INCREASING CLAVULANIC ACID PRODUCTION BOTH IN WILD TYPE AND INDUSTRIAL *STREPTOMYCES CLAVULIGERUS* STRAINS BY AMPLIFICATION OF POSITIVE REGULATOR *CLAR* GENE

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ABSTRACT

INCREASING CLAVULANIC ACID PRODUCTION BOTH IN WILD TYPE AND INDUSTRIAL *STREPTOMYCES CLAVULIGERUS* STRAINS BY AMPLIFICATION OF POSITIVE REGULATOR *claR* GENE

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Streptomyces clavuligerus is a Gram-positive, filamentous bacterium which produces several important secondary metabolites, including isopenicillin N, cephamycin C and the β-lactamase inhibitor clavulanic acid. Among these compounds, clavulanic acid is being used in combination with commonly used β-lactam antibiotics in order to fight against bacterial infections that are resistant to such antibiotics. Among these combinations, Augmentin, composed of amoxicillin and clavulanic acid, is the most widely prescribed drug and has a market value of more than one billion dollars per year. There are two genes that act in regulation of clavulanic acid biosynthesis: *ccaR* located in cephamycin C gene cluster and *claR* located in clavulanic acid gene cluster. The goal of this study is to improve clavulanic acid production capacities of both wild type and industrial *S. clavuligerus* strains by integrating extra copies of *claR* gene into

S.clavuligerus genome and its overexpression via a multicopy plasmid. Although previously has shown to be quite effective on wild type *S. clavuligerus* strains, *claR* overexpression in the industrial strain used in this study yielded only 1.4-fold increase in volumetric and 1.7-fold increase in specific CA production by the recombinant strains MA28 and MA16, respectively.

Keywords: *Streptomyces clavuligerus*, *claR*, clavulanic acid, HPLC, industrial strain.

ÖZ

POZITIF REGULATOR *CLAR* GENININ AMPLIFIKASYONUYLA YABANIL VE ENDÜSTRIYEL *STREPTOMYCES CLAVULIGERUS* SUŞLARINDA KLAVULANIK ASIT ÜRETIMININ ARTIRILMASI

Mutlu, Alper

Yüksek Lisans, Biyolojik Bilimler Bölümü

Tez Yöneticisi: Prof. Dr. Gülay Özcengiz

Eylül 2012, 100 sayfa

Gram-pozitif, ipliksi bir bakteri olan *Streptomyces clavuligerus*, biyoteknolojik açıdan önemli izopenisilin N, sefamisin C ve klavulanik asit gibi birçok sekonder metaboliti sentezleme yeteneğine sahiptir. Bu bileşikler arasında bir β-laktamaz inhibitörü olan klavulanik asit, yaygın olarak kullanılan β-laktam antibiyotiklerine dirençli bakteriyel enfeksiyonların tedavisinde bu antibiyotikler ile bir arada kullanılmaktadır. Bu kombinasyonların arasında amoksisilin ve klavulanik asit karışımı olan Augmentin, yıllık bir milyar doları aşan pazar değeri ile kullanımı en yaygın olan ilaçlardan biridir. Klavulanik asit biyosentezinin regülasyonunda görevli iki gen bulunmaktadır: sefamisin C gen kümesinde yer alan *ccaR* ve klavulanik asit gen kümesinde yer alan *claR*. Bu çalışmanın temel amacı, entegrasyon vektörü kullanılarak *claR* geninin ekstra kopyalarının *S. clavuligerus* genomuna aktarılması ve aynı zamanda bir başka çok kopyalı plasmid vektörü vasıtasıyla hücre içerisinde bu regulatör genin normalin üzerinde ifade

edilmesiyle yabanıl ve endüstriyel *S. clavuligerus* suşlarının klavulanik asit sentez kapasitelerinin artırılmasıdır. Daha önceki çalışmalarda, standart *S. clavuligerus* suşunda *claR* geninin çoklu ifadesinin oldukça etkin olduğu gösterilmesine ragmen bu çalışmada kullanılan endüstriyel suşda genin genoma entegrasyonu ya da çoklu ifadesi durmunda en çok 1.4-katlık volumetrik (MA28 suşu ile) ve 1.7-katlık spesifik (MA16 suşu ile) klavulanik asit üretimi sağlanabilmiştir.

Anahtar kelimeler: *Streptomyces clavuligerus*, *claR*, klavulanik asit, HPLC, endüstriyel suş.

To my father

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LIST OF ABBREVATIONS

ATCC	American Type Culture Collection
NRRL	Agricultural Research Service Collection
bp	Base pair
dH_2O	Distilled water
dNTP	Deoxynucleotide Triphosphate
RE	Restriction endonuclease
ORF	Open Reading Frame
rpm	Revoultion per minute
kb	Kilo base
o/n	Overnight
RT	Room temperature

CHAPTER 1

INTRODUCTION

1.1. The genus *Streptomyces*

Members of the genus *Streptomyces* are gram-positive soil inhabitants and they possess characteristics of the order *Actinomycetales*. Large (>8 Mb) linear chromosomes with high G + C content (69 – 78 mol %) accompanied with large linear plasmids are commonly seen in *Streptomyces* spp. (Paradkar *et al*., 2003). Strong restriction-modification systems are also widespread among *Streptomyces* spp. (MacNeil, 1988), which hinders the transfer of DNA of non-*Streptomyces* origin to target *Streptomyces* host (Matsushima *et al*., 1987). S*treptomycetes* are especially noteworthy because of their capability of producing a wide range of secondary metabolites, including antimicrobials, antifungals, herbicides, anticancer compounds and immunosuppressants. They are also famous for producing enzymes able to decompose organic polymers and thus have a significant role in soil ecology and enzyme industry (Weber *et al*., 2003). Since the discovery of streptomycin in 1944, the search for novel antibiotics from genus *Streptomyces* and related genera led to the discovery of novel antibiotics which now constitute 80% of all practically used microbial metabolites (Olano *et al*., 2008).

In order to survive in harsh soil environment, *Streptomyces* spp. have developed complex morphological and physiological adaptations. The life cycle of the *Streptomyces* spp. involves a series of morphological changes (Figure 1.1).

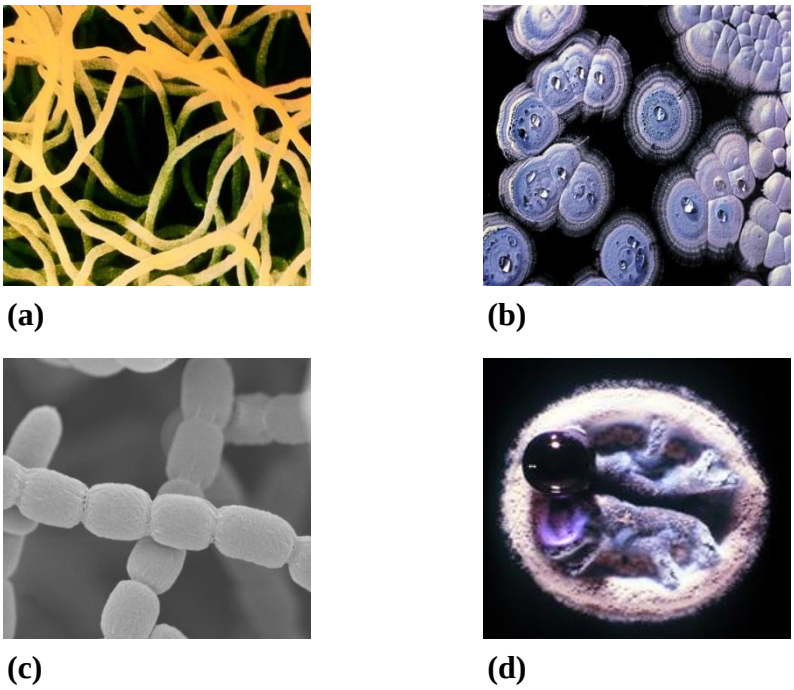


Figure 1.1. Morphological differentiation of *Streptomyces* spp.

(a) *Sreptomyces griseoviridis* mycelial hyphae
(http://www.sciencephoto.com/media/13155/enlarge)

(b) *Streptomyces coelicolor* colonies
(http://mml.sjtu.edu.cn/laotuo/Compressed%20for%20Carton%20CHEN/1/)

(c) Spore chain of *Streptomyces*
(http://www.science.leidenuniv.nl/index.php/ibl/claessen)

(d) Antibiotic droplet secretion from *Streptomyces* colony
(http://microbewiki.kenyon.edu/index.php/Streptomyces)

Colonization of *Streptomyces* spp. in soil starts with formation of multinucleate hyphae to form a mycelial network which facilitates attachment, penetration and feeding from dead plant tissues. After establishing a basal substrate mycelium, hyphae start to grow into the air. The progressing aerial hyphae coil and divide into smaller compartments, which in turn develop into unigenomic spore chains and spread via animals or wind to germinate and form new mycelial colonies (Claessen *et al*., 2006) (Figure 1.2)

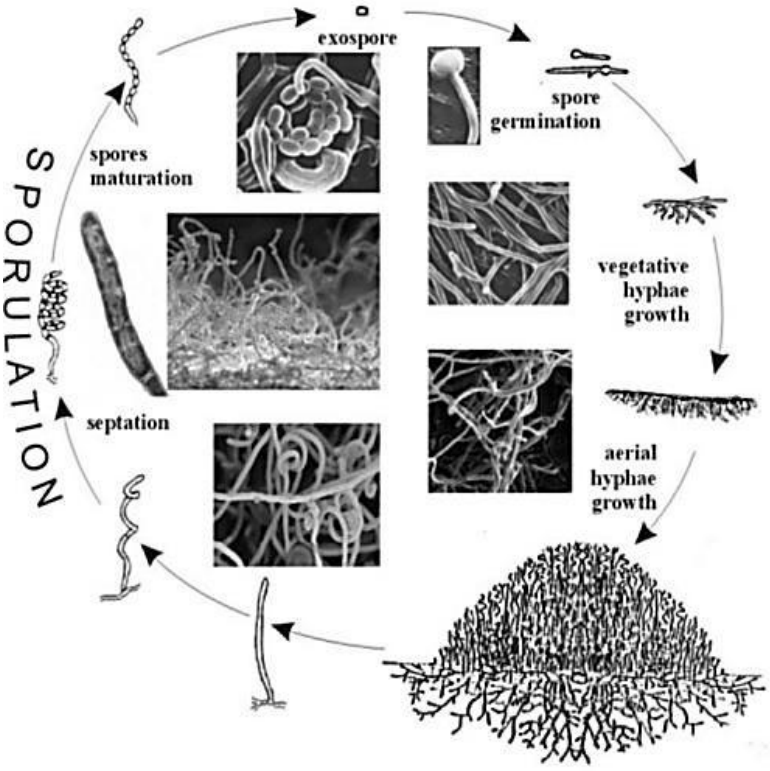


Figure 1.2. The life cycle of *Streptomyces* spp.

(http://www2.iitd.pan.wroc.pl/dept/mic/gb/projects.html)

Depletion of readily available nutrients by germinating spore and vegetative hyphae, presence of a small inducer molecule or decrease in the growth rate of the colonization process are the events that trigger the chemical and morphological differentiation of the colony (Bibb, 2005). The presence of one or more of these three factors triggers a cascade of reaction that activates a "master gene" which controls both morphological differentiation and antibiotic synthesis events. Therefore, it is reasonable to associate the morphological differentiation with the antibiotic production. (Chater, 2006; Demain, 1998).

To sum up, the capability of synthesizing both industrially and clinically important secondary metabolites, the characteristic genetic and growth properties make the *Streptomyces* spp. very remarkable subjects to study.

1.2. *Streptomyces clavuligerus* and its secondary metabolites

S. clavuligerus is well known for its industrially and clinically important β-lactam metabolites. *S. clavuligerus* produces mainly two types of β-lactam metabolites (Figure 1.3). Of these, sulfur containing metabolites usually show antibiotic activity and include isopenicillin N, deacetoxycephalosporin C and cephamycin C while oxygen containing group of β-lactams, collectively called clavams, have various other biological activities with a weak antibiotic activity. One of the most important clavams produced by *S. clavuligerus* is clavulanic acid (CA), which is a potent inhibitor of serine β-lactamases and used in combination with broad-spectrum antibiotics to fight against β-lactam resistant bacterial infections. Commercial products like Augmentin™ and Timentin™, which are combination of CA with amoxicillin and ticarcillin, respectively, are prescribed in more than 150 countries and the annual market value of these products excesses one billion dollars (Paradkar and Jensen, 1995; Saudagar *et al*., 2008). Hence, many efforts

have been dedicated to produce strains with enhanced CA production. Although structurally related, the other clavam products of *S. clavuligerus* do not have β-lactamase activity. β-lactamase activity is being credited to 3R, 5R stereochemistry of the CA which differs from other clavams that show 5S stereochemistry (Tahlan *et al*., 2004). 5S clavams include 2-hydroxymethylclavam, 2-formylmethylclavam, and clavam-2-carboxylate that possess antifungal activity and alanylclavam which has both antifungal and antibacterial activities (Paradkar, Jensen, 1995). Lastly, *S. clavuligerus* produces a β-lactamase which is sensitive to CA, a β-lactamase inhibitory protein (BLIP), and a BLIP-homologous protein (BLP) (Santamarta *et al*., 2002).

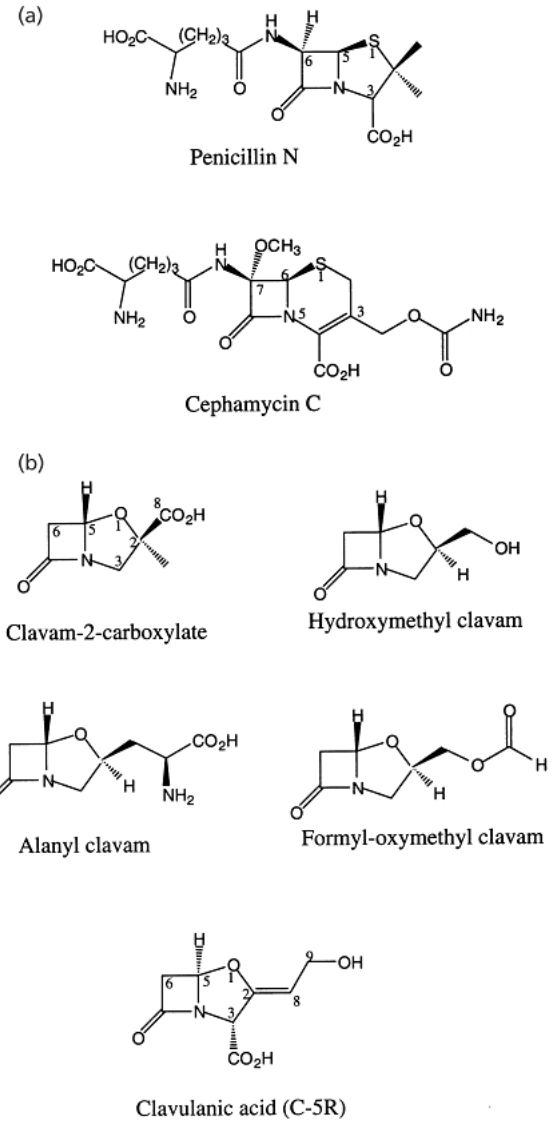


Figure 1.3. β-lactam products of *S. clavuligerus*. (a) Antibiotics. (b) Clavam metabolites (Thai *et al*., 2001)

1.3. β-lactam antibiotics

Alexander Fleming's observation of a contaminated *Staphylococcus aureus* plate by a blue-green mold (*Penicillum notatum*) with an inhibition zone around it,

eventually led to the discovery of penicillin in 1932. Discovery of penicillin is possibly the most important one in the history of the therapeutic medicine due to being the forerunner of antibiotics useage to cure infectious diseases. Penicillin, at that time, was unique since it was quite specific in terms of targeting bacterial infections and not being toxic to patients. In 1945, while new methods to produce larger amounts were being researched, Hodkin and Low revealed the X-ray chrysallographic structure of penicillin containing the β-lactam ring (Brakhage, 1998). Following the success of penicillin, many researchers started to look for new β-lactam antibiotics which led to the discovery of cephalosporin production by *Cephalosporium acremonium*, cephamycin, clavam and carbapenem production by actinomycetes, and monocyclic β-lactam production by actinomycetes (Demain and Elander, 1999). Today, the total world market of β-lactam antibiotics is estimated to be 15 billion dollars, which corresponds to ~65% of the total antibiotic market (Elander, 2003) with the production volumes above 60,000 tons a year (Thykaer and Nielsen, 2003).

As mentioned above, the high specificity of the β-lactam antibiotics originates from their targeted action on the unique bacterial cell wall structure. Most bacterial cell walls contain a thick layer of peptidoglycan whose molecules are crosslinked by amino acids to form a protective network over the cell membrane. The synthesis of the peptidoglycan starts with the formation of precursor molecule *N*-acetylglucosamine-β-1,4-*N*-acetylmuramyl-pentapeptide-pyrophosphoryl-undecaprenol in cytoplasm. This precursor molecule is then transported across the cell membrane where it is incorporated into newly forming glycan strand by transglycosylases. The adjacent glycan strands then undergo extensive crosslinking by the action of transpeptidase enzymes (also termed as penicillin-binding proteins or PBPs). The ring structure of β-lactam antibiotics is capable of binding to the transpeptidase and transglycosylase active sites. Acylation of the enzyme active sites interfere with proper crosslinking reaction, which eventually cause rupturing of the cell wall due to high internal osmotic pressure (Walsh, 2000) (Figure 1.4).

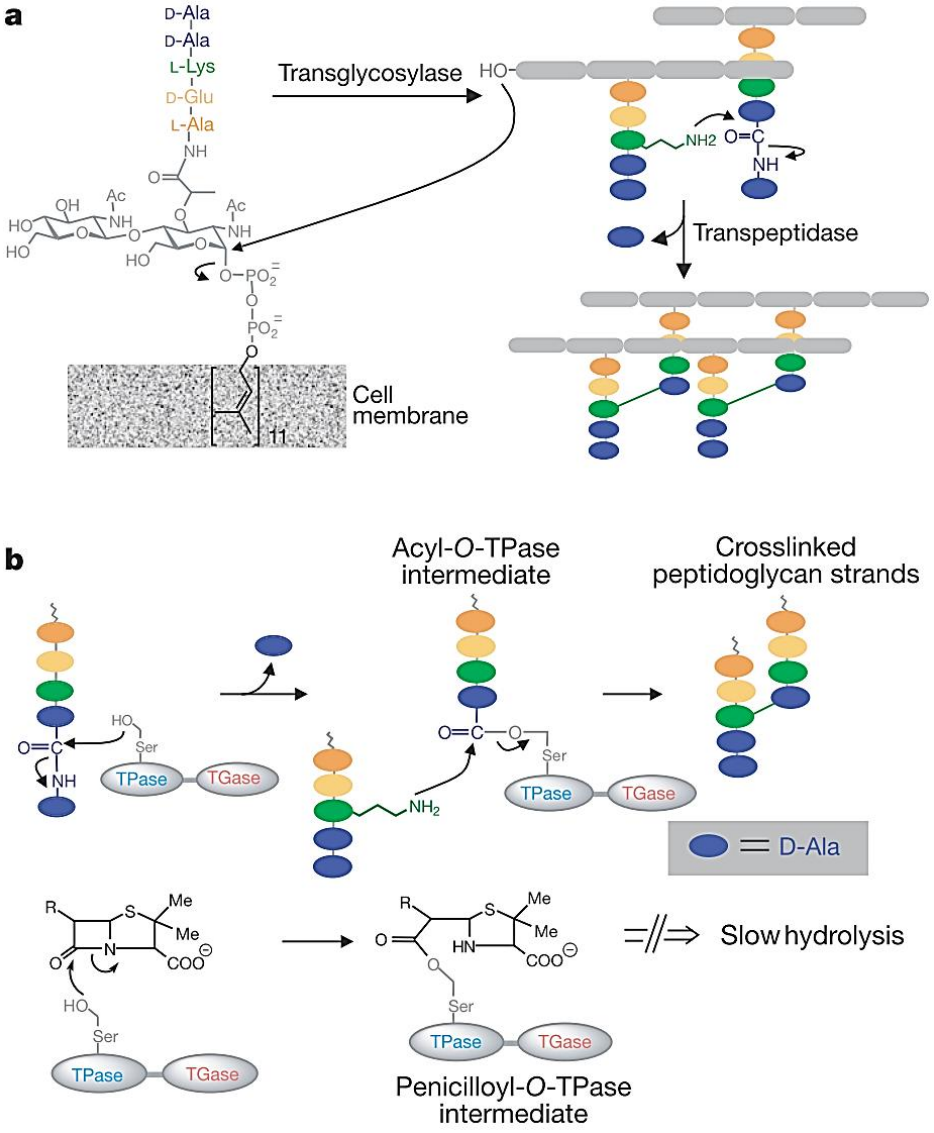


Figure 1.4. Bacterial peptidoglycan biosynthesis and interruption of crosslinking via β-lactams. (a) Transpeptidation and transglycosylation steps of peptidoglycan synthesis (b) Formation of slowly hydrolyzing covalent acyl enzyme intermediate and inhibition of transpeptidation reaction (Walsh, 2000).

1.4. Resistance to β-lactam antibiotics

Since their introduction to medicine in 1940s, β-lactam antibiotics are still being used in treatment of wide range of bacterial infections. However, just after their release, drug resistant bacterial strains started to appear in civil and military hospitals, where most antibiotics were being extensively used (Levy and Marshall, 2004). Antibiotic resistance of microorganisms is a natural phenomenon and it predates the discovery and clinical use of antibiotics yet the selective pressure applied by the overuse of antibiotics for clinical, agricultural and stockbreeding purposes have boosted the emergence rate of resistant strains (Levy and Marshall, 2004; D'Costa *et al*., 2011).

Both gram positive and gram negative bacteria can develop resistance against β-lactam antibiotics by means of manufacturing protein pumps to prevent accumulation of drugs inside the cell, inactivating the antibiotics via β-lactamases or altering the structure of PBPs to prevent binding of antibiotics within the cell. Among these, β-lactamases are the most common and most efficient mechanisms of resistance, and, are now observed in a wide variety of clinically important bacteria (J. Williams, 1999).

β-lactamases can be classified according to their structural or functional characteristics. Based on their conserved amino acid motifs, β-lactamases can be classified into four groups, namely A, B, C and D β-lactamases. A, C and D class enzymes are also called serine β-lactamases due to the presence of an active site serine residue whereas class B metalloenzymes require zinc ions as cofactors. Both serine residues and zinc ions attack the β-lactam ring structure and cause inactivation of the antibiotics (Livermore, 1995) (Figure 1.5). Another method of classification is the functional grouping, where enzymes were aligned based on their ability to hydrolyze specific β-lactam classes and on the inactivation properties of the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam. The functional grouping can be more subjective than the structural

classes, but they help the clinician and laboratory microbiologist to correlate the properties of a specific enzyme with the observed microbiological resistance profile of a clinical isolate. Thus, cephalosporinases, serine β-lactamases and metallo-β-lactamases (MBLs) are the three main classes of functional grouping along with many subgroups (Bush and Jacoby, 2010).

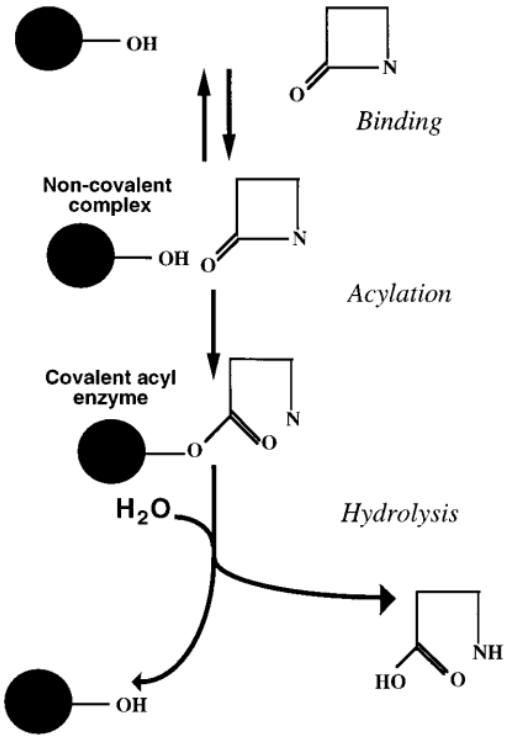


Figure 1.5. General action mechanism of serine β-lactamases (Livermore, 1995).

1.5. The β-lactamase inhibitor clavulanic acid (CA)

In 1971, as part of a screening programme for β-lactamase resistant β-lactam compound producers, *S. clavuligerus* had been isolated for being a cephamycin C producer. However, the further screening for β-lactamase inhibitor production revealed the presence of CA among the secondary metabolites synthesized by the candidate bacteria (Saudagar *et al*., 2008). Although, being effective against a wide range of bacteria, CA by itself has a very low antibacterial activity. As stated earlier, CA is formulated in combination with broad range β-lactam antibiotics to show its effect in full extent (Lee *et al*., 2002). For example, *in vitro* studies indicate that minimum inhibitory concentration (MIC) of ampicillin to exceed 500 μg/mL when measured against a lactamase producing strain of *S. aureus*. The supplementation of ampicillin with 5 μg/mL of CA, resulted in a reduced level of MIC to 0.1 μg/mL (Saudagar *et al*., 2008). In addition, CA has been reported to be 14-20 times more active than an alternative inhibitor, sulbactam, in terms of β-lactamase inhibition (Payne *et al*., 1994).

1.6. β-lactamase inhibition mechanism by clavulanic acid

The X-ray studies revealed that clavulanic acid has a unique structure resembling penicillins but is also significantly different in that the bicyclic β-lactam ring is much more strained compared to penicillins and cephalosporins as a result of (i) the substitution of an oxygen atom for sulfur, (ii) the lack of an amino substituent at C-6, and (iii) the presence of an *exo*-β-hydroxyethylidene function at C-2 (Baggaley and Brown, 1997). CA acts as a very slow substrate for β-lactamases and occupy active site of the enzymes long enough to ensure that the co-administrated anitibiotics can act on their targets. Inactivation of a serine β-lactamase starts with ligand binding, which is followed by the nucleophilic attack on the β-lactam ring of CA by the hydroxyl group of active site Ser-70, resulting in a covalent acyl intermediate and opening of β-lactam ring. After this step, the five-membered oxazolidinic ring opens and at the later stages the inhibitor linearizes as an imine intermediate. The imine intermediate undergoes isomerization and becomes a *cis*-enamine, which can turn into a more stable *trans*-enamine form. In a few hours, the inhibitor regains its active form and the enzyme becomes permanently inactivated by a covalent modification at Ser-130 residue (Padayatti *et al*., 2005) (Figure 1.6).

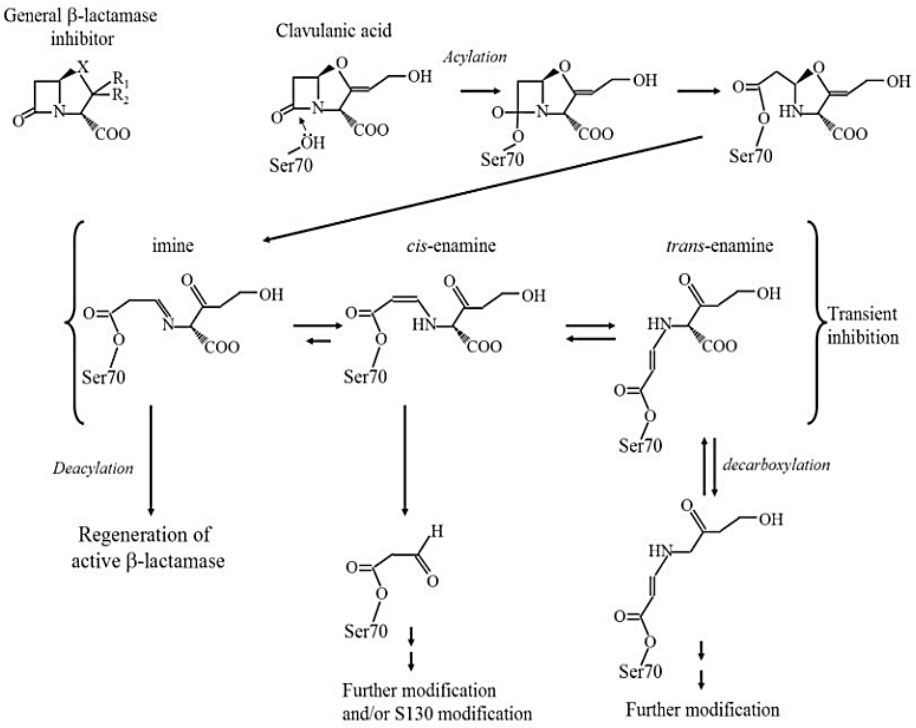


Figure 1.6. Mechanism of β-lactamase inhibition by CA (Padayatti *et al*., 2005).

1.7. Clavulanic acid gene cluster

Although structurally similar, cephamycin C and clavulanic acid biosynthesis pathways do not share any enzymes, yet their respective gene clusters are adjacent to each other in *S. clavuligerus* genome that form a supercluster of about 60 kb in size (Ward and Hodgson, 1993). Clavulanic acid gene cluster is thought to include genes (Table 1.1) that function in biosynthesis, transport, and regulation of CA (Arulanantham *et al*., 2006), however complete characterization of ORFs is still not completed. The biosynthetic pathway leading to CA and 5S clavams are shared. However, the genes encoding both of these compounds reside in three different clusters that are physically apart in *S. clavuligerus* genome. While the

CA gene cluster contains the early shared genes of both pathways and the late genes of only CA biosynthesis pathway, clavam cluster comprises *cas1* gene, a paralogue of *cas2* from CA gene cluster, and the other genes that function exclusively in 5S biosynthesis. Another distinct gene cluster contains paralogues of early shared genes of both CA and 5S clavams that are expressed only in soy-based medium and are differentially regulated (Jensen *et al*., 2000; Tahlan *et al*., 2004) (Figure 1.7). The loci of each cluster were identified from the draft genome sequence of *S. clavuligerus* ATCC 27064. While the supercluster of cephamycin C and clavulanic acid is located in the chromosome, the paralog gene cluster lies on a large linear plasmid, pSCL4 (Song *et al*., 2010)

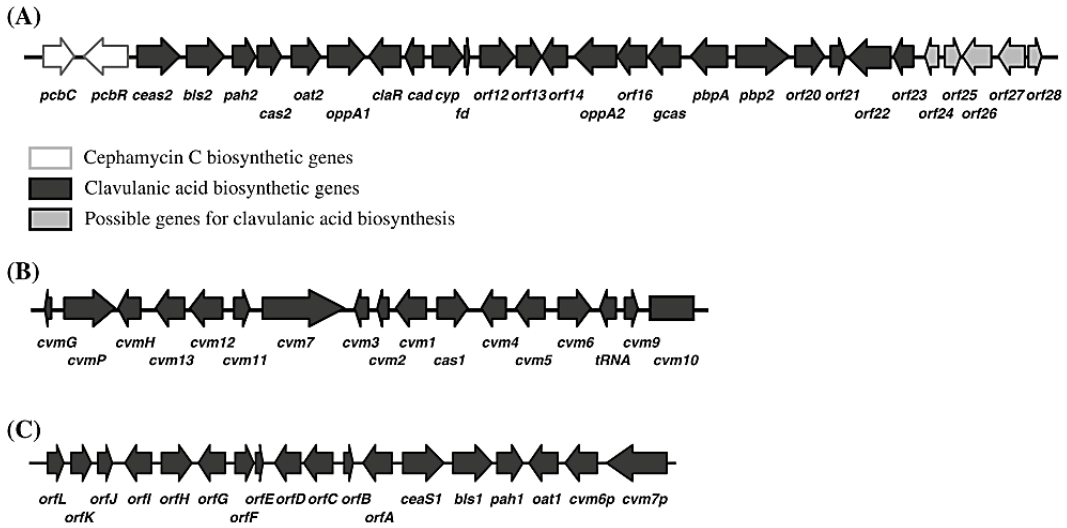


Figure 1.7. Clavulanic acid and other clavam metabolite gene clusters. (A) Clavulanic acid gene cluster. (B) Clavam gene cluster. (C) Paralog gene cluster (Song *et al*., 2010).

Table 1.1 Clavulanic acid gene cluster ORFs and their functions

Gene	Product/ Putative Function.	Reference
ceaS	N2-(2-carboxyethyl) arginine Synthase	(Khaleeli *et al*., 1999)
bls	β-lactam synthase	(Bachmann *et al*., 1998)
pah2	proclavaminate amidinohydrolase	(Wu *et al*., 1995)
cas2	clavaminate synthase	(Elson *et al*., 1987)
oat	ornithine acetyltransferase	(Kershaw *et al*., 2002)
oppA1	oligopeptide binding protein	(Hodgson *et al*., 1995)
claR	ClaR regulatory protein (DNA binding protein)	(Paradkar *et al*., 1998) (Perez-Redondo *et al*., 1998)
cad	clavulanic acid dehydrogenase	(Nicholson *et al*., 1994)
cyp	P450 mono-oxygenase	(S. E. Jensen *et al*., 2000) (Li *et al*., 2000) (Jensen *et al*., 2004) (Mellado *et al*., 2002)
fd	ferredoxin	(R Li et al., 2000) (Jensen *et al*., 2004)
orf12	acetyl transferase	(Jensen *et al*., 2004; Mellado *et al*., 2002)
orf13	efflux pump	(Jensen *et al*., 2004; Mellado *et al*., 2002)
orf14	acetyltransferase	(Jensen *et al*., 2004; Mellado *et al*., 2002)

Table 1.1. (Continued)

oppA2	oligopeptide binding protein	(Hodgson *et al*., 1995; Jensen *et al*., 2000; Jensen *et al*., 2004; Mellado *et al*., 2002)
orf16	hypothetical protein	(Jensen *et al*., 2004; Mellado *et al*., 2002)
gcas	N-glycyl-clavaminic acid synthetase	(Arulanantham *et al*., 2006)
pbpA	PBP	(Jensen *et al*., 2004; Mellado *et al*., 2002)
*pbp*2	PBP	(Jensen *et al*., 2004; Mellado *et al*., 2002)

1.8. Clavulanic acid biosynthesis

Biosynthesis of clavulanic acid differs from conventional β-lactam antibiotics such that the nitrogen atom providing precursor is L-arginine, rather than tripeptide precursors synthesized by non-ribosomal peptide synthases. The first step of synthesis starts with a condensation reaction of L-arginine with glyceraldehyde-3-phosphate via carboxyethylarginine synthase (CeaS). The condensation reaction further catalyzes a thiamine diphosphate (ThDP)-dependent reaction to yield N^2-(2-carboxyethyl)arginine intermediate. Next, β-lactam synthetase (Bls) catalyzes the second step of pathway by conversion of N^2-(2-carboxyethyl)arginine to deoxyguanidinoproclavaminate, which encloses the monocyclic β-lactam ring. Following the action of Bls, clavaminate synthase

(Cas) hyroxylates the deoxyguanidinoproclavaminate to give guanidinoproclavaminate, which is then converted to proclavaminate by proclavaminate aminohydrolyze (Pah) removing the arginine-derived guanidine group. Cas then participates again in the next two reactions to catalyze the formation of the first bicyclic intermediate through oxidative ring closure of proclavaminate to give dihydroclavaminate, followed by desaturation to form clavaminate. The common steps in 5S clavam and clavulanic acid synthesis end up with the formation of clavaminate and they diverge into separate pathways. Next step of clavulanic acid biosynthesis is catalyzed by the ATP dependent enzyme Gcas encoded by *orf17* to produce *N*-glycyl-clavaminic from claviminic acid. A double epimerization and an oxidative deamination reaction are predicted to follow Gcas in the pathway and ultimately to yield clavaldehyde. However, the mechanism by which N-glycyl-clavaminic acid is converted to clavaldehyde is still not clear. Clavaldehyde (3R,5R- clavulanate-9-aldehyde), the last known intermediate of the pathway and the immediate precursor of clavulanic acid, is an unstable α,β-unsaturated aldehyde with β-lactamase inhibitory activity. The observation that clavaldehyde shows β-lactamase inhibitory activity supports the contention that inhibitory activity is a feature associated with the 3R,5R stereochemistry of the β-lactam ring. The highly unstable clavaldehyde intermediate finally gets reduced to give clavulanic acid in an NADPH-dependent reaction catalyzed without degradation of the bicyclic β-lactam ring by clavulanic acid dehydrogenase (Cad) (Song *et al*., 2010) (Figure 1.8)

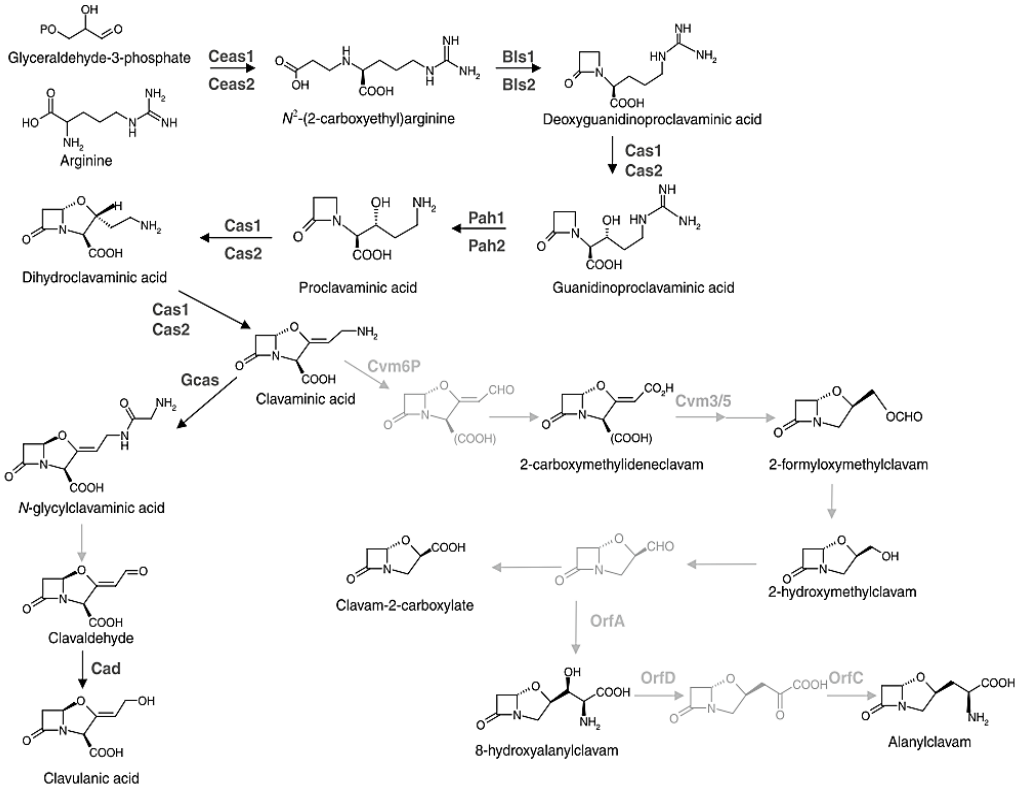


Figure 1.8. Biosynthetic pathway of clavam metabolites and clavulanic acid. Grey lines represent the putative steps and enzymes in the pathways (Song *et al*., 2010).

1.9. Regulation of antibiotic production in *S. clavuligerus*

Knowledge about the regulation of secondary metabolite production in *S. clavuligerus* is far from complete. However, it is well-known that a complex network of global and pathway-specific regulators act in the process (Liras *et al*., 2008) (Fig 1.9)

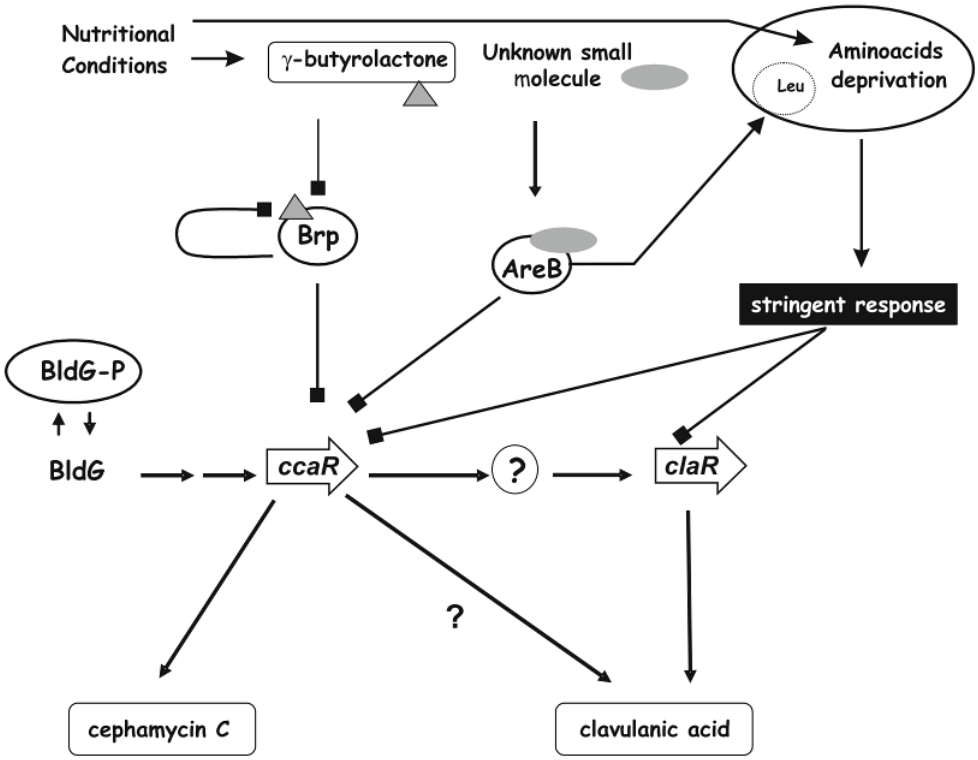


Figure 1.9. Schematic representation of regulatory proteins acting in *S. clavuligerus* cephamycin C and clavulanic acid biosynthesis (Liras *et al*., 2008).

Pleiotropic regulators like products of *bld* genes are the first level of control in antibiotic synthesis and they regulate both secondary metabolism and morphological differentiation in *Streptomyces* spp. *bldA* gene encoding $tRNA^{Leu}$ for translation of rare UUA codon controls expression of almost 150 genes in *S. coelicolor* and *bldA*-null mutants lose their capability to form aerial hyphae and spores (White and Bibb, 1997). The first reported *bld* gene affecting morphological differentiation and secondary metabolism in *S. clavuligerus* is *bldG,* which controls the expression of *ccaR*, a pathway specific activator of

cephamycin C and clavulanic acid production. *bldG* encodes an anti-anti-sigma factor which presumably acts on a an anti-sigma factor regulating the activity of CcaR (Bignell *et al*., 2005).

Many of the pathway-specific regulatory proteins of *Streptomyces* spp. belong to the SARP (*Streptomyces* Antibiotic Regulatory Proteins) family of proteins which usually possess helix-turn-helix DNA binding motifs towards their N-termini. *ccaR* gene identified in the cephamycin gene cluster of *S. clavuligerus* is a member of SARP family which lacks the helix-turn-helix motif yet contain a DNA binding domain similar to that of OmpR-family of proteins (Wietzorrek and Bibb, 1997). According to DNA-binding assays, CcaR interacts with lat, cefD-*cmcI*, and *cefF* promoter regions of the cephamycin C cluster, thus controlling the early, middle and late genes of the pathway (Liras et al., 2008; I Santamarta et al., 2011). It has also been reported that CcaR controls the expression of early clavulanic acid genes by binding to the promoter region of *ceaS2*, whose expression leads to a polycistronic transcript covering the genes *ceaS2, bls2, pah2, and cas2* (Tahlan and Anders, 2004). Another important pathway-specific regulator, ClaR, a LysR type of transcription activator is required for the expression of the late genes of clavulanic acid biosynthesis as *claR*-null mutants accumulate the last intermediate of the pathway (Paradkar *et al*., 1998; Perez-Redondo *et al*., 1998). *claR*-dependent transcription of genes located immediately upstream and downstream of *claR* that have been later characterized as essential for clavulanic acid production. *claR* regulated genes encode an oligopeptide permease (*oppA1*), the clavulanic-9-aldehyde reductase (*car*) and a cytochrome P450 (*cyp*). The binding of CcaR to the promoter of *claR* as well as *ceaS2* and *oppA1* was recently shown Moreover, the decrease in the expression of those genes in *ccaR*-disrupted cells were found as 3173 (for *ceaS2*), 78.1 (for *oppA1*) and 191.4-fold (for *claR*) (Santamarta *et al*., 2011). Therefore, while cephamycin C production is controlled only by CcaR, clavulanic acid production is controlled in at least two different levels: first one with CcaR binding to the promoter region of *ceaS2, claR* and *oppA1*, second via the role of *claR* (Santamarta *et al*., 2011).

γ-butyrolactone type of autoregulators are common in actinomycetes and act as "microbial hormones" that regulate the antibiotic production. The gene encoding a butyrolactone receptor, *blp*, was simultaneously characterized by two different groups in *S. clavuligerus*, showing that Brp represses both cephamycin C and clavulanic acid production as *brp*-null mutants overproduce the substances (Kim *et al*., 2004; Santamarta *et al*., 2005). Palindromic inverted repeats called ARE boxes are specific target sequences of Brp and two important ARE boxes have been identified in *S. clavuligerus*: the first one is located at the promoter of *brp*, suggestive of autoregulation; while the second ARE box is located 815 bp upstream of *ccaR* transcription start point (Liras *et al*., 2008). Recently, another ARE box was discovered that is located upstream of *adpA*, whose expression is increased 2.5-fold in *blp*-null mutants. It was also suggested that the control of *ccaR* expression was mediated directly by Brp and indirectly via Brp-dependent AdpA regulator. Thus, *ΔadpA* mutation in *S. clavuligerus* resulted with a profound decrease in the expression of early step genes of the CA cluster while it led to a lesser extent of decrease in the expression of late step genes of the pathway (López-García *et al*., 2010).

The first example of multiple proteins binding to an ARE box was reported by Santamarta *et al*. (2007). The AreB protein, which belongs to the IclR family of regulators, was isolated from *S. clavuligerus* was shown to interact with the ARE box located upstream of *ccaR*. *S. clavuligerus* AreB is required for effective leucine assimilation and biosynthesis, in addition to its role in fatty acid utilization as carbon source. Δ*areB* mutants of *S. clavulgierus* show increased levels of cephamycin C and clavulanic acid production along with underexpression of leucine biosynthetic genes. Downregulation of leucine biosynthesis increases the pool of valine precursor that is available to produce cephamycin C, indicating a novel role of AreB in connecting the primary and secondary metabolism (Liras *et al*., 2008).

The stringent response mechanism in prokaryotes is essential in sensing nutrient starvation and in adapting to new environmental conditions. Presence of

uncharged tRNAs in the A-site of ribosomes under amino acid deprivation triggers the activity of polyphosphorylated guanine (ppGpp) producer enzyme RelA and decreases the intracellular GTP levels, which result in stress response and amino acid biosynthesis (Liras *et al*., 2008). Stringent response in *Streptomyces* induces both morphological differentiation and secondary metabolism (Ochi, 1986), however studies on *S. clavuligerus* stringent response yielded unexpected results and lack of correlation between ppGpp levels and antibiotic production. This unexpected phenomenon was later confirmed by comparing the expression of structural genes (*ceaS2, cefD*) or regulatory genes (*ccaR, claR*) suggesting that in a typical batch fermentation condition, antibiotic production peaks at the rapid growth phase rather than the stationary phase, where the ppGpp levels start to rise (Liras *et al*., 2008). Surprisingly, recent strain improvement studies on *S. clavuligerus* revealed that *relA*-null mutants overproduce cephamycin C (six-fold) and clavulanic acid (four-fold) which is further explained due to increased expression levels of *cefD* and *ceaS2* biosynthetic genes of the former and the latter one, respectively (Gomez-Escribano *et al*., 2008).

1.10. Strain improvement for *Streptomyces* spp.

Commercially used antibiotics are produced either by fermentation, chemical synthesis or semisynthetic methods. In order to use fermentative production methods, the producer microorganism must be capable of synthesizing high titers of the product. Wild type strains however, are able to produce only limited amounts of secondary metabolites, which brings out the need to improve production capability by means of strain improvement techniques (Olano *et al*, 2008b). Traditionally used strategies were usually based on iterative rounds of random mutagenesis and screening to choose overproducer strains empirically. Although the method has proven itself during the past century, it requires

extensive amount of time and labor. More recently, protoplast fusion and whole genome shuffling methods have also been used in combination with the traditional methods to improve production yields (Baltz, 2011). With the development of new molecular microbiology and recombinant DNA technologies, along with the increased knowledge about the metabolic biosynthesis pathways and regulatory networks of antibiotics, a new approach of rational strain improvement called "metabolic engineering" has been developed (Chen *et al*., 2010).

Better understanding of clavulanic acid synthesis pathway in *S. clavuligerus* has led to metabolic engineering of the species to improve CA yields, mostly by altering the expression levels of biosynthetic or regulatory genes. Overexpression of *ceaS2*, encoding the first enzyme in the CA pathway increased the production capability of the wild type *S. clavuligerus* strains by 100% (Pérez-Redondo *et al*., 1999). Although functions of specific enzymes are not clear, overproduction of biosynthetic genes such as *cyp*, *fd*, *orf12*, and *orf14*A also increased the CA production about 100% in *S. clavuligerus* (Mellado *et al*., 2002). Amplification of regulatory gene *ccaR* yielded about three fold increase in both clavulanic acid and cephamycin C levels of *S. clavuligerus* (Pérez-Llarena *et al*., 1997) whereas overexpression of *claR*, the specific regulator of clavulanic acid pathway, on a multicopy plasmid vector resulted in strains with 2-fold increased CA and alanylclavam yields (Perez-Redondo *et al*., 1998). Recently, combined overexpression of two regulatory genes, *ccaR* and *claR*, and a rate limiting enzyme of the pathway, *cas2*, by means of integration into *S. clavuligerus* chromosome have induced a significant improvement of 23.8-fold CA production (Hung *et al*., 2007). Aside from pathway specific regulators, increased dosage of pleiotropic regulator AdpA enhanced both cephamycin C and clavulanic acid productions almost 2-fold (López-García *et al*., 2010). Channeling the carbon flux towards CA synthesis by means of inactivating *lat* gene of the cephamycin C biosynthesis pathway also led to an increase in CA production by 2- to 2.5-fold (Paradkar *et al*., 2001). In another study, Li and Townsend (2006) focused on the glycolytic pathway to overcome glyceraldehyde-3-phosphate which was

suggested to be a limiting factor in clavulanic acid biosynthesis. Two genes, *gap1* and *gap2*, encoding glyceraldehyde-3-phosphate dehydrogenases were inactivated by genetic engineering and consequently, a *gap1* disrupted mutant showed a 2-fold improvement in its clavulanic acid production, suggesting that the rational engineering was achieved by glyceraldehyde-3-phosphate flux channeled to the clavulanic acid biosynthesis rather than to the glycolytic pathway. Moreover, when arginine was fed to the mutant where the intracellular arginine pool might be decreased, clavulanic acid production increased a further threefold over the wild type strain (Song *et al*., 2010). More recently, overexpression and chromosomal integration of both c*caR* and *claR* in the *gap1* deletion mutant further enhanced the clavulanic acid production by 2.59-fold and 5.85-fold, respectively (Jnawali *et al*., 2010).

In addition, after the identification of a glycerol utilizing cluster (*gylR-glpF1K1D1*) in *S. clavuligerus*, transformants having extra copies of *glpF1K1D1* that were fed with high glycerol concentrations produced 7.5-fold higher CA as compared to wild type strains (Baños *et al*., 2009).

Another option to obtain an appropriate expression host for industrial purposes is to develop a superhost by manipulating already overproducing industrial strains. However, the use of industrial strains usually has its restrictions basically due to being patented and lack of information about the genetic background of the strains (Smolke, 2010).

1.11. The present study

As mentioned earlier, *S. clavuligerus* is a well-known producer of many industrially and medicinally important secondary metabolites, especially the potent β-lactamase inhibitor, clavulanic acid. In this study, the specific regulator of the clavulanic acid pathway, *claR*, was overexpressed in the wild type and an industrial overproducer strain of *S. clavuligerus* by using two different methods:

(i) constitutive expression of *claR* via the chromosomal integration by using the vector, pSET152^{ermE*}; and (ii) its enhanced expression under a strong glycerol promoter by using multicopy *Streptomyces* expression vector, pSPG. The aim of these manipulations was to further improve the CA production capability of the industrial *S. clavuligerus* strain.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, media and culture conditions

Table 2.1. shows the list of bacterial strains and plasmids used in this study along with their specific properties. Luria broth liquid medium (LB) and agar plates (LA) were used to grow *Escherichia coli* cells (Appendix A) at 37°C. *E. coli* cells were preserved on agar plates, whereas for long term storage 20% glycerol stocks were prepared and kept at -80°C. *S. clavuligerus* cultures were grown in Tryptic soy broth (TSB) or agar (TSA) (Appendix A) by incubating on a rotary shaker (220 rpm) at 28°C and mycelium cultures were stored as %20 glycerol stocks at -80°C. *S. clavuligerus* fermentations were performed in CC2 vegetation and CC3 fermentation media (Appendix A) in 500 mL baffled flasks (240 rpm) at 23.5°C or in Starch-Asparagine (SA) media (Appendix A) in 500 mL baffled flasks (220 rpm) at 28 °C.

pGEM-T Easy (Promega) vector was used for cloning of *claR* gene into *E. coli* (Figure 2.1). For intergeneric conjugation of *claR* from *E.coli* to *Streptomyces*, shuttle vectors pSET152^{ermE*} and pSPG were used (Figure 2.2).

Table 2.1. List of bacterial strains and plasmids used in this study

Strains & plasmids	Description	Source or reference
S. clavuligerus		
NRRL 3585	Wild type, clavulanic acid and cephamycin C producer	Prof. J. Piret, Northeastern University, USA
Industrial strain	Clavulanic acid overproducer	DEPA Pharmaceuticals, İzmit, Turkey
MA11 MA16 MA28	Industrial *S. clavuligerus* strains carrying an extra copy(ies) of *claR* in their chromosome	This study
MAG1 MAG2 MAG4	Industrial *S. clavuligerus* strains carrying pMAG03	This study
WMA1 WMA2 WMA4	Wild type *S. clavuligerus* strains carrying an extra copy(ies) of *claR* in their chromosome	This study
WMAG1 WMAG3 WMAG4	Wild type *S. clavuligerus* strains carrying pMAG03	This study
Klebsiella pneumoniae		
ATCC 29665	Indicator organism	Prof. P. Liras, INBIOTEC, Leon, Spain

Table 2.1. (Continued)

E. coli		
DH5α	F` ϕd*lac*ZM15 (*lac*ZYA *arg*F), U169, *sup*E44λ-, *thi*-1, *gyr*A, *rec*A1, *rel*A1, *end*A1, *hsd*R17	*E. coli* Genetic Stock Center
ET12567	dam^{-} 13::Tn9 dcm-6 hsdM hsdR, *lac*YI	
Plasmids		
pGEM-T® -Easy	Amp^{R}, *lacZ*'	Promega
pSET152^{ermE*}	*lacZ*, *rep*^{puc}, *att*$^{\Phi C31}$, *oriT*, *ermE**	Combinature, Biopharma
pAK23	*ccaR* carrying pSPG	(Kurt, 2011)
pMAE01a	pGEM-T with *S. clavuligerus claR* gene including *Eco*RI-*BamH*I sites	This study
pMAE01b	pGEM-T with *S. clavuligerus claR* gene including *Nde*I-*Spe*I sites	This study
pMAE02	pSET152^{ermE*} with S. *clavuligerus claR* gene at its *Eco*RI-*BamH*I site	This study
pMAG03	pSPG with S. *clavuligerus claR* gene at its *Nde*I-*Spe*I site	This study

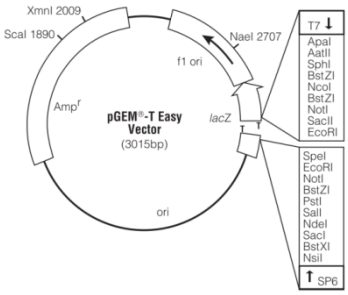
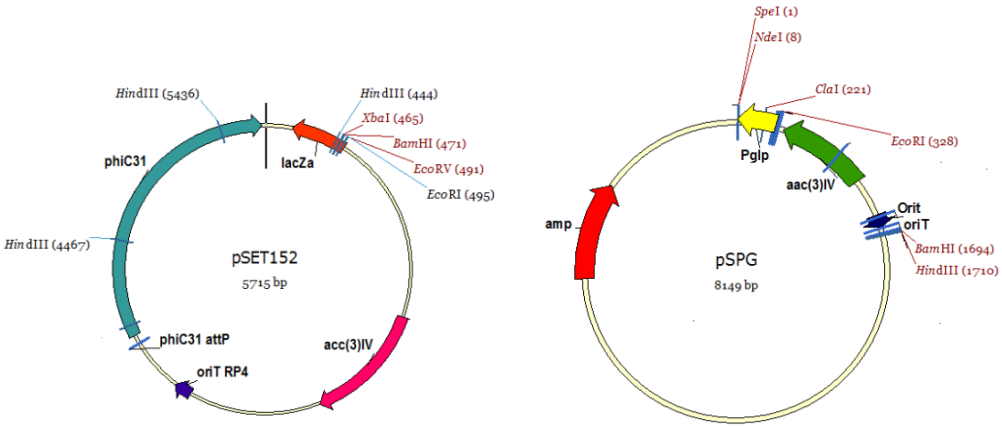


Figure 2.1. pGEM-T Easy plasmid map**.**



(a) **(b)**

Figure 2.2. Maps of plasmids (a) pSET152^{ermE*} and (b) pSPG.

2.2. Culture media

The ingredients and preparation of culture media can be found in Appendix A.

2.3. Buffers and solutions

The ingredients and preparation of buffers and solutions are listed in Appendix B.

2.4. Chemicals and enzymes

Appendix C contains information about the chemicals and enzymes used, along with their suppliers.

2.5. *S. clavuligerus* genomic DNA isolation

S. clavuligerus genomic DNA was isolated by salting out method (Pospiech and Neumann, 1995). 50 mL TSB (Appendix A) was inoculated with 50 µL of mycelium stock and incubated at 28°C (220 rpm) for 48-60h. 30 mL of this suspension was precipitated by centrifugation at 2000 rpm for 15 min, re-suspended in 5 mL of STET buffer (Appendix B) and treated with 100 µL of lysozyme (Appendix C) for 30-60 min at 37°C. 140 µL proteinase K (Appendix C) and 600 µL 10% SDS (Appendix C) was added and mixed. After this step, 2 mL of 5M NaCl (Appendix C) was added and thoroughly mixed by inversions. Next, 5 mL of chloroform (Appendix C) was added and incubated for 30 min with regular inversions at 20°C. After incubation, the solution was centrifuged at 6000 rpm for 15 min and the supernatant was transferred to a new tube. Following the transfer, 0.6 vol isopropanol was added and mixed again by inverting the tube several times. Visible DNA was spooled out and washed with 5 mL 70% ethanol.

After washing, ethanol was removed and left for air drying. Finally, DNA was dissolved in 1-2 mL TE (Appendix B) at 55°C (Kieser *et al*., 2000).

2.6. Plasmid isolation from *E. coli*

Fermentas Plasmid Purification Mini Kit (Fermentas) was used for isolation of plasmids from *E. coli* according to the manufacturer's instructions. Manual isolation of plasmids from *E. coli* was performed according to the plasmid miniprep method described by Hopwood *et al*. (1986). According to this, colonies were grown on selective LA solid media as patches and 1 square cm of cell mass was collected with a sterile pipette tip. Next, the cells were re-suspended in 100 µL cold SET buffer (Appendix B) and the tip was discarded. Then, the tubes were kept on ice for 20 min. 0.6 vol of lysis solution (Appendix B) was added on top of the cell suspension and vortexed. After 10 min incubation at RT, lysed cell suspension was incubated at 70°C for 10 min to denature the DNA. After denaturation, tubes were rapidly cooled with cold water. Following this step, an equal amount of phenol-chloroform solution (water-saturated, Appendix B) was added and vortexed until a homogenous and milky solution was obtained. Finally, the samples were centrifuged at 13000 rpm for 5 min to obtain distinct phases. The upper aqueous phase was transferred to a new tube and 25 µL of it was loaded on agarose gel for investigation.

2.7. *E. coli* competent cell preparation

For preparation of competent *E. coli* cells, the protocol described by Hanahan *et al*. (1983) was being used. Single *E. coli* DH5α/E.coli ET12567/pUZ8002 colony from fresh LA (Appendix A) culture was inoculated into 3 mL of LB (related

antibiotics were added if necessary) and incubated by shaking at 37 °C for o/n. 3 mL of this culture was transferred to 200 ml of LB in 500 mL Erlenmayer flask (related antibiotics were added if necessary) and incubated on an orbital shaker at 37 °C until OD_{600} reaches to 0.4-0.7. The culture was then incubated on ice for 15 min. and centrifuged at 3500 rpm and at 4 °C for 5 min. After decanting the supernatant, the pellet was dissolved in 20 mL of ice cold Buffer 1 (Appendix B). The cells were kept on ice throughout the procedure. The resuspended cells were centrifuged as above and the supernatant was discarded again. The cells were resuspended in 8 mL of ice cold Buffer 2 (Appendix B) and this cell suspension was aliquoted in 100 µL volumes to the 1.5 mL Eppendorf tubes. They were incubated on ice for 15-30 min. Finally, the tubes were frozen in liquid nitrogen and stored at -80 °C until use.

2.8. Transformation of competent *E. coli* cells

Competent *E. coli* cells were transformed using the "heat-shock" method as described by Sambrook *et al*. (1989). -80°C competent cell stocks were thawed on ice and 1-50 ng plasmid DNA or ligation product was added into the suspension. After 30 min incubation on ice, the tubes were placed in a 42°C water-bath for 90 sec to apply heat-shock. Immediately after the heat-shock, the tubes were placed on ice for 5 min. 900 µL sterile LB was added to each tube and incubated at 37°C for 90 min. Following incubation, the cells were centrifuged at 3500 rpm for 15 min and most of the supernatant was discarded, leaving only 100 µL of LB to re-suspend the pellet. Finally, the cells were spread onto selective LA plates and left for overnight incubation at 37°C. The next day, single colonies were picked and screened for possible recombinants.

2.9. Intergeneric conjugation between *E. coli* and *Streptomyces*

The original method was described by Mazodier *et al*. (1989) and modified by Flett *et al*. (1997) using non-methylating *E. coli* as donor. pUZ8002 and *dam* mutation of competent *E. coli* ET12567/pUZ8002 cells were maintained by kanamycin (Km) (25 μg/mL) and chloramphenicol (Cm) (25 μg/mL) (Appendix C), respectively. Apramycin (Apr) (50 μg/mL) (Appendix C) was also added to maintain the *ori*T-containing vectors. Recombinant *E. coli* ET12567/pUZ8002 with the desired plasmid was inoculated into 10 mL of LB media containing Km, Cm and Apr and grown o/n at 37°C at 200 rpm. The next day, cells were diluted 1:100 in fresh LB containing three antibiotics and grown at 37°C to an OD_{600} of 0.4 – 0.6. After incubation, the cells were washed twice with an equal volume of fresh LB and re-suspended in 0.1 volume of LB. *Streptomyces* mycelia were harvested from 3-4 days old culture growing on TSA (Appendix A) using 3-4 mL of 20% glycerol, vortexed thoroughly and 0.5 mL of it was mixed with 0.5 mL of *E. coli* culture. The cell mixture was mixed and spinned down briefly. Supernatant was mostly poured off and the pellet was re-suspended in residual fluid. The cells were plated out on MS agar (Appendix A) containing 10 mM $MgCl_2$ and incubated at 30°C for 16-20 hours. After incubation, the plates were overlaid with 1 mL of distilled water containing 0.5 mg of nalidixic acid (Appendix C) and 1mg of apramycin. Nalidixic acid kills *E. coli* but has no effect on *Streptomyces* viability due to its natural resistance (Keiser *et al*., 2000). After 3-4 more days of incubation at 30°C, potential exconjugants were picked off to selective TSA plates containing nalidixic acid (25 μg /mL) and apramycin (50 μg/mL) (Kieser *et al*., 2000).

2.10. Manipulation of DNA

2.10.1. Digestion with restriction enzymes

The conditions specified by the manufacturers were employed while doing restriction enzyme digestions.

2.10.2. Agarose gel electrophoresis

The most frequently used concentration of agarose was 1 %; however, depending on the size of fragments to be separated, the concentration was modified accordingly. 1X TAE (Appendix B) was used as electrophoresis buffer and ethidium bromide solution (Appendix C) as a final concentration of 0.4 ng/mL was used for staining of DNA for 15-20 min at RT. The bands were visualized on a UV transilluminatior (UVP) and photographed via Vilber Lourmat Gel Imaging System. The molecular weights of DNA bands were determined using Lambda (λ)/*Pst*I and O'GeneRuler 100 bp DNA ladder plus DNA size markers (Appendix C).

2.10.3. DNA fragment extraction from agarose gel

Genemark Gel Extraction kit (http//www.genemark.com.tr) was used to extract DNA fragments from agarose gels. The slice of gel that contains the target DNA fragment was extracted according to the manufacturer's instructions. The extraction yield was measured with a NanoDrop® ND-2000 (ThermoScientific) spectrophotometer.

2.10.4. Ligations

Gel extracted PCR products were ligated with pGEM-T Easy vector via TA cloning. The ligation mixture was composed of 55 ng pGEM-T Easy vector, 50 ng insert DNA, 1X ligation buffer and 1-3 u T4 DNA ligase (Promega) in a total volume of 10 µL. Ligation was performed at 4 °C for 16 h.

Following excision from the pGEM-T vector, *claR* was ligated with target vectors pSET152ermR and pSPG as follows: 1 µL of 1-3 u/µL T4 DNA ligase (Promega), 5 µL of 2X reaction buffer, 50 ng vector DNA, 150 ng insert DNA mixed and volume of the mixture was completed to 10 µL with dH_2O. Overnight incubation at 4 °C was provided for ligation reactions to take place.

2.11. Primer design

Table 2.2. shows the nucleotide sequences of PCR primers used in this study. Forward and reverse primers included *Eco*RI-*Bam*HI and *Nde*I-*Spe*I sites, for pSET152^{ermE*} and pSPG vectors respectively, and they amplify a sequence of 1426 bp which includes the *claR* gene.

Two extra sets of primers were also designed to confirm the presence or absence of the recombinant plasmids in possible exconjugants via colony PCR. One of the primers in these sets matches with a sequence within *claR* gene (claRint) and the other one (psetint and pspgint) matches with the vector sequences flanking *claR* gene. Expected amplicon sizes are 858 bp and 605 bp when internal primer pairs are used in PCR reaction, respectively. The designed primers were synthesized by the Alpha DNA (Quebec, Canada). The nucleotide sequence of *claR* gene and positions of the primers are shown in Figure 2.3.

Table 2.2. Primers used in this study.

Primers	Sequence	Tm (°C)
Pset152ermF	**gaattc**gccgatgcgatctgtcttta	67.5
Pset152ermR	**ggatcc**gccccgggaccgtatgtc	73.1
pspgF	**catatg**gccgatgcgatctgtcttta	67.1
pspgR	**actagt**gccccgggaccgtatgtc	71.0
claRint	gggccacttccatgaggtct	55.1
psetint	tagtcctgtcgggtttcgccac	59.8
pspgint	tgcctttgctcggttgatcc	57.3

Figure 2.3. Nucleotide sequence of *claR* (GenBank accession number U87786.2) and the location of the primers.

2.12. Polymerase chain reaction

Amplification of *claR* from *S. clavuligerus* genomic DNA was performed under the following conditions: Initial denaturation at 98 °C for 2 min followed by 15 sec at 98 °C for denaturation, 15 sec at 57 °C for annealing, 30 sec at 72 °C for extension. The last three steps were repeated 30 times and the reaction was

finalized with a final extension step at 72 °C for 10 min. The reaction mixture was composed of: 5 µL of 5X HF buffer (Finnzyme), 1 µL of 50 mM $MgCl_2$ (Finnzyme), 1 µL of 10mM dNTP mix (Fermentas), 1.25 µL DMSO, 1.25 µL from each 10mM primer stock, 2 µL of DNA (Genomic or plasmid DNA), 0.5 µL of 2 u/µL Phusion DNA polymerase (NEB) and 11.75 µL dH_2O.

Colony PCR was performed as described by Asano *et al*. (1998) with slight modifications to adapt the procedure to *S. clavuligerus*. Samples taken from the colonies on agar plate were re-suspended in 50 µL dH_2O and lysed at 95 °C for 15 min. Then, 5 µL cell lysate was used as template in the standard PCR mixture described above. The conditions of colony PCR were as follows: Initial denaturation at 98 °C for 10 min followed by 40 sec at 98 °C for denaturation, 30 sec at 55 °C for annealing, 30 sec at 72 °C for extension. The last three steps were repeated 30 times and the reaction was finalized with a final extension step at 72 °C for 10 min. The PCR products were then run on 1% agarose gel and results were evaluated after DNA staining.

2.13. Growth determination via DNA quantification

The procedure described by Burton *et al*. (1968) was performed in order to measure the growth of *S. clavuligerus* cultures. 1 mL of culture was collected at 24 h intervals and centrifuged at 13200 rpm for 10 min. The pellet was kept at -20 °C for storage. After thawing on ice, the pellets were re-suspended in 2 mL 0.85% NaCl (saline) solution and diluted 1:20 in 400 µL saline in a new tube. 400 µL of 1N $HClO_4$ (Appendix B) was added on top of the samples and mixed by inverting the tubes. After that, the samples were incubated at 70 °C for 20 min. Next, 800 µL of diphenylamine reagent (Appendix B) was added and mixed several times with inversions, followed by incubation of samples at 30 °C for 15-17 h. Samples were taken out and centrifuged at 13200 rpm for 10 min. Supernatant absorbance

values were measured at 600 nm and recorded along with the absorbance of standard Herring Sperm DNA (Appendix C) samples. The amount of DNA in the samples was determined as μg DNA/mL of culture according to the standard curve drawn by using the standard solutions.

2.14. Clavulanic acid fermentation

Since most secondary metabolites are produced during late exponential to stationary growth phase of microbial producers, as the cheapest method to grow cells up to the desired phase of growth, shake flask fermentations are performed. The aim of using small volumes of culture compared to batch fermentations is usually to extract or detect the desired metabolite from the culture media and compare the production capacities of two or more strains. Hence, the comparison of CA titers of recombinant and parental strains was performed by taking samples from *S. clavuligerus* cells fermenting for a total of 168 h.

The media, cultivation conditions and the protocols were identical with those employed for the commercial production of CA in DEPA Pharmaceuticals Co., Clavulanic Acid Manufacturing Plant, Köseköy, İzmit, except for a proper scaling down to apply in our laboratory.

Since growth phase is a crucial parameter in antibiotic production in Streptomycetes (Demain and Fang, 1995), parental and recombinant strains were brought to the rapid growth phase for fermentation where CA production is maximum. First, the mycelia of all strains were inoculated into TSB media by using sterile ear sticks and incubated at 28 °C and 220 rpm for 2-3 days to reach an OD_{600} of 3 or 4. Then, 500 μL of each culture was inoculated into 50 mL CC2 media and incubated at 23.5 °C for 40 h. After 40 h incubation the cultures reach an OD_{600} around 6-7. 400 μL seed culture from each flask was inoculated into 40 mL CC3 media and incubated at 23.5 °C, 240 rpm for 168 h. 1 mL samples from

each culture flask was collected at 24 h intervals and centrifuged at 13200 rpm for 10 min. The pellets were stored at -20 °C for DNA quantification whereas the supernatants were kept at -80 °C until they were used for HPLC or bioassay analyses.

2.15. Sample collection and conditions of HPLC for CA concentration measurement

In order to observe the direct effect of *claR* overexpression on the CA yields of recombinants as compared to their parent strains, samples taken from fermentation cultures were prepared for HPLC analysis of CA as its retention time can be specified by using standard (potassium salt of CA) solutions.

The -80 °C samples were thawed on ice and diluted 1:5 in sodium acetate solution (Appendix B). Diluted samples were filtered through nylon syringe filters of 0.4 μm diameter to remove insoluble ingredients and clumps of mycelia. Pursuit C18 column (A3000150x046, Serial No: 318437) was used in this study. Sample elution was performed with a mobile phase containing aqueous solution of sodium dihydrogen phosphate, pH 4 (Appendix B) and HPLC grade methanol (Appendix C), mixed in a 95:5 ratio. The column was washed and saturated with the mobile phase prior and after the injection of samples. Mobile phase flow-rate was 1.0 mL/min and the injection volume was 50 μL for all samples and standards. Column eluent was monitored with VARIAN PDA Detector Model 330 at 210 nm wavelength. Total run time was 7 min for all samples and all injections were performed at 4 °C.

2.16. Construction of CA calibration curve

Pure CA (potassium clavulanate salt), provided by DEPA Pharmaceuticals, İzmit, Turkey, was used in construction of the calibration curve. 10 mg/mL CA stock solution was prepared in sodium acetate buffer and serially diluted in sodium acetate buffer to obtain CA solutions with final concentrations of 0.001, 0.002, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25 mg/mL. Double injection of each standard solution was performed and peak area results were used in construction of CA calibration curve (Figure 2.4).

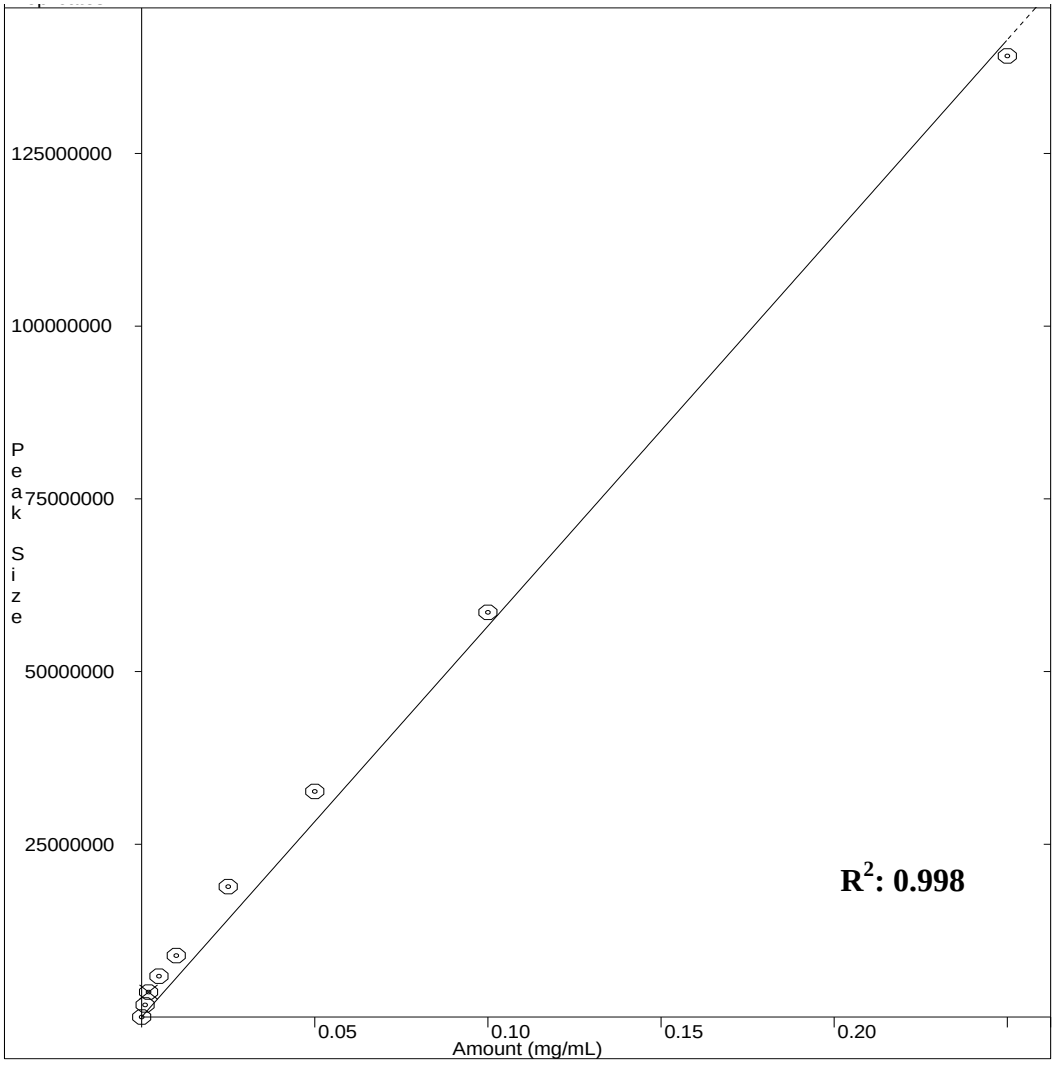


Figure 2.4. CA concentration vs peak area calibration curve.

2.17. Bioassay of CA

The agar plate diffusion method used by Romero *et al*. (1984) was adopted for CA bioassay procedure. Supernatants collected from each 1 mL sample were kept at -80 °C and thawed on ice on the day of assay. *K. pneumoniae* was grown in TSB up to an OD_{600} value of 0.9 – 1.0. 3.3 mL of the cell culture was mixed with 100 mL molten TSA at 47 °C. 100 µL of penicillin G (Appendix B) was added,

immediately mixed and poured into petri plates. Following this, holes were loaded with 60 µL of sample along with CA standards. All samples and standards were prepared and diluted in 1 M MOPS (pH 6.8) (Appendix B). Following 2 h incubation at 4 °C, plates were placed in a 30 °C incubator for 12-15 h incubation. CA concentrations of the samples were calculated using the linear regression curves obtained from Log [CA standards] (10, 7.5, 5, 3.75, 2.5, 1.25, 0.625, 0.3125, and 1.156 mg/mL) vs zone diameter (mm) plots (Figure 2.5).

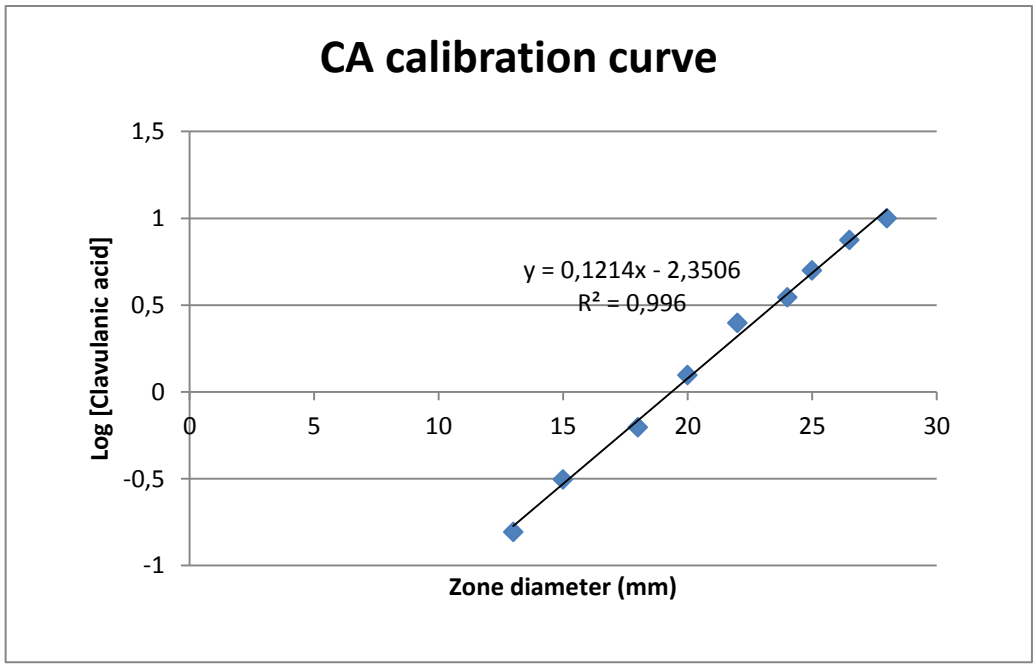


Figure 2.5. CA bioassay calibration curve.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Overxpression of *claR* gene in pSPG expression vector and its integrative expression under the control of *ermE** promoter in pSET152^{ermE*}

3.1.1. Cloning of *claR* into pSET152^{ermE*} for its chromosomal integration

Specific primers to amplify *claR* gene from *S. clavuligerus* NRRL 3585 genomic DNA were designed according to the sequence obtained from GenBank accession number U87786.2. Amplification of *claR* with pset152ermF and pset152ermR primer pair generated a 1426 bp PCR product that presumably included *claR* gene (Figure 3.1). Gel eluted PCR product with predicted size was then ligated to pGEM-T easy cloning vector and transferred into *E. coli* DH5α cells via transformation. After blue-white selection of the putative recombinant colonies on ampicillin (100μg/mL) plate, overlaid with X-Gal-IPTG (Appendix B), one of them was selected and the recombination was further verified with restriction enzyme (RE) digestion, PCR (Figure 3.2), and finally with DNA sequencing analysis. The recombinant plasmid was designated as pMAE01a (Figure 3.3).

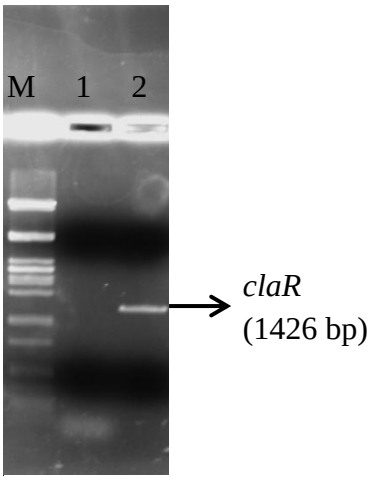
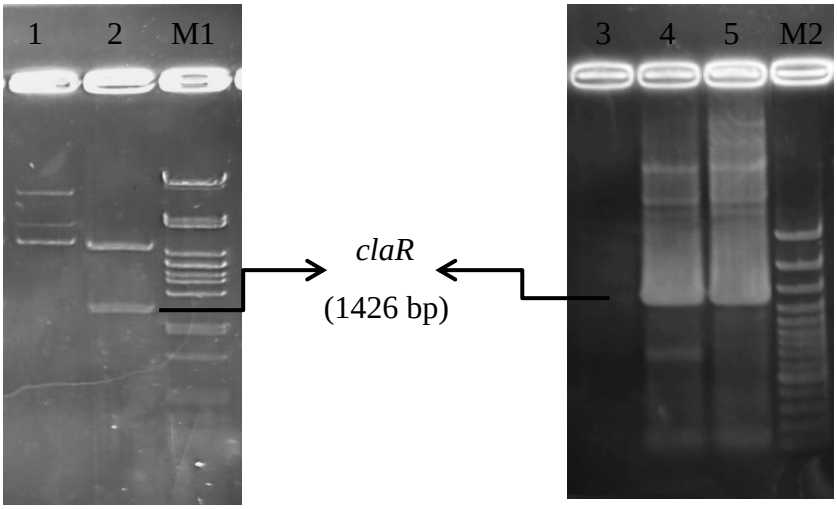


Figure 3.1. PCR amplification of *claR*. M: λ *Pst*I DNA marker, 1: No template control for PCR, 2: PCR product obtained from *S. clavuligerus* genomic DNA template with pset152ermF-pset152ermR primer pair.



(a) **(b)**

Figure 3.2. Recombinant plasmid verification via (a) RE digestion and (b) PCR. 1: Undigested recombinant plasmid, 2: *Eco*RI-*Bam*HI digested recombinant

plasmid, releasing *claR*, M1: λ PstI DNA marker, 3: No template control, 4: *claR* PCR product obtained by using the recombinant plasmid as template DNA, 5: *claR* gene amplified from *S. clavuligerus* genomic DNA by PCR M2: O'GeneRuler 100 bp DNA ladder plus.

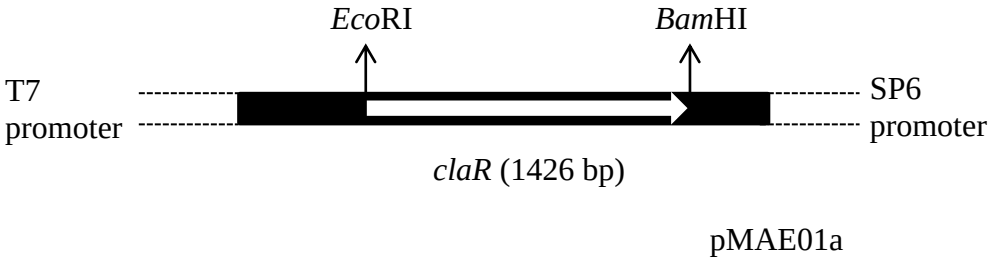


Figure 3.3. Orientation of *claR* in polylinker site of pMAE01a.

*Eco*RI-*Bam*HI digestion of the pMAE01a released *claR* gene from the vector which allowed ligation with the linearized pSET152^{ermE*} having compatible ends with the gene (Figure 3.4). As a result of ligation reaction, *claR* was inserted just downstream of the constitutive promoter *ErmE** of pSET152^{ermE*}. The new construct was introduced into *E. coli* DH5α cells via transformation and the recombinant colonies were selected in the presence of apramycin (50 μg/mL). The confirmation of recombination was performed by PCR (Figure 3.5). This newly formed recombinant plasmid was named as pMAE02 (Figure 3.6).

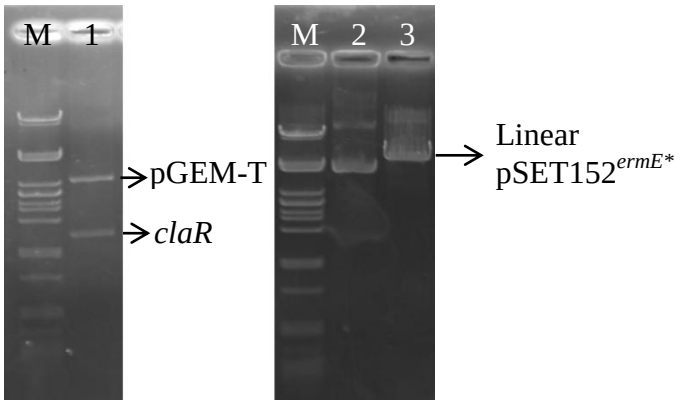


Figure 3.4. *Eco*RI-*Bam*HI digestion of (a) pMAE01a and (b) pSET152^{ermE*}. M: λ *Pst*I DNA marker, 1: Linearized pGEM-T easy vector and the released *claR* gene as a result of *Eco*RI-*Bam*HI digestion, 2: Undigested pSET152^{ermE*}, 3: *Eco*RI-*Bam*HI digested linear pSET152^{ermE*}.

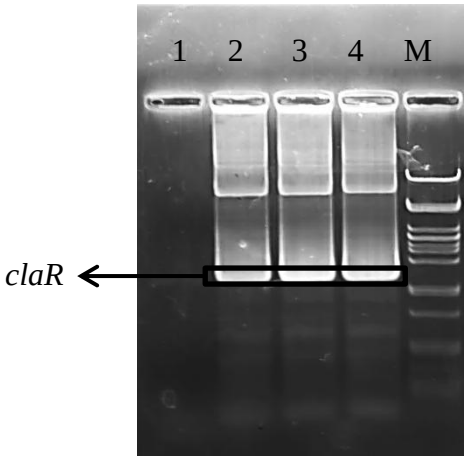


Figure 3.5. PCR verification of cloning of *claR* into pSET152^{ermE*} integration vector. 1: Negative control reaction of PCR with no template DNA, 2-4: *claR* PCR product obtained from pMAE02 as template, M: λ *Pst*I DNA marker.

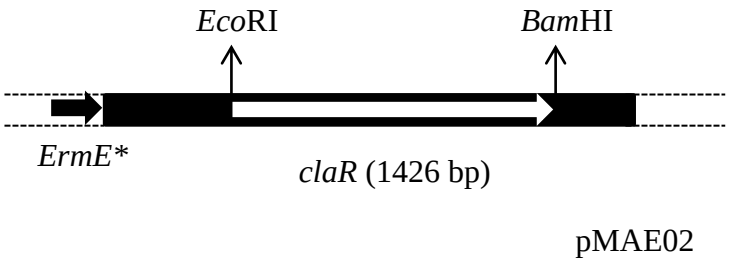


Figure 3.6. Orientation of *claR* in MCS of pMAE02 vector.

3.1.2. Cloning of *claR* into pSPG expression vector containing strong glycerol promoter (PglP)

claR gene with specific primers (pspgF and pspgR) was amplified from the industrial *S. clavuligerus* genomic DNA (Figure 3.7). Gel eluted DNA fragment containing *claR* was ligated to pGEM-T easy vector and introduced into *E. coli* DH5α via transformation and recombinants were confirmed by PCR amplification, RE double digestion (Figure 3.8), and sequencing analysis. The recombinant plasmid was designated as pMAE01b (Figure 3.9)

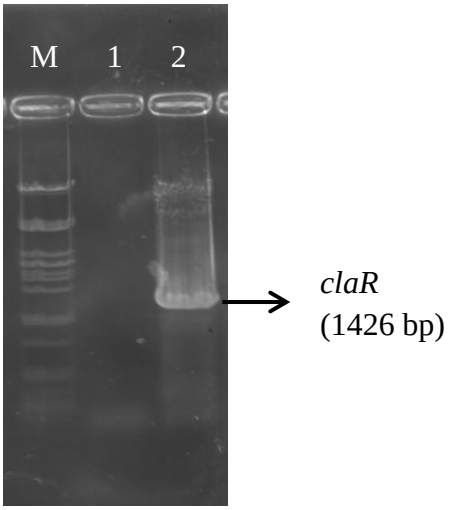


Figure 3.7. *claR* gene amplification. M: λ PstI DNA marker, 1: No template negative control, 2: *claR* PCR product by using *S. clavuligerus* genomic DNA as template.

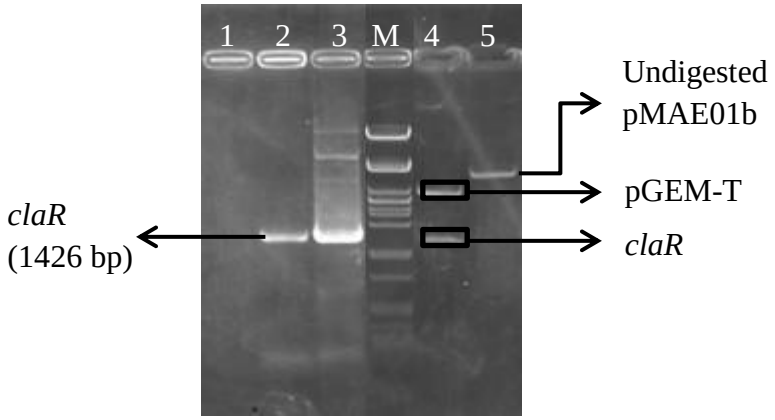


Figure 3.8. Recombinant plasmid verification via PCR and restriction endonuclease digestion. 1: No template control, 2: *claR* amplified from *S. clavuligerus* genomic DNA, 3: *claR* amplified from pMAE01b template, M: λ PstI DNA marker, 4: Linearized pGEM-T easy vector and released *claR* gene as a result of *Nde*I-*Spe*I digestion of pMAE01b. 5: Undigested pMAE01b plasmid.

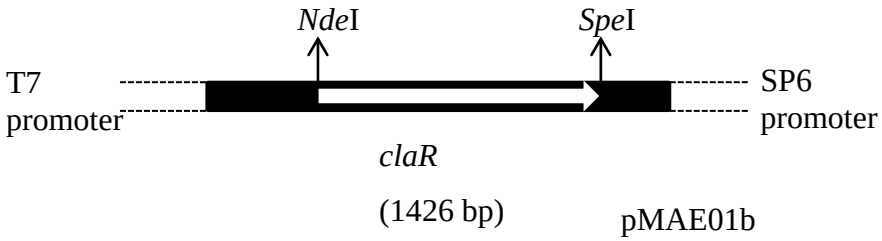


Figure 3.9. Orientation of *claR* in MCS of pMAE01b.

Double digestion with *Nde*I and *Spe*I released *claR* gene from pMAE01b and linearized the pSPG vector as the 876 bp insert DNA was removed from the MCS of pAK23 (Figure 3.10). Afterwards, transformation was performed to introduce

claR and pSPG ligation product into *E. coli* DH5α cells. The resulting recombinant cells were selected from LA plates containing 50 μg/mL apramycin and verification of recombination was performed by PCR (Figure 3.11). The recombinant plasmid was designated as pMAG03 (Figure 3.12).

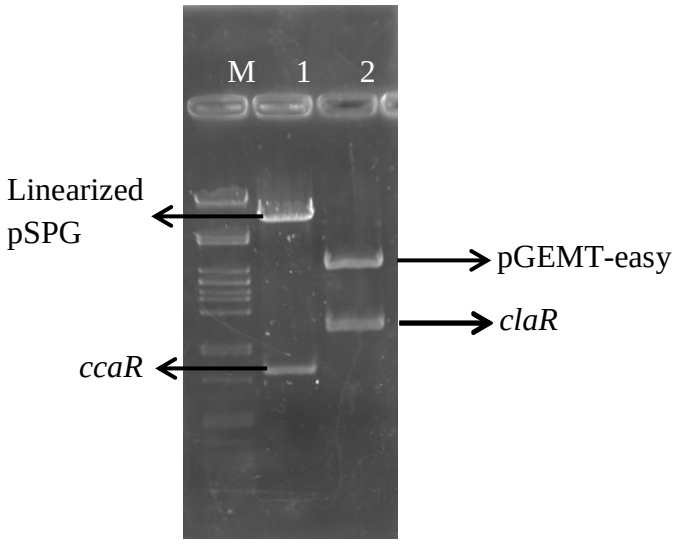


Figure 3.10. *Nde*I-*Spe*I digestion of pAK23 and pMAE01b. M: λ *Pst*I DNA marker, 1: Linearized pSPG vector and the released *ccaR* gene, 2: Linearized pGEMT-easy and the released *claR* gene.

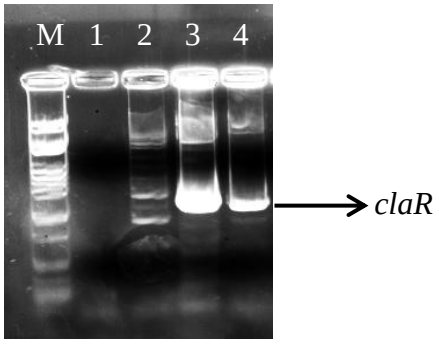


Figure 3.11. Verification of recombinant pMAG03 via PCR. M: λ *Pst*I DNA marker, 1: No template negative control, 2: PCR product of empty pSPG vector used as template DNA, 3: *claR* amplicon obtained by using pMAG03 as template, 4: *claR* gene amplified from *S. clavuligerus* genomic DNA.

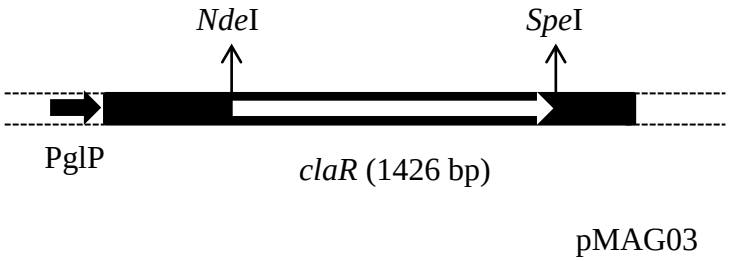


Figure 3.12. Orientation of *claR* gene inside the polylinker of pMAG03.

3.1.3. Intergeneric conjugation between *E. coli* and *S. clavuligerus*

There are several ways of introducing a foreign DNA fragment into *Streptomyces* spp. including protoplast transformation, transfection, electroporation, and

intergeneric conjugation with *E. coli*. Although all of these techniques can be applied to specific strains, conjugational transfer with *E. coli* is a more common technique that can be applied to nearly all *Streptomyces* spp. (Kieser *et al*., 2000). The advantages of using intergeneric conjugation over other techniques include: (i) the simplicity of the technique, so that it does not require protoplast formation and regeneration, (ii) the success of the technique in evading the restriction barrier by the transfer of single-stranded concatamers of plasmid DNA (Matsushima *et al*., 1994), (iii) availability of various useful *ori*T vectors that facilitate site-specific or insert-directed chromosomal integration, and (iv) the increased amount of vector that can replicate in *E. coli* host (Kieser *et al*., 2000). Thus, pMAE02 and pMAG03 recombinant plasmids were introduced into *E. coli* ET12567/pUZ8002 methylation deficient cells prior to conjugation. The importance of using methylation deficient *E. coli* in conjugation reaction is to bypass the restriction barrier of *S. clavuligerus* cells. The plasmid pUZ8002 is a RK2 derivative transfer plasmid that helps mobilization of recombinant plasmids during conjugation as the pUZ8002 itself cannot be transferred efficiently due to presence of a mutant *ori*T site (Bierman *et al*., 1992; Paget *et al*., 1999; Paranthaman and Dharmalingam, 2003). After conjugation, *S. clavuligerus* exconjugants picked from MS agar plates were grown on TSA plates containing nalidixic acid (25 μg/mL) and apramycin (50 μg/mL). Recombinant *S. clavuligerus* colonies were screened for the presence of pMAE02 integrated into the chromosome (Figure 3.13) and pMAG03 expressed inside the cell (Figure 3.14) via colony PCR by using the primer pairs clarint-psetint and clarint-pspgint, respectively. Recombinant *S. clavuligerus* cells that have pMAE02 integrated into their chromosomes were named as MA11, MA16, MA28 and WMA1, WMA2, WMA5 depending on the strain that they have originated from. Similarly, pMAG03 containing recombinant cells were designated as MAG1, MAG2, MAG4 and WMAG1, WMAG3, WMAG4, referring to their parental strains.

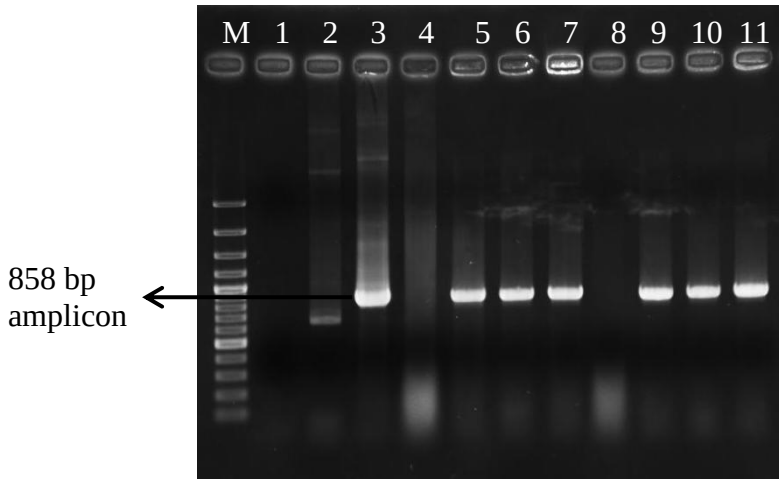
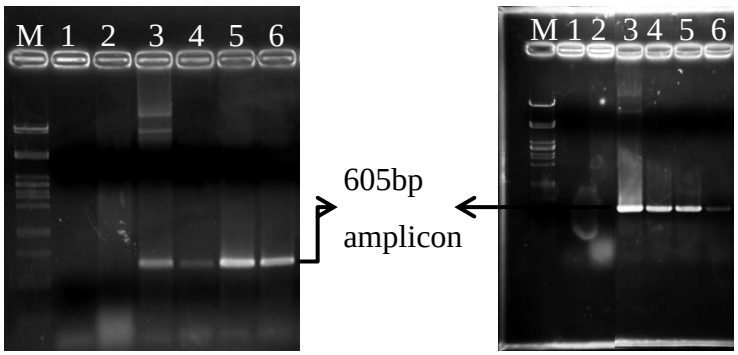


Figure 3.13. Verification of pMAE02 integration into *S. clavuligerus* chromosome by colony PCR. M: O'GeneRuler 100 bp DNA ladder plus, 1: No template control, 2: Negative control with empty pSET152^{ermE*} used as template DNA, 3: Positive control with pMAE02 used as template DNA, yielding 858 bp amplicon, 4,8: Negative control with the wild type *S. clavuligerus* genomic DNA used as template, 5-6-7: PCR amplicon obtained by using MA11, MA16 and MA28 colonies as source of template DNA, respectively, 9-10-11: PCR amplicon obtained by using WMA1, WMA2 and WMA5 colonies as source of template DNA, respectively.

The integration vector pSET152^{ermE*} is able to replicate in *E. coli* host but cannot multiply inside *Streptomyces* host; thus, stable exconjugants can only be maintained in selective media provided that chromosomal integration takes place by the aid of *φ*C31 *attP-int* locus in its sequence, which recognizes the *attB* attachment sites in *Streptomyces* chromosome (Paranthaman and Dharmalingam, 2003).



(a) **(b)**

Figure 3.14. PCR verification of recombinant *S. clavuligerus* colonies containing pMAG03 expression vector. (a) M: λ *Pst*I DNA marker, 1: No template control, 2: Negative control by using wild type genomic DNA as the template, 3: Positive control with pMAG03 used as template DNA, yielding 605 bp amplicon, 4-5-6: PCR product obtained by using MAG1, MAG3 and MAG4 colonies as source of template DNA, respectively, (b) M: λ *Pst*I DNA marker, 1: No template control, 2: Negative control by using wild type genomic DNA as the template DNA, 3: Positive control with pMAG03 used as template DNA, yielding 605 bp amplicon, 4-5-6: PCR product obtained by using WMAG1, WMAG3 and WMAG4 colonies as source of template DNA, respectively.

3.2. Clavulanic acid titers of the recombinant and parental *S. clavuligerus* strains

CA production by both parental and recombinant *S. clavuligerus* strains was compared via fermentation studies by using CC2-CC3 fermentation media and SA defined media. The reason for using two distinct fermentation media was the incapability of the standard strain NRRL 3585 to grow in CC2-CC3 media which was manufactured specifically to increase the CA yields of the industrial strain (Vanli, 2010). CA production capabilities of cells were compared as based on volumetric and specific CA production titers. While the former is important for industrial CA production and can be increased significantly via enhanced cell densities, the latter provides better differentiation of cells based on their fermentative performances as it gives the μg CA produced per mg DNA of bacteria (Hahn – Hägerdal *et al*., 2001).

Previously in our laboratory, specific production of CA was found to be 5-fold higher in the industrial strain as compared to that in the wild type *S. clavuligerus* ATCC 27064 grown in CC3 fermentation media. Moreover, the increase was reported as much greater (at least 7-fold) when HPLC analyses were performed (Vanli, 2010).

Prior to fermentation studies with the recombinants obtained in this study, the growth and CA production titers of the wild type NRRL3585 and industrial *S. clavuligerus* strains grown in SA-defined media were compared via HPLC analyses (Figure 3.15 and Figure 3.16).

The growth of the industrial strain was slightly slower than that of the wild type till T_{72} of cultivation and became almost the same at T_{72}. Then, the growth of both strains gradually decreased till the end of fermentation while the growth of the industrial strain was higher relative to that of the standard strain. Regarding to CA yields, volumetric CA production by industrial *S. clavuligerus* strain (151.5

μg/mL at T_{72} and 133.0 μg/mL at T_{96}) was found to be 3- and 4.3-fold higher than the wild type strain (46.75 μg/mL at T_{72} and 31.25 μg/mL at T_{96}) ($p<0.05$). Specific CA titer of industrial *S. clavuligerus* strain reached its maximum value at T_{72} with 447.40 μg/mg corresponding to 3-fold increase with respect to that in the wild strain (152.2 μg/mg) while its specific CA yield gradually increased by time with a maximum of 4.3-fold higher titer (355.7 μg/mg) as compared to that of the wild type strain (83.5 μg/mg) at T_{168} ($p<0.05$).

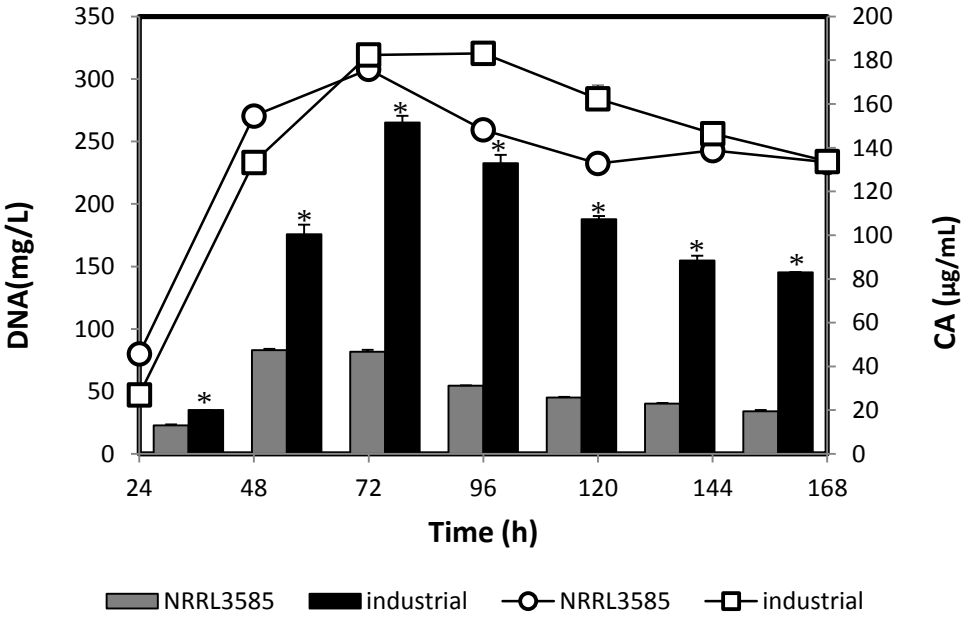


Figure 3.15. Time-dependent growth (symbols) and volumetric CA titers (bars) of NRRL 3585 and the industrial *S. clavuligerus* strain grown in SA medium, as determined by HPLC. (*: $p < 0.05$)

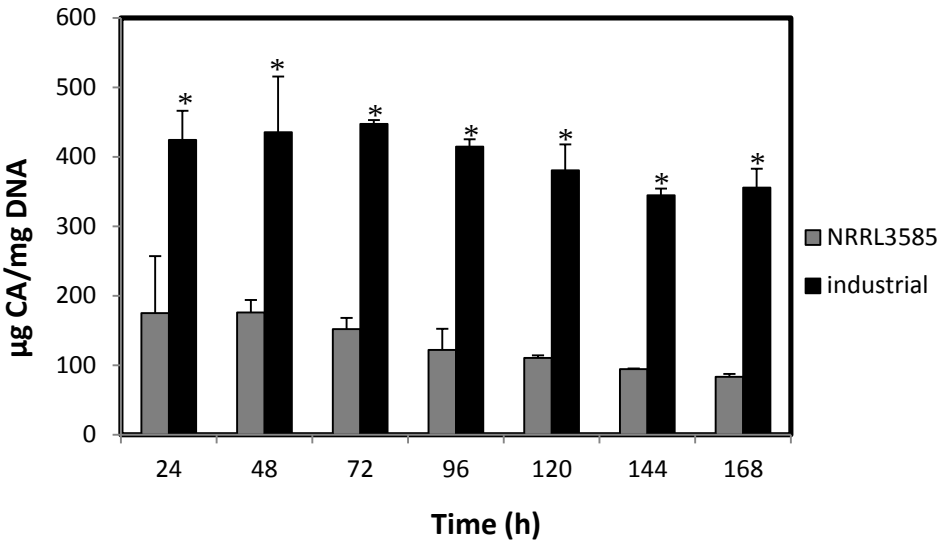


Figure 3.16. Comparison of specific CA titers of wild type and industrial strains, as determined by HPLC. (*: $p < 0.05$)

As mentioned in previous sections, *claR* overexpression has been shown to increase CA production in wild type *S. clavuligerus* cells by 2-fold when cloned in a multicopy plasmid vector (Perez – Redondo *et al*., 1998). During 168 h fermentation in SA medium, volumetric and specific CA titers of the recombinants WMA1, WMA2, WMA4, WMAG1, WMAG3 and WMAG4 were measured by bioassay. Growth determination via DNA quantification assays revealed no negative effect of the recombinant plasmid on the viability of recombinant strains (Figure 3.17).

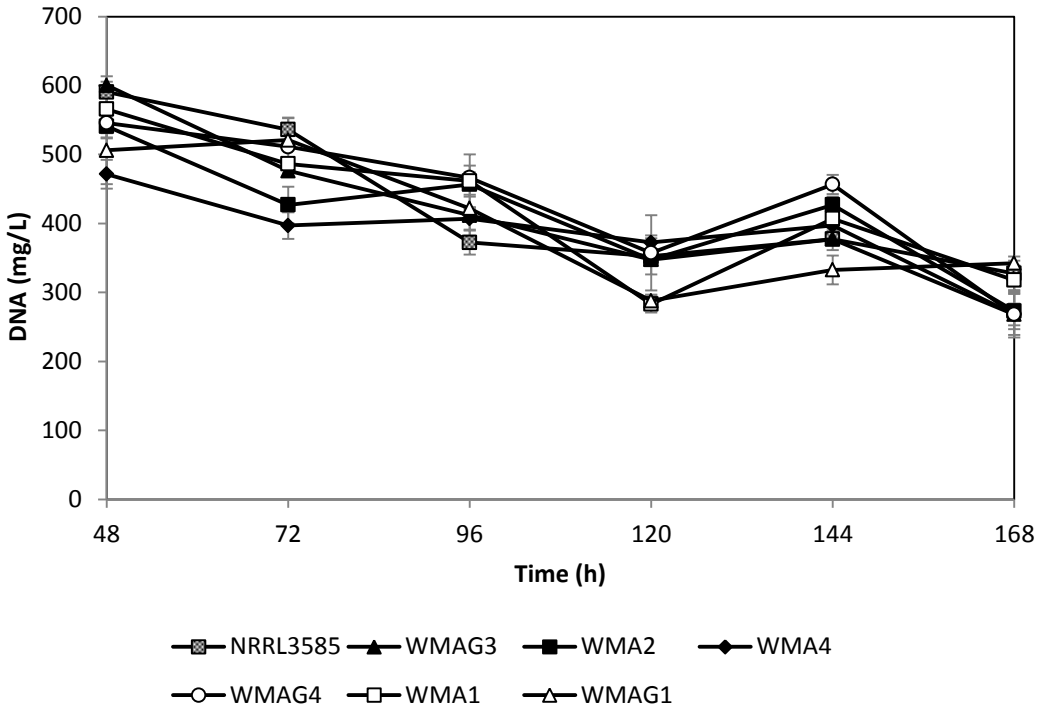


Figure 3.17. Time-dependent growth profile of NRRL 3585 and its recombinants, WMA4, WMA2, WMA1, WMAG4, WMAG3 and WMAG1 in SA defined medium.

Volumetric and specific CA titers of recombinants displayed variations such that two out of three recombinants bearing the plasmid pMAE02 (WMA2 and WMA4) and only one out of three recombinants having the plasmid pMAG03 (WMAG3) gave an elevated CA yield at various stages of fermentation (Figure 3.18 and 3.19). On the other hand, WMA4, WMA2 and WMAG3 exhibited ca. 2.7 (T_{48}), 2.8 (T_{48}) and 1.6-fold (T_{48}) increase in volumetric and 3.1 (at T_{48}), 2.4 (at T_{48}) and 1.6-fold (at T_{48}) increase in specific CA production ($p < 0.05$).

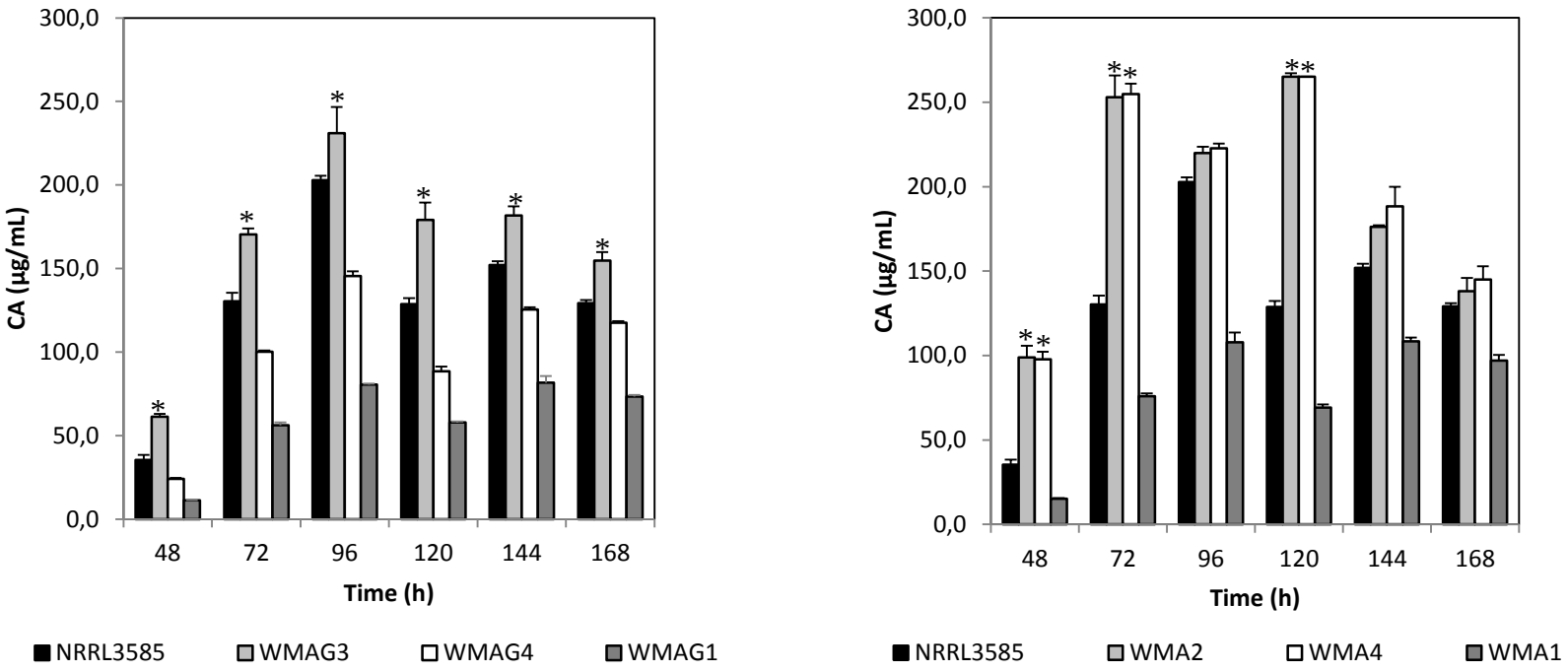


Figure 3.18. Comparison of volumetric CA titers of NRRL 3585 and recombinant strains WMA4, WMA2, WMAG3, WMAG4, WMA1 and WMAG1 when grown in SA defined medium, as determined by bioassay (*: $p < 0.05$).

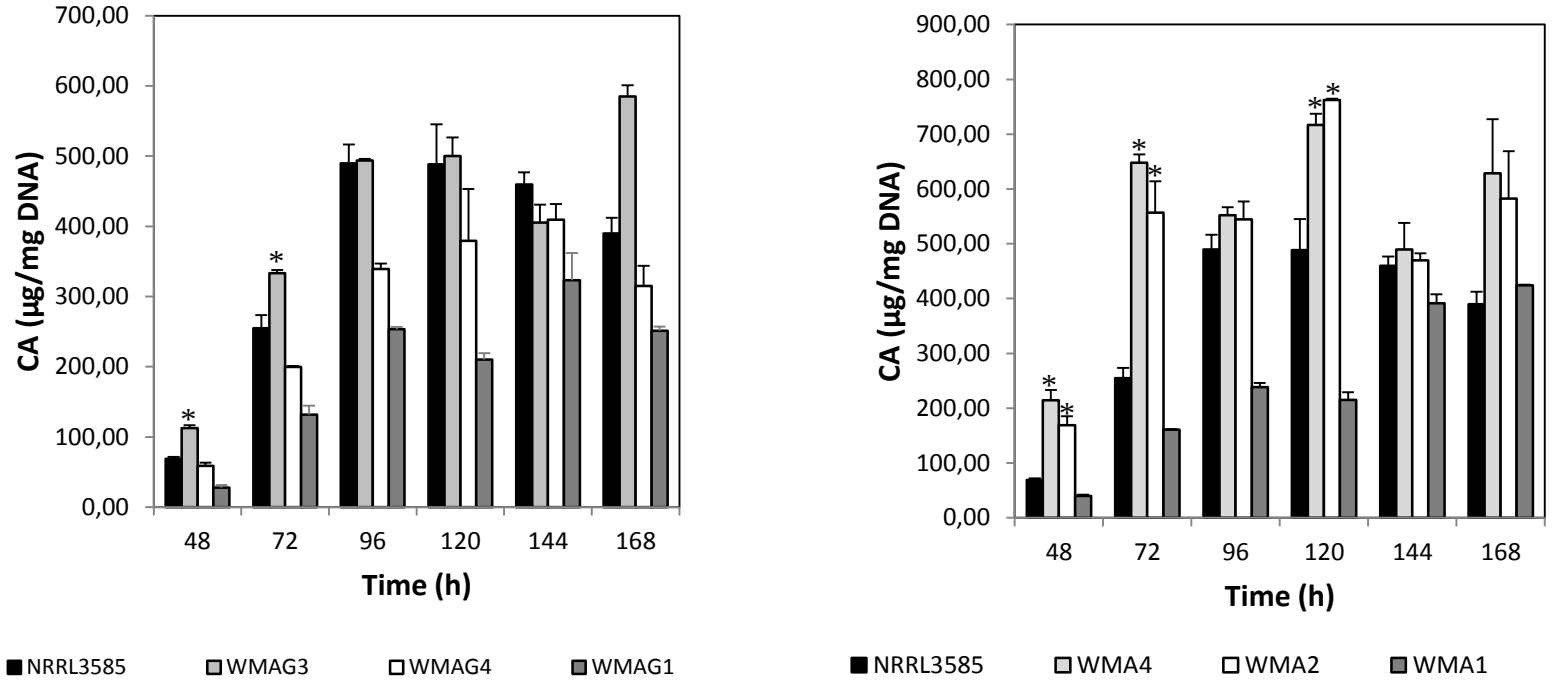


Figure 3.19. Comparison of specific CA titers of NRRL 3585 and recombinant strains WMA4, WMA2, WMA1, WMAG3, WMAG4 and WMAG1 grown in SA medium, as determined by bioassay.

Both volumetric and specific CA titers of WMA1, WMAG1 and WMAG4 were significantly lower or equal to those of NRRL 3585 and showed variations throughout the fermentation (Figure 3.18 and 3.19), which can be explained by the fact that overexpression of a recombinant gene on a plasmid vector may significantly affect the host cell physiology and hinder the secondary metabolite production. This phenomenon, also known as the "metabolic burden", is thought to arise due to the extra cost to synthesize and maintain the plasmid vector within the cell (Baltz, 1998; Balderas-Hernandez *et al*., 2009; J. A. Williams *et al*., 2009; Özcengiz *et al*., 2010). Moreover, the variable number of *attB* sites in *S. clavuligerus* chromosome (Bierman *et al*., 1992) may affect the overexpression of *claR* by integration vector pSET152^{ermE*} because of the differences in the insert copy of the gene integrated into the chromosome. The mycelial nature and the morphological differentiation of *Streptomyces* spp. are among the main reasons of fluctuation in antibiotic fermentations and this situation has been frequently experienced in our laboratory (Yılmaz *et al*., 2008; Vanli, 2010; Özcengiz *et al*., 2010; Kurt, 2011) as well as in other laboratories (Minas *et al*., 2000; Büchs, 2001). Hence, it has been hypothesized that splashing of the liquid medium, resulting with the growth of cells through the walls of baffled Erlenmeyer flasks in shaken cultures might have also contributed to fluctuations (Büchs, 2001; Betts and Baganz, 2006).

In order to observe the effect of *claR* overexpression in the industrial *S. clavuligerus* strain, recombinants and the parental industrial strain were grown in CC3 fermentation media and their CA yields were initially compared by bioassay. Of 35 total recombinants screened, MA11, MA16, MA28, MAG1, MAG2 and MAG4 strains exerted significantly higher specific CA titers with respect to the industrial strain as determined by CA bioassays (Figure 3.20 and 3.21), and were regrown in CC3 fermentation medium to measure their CA titers via HPLC analysis. CC2 and CC3 media contain soybean flour (20g/L) as the only nitrogen

source and dextrin (10g/L) plus glycerol triolate (GTO) as carbon sources. L-arginine, which is present in high amounts in soybean flour is a precursor of CA biosynthesis and has been shown to be the most essential nutrient that favors CA biosynthesis (Butterworth, 1984; Mayer and Deckwer, 1996).

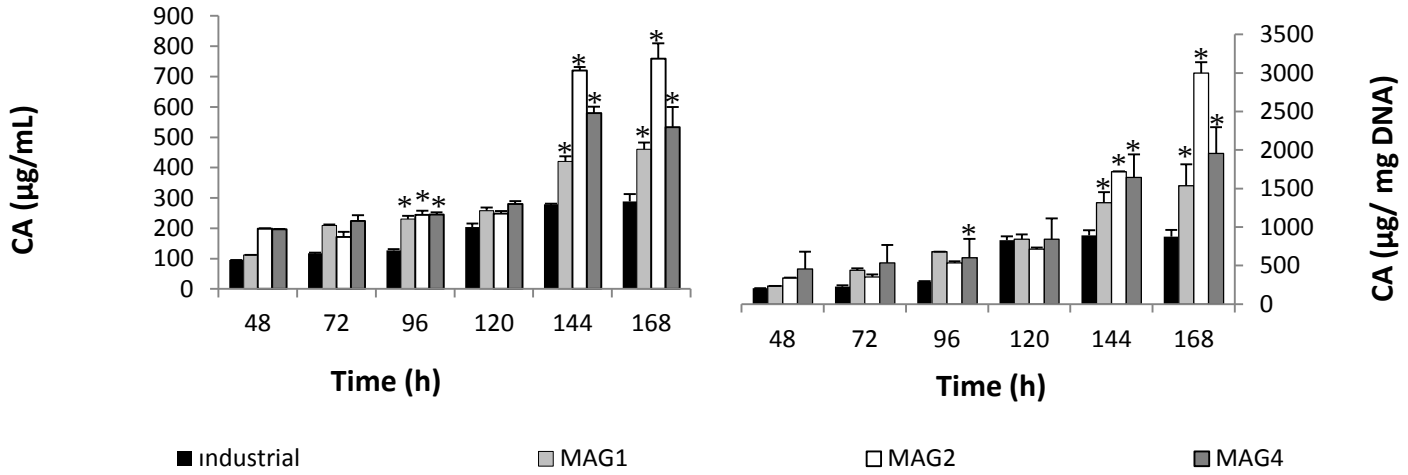


Figure 3.20. Comparison of volumetric and specific CA titers of recombinant strains MAG1, MAG2, MAG4 and their parental industrial strain when grown in CC3 medium, as determined by bioassay (*: $p < 0.05$).

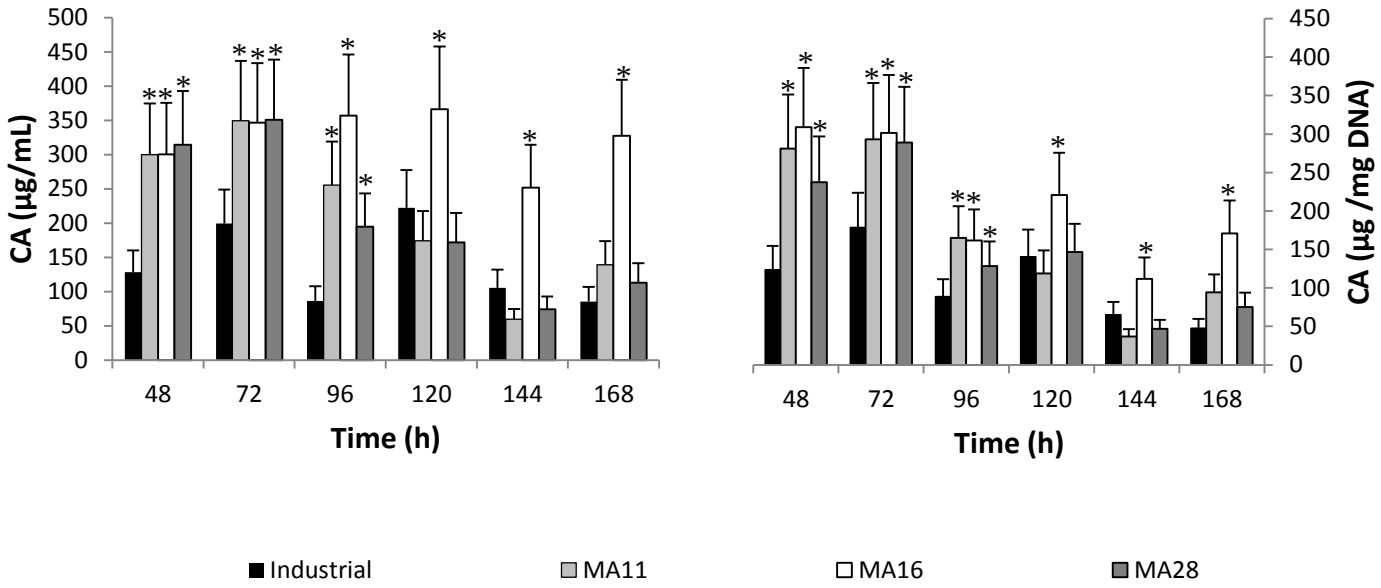


Figure 3.21. Comparison of volumetric and specific CA titers of recombinant strains MA11, MA16, MA28 and their parental industrial strain when grown in CC3 mediaum, as determined by bioassay (*: $p < 0.05$).

DNA quantification of samples revealed significantly enhanced growth along with a significant fluctuation in DNA levels of samples in comparison to that in the SA medium (Figure 3.22). Although CC2 and CC3 fermentation media favor the production of CA and provide a rich nutrient environment for cells, the nature and the exact composition of several ingredients are not completely defined like those in SA medium. Using inexpensive carbon and nitrogen sources to enhance commercial fermentations is a common approach; however, this may alter the fermentation performance because of lot-to-lot variation inherently associated with these ill-defined components (Zhang and Greasham, 1999).

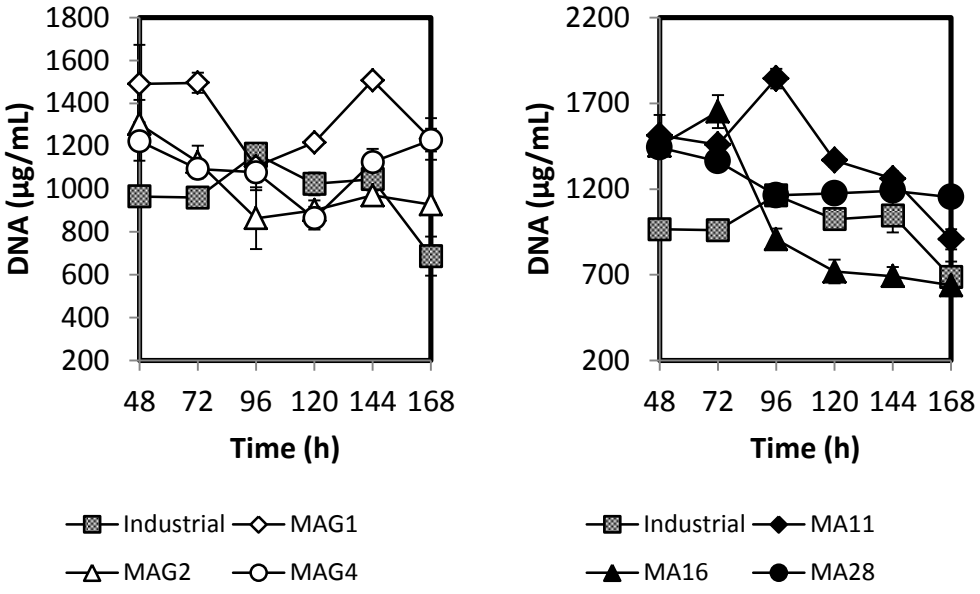


Figure 3.22. Time-dependent growth profile of industrial and recombinant *S. clavuligerus* strains MAG1, MAG2, MAG4, MA11, MA16 and MA28 when grown in CC3 fermentation medium.

Although a significant increase in volumetric CA yields were observed in the recombinants derived from wild type NRRL 3585 strain, a similar case were not observed with the industrial strain and its derived recombinants, especially after HPLC analysis results were evaluated. The most significant increase in the volumetric CA titers were provided by MA28 strain after 96^{th} and 120^{th} h of fermentation as being 1.3-fold and 1.4-fold ($p < 0.05$), respectively, with respect to that in the industrial strain (Figure 3.23). However, analysis of the data belonging to MA28 was failed to show statistical significance at $\alpha = 0.05$ and requires re-measurement with increased sample numbers to confirm the elevated CA levels.

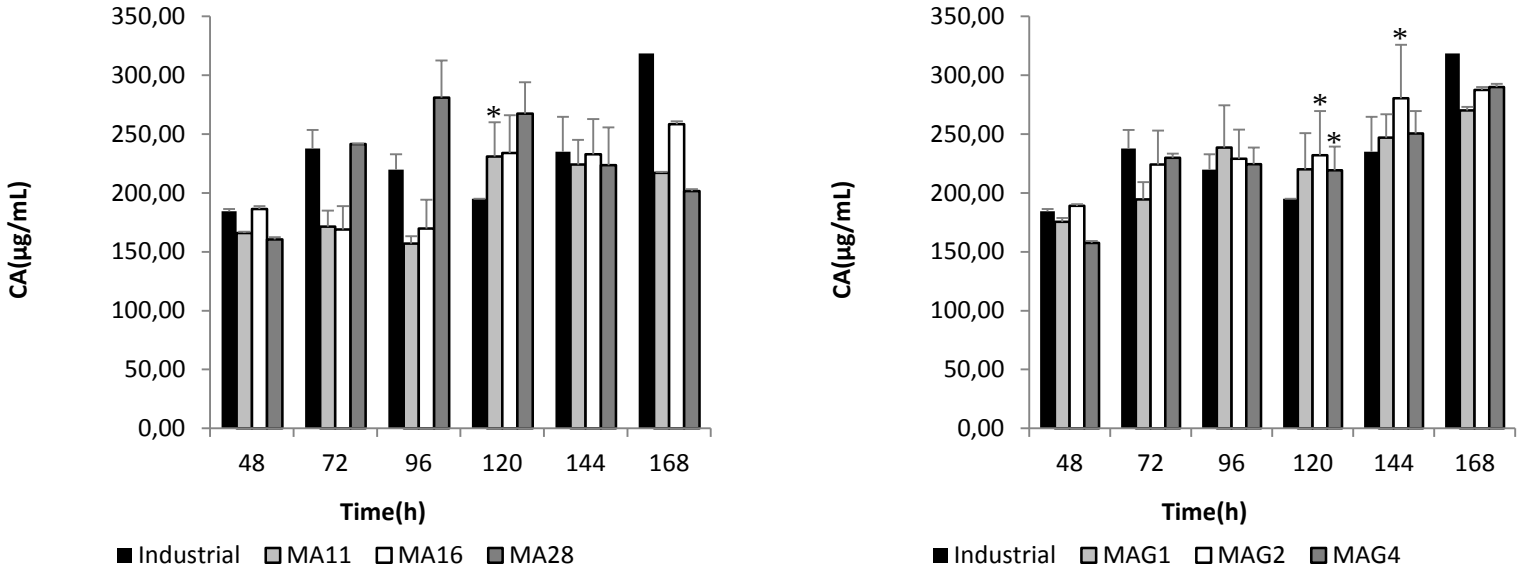


Figure 3.23. Comparison of volumetric CA titers of industrial parental strain and recombinant MA11, MA16, MA28, MAG1, MAG2 and MAG4 strain when grown in CC3 fermentation medium, as determined by HPLC (*: $p < 0.05$).

Since specific production is a better representation of production capabilities of recombinant cells, it provides a more reliable comparison between the recombinants and the industrial *S. clavuligerus* strain regarding CA yields. While the fluctuation of CA levels is a major drawback for comparison, it can be clearly observed that the recombinant strain MA16 had significantly higher levels of specific CA production, with 1.7-fold (325.48 μg/mg) and 1.5-fold (336.64 μg/mg), higher levels than that of the industrial strain (190.63 μg/mg and 224.38 μg/mg); especially after 120^{th} and 144^{th} h of fermentation. Another strain exerting high level of CA was MAG2 with 1.4-fold higher CA titer as compared to that of the industrial strain after 120^{th} h of fermentation. However, statistical analysis of the data failed to show the significance at $\alpha = 0.05$ except for the MAG2 at T_{120}. Thus, increasing biological replicate numbers for MA16 in future fermentation is necessary to confirm the specific CA titer increases (Figure 3.24).

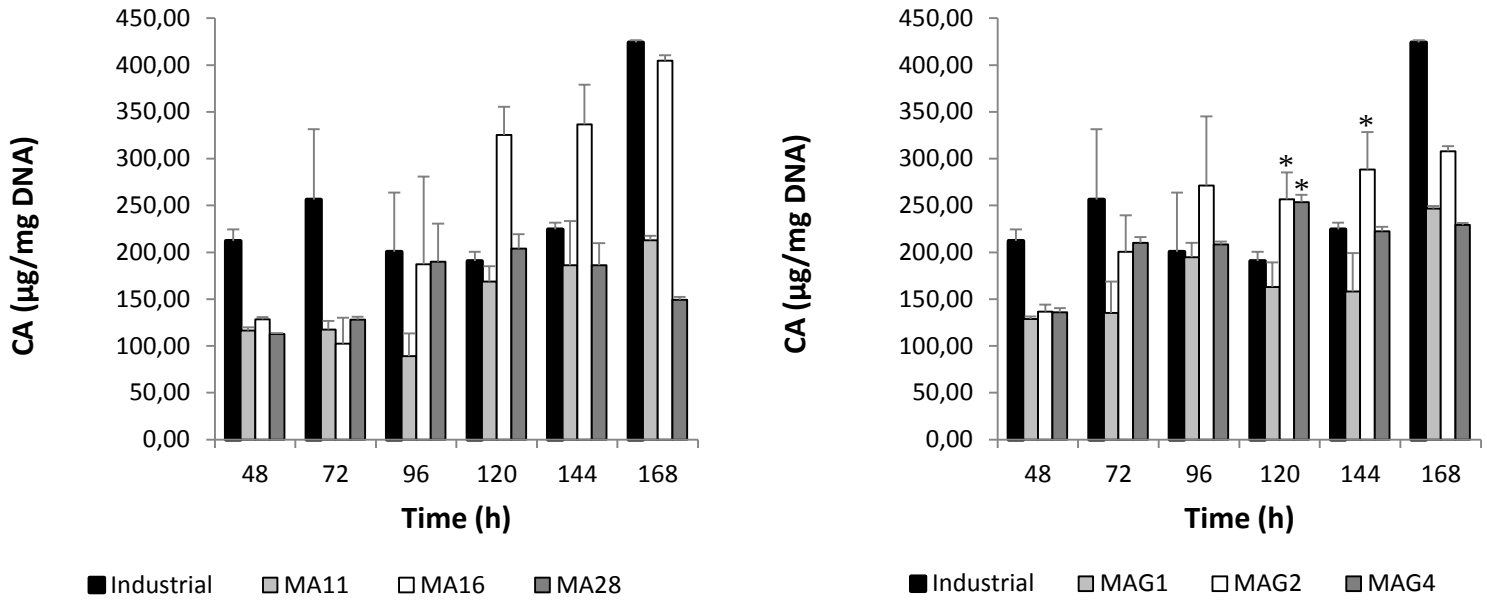


Figure 3.24. Comparison of specific CA titers of industrial parental strain and recombinant MA11, MA16, MA28, MAG1, MAG2 and MAG4 strains when grown in CC3 fermentation medium, as determined by HPLC (*: $p < 0.05$).

Taken together, it can be stated that the recombinant strains containing the pMAE02 and pMAG03 were partially successful in overproducing CA as compared to their parental counterparts. Despite the attempts to reduce the fluctuations in the growth and CA levels, species specific and method specific variations are major drawbacks for comparison of CA titers of the strains which are the most puzzling part of inspecting antibiotic production (Minas *et al*., 2000). In addition to the factors mentioned earlier, studying with an industrial overproducer strain has its own challenges due to lack of genetic and metabolic setup after being subjected to numerous cycles of random mutation and selection procedures (Smolke, 2010). At the molecular level, such directed evolution studies may result in increase in the stability or catalytic activity of proteins via single amino acid mutations; or elevated expression levels of either single genes or whole gene clusters (Dean and Thornton, 2007). The former scenario is hardly possible in the industrial strain that is used in this study since DNA sequencing analysis of *claR* gene showed no difference in nucleotide sequence as compared to that of wild type gene. However, it is still possible that the cis-acting elements where *claR* interacts with late clavulanic acid genes may have altered in a way to enhance the function of *claR*. In a recent study, transcriptome analysis of an industrial clavulanic acid overproducer *S. clavuligerus* strain has been performed as compared to wild type ATCC 27064 strain. The results were intriguing in the sense that most of the changes were located in the genes of clavulanic acid/cephamycin C supercluster, where most of the rational strain improvement studies have focused earlier on. Moreover, the expression of pathway specific regulatory *claR* and *ccaR* genes were also elevated in the overproducer strain (Medema *et al*., 2011). Hence, it is possible to deduce that the industrial strain used in our study may already have increased levels of *claR* expression or a more potent activator, such as *ccaR* or *AdpA*, which would hinder further manipulation of expression or makes its effect on CA yield harder to detect as it might not be reflected in the phenotype as it would be in wild type strain. Therefore, performing transcriptional analysis of *claR* prior to fermentation would be a better

approach in selection of recombinants which can be integrated into further studies on candidates obtained in this study.

CHAPTER 4

CONCLUSION

- Wild type *S. clavuligerus* NRRL 3585 and CA overproducer industrial strain were engineered either by *claR* overexpression via chromosomal integration with pSET152^{ermE*} vector or expressing it under strong glycerol promoter of pSPG expression vector. The recombinants were designated as *S. clavuligerus* WMA1, WMA2, WMA4, WMAG1, WMAG3, WMAG4 and MA11, MA16, MA28, MAG1, MAG2 and MAG4, referring to their respective parental strains and the vector constructs they contain. Then, the CA production capabilities of recombinants were compared with respect to those of their parental strains either via CA bioassay or HPLC analyses.

- Prior to fermentation experiments done with recombinant strains, CA titers of wild type and industrial *S. clavuligerus* strain were compared by HPLC analysis and 3-fold volumetric and 4.3-fold specific higher CA production was observed in the industrial strain.

- The effect of *claR* overexpression on wild type NRRL 3585 cells were tested by performing CA bioassay with samples obtained from cultures grown in SA defined media. Of 6 recombinants screened, WMA4, WMA2 and WMAG3 recombinant strains derived from NRRL 3585, exhibited 3.1-, 2.4- and 1.6-fold increase in specific CA production at T_{48}.

- Based on the preliminary bioassays performed on total of 35 recombinant strains derived from the industrial *S. clavuligerus* strain, MA11, MA16, MA28, MAG1, MAG2 and MAG4 were selected as candidates for HPLC analysis as they exerted significantly higher CA titers with respect to their parental strain.

- Fine measurement of CA titers via HPLC analyses have failed to match with the results obtained via bioassay studies as the highest improvement in volumetric CA titers was obtained with MA28 strain as 1.4-fold (T_{120}). However, specific CA titers of MA16 and MAG2 were fairly high as 1.7-fold (325.48 µg/mg at T_{120}) and 1.3-fold (256.75 µg/mg at T_{120}) with respect to the parental industrial strain (190.63 µg/mg at T_{120}), while the remaining recombinant strains were not able to match with the industrial strain in terms of CA yields.

- Chromosomal integration and expression of *claR* under strong glycerol promoter was partially successful in obtaining superhosts from CA overproducer industrial strains as there is lack of information in terms of metabolic and genetic background of the host strain and fluctuations resulting from strain specific and method specific factors.

- In future studies, to gain a better perspective about the result of overexpression of *claR* in the industrial strain, expression levels of *claR* as well as the structural genes of clavulanic acid biosynthetic cluster can be analyzed in exconjugants prior to fermentation.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

A.1. Liquid Media

Luria Broth (LB)	**g/L**
Luria Broth	25

15 min sterilization at 121°C

Tryptic Soy Broth (TSB)	**g/L**
Tryptic Soy Broth	30

15 min sterilization at 121°C

Starch-Asparagine (SA) Medium (Aharonowitz and Demain, 1979)

Following components were added into 600 mL boiling dH_2O with continuous stirring

	g/L
Starch	10
MOPS	21
K_2HPO_4	4.4

After completely dissolving the components, the solution was cooled to RT, pH was adjusted to 6.8 and the volume was completed to 800 mL, followed by 15 min sterilization at 121°C

After sterilization, the following sterile components were added

	mL/L
L-Asparagine (10 g/L)	200
$MgSO_4$. $7H_2O$ (0.6 g/mL)	2
Trace element solution*	1

Trace element solution	**g/L**
$FeSO_4$. $7H_2O$	1
$MnCl_2$. $4H_2O$	1
$ZnSO_4$. $7H_2O$	1
$CaCl_2$. $3H_2O$	1.3

CC2 Vegetation Medium	**g/L**
Soy flour	20
Dextrin	10
KH_2PO_4	0.6
GTO	5
pH was adjusted to 7.6 – 8.10 with $NaOH/H_2SO_4$	
20 min sterilization at 121°C	

CC3 Fermentation Medium	**g/L**
Soy flour	20
Dextrin	10
KH_2PO_4	0.6
GTO	5
MOPS	10.5
Oligo elements solution*	10mL
pH was adjusted to 6.8 – 7.2 with $NaOH/H_2SO_4$	
20 min sterilization at 121°C	

*Oligo elements solution	**g/L**
$CaCl_2$	10
$MgCl_2 .6H_2O$	10
$FeCl_3$	3
$ZnCl_2$	0.5
$MnSO_4. H_2O$	0.5
NaCl	10

A.2. Solid Media

LB agar	**g/L**
Luria Broth	25
Agar	15
15 min sterilization at 121°C	

TSA	**g/L**
Tryptic Soy Broth	30
Agar	20
15 min sterilization at 121°C	

Mannitol Soya flour (MS) agar (Hobbs *et al*. 1989)

	g/L
Soy flour	20
Agar	20
Mannitol	20
15 X 2 min sterilization at 121°C	

APPENDIX B

BUFFERS AND SOLUTIONS

B.1. Plasmid and Chromosomal DNA Isolation

SET buffer

NaCl	75 mM
EDTA (pH 8.0)	25 mM
Tris – HCl (pH 7.5)	20 mM

TE buffer

Tris – HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

STET buffer

Sucrose	0.3 mM
Tris – HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM

Lysis solution

NaOH	0.3 mM
SDS	2 %

Phenol – chloroform solution (water saturated) (Hintermann, 1981)

Phenol	500 g
Chloroform	500 mL
dH_2O	400 mL

Stored at RT and kept in dark.

B.2. Agarose Gel Electrophoresis

Tris-Acetate-EDTA (TAE) buffer (50X)	
Tris base	242 g
Glacial acetic acid	57.1 mL
EDTA (0.4 M, pH 8.0)	125 mL
dH_2O added to	1000 mL

Loading buffer (6X)	
Bromophenol blue	0.25 %
Xylene cyanol FF	0.25 %
Sucrose in water	40 %

Ethidium bromide solution	
Ethidium bromide (10 mg/mL)	100 µL/L
TAE buffer (1X)	1 L

B.3. Selection of Recombinant Colonies

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)

X-Gal	20 mg
Dimethylformamide	1 mL

Stored in -20 °C and protected from light

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
dH_2O	1 mL

Filter sterilized and kept at -20 °C

B.4. Competent *E. coli* Preparation

Buffer 1

Potassium acetate	30 mM
$RuCl_2$	100 mM
$CaCl_2$	10 mM
Glycerol (87 %)	8.6 mL

Complete to 50 mL with dH_2O and filter sterilize

Buffer 2

MOPS	10 mM
$RuCl_2$	10 mM
$CaCl_2$	75 mM
Glycerol (87 %)	8.6 mL

Adjust pH to 6.5 with 0.2M KOH. Complete to 50 mL with dH_2O and filter sterilize

B.5. Growth determination via DNA quantification

Diphenylamine reagent

Diphenylamine	1.5 g
Glacial acetic acid	100 mL
Cover and protect from light	
Add the following reagents prior to use	
Concentrated H_2SO_4	1.5 mL/100 mL
Acetaldehyde (1.6 %)	0.1 mL/20 mL

Aqueous acetaldehyde

Acetaldehyde	1 mL
dH_2O	49 mL

1 N $HClO_4$

$HClO_4$ (80%)	10.87 mL/100 mL
or	
$HClO_4$ (60 %)	16.74 mL/100 mL

B.6. Bioassay and HPLC Analysis

1 M MOPS	**g/L**
MOPS	20.9 g
dH_2O	1000 mL

Adjust pH to 6.8 with NaOH, filter the solution and protect from light

Sodium acetate solution	**g/L**
Sodium acetate	4.1 g

Adjust pH to 6.0 with acetic acid and filter the solution

Sodium dihydrogen phosphate solution	**g/L**
NaH_2PO_4. H_2O	15 g

Adjust pH to 4.0 with 50% phosphoric acid and filter the solution

Mobile phase	
NaH_2PO_4 solution (pH 4.0)	95 %
Methanol (HPLC grade)	5 %

B.7. Antibiotic Stocks

	Stock concentration	**Final concentration**
Ampicillin	100 mg/ mL dH_2O	100 µg/ mL
Kanamycin	50 mg/ mL dH_2O	25 or 50 µg/ mL
Chloramphenicol	25 mg/ mL ethanol	25 µg/ mL
Penicillin G	10 mg/ mL dH_2O	10 µg/ mL
Apramycin	50 mg/ mL dH_2O	50 µg/ mL

APPENDIX C

CHEMICALS AND SUPPLIERS

C.1. Chemicals

Acetaldehyde	Sigma
Agar	Sigma
Agarose	Prona
Ampicillin	Sigma
Apramycin	Sigma
Bromophenol blue	Sigma
$CaCl_2$. $2H_2O$	Merck
Chlorofom	AppliChem
Chloramphenicol	Sigma
Dimethylformamide	Merck
Diphenylamine	Sigma
DMSO	Sigma
EDTA	Sigma
Ethanol	Botafarma
Ethidium bromide	Sigma
$FeSO_4$. $7H_2O$	Merck
Glacial acetic acid	Merck
Glycerol	Merck

Herring sperm DNA	Sigma
HCl	Sigma
$HClO_4$	Merck
H_2SO_4	Merck
IPTG	Sigma
Isoamylalcohol	AppliChem
Isopropanol	Merck
Kanamycin	Sigma
K_2HPO_4	Merck
KH_2PO_4	Merck
L-Asparagine	Sigma
Luria Broth	Sigma
Mannitol	Merck
Methanol (HPLC grade)	Sigma
$MgCl_2$	Merck
$MgSO_4$. $7H_2O$	Merck
$MnCl_2$. $4H_2O$	Merck
MOPS	Sigma
Sodium acetate	Merck
NaCl	Sigma
NaH_2PO_4	Merck
Nalidixic acid	Sigma
NaOH	Merck
Orto-phosphoric acid	Merck
Penicillin G	Sigma

Phenol (water-saturated)	AppliChem
$RuCl_2$	Merck
SDS	Merck
Soybean flour	Commercial
Starch	Merck
Sucrose	Merck
Tris-HCl	Merck
Tryptic Soy Broth	Oxoid
X-Gal	Fermentas
Xylene cyanol FF	Sigma
$ZnSO_4$. $7H_2O$	Merck

C.2. Enzymes

Lysozyme	Sigma
Proteinase K	Sigma
*Eco*RI	Roche
*Bam*HI	Roche
*Nde*I	Roche
*Spe*I	Roche
Taq polymerase	Fermentas
Phusion DNA polymerase	NEB
T4 ligase	Fermentas
T4 ligase of pGEM-T Easy	Promega

C.3. DNA size markers

*Pst*I digested/ Lambda DNA ladder	Fermentas
O'GeneRuler 100 bp DNA ladder	Fermentas

C.4. Kits

Plasmid isolation kit (Mini)	Fermentas
Gel elution kit	GeneMark
pGEM-T Easy vector	Promega