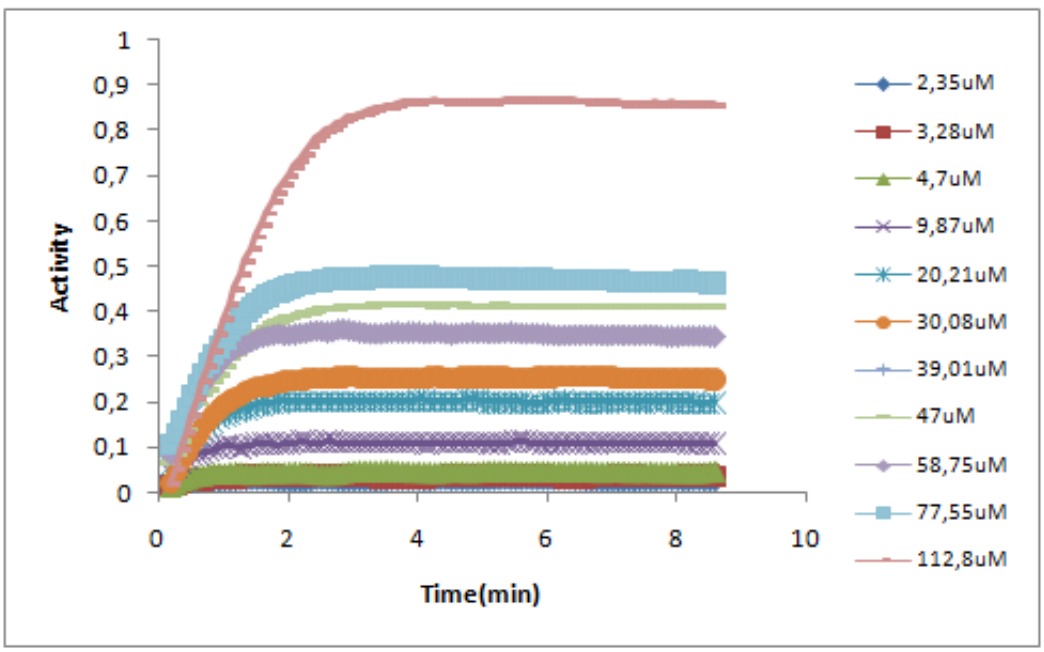

Figure 5.17. Activity Graphs of enzyme 2 with different CENTA concentrations in the presence of 25μM P2

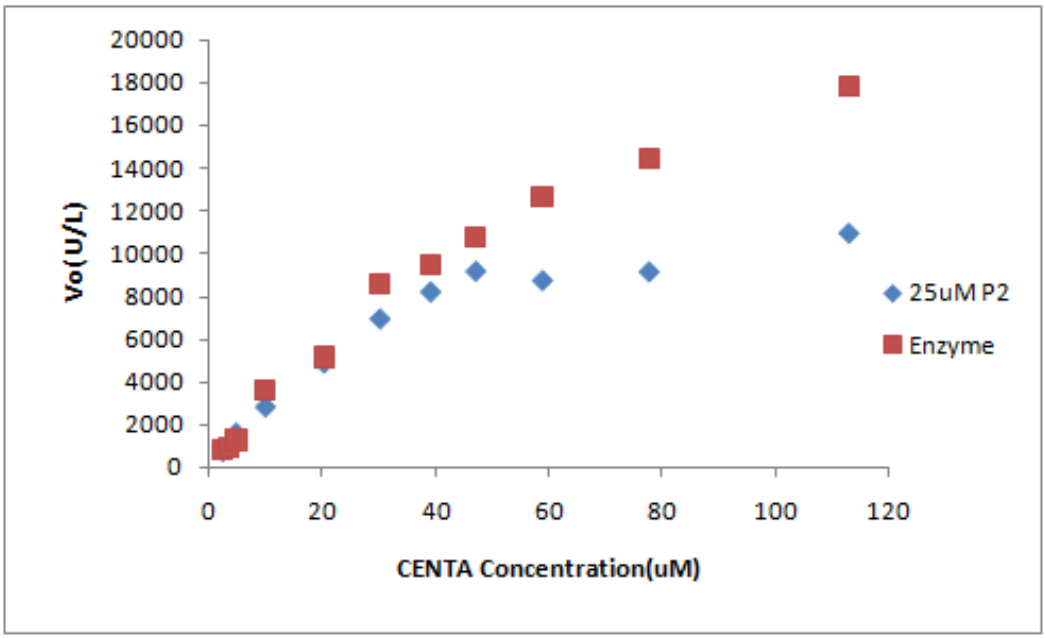

Figure 5.18. Activity Values of Enzyme 1 (Vo) with and without 25μM P2 for selected CENTA concentrations

together in Figure 5.18. Until 50μM CENTA concentration, not much difference in the activities were observed between the two cases. After that point the inhibition became more apparent.

While constructing the Lineweaver-Burk and Hanes-Woolf plots, the measurements using 2.35μM, 3.28 μM and 4.7 μM were not used. Measurements obtained using small CENTA substrate concentrations could include more mistakes as it was difficult to get the same amount of CENTA at each run. As the activity values diverged from the pure enzyme at high CENTA concentrations, it also made more sense not to use small CENTA concentrations to emphasize the inhibition at higher CENTA substrate concentration values.

The Lineweaver-Burk plots beta lactamase enzyme with and without 25μM P2 were shown together in Figure 5.19. Using Equation 4.1, V_{max} was found as 14285 U/L and K_m as 32.7 μM in the presence of P2. V_{max} for pure enzyme 1 was calculated as 25000 U/L and the K_m was calculated as 53.8 μM. When these values were compared,

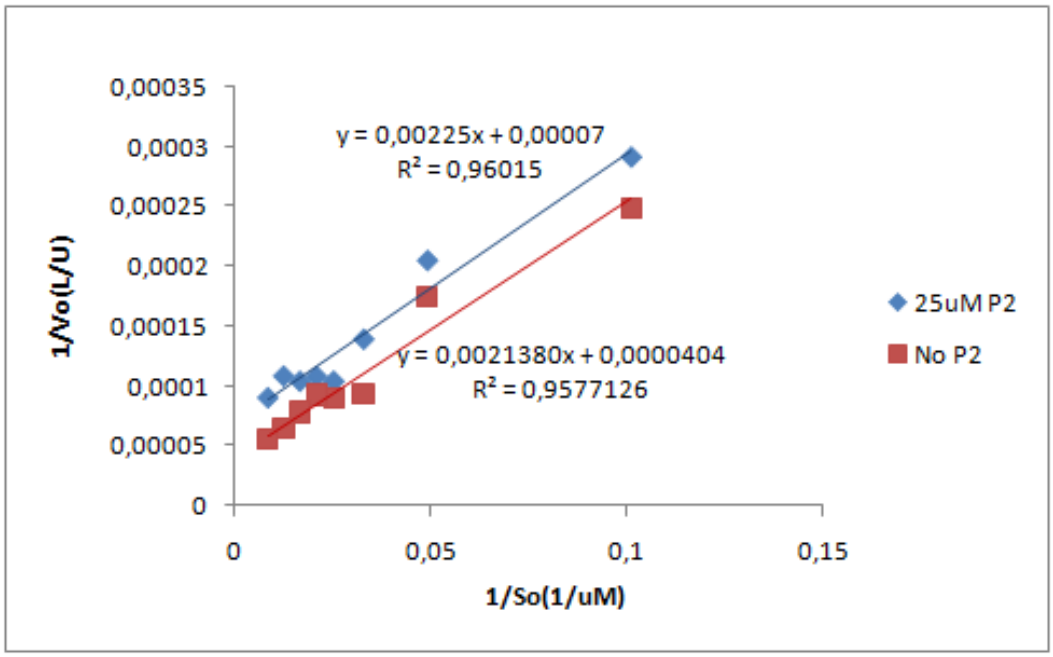

Figure 5.19. Lineweaver-Burk plot for enzyme 1 with and without 25μM P2

it was observed that both K_m and V_{max} decreased.

The kinetic parameters were calculated using also the Hanes-Woolf plot. K_m value of the enzyme was found as 25000 U/L and K_m was calculated as 57.25μM. When 25μM P2 was present, V_{max} was found as 14285 U/L and K_m was decreased to 28.71μM. The kinetic parameters calculated using two plots were close to each other. V_{max} values did not show any differences, K_m was calculated as slightly lower in Hanes-Woolf plot.

The two plots and the kinetic parameters indicated uncompetitive inhibition where the inhibitor complex only binds to the enzyme substrate complex. In the presence of P2, both K_m and V_{max} decreased.

In the case of uncompetitive inhibition, both K_m and V_{max} are expected to decrease by the same amount. For that reason, K_m/V_{max} ratio was calculated for the two cases and compared with each other. In the Lineweaver-Burk plot, the ratio was found as 0.002138 for the enzyme and 0.00225 after the addition of 25μM P2. For the

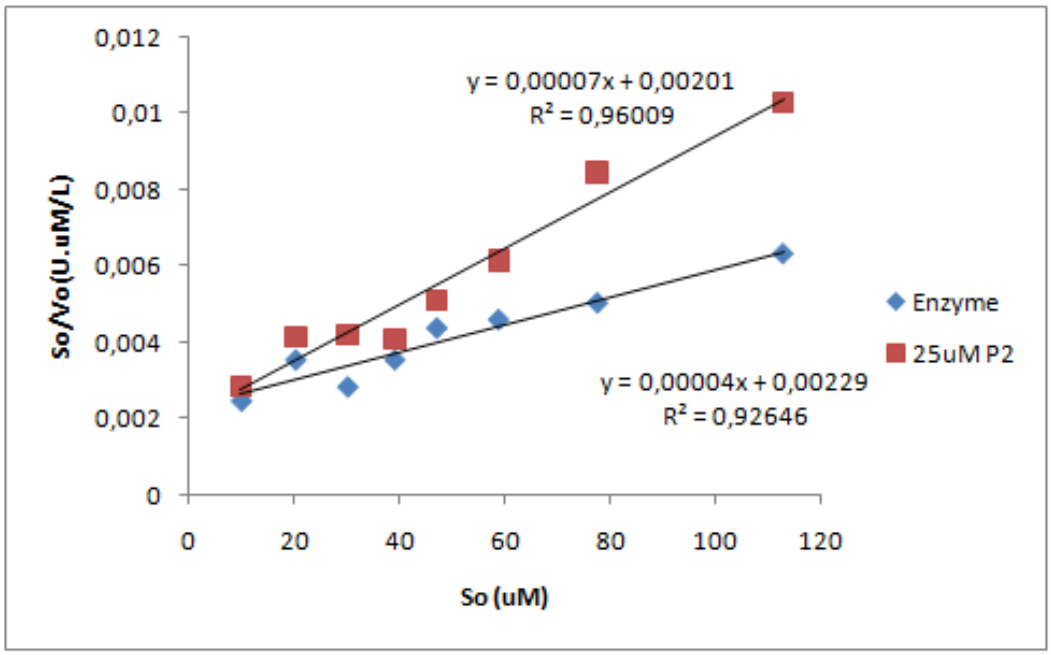

Figure 5.20. Hanes-Woolf plot for enzyme 1 with and without 25μM P2

Hanes-Woolf plot, these ratios were calculated as 0.00229 and 0,00201 respectively. The ratios were close enough to suggest uncompetitive inhibition. The KI was calculated using K_m and V_{max} separately using the two plots and shown in Table 5.2.

Table 5.1. Kinetic parameters of enzyme 1 with and without 25μM P2

	$\mathbf{V_{max}(U/L)}$	$\mathbf{K_m(\mu M)}$	$\mathbf{K_m/V_{max}}$	**Ki(μM)**
Enzyme 1 (Lineweaver-Burk)	25000	53.8	0.00225	
Enzyme 1 with 25μM P2 (Lineweaver-Burk)	14285	32.14	0.00214	36.39
Enzyme 1 (Hanes-Woolf)	25000	57.25	0.00229	
Enzyme 1 with 25μM P2 (Hanes-Woolf)	14285	28.71	0.00201	29.25

5.3. Experimental Results In The Presence of P3

P3 was a known beta lactamase inhibitor. It was expected to see inhibition in cell growth of resistant *E.coli* cells. Also, inhibition was anticipated for enzyme activity experiments.

The P3 aliquot was prepared in 5mM concentrations using sterile distilled water. No solubility problems interfering with the results were observed.

Cell growth experiments using wild type K12 and resistant, K12pUC18 *E.coli* cells were performed. Also kinetic experiments of the enzyme in the presence of selected peptide concentrations were performed to understand inhibition level and inhibition type. The results were compared with the P3 free control samples. The experiments were repeated twice to increase reliability.

5.3.1. Effect of P3 on Cell Growth

Wild type *E.coli* K12 cells lacked of beta lactamase production ability and thus were used as control OD data was taken at 600nm for 20 hours at 37°C and 180 rpm. Growth curves were compared with the peptide free control sample.

Until 6^{th} hour, no inhibition was encountered for both cell types. After hour 6, optical densities started to decrease compared to the control samples. For resistant cells, 15% decrease in the presence of 62.6μM, 24% in the presence of 125μM, 17% in the presence of 250μM and %16 decrease in the presence of 500μM was observed . The growth curves of wild type cells decreased by an average of 11%. Changing peptide concentration did not lead to any decreases.

5.3.2. Viable Cell Count in the Presence of P3

To determine the cell colony numbers, the samples were plated after 4 and 9 hours of incubation. The petri dishes were kept at 37°C for 20 hours, than cell colonies

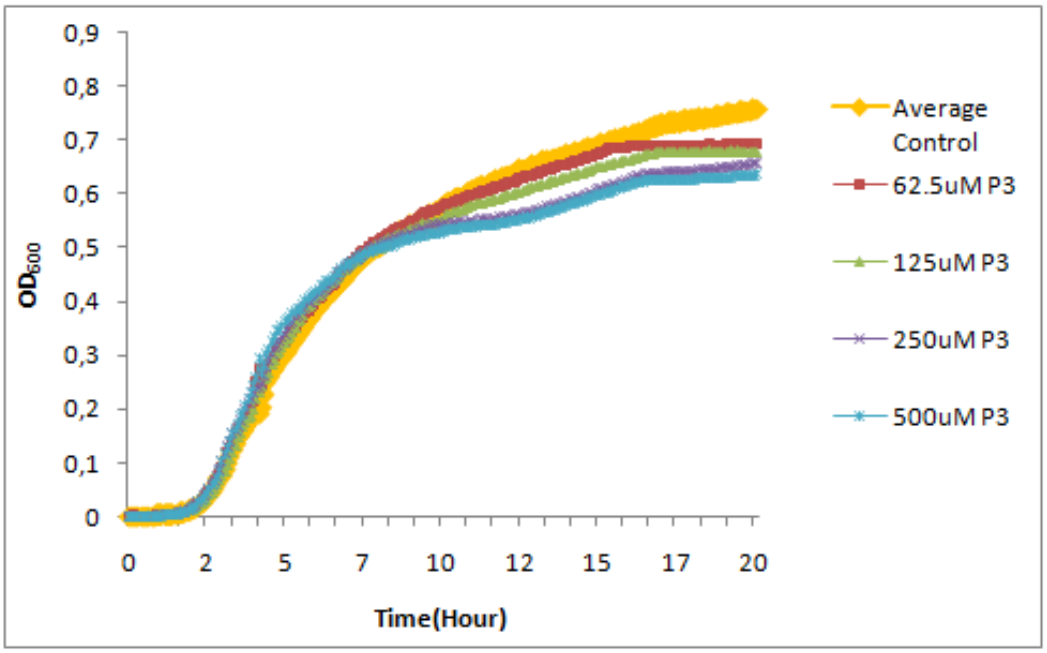

Figure 5.21. The growth profiles of wild type *E.coli* K12 cells in the presence of 62.5μM, 125μM, 250 μM and 500μM P3 and the average control sample

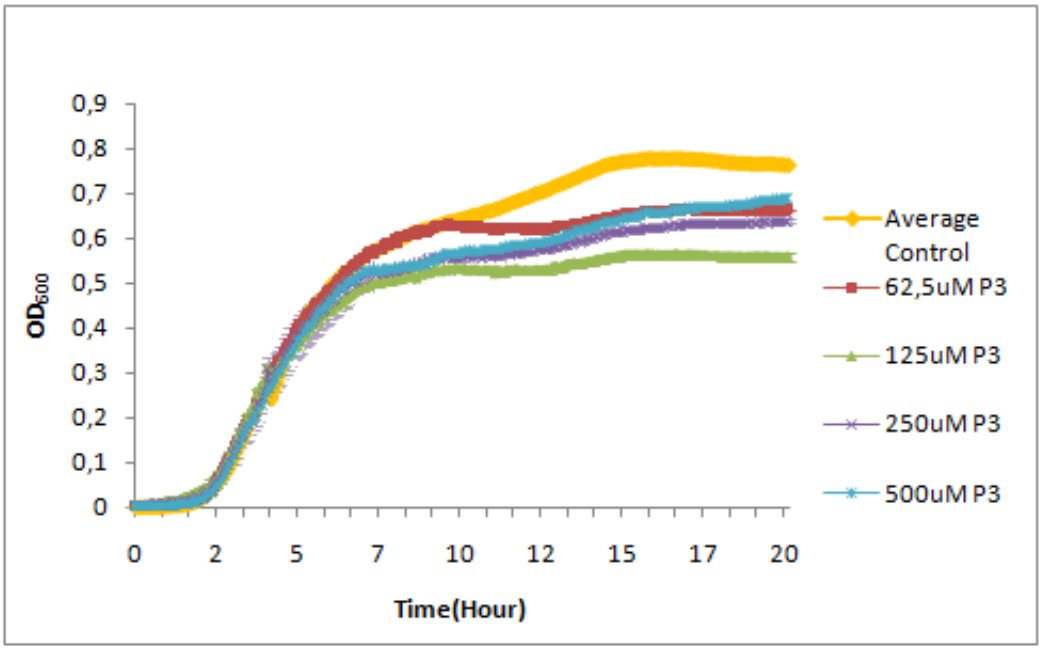

Figure 5.22. The growth profiles of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250 μM and 500μM P3 and the average control sample

were counted. The results of P3 containing samples were compared with the peptide free control samples to see the effect of the peptide.

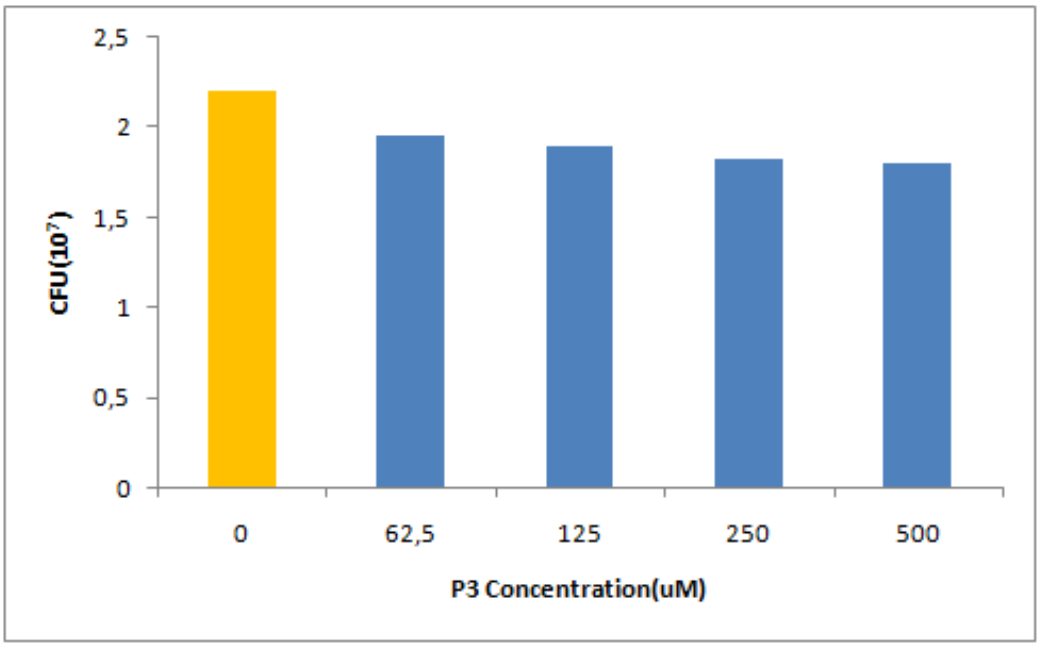

Figure 5.23. Viable cell counts of wild type *E.coli* K12 cells in the presence of 62.5μM 125μM, 250μM, 500μM P3 and the average control sample

The viable cell counts of wild type cells after 4 hours of incubation indicated 13% decrease for 62.5μM, 18% for 125μM, 20% for 250μM and 22% for 500μM P3. The viable cell counts of resistant cells did not change depending on the peptide concentration and showed an average of 27% decrease. As the growth curve experiment results were analyzed, it was decided to repeat viable cell count experiment by taking sample at 9^{th} hour . The experiment was performed using the sama procedure and the results were shown in Figure 5.25.

The CFUs of the samples taken after 9 hours of microplate incubation showed almost no decrease in cell counts when compared the control sample. An average of 5% of the cells died in the presence of P3 where increasing peptide concentration resulted in no additional change.

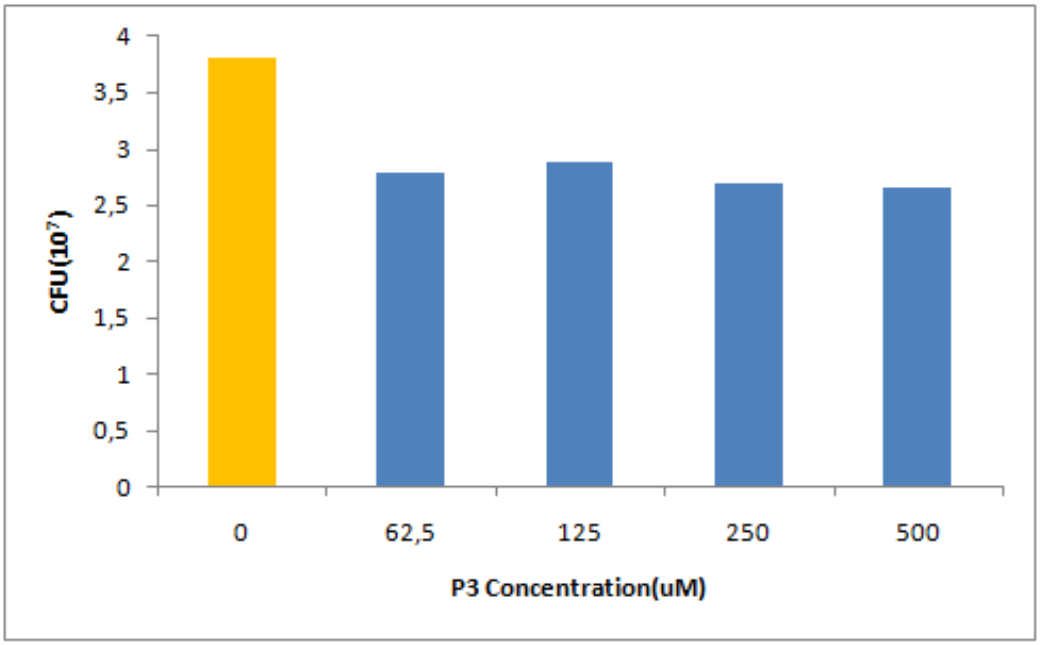

Figure 5.24. Viable cell counts of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250μM, 500 μM P3 and the average control sample at the end of 4^{th} hour

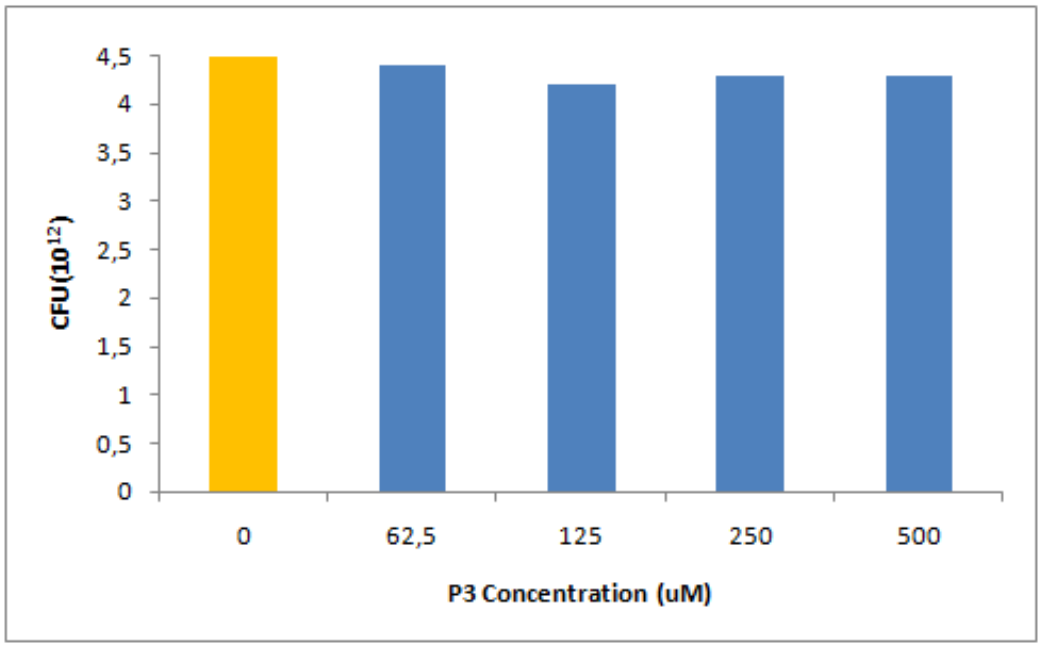

Figure 5.25. Viable cell counts of resistant *E.coli* cells in the presence of 62.5μM 125μM, 250μM, 500μM P3 and the average control sample at the end of 9th hour

5.3.3. Kinetic Characterization of Beta Lactamase in the Presence of P3

5.3.3.1. Activity Results of Beta Lactamase Enzyme. Enzyme activity was detected by monitoring CENTA hydrolysis. CENTA hydrolysis was followed by taking OD data for 10 minutes at a wavelength of 405 nm. At each run, 2μg enzyme in 5μl volume was used where the total enzyme concentration was 12.12uM. The experiments were repeated twice for each CENTA concentration. The activity graphs of beta lactamase

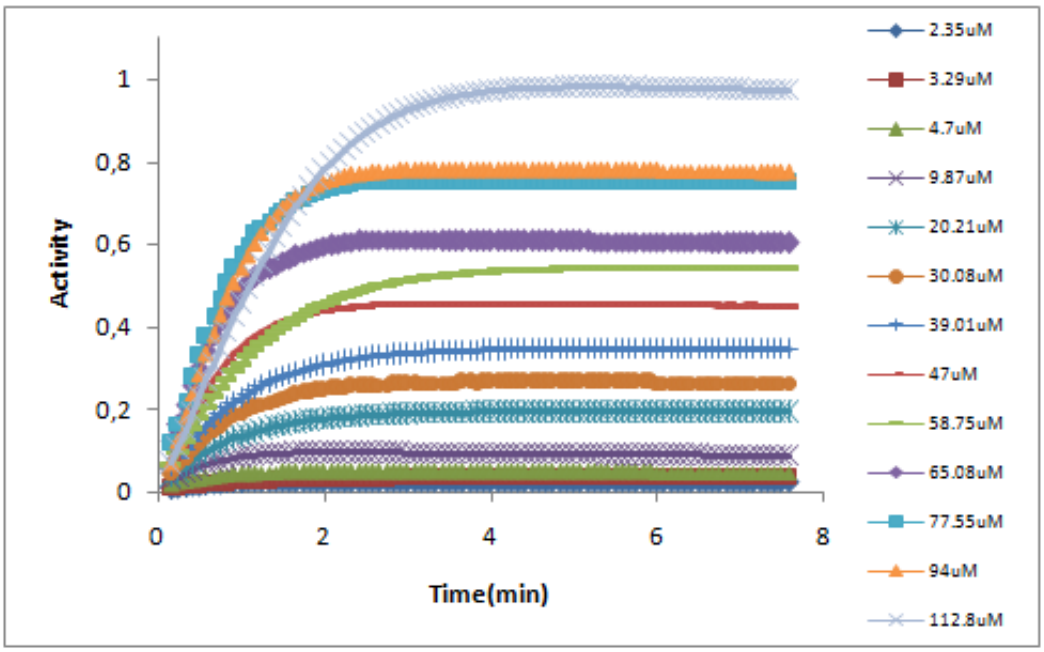

Figure 5.26. Activity graphs of enzyme 2 using selected CENTA concentrationsin the presence of 25μM P3

enzyme 2 for different CENTA substrate concentrations were shown in Figure 5.26. It was expected to see higher activities and slopes with increasing CENTA concentrations. The plots showed a similar pattern which indicated that the enzyme results were reliable.

The relationship between the activities of enzyme 2 and the CENTA substrate concentrations were shown in Figure 5.27. The figure showed a stable increase in the activities with increasing substrate concentration.

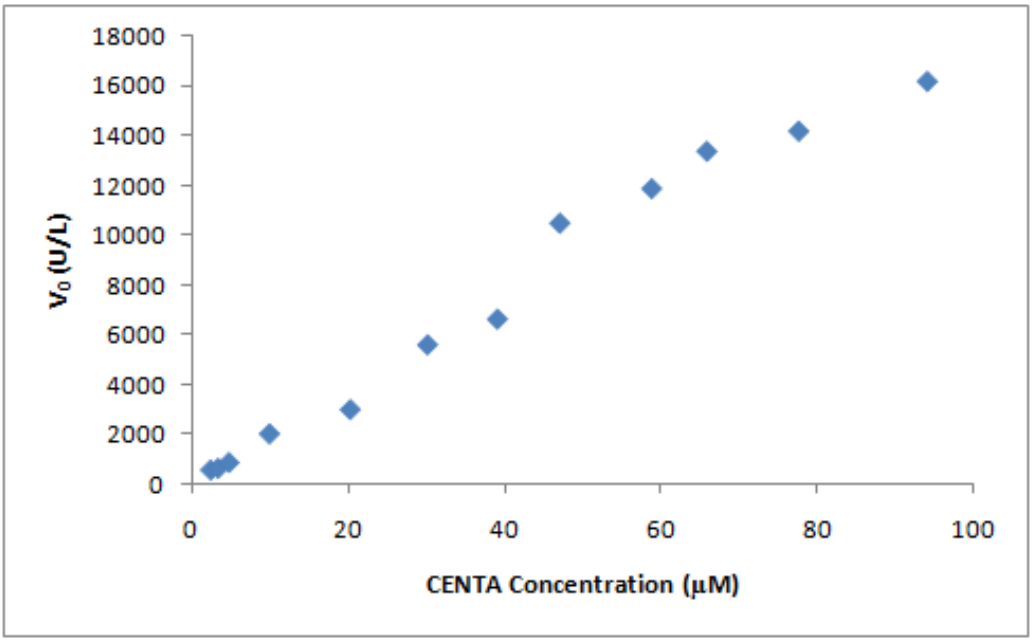

Figure 5.27. Activity Values of Enzyme 2 (Vo) for selected CENTA concentrations

To construct Lineweaver-Burk plot data from low CENTA concentrations were not used. For that purpose the measurements using 2.35 μM, 3.28 μM and 4.7 μM CENTA were ignored because they showed more fluctuations and they were more open to mistakes and human error.

The Lineweaver-Burk plot was used to detect K_m and V_{max}. R^2 value was calculated as 0.97950 which was sufficient enough to consider the results as reliable. Using Equation 4.1, V_{max} was found as 58823.5 U/L and K_m as 239.7μM.

5.3.3.2. Beta Lactamase Enzyme Activity In the Presence of P3. Beta lactamase enzyme activity measurements were performed using 25μM P2, 2 μg enzyme and chromagenic CENTA substrate. Its hydrolysis at different concentrations were followed by measuring OD at 405nm. By using activity data, Lineweaver-Burk and Hanes-Woolf plots were drawn to obtain kinetic parameters.

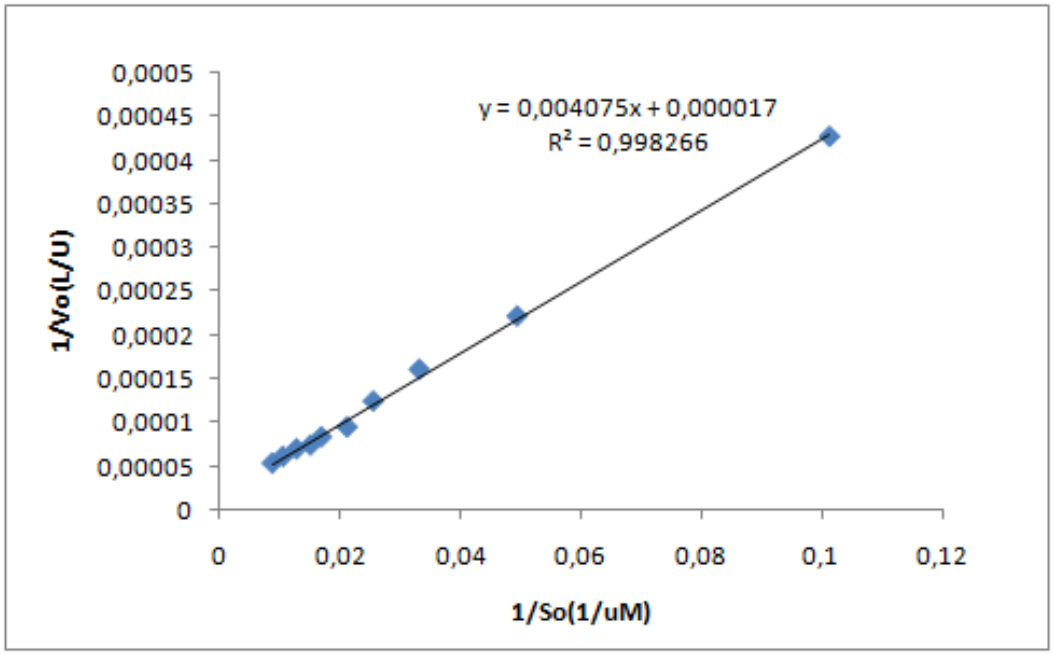

Figure 5.28. Lineweaver-Burk plot for enzyme 2

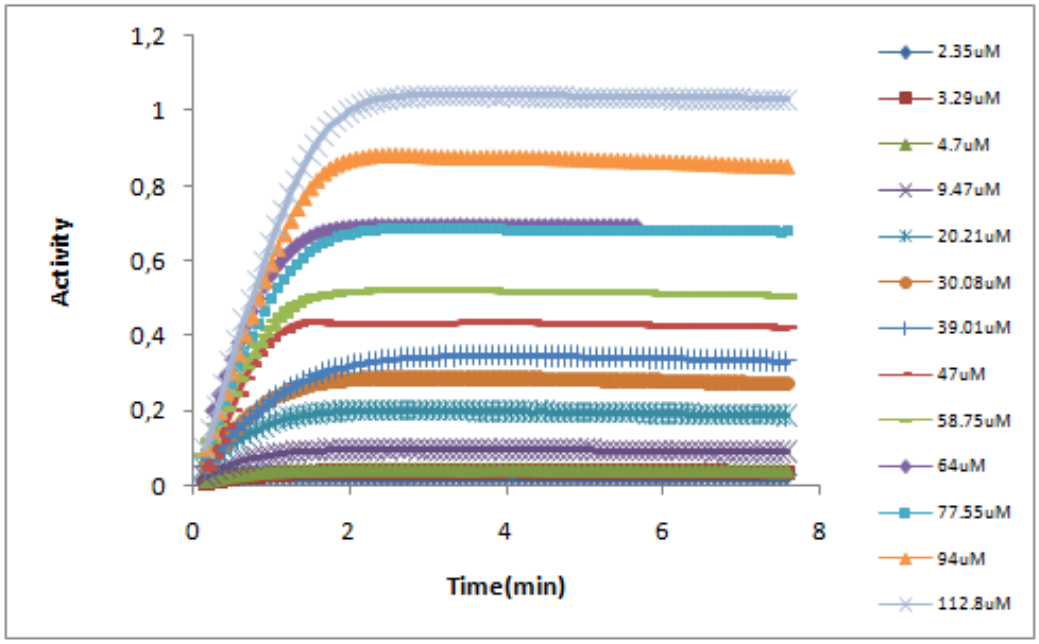

Figure 5.29. Activity graphs of enzyme 2 with different CENTA concentrations in the presence of 25μM P3

The activity curves for several CENTA concentrations were shown in Figure 5.29. The activity curves reached higher values with the increase of CENTA concentrations. This situation matched the expectations, thus the experiment was considered reliable. The maximum activities of enzyme 2 for selected CENTA concentrations with and

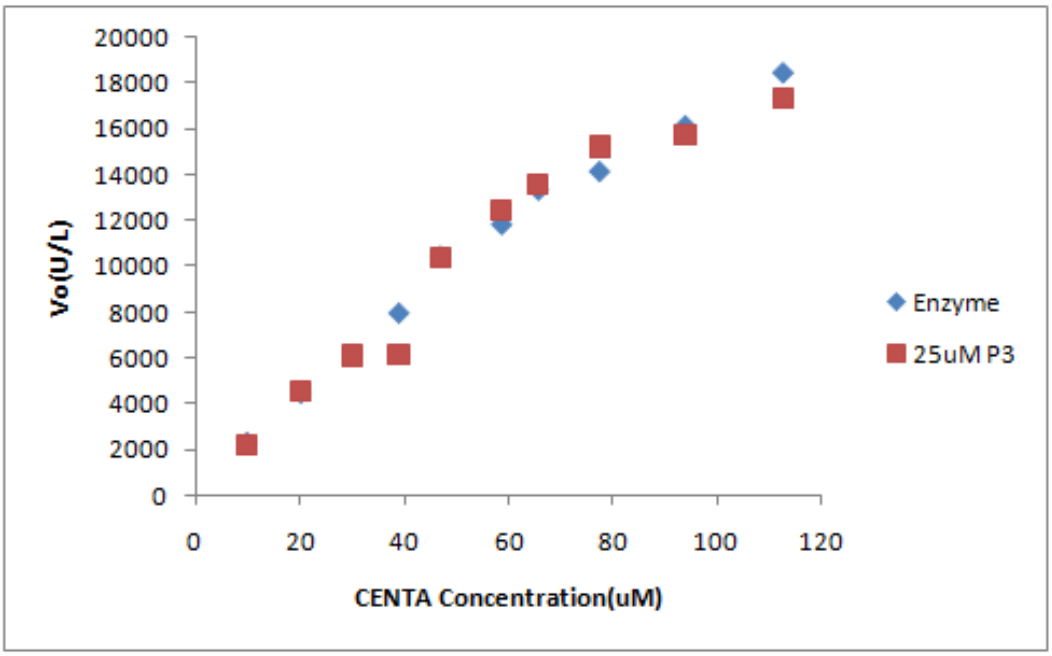

Figure 5.30. Activity Values of Enzyme 2 (Vo) with and without 25μM P3 for selected CENTA concentrations

without 25μM P3 were calculated and shown in Figure 5.30. From the figure, it was observed that for most CENTA concentrations, addition of P3 did not result in significant changes on maximum activities of the enzyme. To avoid errors, measurements using small CENTA concentrations were neglected when plotting Linewiever-Burk and Hanes-Woolf plots.

The inhibitory effect of P3 was analyzed using Lineweaver- Burk and Hanes-Woolf plots with and without P3. V_{max} of the enzyme was calculated as 58823.53U/L and K_m as 239.7 μM. In the presence of 25μM P3, V_{max} was found the same and K_m was calculated as 254.23μM.

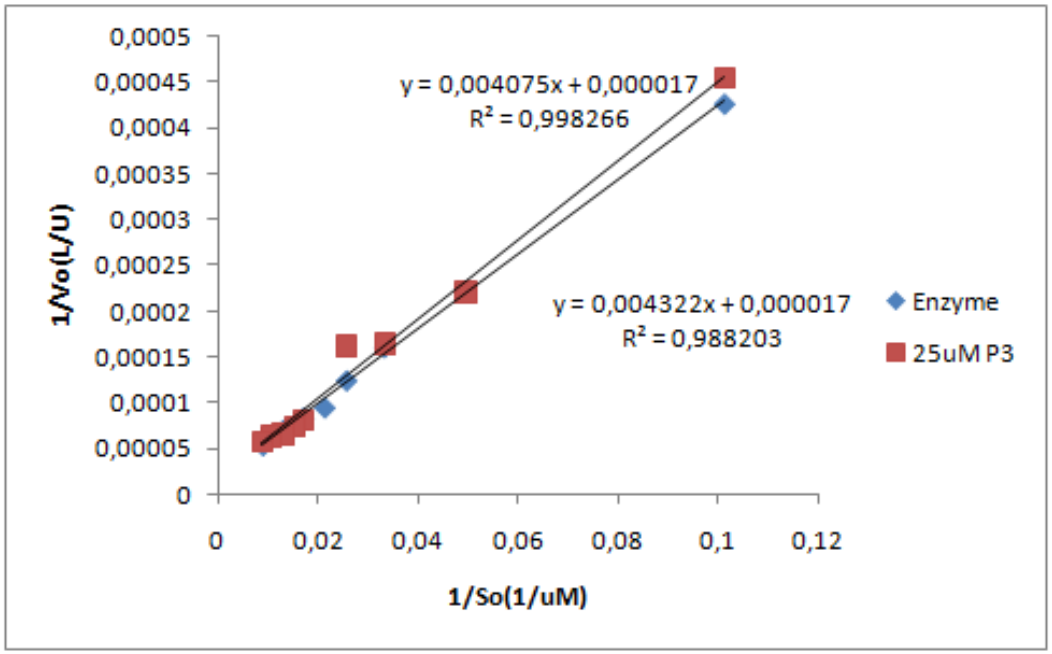

Figure 5.31. Lineweaver-Burk plot for enzyme 2 with and without 25μM P3

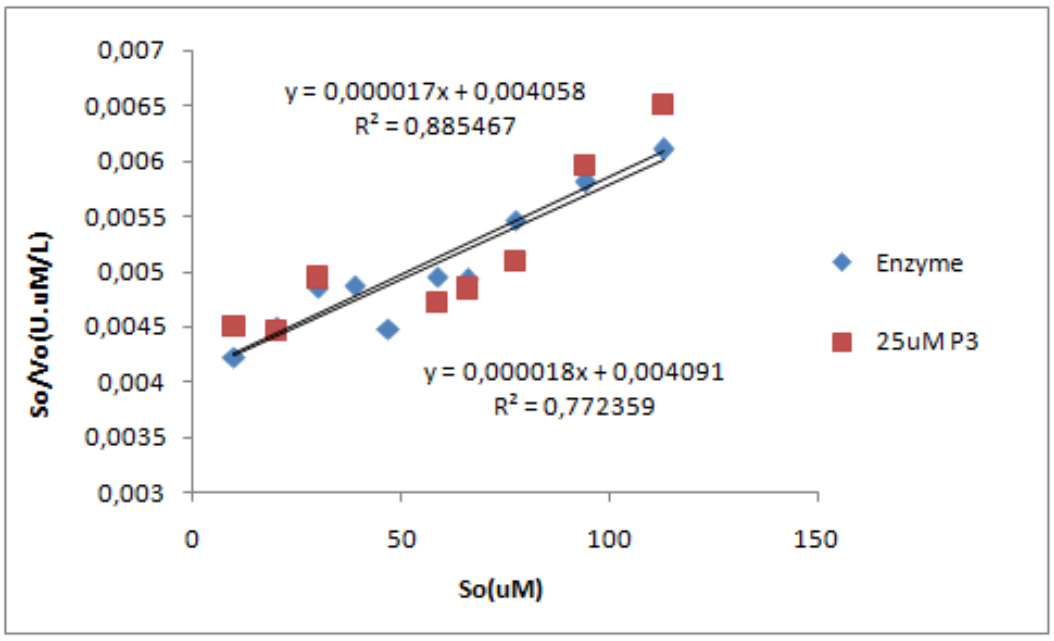

Figure 5.32. Hanes-Woolf plot for enzyme 2 with and without 25μM P3

Figure 5.32 plot in the presence of P3 peptide. Using the plot V_{max} for the enzyme was calculated as 58823.53 U/L and K_m was calculated as 238.70μM. When P3 was added, V_{max} decreased to 55555.55 U/L and K_m to 227.27μM.

To understand the inhibition type, the figures were investigated together. The Lineweaver-Burk plot indicated competitive inhibition as V_{max} stayed the same after the addition of P3 and K_m increased. Hanes-Woolf plot showed decrease in both V_{max} and K_m. As the R^2 of the Lineweaver-Burk plot was higher than the one in Hanes-Woolf, Lineweaver-Burk plot was found more reliable and used for the calculation of K_i.

As the V_{max} value stayed the same and K_m decreased according to the Lineweaver-Burk plot, the inhibition type was detected as competitive inhibition. The K_i was calculated using equation 2.1 and found as 412.44μM. The K_i found from the literature was 136μM. As higher inhibition constant means low potency, the results found in the experiments indicated that the potency of P3 was lower than expected.

Table 5.2. Kinetic parameters of enzyme 1 with and without 25μM P3

	$\mathbf{V_{max}(U/L)}$	$\mathbf{K_m(\mu M)}$	$\mathbf{K_i(\mu M)}$
Enzyme 2 (Lineweaver-Burk)	58823.53	239.7	
Enzyme 2 with 25μM P2 (Lineweaver-Burk)	14285	254.23	412.44
Enzyme 2 (Hanes-Woolf)	58823.53	238.7	
Enzyme 2 with 25μM P2 (Hanes-Woolf)	55555.55	227.27	

5.4. Experimental Results In The Presence of P3A

P3A peptide was designed by adding alanine to the C terminus of P3 peptide. Alanine is an aliphatic, nonpolar, hydrophobic molecule. Its hydrophobic structure

may lead to better interactions with the cell wall of bacteria and improve cell penetration and antimicrobial properties. For that purpose, cell growth and viable cell count experiments were performed using resistant *E.coli* cells and the results were compared with the results of P3.

5.4.1. Effect of P3A on Cell Growth

OD_{600} data were taken for 20 hours using microplate reader to construct growth curves of P3A containing wells. The growth curves were analyzed and compared with the peptide free control sample. From Figure 5.33, it was seen that all the growth curves

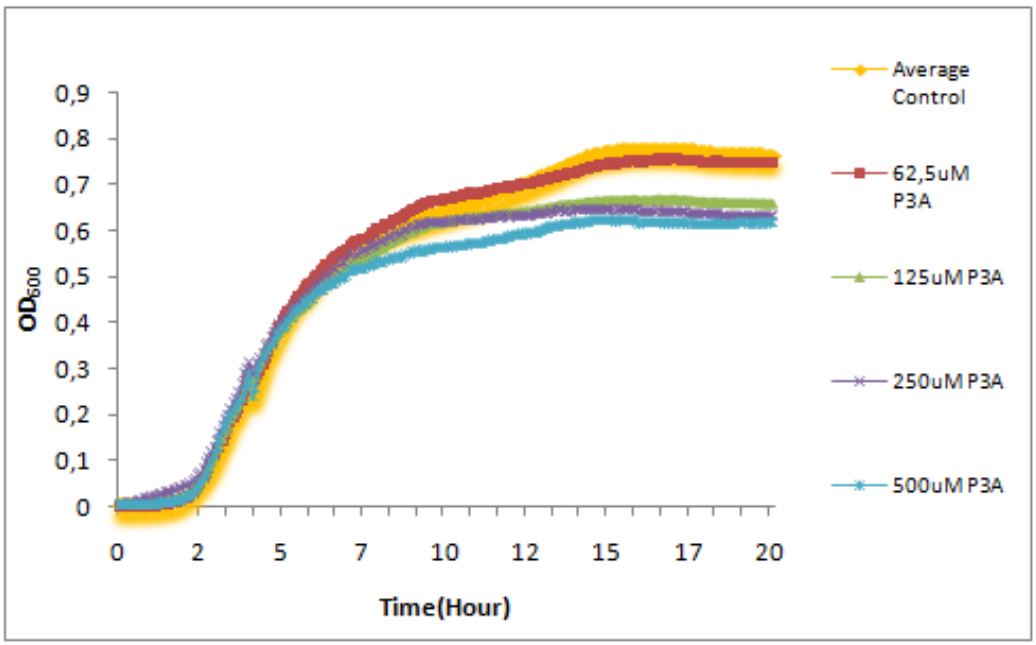

Figure 5.33. The growth profiles of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250 μM and 500μM P3A and the average control sample

were highly similar until 7^{th} hour which led to the conclusion that the growth in the presence of different P3A concentrations were similar. After 7^{th} hour, the growth rates of the wells that contain 62.5μM P3A still showed no decrease. The wells that contain 125μM P3A led to 13.7%, 250μM 17% and 500μM 25% decrease which indicated that P3A was more effective than P3.

5.4.2. Viable Cell Count in the Presence of P3A

The CFU experiments were performed to detect viable cell counts. After 4 hours of incubation in microplate reader which operated at 37°C and 180 rpm, samples from each well were plated using LB-agar medium and cell colonies were counted after 16 hours. Figure 5.34 shows the viable cell counts of resistant *E.coli* cells in the

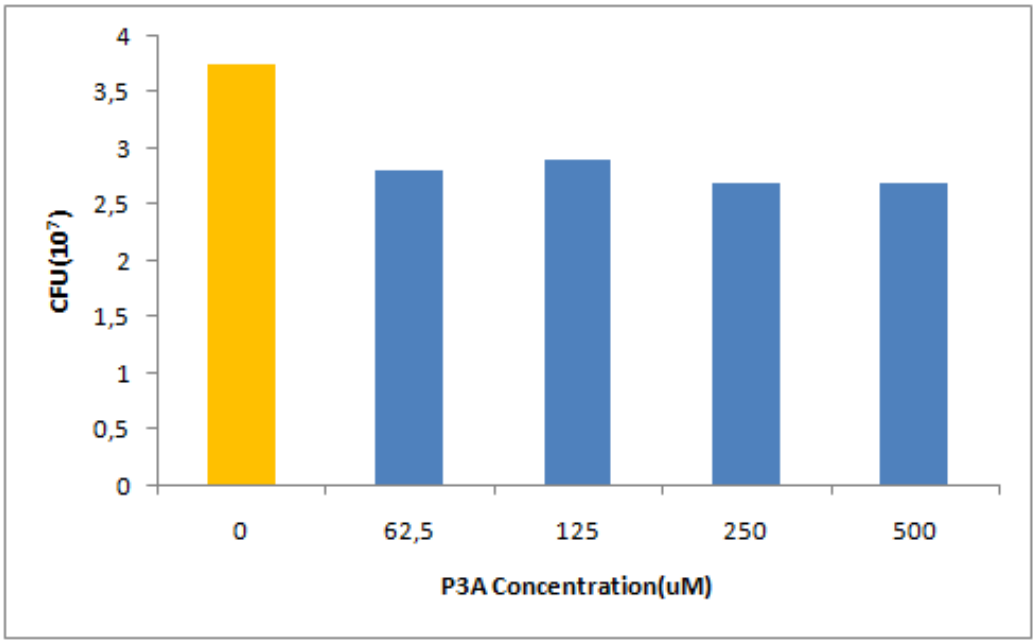

Figure 5.34. Viable cell counts of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250μM, 500μM P3A and the average control sample

presence of selected P3A concentrations. The peptide concentration did not effect CFUs significantly and an average of 22% decrease was detected which was only 1% lower than the CFU counts of P3.

5.5. Experimental Results In The Presence of P4

P4 is designed by adding five hydrophobic residues (LLIIL) to P3 peptide. Cell growth experiments on resistant *E.coli* cells in the presence of selected P4 peptide concentrations were performed.. In addition, kinetic characterization of beta lactamase in

the presence of P4 was performed. For the cell growth and viable cell counts experiments, cells were incubated using the microplate reader for 9 hours at 37°C and 180 rpm.

5.5.1. Experimental Results In The Presence of P4

Cell growth experiment were performed on resistant *E.coli* cells. The growth profiles in the presence of 62.5 μM, 125 μM , 250 μM and 500 μM P4 peptide were analyzed by monitoring OD values at 600nm in microplate reader which operated at 37°C and 180 rpm. The results were shown in Figure 5.35.

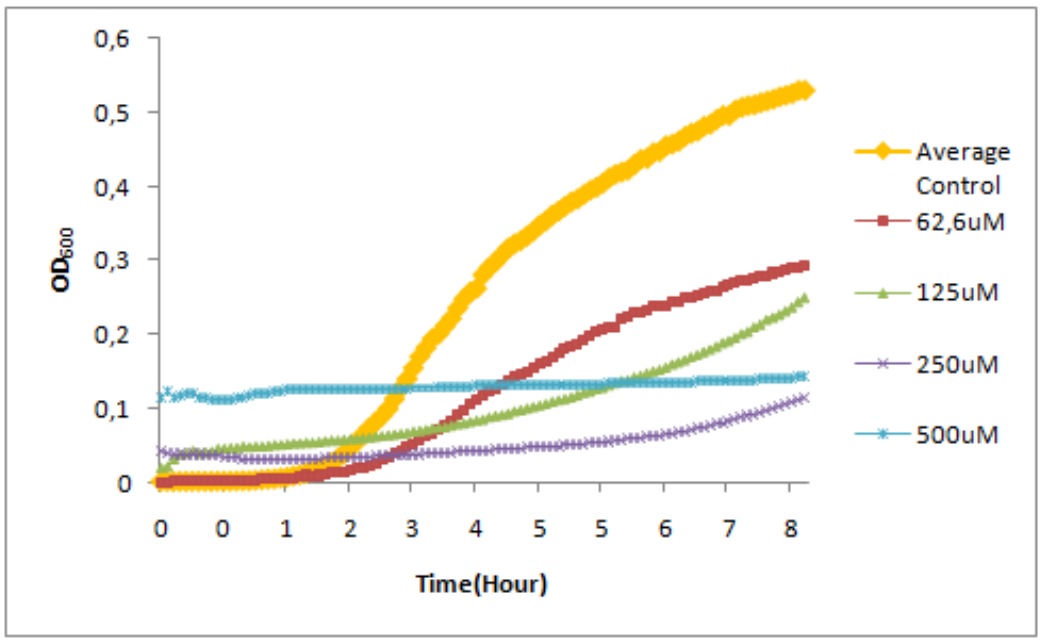

Figure 5.35. The growth profiles of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250μM and 500 μM P4 and the average control sample

The results of the cell growth experiment clearly indicated high inhibition levels even in the presence of lowest P4 concentration of 62.5 μM The inhibition became more distinguishable as the peptide concentration was increased. In the presence of 500 μM P4, no cell growth was observed which showed a high possibility of observing MIC (minimum inhibitory concentration) at a concentration between 250 μM and 500 μM.

5.5.2. Viable Cell Count in the Presence of P4

After 9 hours of microplate incubation , samples were subjected to serial dilutions from 10^7 to 10^12 using LB medium and spread onto petri dishes, then ampicillin containing LB-agar growth medium was poured. The results were demonstrated in Figure 5.36.

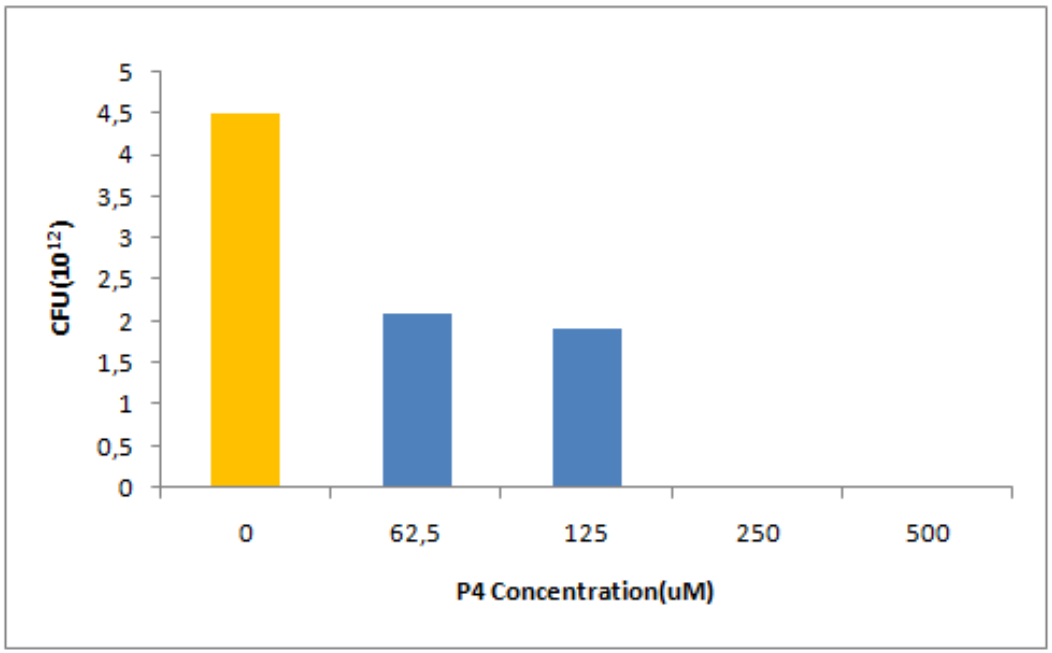

Figure 5.36. Viable cell colony numbers of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250μM, 500μM P4 and the average control sample at the end of 9^{th} hour

The viable cell counts emphasized the potential of P4 peptide as a very effective potent beta lactamase inhibitor. In the presence of 62.5 μM P4, the cell growth decreased by 53% and in the presence of 125 μM this decrease reached to 57%. The most significant fall was encountered after 250 μM where the decrease was 99.99%. Cell growth experiments also indicated significant decrease after that concentration, so the inhibition levels were reasonable. To see the kinetic characterization of beta lactamase in the presence of P4 peptide, enzyme kinetics experiments were conducted and shown in section 5.5.3.

5.5.3. Kinetic Characterization of Beta Lactamase in the Presence of P4

To calculate the kinetic parameters, 25μM P4 peptide and 5μg TEM-1 beta lactamase enzyme (enzyme2) was used at each run. OD_{405}data was taken for 10 minutes for 13 selected CENTA substrate concentrations. The experiments were repeated 3 times for each CENTA concentration to see if the results were matched. The results were found reliable.

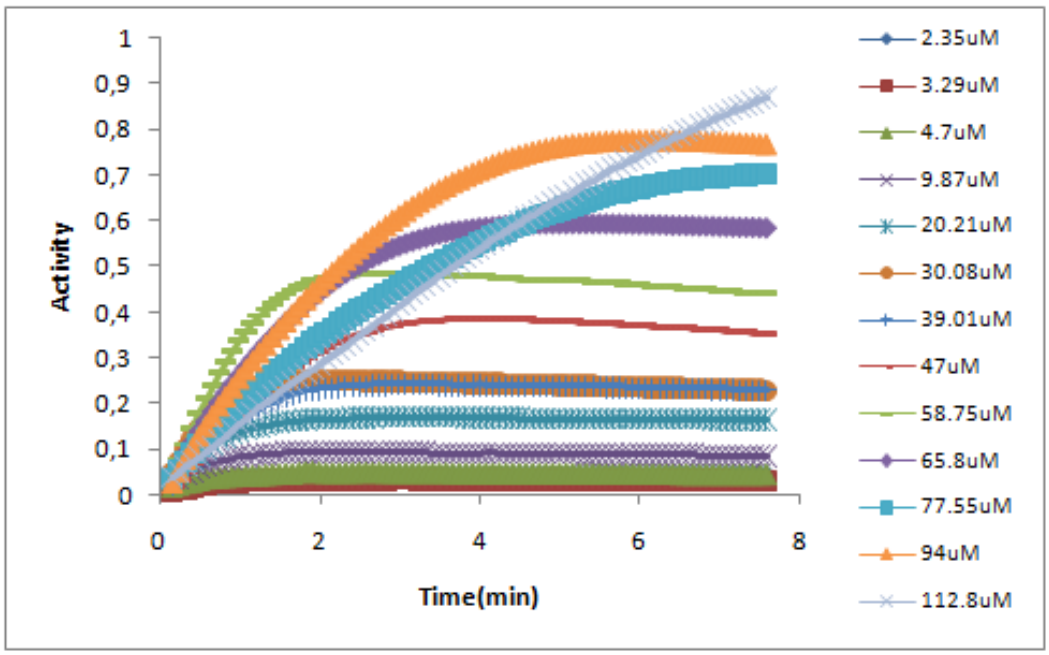

Figure 5.37. Activity graphs of enzyme 2 with selected CENTA concentrations in the presence of 25μM P4

The activity graphs containing CENTA at 13 different concentrations were shown in Figure 5.37. When the graphs were considered together, it was seen that the maximum activities increased with increased CENTA.

Figure 5.38 shows that the maximum activities in the presence of P4 started to decrease drastically after 58.75μM CENTA concentration. In addition to this, the activity gap of the enzyme with and without P4 became bigger with higher concentrations. This situation became more apparent at higher CENTA substrate concentrations, meaning that the beta lactamase was inhibited especially at high concentrations. When com-

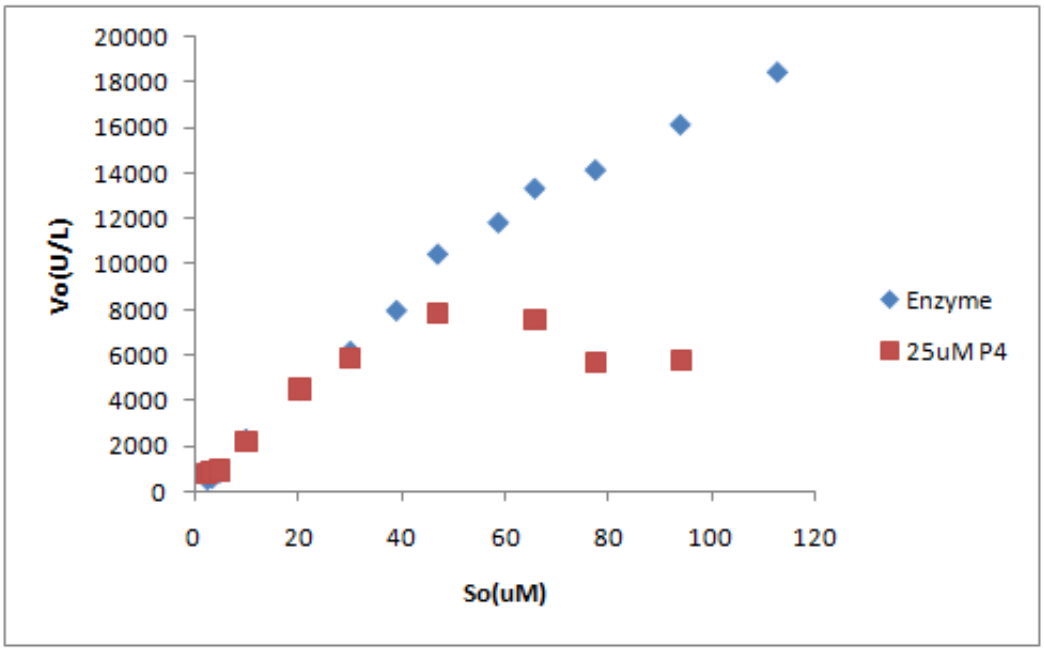

Figure 5.38. Activity Values of Enzyme 2 (Vo) with and without 25μM P4 for selected CENTA concentrations

pared to P3 peptide that lacked of hydrophobic LLIIL residues, this phenomenon was considered as a significant improvement. With P3, low inhibition was detected. To compare the kinetic parameters, Lineweaver-Burk and Hanes-Woolf plots were drawn and analyzed. From Figure 5.39 K_m and V_{max} were detected. The V_{max} and K_m of enzyme without P4 was 58823.53 U/L and 239.70μM. These values were calculated in the presence of 25μM P4 as 10752.69 U/L and 35.20μM.

Hanes-Woolf plot was also used to calculate K_m and V_{max}. K_m was found as 9μM and V_{max} was calculated as 6944.44 U/L. When the values found using Lineweaver-Burk and Hanes-Woolf plots were compared, huge differences were observed.

For the inhibition to be considered as noncompetitive, the ratios of K_m/V_{max} must stay the same for the inhibited and the uninhibited case, so K_m/V_{max} were calculated for both pure beta lactamase enzyme and after the addition of P4. However they were very different from each other. However, as both K_m and V_{max} decreased, it was considered to be closest to uncompetitive inhibition. K_i was calculated using

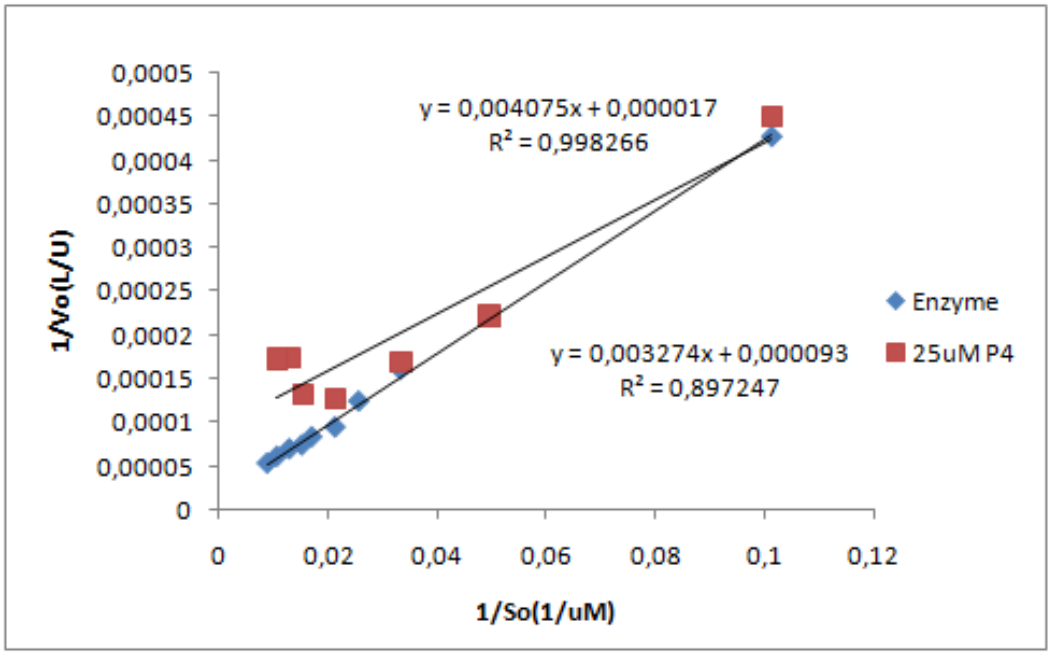

Figure 5.39. Lineweaver-Burk plot for enzyme 2 with and without 25μM P4

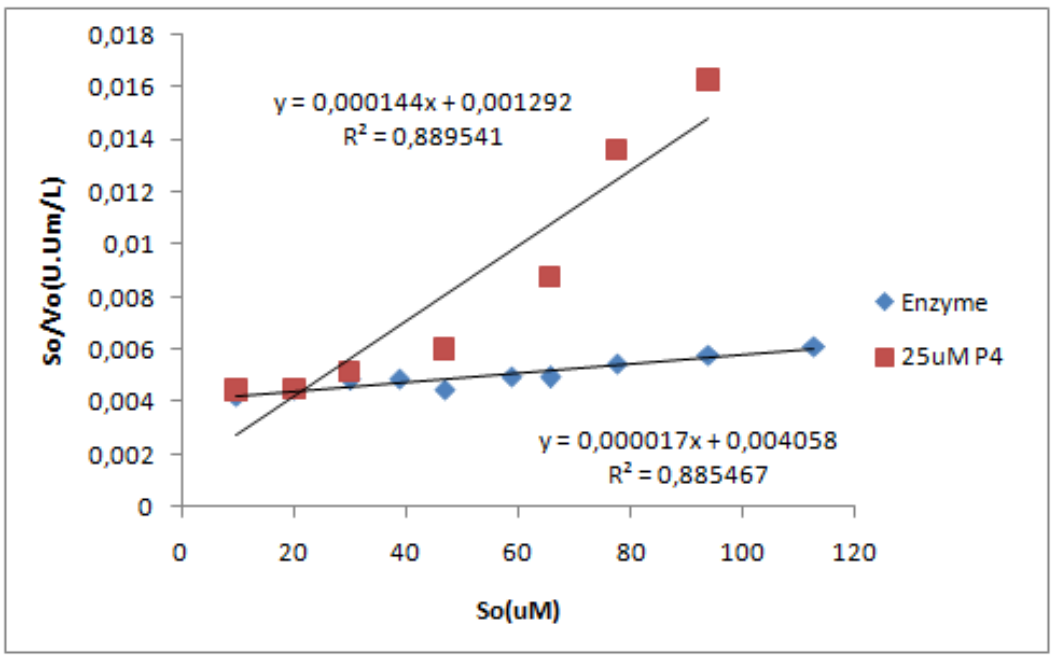

Figure 5.40. Hanes-Woolf plot for enzyme 2 with and without 25μM P4

Lineweaver-Burk accordingly and found as 4.95μM which indicated that it was a significantly more effective beta lactamase inhibitor than P3. The results were shown in Table 5.3.

Table 5.3. Kinetic parameters of enzyme 2 with and without 25μM P4 .

	$\mathbf{V}_{max}$**(U/L)**	$\mathbf{K}_m$**(**μ**M)**	$\mathbf{K}_m/\mathbf{V}_{max}$	**Ki(**μ**M)**
Enzyme 2 (Lineweaver-Burk)	58823.53	239.79	0.004075	
Enzyme 2 with 25μ**M P4 (Lineweaver-Burk)**	10752.69	35.20	0.003274	4.95
Enzyme 2 (Hanes-Woolf)	58823.53	238.70	0.004058	-
Enzyme 2 with 25μ**M P4 (Hanes-Woolf)**	6944.44	9.0	0.001292	

5.6. Experimental Results In The Presence of P5

5.6.1. Effect of P5 on Cell Growth

The effect of P5 on *E.coli* cells were investigated through wild type *E.coli* K12 cells and resistant *E.coli* cells. The P5 aliquot were prepared in 1mM concentrations using sterile distilled water and 3.84% DMSO. It was observed that the solution was not homogeneous and did not dissolve properly which resulted in visible P5 particles and failure to reach target peptide concentration in wells. The growth was monitored for 20 hours by taking OD data at 600nm by microplate reader which operated at 37°C and 180rpm. The growth curves are shown in Figure 5.41 and 5.42.

From Figure 5.41 it was seen that the starting OD_{600} values of the wells which contained more than 100μM P5 were extraordinarily higher. This was a clear result of peptide heterogeneity. To overcome this problem, the experiment was repeated with

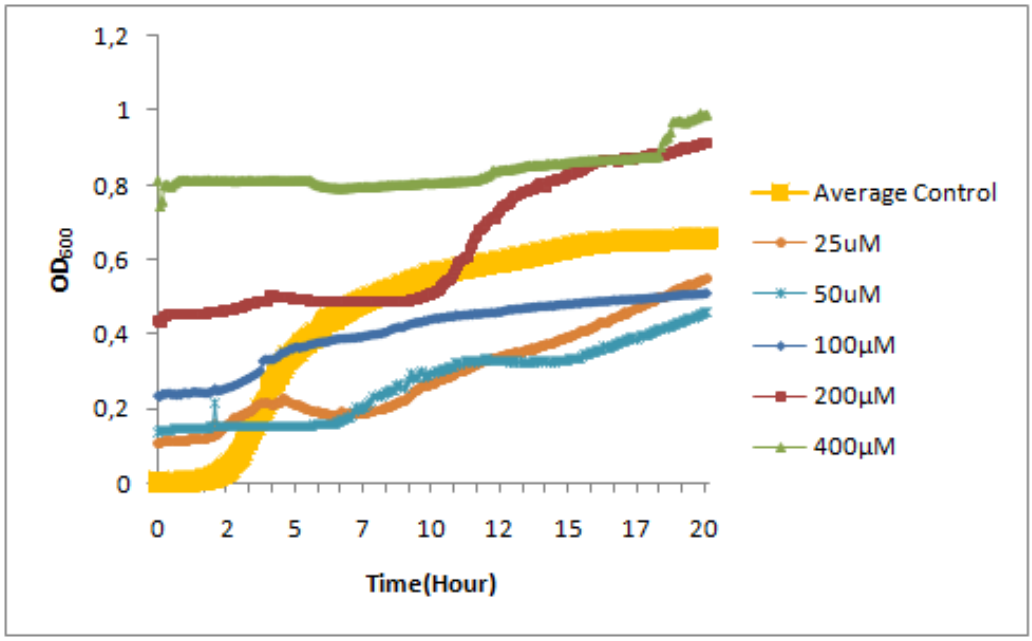

Figure 5.41. The growth profiles of wild type *E.coli* K12 cells in the presence of 25μM, 50μM, 100μM 200μM and 400 μM P5 and the average control sample

lower P5 concentrations which helped overcome visible problems.

The growth was inhibited in all concentrations. The inhibition became more apparent after 100μM. No growth was observed for 200μM well after 10 hours, then the cells started to grow. However, for 400μM P5, no growth was observed, however OD fluctuations were seen due to the solubility problem with the peptide. The growth curves indicated a Minimum Inhibitory Concentration (MIC) value between 200μM and 400μM concentration. To be certain about the cell death visible cell counts in the presence of P5 was also performed for resistant cells.

Figure shows the growth of resistant *E.coli* cells in the presence of different concentrations of P5. As seen from the figure, the OD600 of the wells with higher P5 concentrations were significantly higher. The growth were inhibited after 25μM peptide concentration, however no additional inhibition was observed when the P5 concentration was increased.

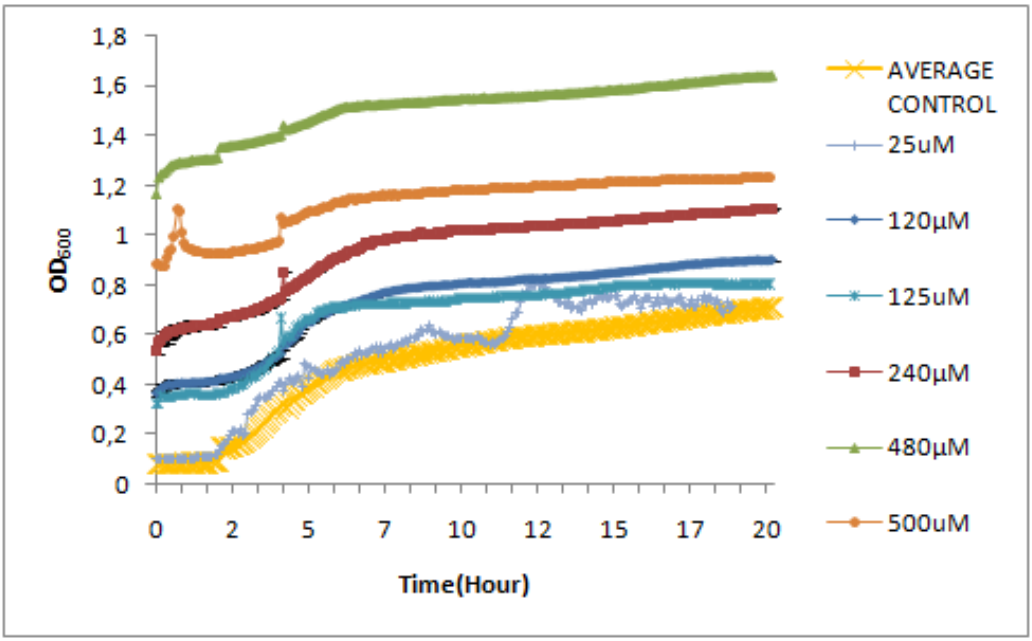

Figure 5.42. The growth profiles of resistant *E.coli* cells in the presence of 62.5μM, 125μM, 250 μM and 500μM P5 and the average control sample

5.6.2. Viable Cell Count In the presence of P5

Wild type and resistant *E.coli* cells in the presence pf P5 were plated after 4 hours of incubation in the miroplate raader to determine the colony forming units (CFU). The petri dishes were kept at 37°C and counted after 16 hours. The results were shown in Figure 5.43 and Figure 5.44.

Figure 5.43 shows the viable cell counts of P5 containing wells and the average of P5 free control samples. The results indicated significant decrease in CFU for all P5 concentrations compared to the P5 free control sample. After 240μM, the colony forming units decreased even more dramatically. 81% decrease was detected in the presence of 25μM to 250μM P5 concentrations. The decrease reached to 91% in the presence of of 400μM P5. The highest decrease compared to peptide free sample was detected for 480μM P5 in which the viable cells were reduced 99.9%. No such dramatic decrease in CFUs was anticipated as wild type *E.coli* K12 cells lacked of beta lactamase formation ability. To check the reliability of the results, the experiments for

each concentration were repeated twice. The results showed high similarity and did not fluctuate much.

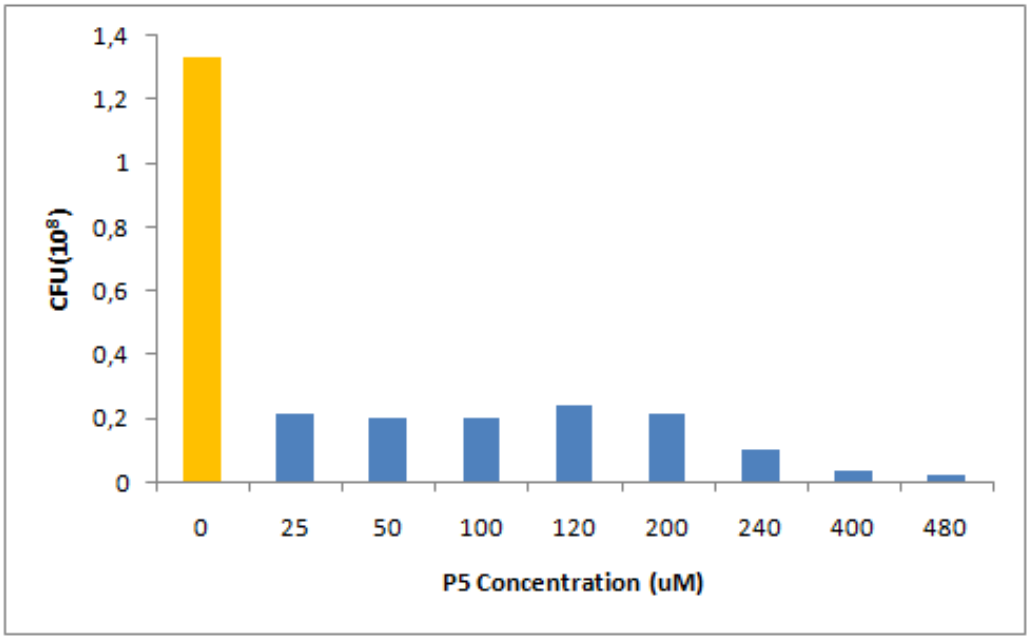

Figure 5.43. Viable cell colony counts of wild type *E.coli* K12 cells in the presence of 25 μM, 50 μM, 100 μM, 120μM, 125μM, 200 μM, 240 μM, 400 μM 480μM P5 and the average control sample

As the CFU results were investigated together with the cell growth experiment, no conflicting outcomes were observed. The cell growth experiment indicated a MIC value between 200μM and 400μM P5 concentration. The CFU results also showed more drastic decreases after more than 240μM P5 was introduced.

The viable cells of resistant *E.coli*cells were counted using the same method. The results were shown in Figure 5.44. Compared to the control sample, no difference was observed for the P5 concentrations up to 100μM . The 125μM P5 containing sample showed 40%, 250 μM showed 43% and 500 μM showed 50% decrease in colony forming units. As these results were compared with the growth experiments, it was seen that CFU results matched the expectancy. After 125 μM they both showed sharp decreases in cell growth rate. Below that concentration, they were both similar to the peptide free control sets.

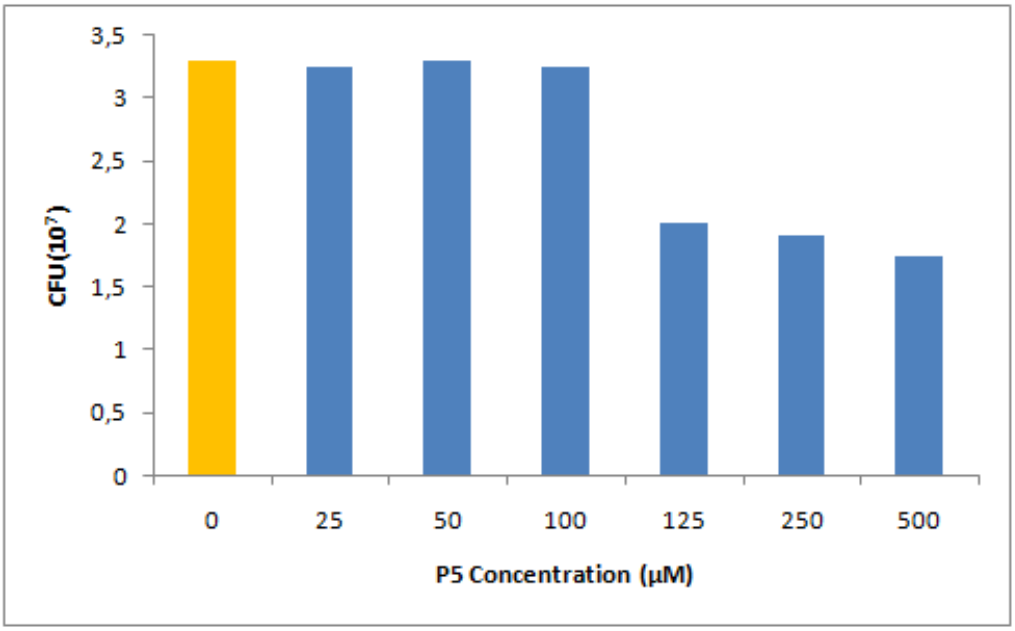

Figure 5.44. Viable cell colony counts of resistant *E.coli* cells in the presence of 25 μM, 50 μM, 100 μM, 125μM, 250μM, and 500μM P5 and the average control sample

The P5 aliquot showed high levels of heterogeneity; therefore kinetic characterization of beta lactamase in the presence of P5 could not be performed due to inconsistent results. It was understood from the in vivo experiments that wild type *E.coli* K12 cells were affected by P5 more than the antibiotic resistant *E.coli* cells. This result indicated that P5 could also lead to cell death in ways other than beta lactamase inhibition. The peptide may also have failed to penetrate into the periplasmic space of the resistant cells. Also the cell wall structure of wild type cells might be more prone to damage of P5 which might act as an antimicrobial peptide by destroying the cell wall of wild type cells.Unfortunately, the concentrations in the wells was not known precisely, as the peptide solution was not homogeneous. As a result, there might be concentration differences in the wells of resistant cells and wild type cells which might have led to significant differences in the results of viable cells. With optimized conditions, the results may change completely.

5.7. Experimental Results In The Presence of Acetic Acid

Acetic acid was thought to be a good option for dissolving peptides as it is commonly used as a hydrophilic polar solvent. It has the ability to dissolve both polar and non-polar compounds. In order to understand its effect on bacteria, cell growth experiment was performed with antibiotic resistant *E.coli* cells. OD_{600} data was taken for 20 hours at 37°C and 180 rpm using a microplate reader. 1%, 2%, 5%, 8% and 10% acetic acid was added to the microplate wells at the beginning of the incubation .The results were compared with the acetic acid free control well.

5.7.1. Effect of Acetic Acid on Cell Growth

The growth curves of antibiotic resistant *E.coli* cells were determined under different acetic acid concentrations with microplate ncubation at 37°C and 180 rpm. OD_{600} data was taken for 20 hours. Figure 5.45 shows the growth curves of resistant *E.coli*

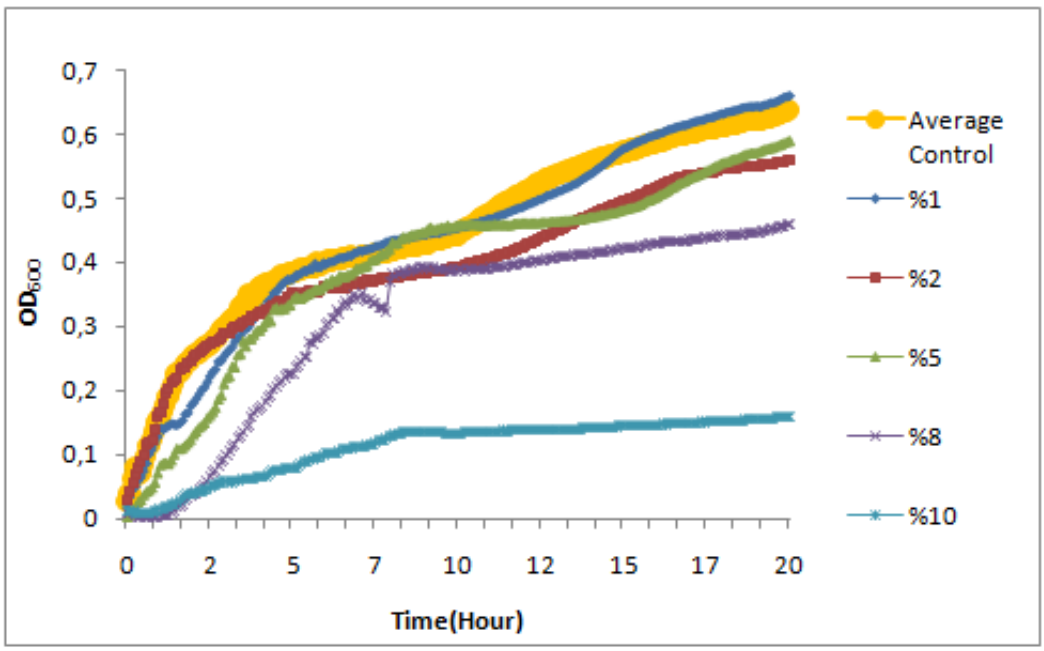

Figure 5.45. The growth profiles of resistant *E.coli* cells in the presence of 1%, 2%, 5%, 8% and 10% acetic acid

cells in the presence of various acetic acid concentrations. It was seen that only at 1% concentration, the growth curve was not negatively affected. At higher, concentrations the growth was inhibited. When the concentration reached to 10% , the cell growth decreased drastically. 82% decrease was observed compared to the acetic acid free sample. As a result it was decided not to use acetic acid as a solvent due to high sensitivity of the cells to acetic acid.

The overall results of the experiments were shown in Table 5.4 and Table 5.5 and Table 5.6. Each sample that contain different peptide concentrations contain same amount of DMSO, so there was no effect of solvents on cell growth. The percentage values represent the decrease in optical density compared to the peptide free control wells in Table 5.5 and decrease in cell counts compared to the peptide free control sample in Table 5.6.

Table 5.4. In Vitro Results

	V_{max}(U/L)	**K_m(μM)**	**Inhibition Type**	**Ki(μM)**
Enzyme 1 (Lineweaver-Burk Plot)	24752.83	52.92		
Enzyme 1 (Hanes-Woolf Plot)	25000	57.25		
Enzyme 1 with 25μM P2 (Lineweaver-Burk)	14285.71	32.14	uncompetitive	36.39
Enzyme 1 with 25μM P2 (Hanes-Woolf Plot)	14285	28.71	uncompetitive	33.33
Enzyme 2 (Lineweaver-Burk Plot)	58825.53	239.705		
Enzyme 2 (Hanes-Woolf Plot)	58825.53	238.70		
Enzyme 2 with 25μM P3 (Lineweaver-Burk Plot)	58825.53	254.23	competitive	412.44
Enzyme 2 with 25μM P3 (Hanes-Woolf Plot)	55555.56	227.27	competitive	412.44
Enzyme 2 with 25μM P4 (Lineweaver-Burk Plot)	10752.69	35.20	-	4.95
Enzyme 1 with 25μM P4 (Hanes-Woolf Plot)	6944.44	8.97	-	-

Table 5.5. Cell Growth Results

	25μM	**62.5μM**	**100μM**	**125μM**	**200μM**	**250μM**	**400μM**	**500μM**
C.acid (wild type)	0%		0%	15%				33%
C.acid (resistant)			16.6%	16.6%	16.6%		30%	73%
P2 (wild type)		14%		25%		30%		24%
P2 (resistant)		40%		50%		41%		42%
P3 (wild type)		10%		11%		12%		12%
P3 (resistant)		15%		24%		17%		16%
P3A (resistant)		0%		15%		15%		16%
P4 (resistant)		44%		60%		89%		no growth
P5 (wild type)	14%		65%			no growth		
P5 (wild type)	0%			13%	30%		35%	

Table 5.6. Viable Cell Counts

	25μM	**62.5μM**	**100μM**	**125μM**	**200μM**	**250μM**	**400μM**	**500μM**
C.acid (wild type, 4h)			18%		30%		40%	
C.acid (resistant, 4h)			33%		63%		75%	96%
P2 (wild type, 4h)		0%		0%		32%		0%
P2 (resistant, 4h)		25%		27%		30%		31%
P2 (resistant, 9h)		42%		44%		47%		53%
P3 (wild type, 4h)		13%		18%		20%		22%
P3 (resistant, 4h)		26%		24%		28%		29%
P3 (resistant, 9h)		2.2%		6.6%		4.4%		5%
P3A (resistant, 4h)		24%		22%		27%		27%
P4 (resistant, 9h)		53%		57%		no growth		no growth
P5 (wild type, 4h)	82%		82%	80%	81%	91%	99.9%	
P5 (resistant, 4h)	0%		0%	40%		43%		50%

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The effect of adding 5 hydrophobic residues (LLIIL) to beta lactamase inhibitory peptides were investigated. LLIIL residues were added to the N terminus of beta lactamase inhibitory protein (BLIP) and two potent TEM-1 beta lactamase inhibitors, RRGHYY and YHFLWGP, and novel inhibitory peptides with improved cell penetration properties were designed. For all peptides, cell growth and viable cell counts on *E.coli* cells and also beta lactamase enzyme inhibition experiments were performed.

The cell growth experiments on P2 showed a decrease between 40% and 50% in viable resistant cells for the presence of 62.5μM to 500μM peptide. This decrease was in the range from 13% to 22% for wild type cells. The viable cell counts decreased by 25% to 31% when the sample was taken after 4 hours of microplate incubation and by 42% to 53% after 9 hours of incubation for the same P2 concentration range. It was seen that P2 was more effective on resistant cells which indicated the cell decrease was due to beta lactamase inhibition. Alaybeyoglu et al detected 30% decrease in viable cells in the presence of 100μM P2 and observed no decrease for HAAGDYYAY which differs from P2 in the lack of hydrophobic LLIIL part [29]. The experimental results show more decrease in viable cells than the findings of Alaybeyoglu et al for both viable cell counts and growth curves.

In vitro beta lactamase inhibition on P2 experiments indicated uncompetitve inhibition with a K_i value between 33.33μM and 36.39μM. The mechanism of HAAGDYYAY was found as competitive inhibition and the K_i was calculated as 58μM in the experiments of B.Alaybeyoglu [29]. The results showed that adding LLIIL part improved the in vitro inhibition up to 43%.

In the presence of 62.5μM to 500μM P3, the growth curves of resistant cells showed decreases between 15% to 24% where for wild type cells,only 10% to 12%

decrease was observed. The viable cell counts of resistant *E.coli* cells resulted in 26% to 29% decrease with the same concentration range after 4 hours of microplate incubation. After 9 hours of incubation 6% decrease in resistant cells were observed. The viable cell count decreases of wild type cells were between 13% and 22% after 4 hours of microplate incubation.

The kinetic experiments on P3 indicated competitive inhibition and K_i was calculated as 412.44μM. Huang et al also observed competitive inhibition, but found K_i as 136μM [33].

In vivo experiments of P3A were performed only on resistant *E.coli* cells. P3A differs from P3 in an additional alanine on the C terminus. Alanine is an aliphatic, nonpolar, hydrophobic amino acid. Its hydrophobic structure may lead to better interactions with the cell wall of bacteria, thus possible improvements in cell penetration. Th growth curves showed a steady 15% decrease in the presence of 62.5μM to 500μM peptide. The viable cell counts however, resulted in decreases between 24% to 27% compared to the peptide free control sample. No in vitro experiments were performed.

Cell growth and viable cell count experiments were performed for resistant *E.coli* cells in the presence of 62.5μM to 500μM P4. The growth curve experiments resulted in 44% decrease for 62.5μM P4, 60% for 125μM, 89% for 250μM P4. No cell growth was observed in the presence of 500μM P4. When the viable cells were counted in the presence of 62.5μM P4, 53% decrease was encountered and 57% decrease was observed in the presence of 125μM P4. No cell growth was seen in the presence of 250μM and 500μM peptide. P4 showed better results than P3 for in vivo experiments. A maximum of 24% decrease in viable cells was observed in the presence of P3. However MIC values were detected in the presence of P4. In order to detect the MIC more precisely, series of experiments should be conducted for more P4 concentrations between 200μM and 500μM.

The results of in vitro TEM-1 inhibition experiments did not indicate a certain type of inhibition. However, it was most close to uncompetitive inhibition. When

it was assumed as uncompetitive and K_i was calculated accordingly, it was found as 4.95μM which is indicated that it was a significantly more effective beta lactamase inhibitor than P3. The V_{max} and K_m were calculated as 58825.53 U/L and 238.70μM for a final enzyme concentration of 12.12 nM. When P4 was added, V_{max} decreased to 10752.69 U/L and K_m decreased to 35.20μM. From the results, it was calculated that the addition of P4 resulted a 82% decrease in V_{max} and 85% decrease in K_m.

Cell growth experiments in the presence of P4 with concentrations between 25μM to 480μM. 14% decrease was detected for 25μM P5 and 65% for 100μM P5. No cell growth was seen after 250μM P5 concentration. No decrease in viable resistant *E.coli* cells was observed in the presence of 25% P5, 13% was seen for 125μM and 35% was for 400μM P5. From the results it was seen clearly that P5 was more effective on wild type cells than the resistant ones. The viable cell counts of wild type *E.coli* cells showed decreases in a range of 82% to 99% for the same P5 concentrations. However a maximum of 50% decrease was detected for resistant cells where no change was observed for P5 concentrations less than 100μM. The in vivo experimental results of P5 indicated the opposite of the expectations. Both viable cell counts and growth experiments concluded that P5 was significantly more effective on wild type cells. However, P5 faced solubility problems during the experiments. The aliquot was completely heterogeneous and formed precipitates when transferred to the wells. This condition caused several problems throughout the project. Firstly, the exact concentrations in the wells were unknown as the peptide solution was not homogeneous. Consequently, there might be concentration differences in the wells of resistant cells and wild type cells which might have caused the significant differences in viable cells. Secondly the heterogeneity might have affected the cell penetration properties. Without proper penetration, the peptide could not reach beta lactamase in the periplasmic space and lost its effectiveness. Also the cell wall structure of wild type cells might be more prone to damage of P5 which might act as an antimicrobial peptide by destroying the cell wall of wild type cells.

Clavulanic acid, as expected almost completely inhibited bata lactamase enzyme. Also the growth experiments and CFUs showed that in the presence of 1mM ampicillin, 480μM clavulanic acid resulted in more than 95% decrease which was significantly

higher than lower concentrations. Below 480μM, it was observed that the cell growth negatively depended on clavulanic acid; and growth was inhibited at every concentration to some extent.

6.2. Recommendations

For the future studies, some recommendations can be made to improve performance and obtain prospective results.

Throughout the project, the biggest problem was to achieve good peptide solubility. Mostly sterile distilled water and DMSO were used to prepare aliquots. P3, P3A and P4 were dissolved perfectly. However, P2 and P5 faced problems which affected the results at high extent. Heterogeneity affected OD values and the state of certainty in peptide concentrations in the wells, but most importantly it might have even affected the level of cell penetration. Consequently, it might have had huge impact on the results. In the future studies, different anionic and cationic solvents can be used depending on the charge of the peptides; also MIC values of the solvents should be determined in order to find the maximum solvent concentration which does not affect the cell growth.

RRGHYY was a potent beta lactamase and used as control. However its effect on *E.coli* K12pUC18 was below the expected values. The reason might be poor cellular uptake or poor inhibition. Another beta lactamase inhibitory peptide would be better to see the effect of LLIIL in the future studies.

While reporting enzyme kinetics results, activity values with different CENTA concentrations ranging from 2.35μM to 112.8 μM were measured. However, while constructing Linewiever-Burk plots to obtain K_m and V_{max}, it was seen that results of lower CENTA concentrations were the most effective ones on the calculations. Lower concentrations require low amounts of CENTA, thus the chance of encountering mistakes is significantly higher. Also it was observed from the enzyme plots that the OD405 difference was quite low for lower concentrations that made the results unreliable. It

is advised for the future studies to use CENTA concentrations higher than 10μM and choose at least 10 concentrations for healthier results. During the project, usually two repeats were performed for each concentration to avoid mistakes. Even more repeats may be performed with the same enzyme set with different peptide concentrations.

When the growth curves were analyzed it was understood that the behaviours of growth curves in the presence of selected concentrations diverged after 8 hours of microplate incubation. In order to obtain more realistic results in CFU counts, samples from the microplate reader should be transferred to petri dishes after at least 8 hours of incubation. Otherwise the results may be misleading

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APPENDIX A: BSA CALIBRATION CURVE

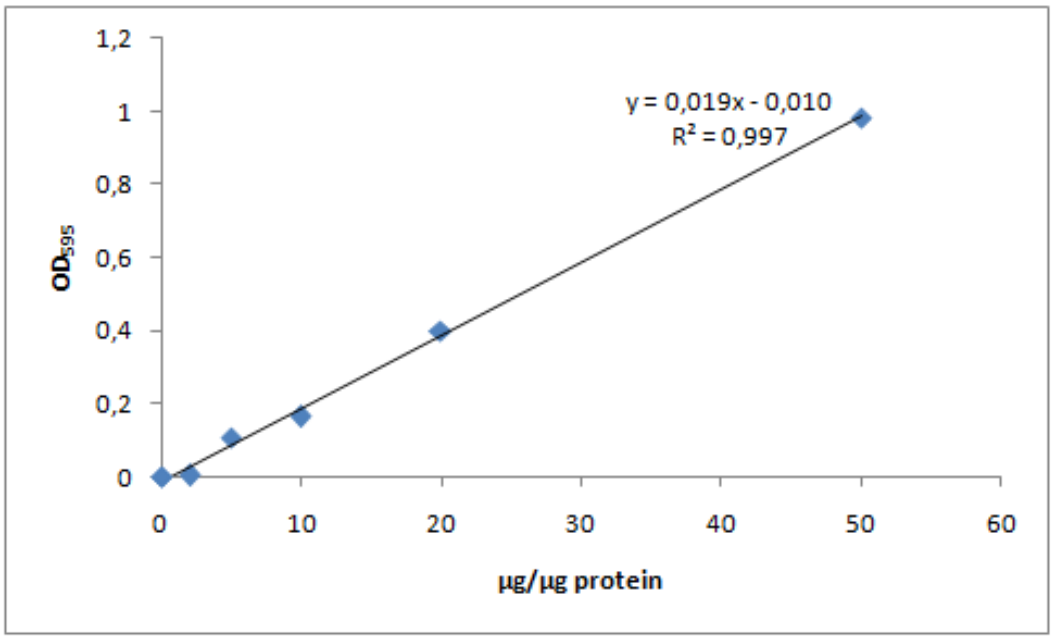

Figure A.1. BSA Calibration Curve