INTEGRATION OF CLAVAMINATE SYNTHASE 2 GENE INTO THE CHROMOSOME OF AN INDUSTRIAL STRAIN OF *STREPTOMYCES CLAVULIGERUS* FOR ENHANCED CLAVULANIC ACID PRODUCTION

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INTEGRATION OF CLAVAMINATE SYNTHASE 2 GENE INTO THE CHROMOSOME OF AN INDUSTRIAL STRAIN OF *STREPTOMYCES CLAVULIGERUS* FOR ENHANCED CLAVULANIC ACID PRODUCTION

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ABSTRACT

INTEGRATION OF CLAVAMINATE SYNTHASE 2 GENE INTO THE CHROMOSOME OF AN INDUSTRIAL STRAIN OF *STREPTOMYCES CLAVULIGERUS* FOR ENHANCED CLAVULANIC ACID PRODUCTION

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Streptomyces clavuligerus is a gram-positive, filamentous bacterium which has a great ability to produce secondary metabolites including isopenicillin N, cephamycin C and a beta-lactamase inhibitor clavulanic acid. Clavulanic acid (CA) which is a bicyclic beta-lactam, inhibits most of class A beta-lactamases by binding irreversibly to the serine hydroxyl group at the active center of beta-lactamases and resulting in the stable acyl-enzyme complexes. Biosynthesis of CA is an eight step pathway and uses L-arginine and 3-phosphoglyceraldehyde as precursors. In *Streptomyces clavuligerus* a hierarchy of regulatory genes act in the regulation of secondary metabolite biosynthesis and sporulation. Gamma-butyrolactone and stringent response, as the global regulatory systems, are at the top of this regulation hierarchy, where pathway-specific transcriptional regulators are at the bottom of the cascade.

Clavaminate synthase (CAS) is one of the best characterized enzymes in the CA biosynthesis pathway regarding its mechanism, activity and structure. It exists as two isoenzymes, CAS1 and CAS2. *cas1* gene is located in clavam biosynthetic gene

cluster and its expression is nutritionally regulated. *cas2* gene encodes for the rate limiting CAS2 isoenzyme which catalyzes the three distinct oxidative transformations. Conversion of deoxyguanidinoproclavaminic acid into guadinoproclavaminic acid takes part in early steps of CA biosynthesis and is catalysed by CAS2. Similarly, proclaviminic acid is converted into claviminic acid by a two-step reaction and also catalysed by CAS2.

Integration of an extra copy of the *cas2* gene into the chromosome of a clavulanic acid over-producer industrial strain of *Streptomyces clavuligerus* by means of an integration vector to improve CA production capacity of the industrial strain is the main goal of this study. Via comperative CA analysis based on HPLC, it was estimated that the recombinant strains produced higher amount of CA in comparison to the parental industrial *S. clavuligerus* strain. The highest CA production achieved by the recombinant strain, namely *S. clavuligerus* GV61 (1583.3 μg/g) corresponded to more than 2 fold increase in the maximal CA titer of the parental strain.

Keywords: *Streptomyces clavuligerus*; clavaminate synthase 2, clavulanic acid, HPLC, choromosomal integration.

ÖZ

KLAVAMİNAT SENTEZ 2 ENZİM GENİNİN KLAVULANİK ASİT BİYOSENTEZİİNİ ARTIRMAK AMACIYLA *STREPTOMYCES CLAVULIGERUS*' UN ENDÜSTRİYEL BİR SUŞUNA KROMOZOMAL ENTEGRASYONU

Vanlı, Güliz

Yüksek Lisans, Biyoteknoloji Bölümü

Tez Yöneticisi: Prof. Dr. Gülay Özcengiz

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Gram-pozitif ve filamentöz bir bakteri olan *Streptomyces clavuligerus*, isopenisilin N, sefamisin C ve bir beta-laktamaz inhibitörü olan klavulanik asit (KA) gibi bir çok sekonder metaboliti sentezleyebilme yeteneğine sahiptir. 3R,5R stereokimyasına sahip bisiklik bir beta laktam olan klavulanik asit, beta-laktamazların aktif merkezinde bulunan serin hidroksil grubuna tersinmez olarak bağlanıp, ortaya stabil asil-enzim kompleksleri çıkarmak suretiyle A sınıfı bir çok beta-laktamazı inhibe eder. Klavulanik asit biyosentez yolu, öncül metabolitler olarak arjinin ve 3-fosfogliseraldehit kullanır. *Streptomyces clavuligerus*' da sekonder metabolit biyosentezi ve sporlanmanın kontrolü regülatör gen hiyerarşisiyle sağlanır. Bu regülasyon hiyerarşisinin en üst basamaklarını global regülatör sistemler olan gama-butirolakton ve stringent response, alt basamaklarını da biyosentez yoluna özgü transkripsiyonel regülatörler oluşturmaktadır.

Klavaminat sentez (CAS); mekanizması, aktivitesi ve yapısı göz önünde bulundurulduğunda KA biyosentez yolunun en iyi karakterize edilmiş enzimlerinden birisidir. CAS1 ve CAS2 izoenzimleri vardır. *cas1* geni klavam gen kümesinde yer alır ve ekspresyonu besiyeri içeriğine bağlı olarak düzenlenir. CAS2 üç farklı oksidatif transformasyonu katalizleyen hız sınırlayıcı bir enzimdir ve *cas2* geni tarafından kodlanmaktadır. KA biyosentez yolunun ilk reaksiyonlarından biri olan deoksiguanidinoproklavaminik asidin guadinoproklavaminik aside dönüştürülmesi CAS2 tarafından katalizlenir. Benzer şekilde, proklaviminik asit klaviminik asite iki aşamalı bir reaksiyonla dönüştürülür ve bu reaksiyon da CAS2 tarafından katalize edilir.

Bu çalışmanın ana hedefi *cas2* geninin çoğaltılıp, endüstriyel bir *Streptomyces clavuligerus* suşunun kromozomu içerisine bir integrasyon vektörü aracılığıyla aktarılması, ve endüstriyel *S. clavuligerus* suşunun KA üretim kapasitesinin artırılmasıdır. HPLC'ye dayalı karşılaştırmalı KA analizleri sonucunda elde edilen rekombinant suşların endüstriyel *S. clavuligerus* suşuna kıyasla daha yüsek oranda KA ürettiği saptanmıştır. En yüksek KA üretimi, 1583.27 µg/g ile *S. clavuligerus* GV61 adı verilen bir rekombinant suşa ait olup parental suş tarafından üretilen KA miktarında 2 kattan daha fazla bir artışa karşılık gelmektedir.

Anahtar Kelimeler: *Streptomyces clavuligerus*; clavaminat sentetaz 2, klavulanik asit, HPLC, kromozomal entegrasyon.

To My Family

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ATCC:	American Type Culture Collection
bp:	Base pairs
CA:	Clavulanic acid
CAS2:	Clavaminate synthase 2 enzyme
*cas*2:	Clavaminate synthase 2 gene
HPLC:	High performance liquid chromatography
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
kb:	Kilobase
NCBI:	National Center for Biotechnology Information
o/n:	Over night
RT:	Room temperature
X-Gal:	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
β-lactam:	Beta-lactam
γ-butyrolactone:	Gamma-butyrolactone

CHAPTER 1

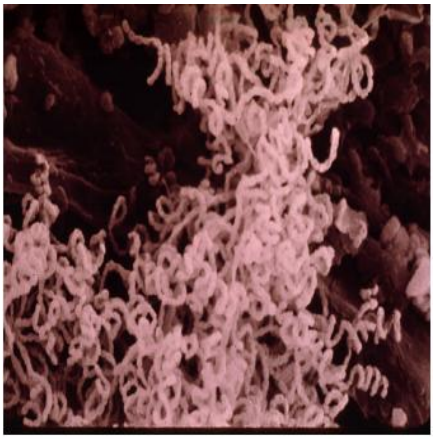
INTRODUCTION

1.1. The genus *Streptomyces*

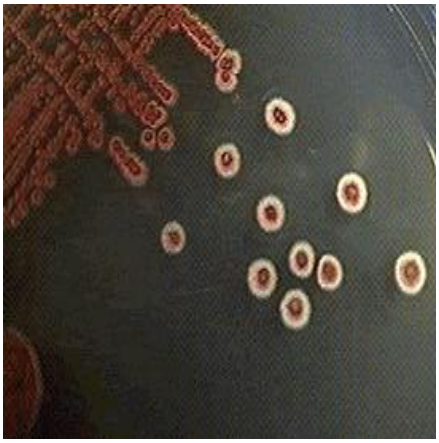
Streptomycetes are gram positive, spore forming soil bacteria and they belong to the order Actinomycetes (Paradkar *et al*., 2003). The actinomycetes are famous for their capability to synthesize great variety of secondary metabolites that have a wide range of bioactivities and thereby may find use as antibacterial and antifungal antibiotics, immunosuppressants, and anti-cancer agents (Paradkar *et al*., 2001; Challis and Hopwood, 2003). Members of the genus *Streptomyces* account for approximately 80% of the production of actinomycete antibiotics (Keiser *et al*., 2000).

The sequencing of *Streptomyces coelicolor* (A3) (Bentley *et al.*, 2002) and *Streptomyces avermitilis* (Ikeda *et al.*, 2003) reveals a large genome (8.8 Mbp and 9.0 Mbp, respectively), and the protein coding sequences (ORFs) are typified by possessing a high G + C content (>70%). Large linear plasmids (10-600 kb) are distinctive for the genus (Keiser *et al*., 2000, Paradkar *et al*., 2003).

A remarkable feature of *Streptomyces* spp. is their presence of strong restriction modification systems, constituting a barrier to transfer DNA from non streptomycetes origins to *Streptomyces* hosts, which makes them hardly transformable (Matsushima *et al*., 1987). The life cycle of *Streptomyces* spp. involves a series of morphological changes (Figure 1.1). The biosynthesis of secondary metabolites, including antibiotics, is coinciding and coordinately regulated with morphological changes (Paradkar *et al*., 2003).



(a)



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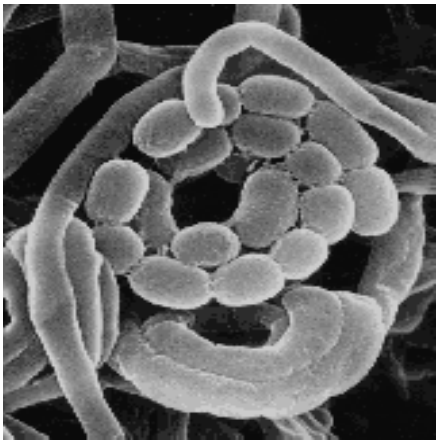


(c)

(d)

Figure 1.1. Morphological differentiation of *Streptomyces* spp.

(a) The filamentous mycelium of *Streptomyces* (b) *Streptomyces* colonies, (c) *Streptomyces* spores, (d) An antibiotic droplet secreted from a *Streptomyces* colony.

(a) http://biology.kenyon.edu/Microbial_Biorealm/bacteria/grampositive/streptomyces/streptomyces.html.

(b) and (c) Brock and Madigan, 1988.

(d) http://www.micro. msb.le.ac.uk/video/Streptomyces.html.

Streptomycetes are strikingly different from most of other bacteria; however, they are apparently more evocative of filamentous fungi. They grow by tip extension to form a mycelium of branched hyphae (Flärdh, 2003). Their life cycle starts with the germination of a single spore (Figure 1.2). This spore produces one or more multi-nucleoid filaments. This complex network of filaments creates a flat colony of multinucleoid hyphae, known as the substrate mycelium. This phase is usually referred as the vegetative growth phase. In response to appropriate signals, the substrate mycelium breaks the surface barrier and produces aerial hyphae, sometimes deforming into helical structures (Goriely and Tabor, 2003). Aerial growth is accompanied by the generation of secondary metabolites in cultures grown on solid media (Chater, 1989). The aerial hyphae undergo extensive cellular division, eventually dividing into uni-nucleoidal compartments, which then mature into a spore (Goriely and Tabor, 2003).

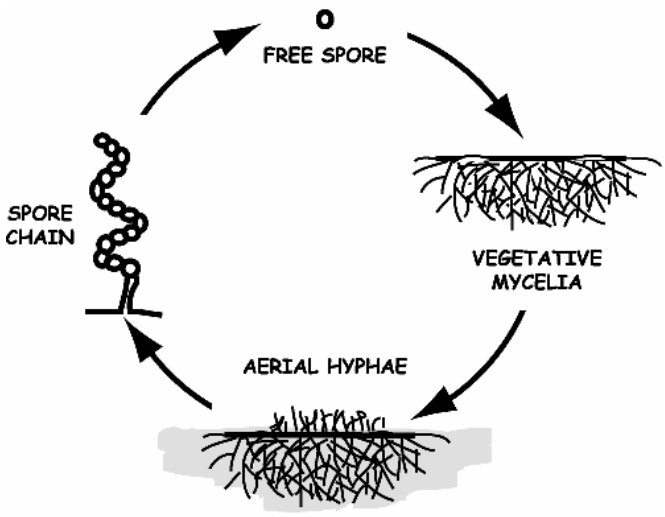


Figure 1.2. Life cycle of *Streptomyces* spp. (http://biology.kenyon.edu/courses/biol114/Chap11/Chapter_11A.html).

Secondary metabolite production in *Streptomyces* spp. is associated with low specific growth rates, and it is the lowering of specific growth rate that signals a cascade of regulatory events resulting in chemical and morphological differentiation (Jonsbu *et al*., 2002).

1.2. Secondary metabolites of *Streptomyces clavuligerus*

Streptomyces clavuligerus produces two major groups of β-lactam compounds as secondary metabolites; the sulphur containing and oxygen containing. The sulphur-containing β-lactam compounds have antibiotic properties and include isopenicillin N, desacetoxycephalosporin C and cephamycin C (Figure 1.3). The oxygen-containing β-lactam compounds are called as clavams and include clavulanic acid as well as a number of related compounds.

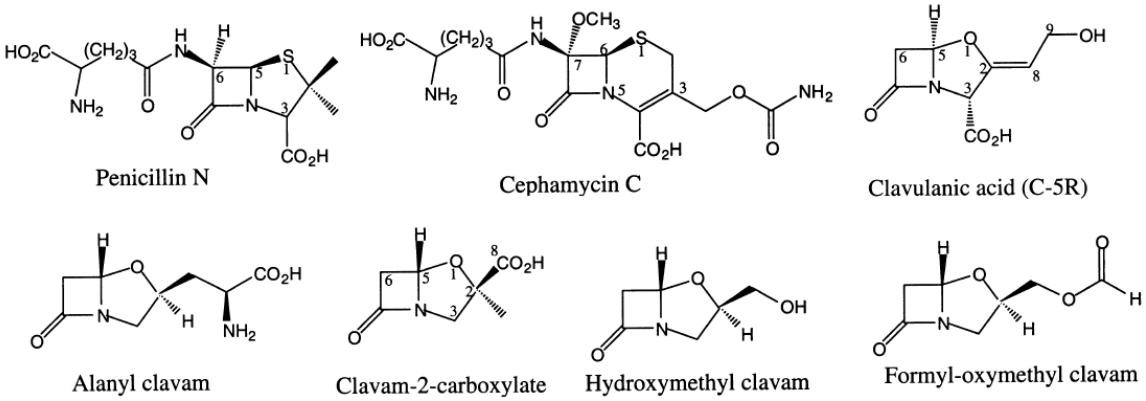


Figure 1.3. β-lactam products of *S. clavuligerus* (Thai *et al*., 2001).

S. clavuligerus also produces the non-β-lactam antibiotics such as N-propionylholothin, tunicamycin and holomycin with a pyrrothine structure, which has antitumor activity (Okamura *et al*., 1977; Kenig and Reading, 1979).

1.2.1. Importance of β-lactam antibiotics

β-lactams constitute probably the most important class of antibiotics for several reasons. First of all, penicillin, the first antibiotic discovered by Alexander Fleming in 1928, is a β-lactam. Despite a growing number of antibiotics and the incidence of penicillin-resistant isolates, β-lactams are still the most frequently used antibiotics. Second, β-lactams have low tocixity and high specifity (Brakhage, 1998). This is because they inhibit peptidoglycan synthesis via a reaction that does not exist in the eukaryotic world (Glazer and Nikaido, 1998). β-lactams inhibit the transpeptidation reaction required for peptidoglycan cross-linking, by acting as structural analogues of the D-alanyl-D-alanine terminus of the stem peptide, which is the substrate of the transpeptidase enzyme (Tipper and Strominger, 1965).

The discovery and development of β-lactam antibiotics were significant achievements for the pharmaceutical industry in the 20th century. They have been used widely for treatment of several bacterial infections since 1940s. Today, penicillins are commodity-type products with annual production volumes exceeding 60,000 tons (Thykaer and Nielsen, 2003). However, the widespread use of β-lactam drugs has applied selection pressure to accelerate the evolution and spread of resistance to these drugs (Blazquez *et al*., 2002).

In both gram positive and gram negative bacteria, resistance against to β-lactams can develop via; (i) the decreased access to the bacterial cell, (ii) the destruction of the antibiotics by β-lactamases or, (iii) altered penicillin-binding proteins (PBPs) as in the case of the presence of low-affinity PBPs (Williams, 1999). Low affinity PBPs are modified versions of cell wall biosynthetic enzymes that fail to bind to the antibiotics due to their low binding affinity (Paradkar *et al*., 1996). Yet, the production of β-lactamases and the chemical transformation of β-lactams are the most prevalent cause of resistance to these antibiotics. The β-lactamases are structurally related to PBPs (Massova and Mobashery, 1998), and can be classified according to their sequence similarities, substrate preference, and whether they are encoded for by genes on a plasmid or chromosome. They can also be classified by

their mechanisms of action, such that class A (penicillinases), class C (cephalosporinases) and class D (oxacillinases) β-lactamases (Ambler, 1980). Members of classes A, C and D are active site serine enzymes and share a similar folding. Class B β-lactamases are metallo-enzymes and are believed to be evolved independently of the serine enzymes. A potent β-lactamase inhibitor clavulanic acid is active against certain β-lactamases of class A and D, but ineffective against class B β-lactamases (Walsh, 2003). Class A β-lactamases such as TEM-1 and SHV-1 are the most frequent plasmid-encoded enzymes in gram negative bacteria (Maiti *et al*., 2006). They catalyse very efficiently the irreversible hydrolysis of amine bond of the β-lactam ring, thus yielding biologically inactive compounds (Figure 1.4). Firstly, the enzyme interacts noncovalently with the antibiotic and forms the Michaelis complex. The β-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally liberates active enzyme and the hydrolyzed, inactive drug. This mechanism is used by β-lactamases of molecular classes A, C, and D, but class B enzymes utilize a zinc ion to attack the β-lactam ring (Livermore, 1995).

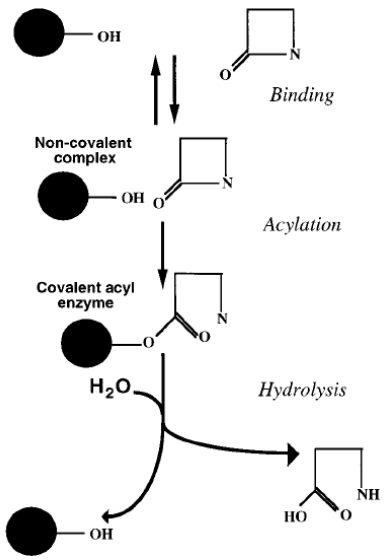


Figure 1.4. General catalytic pathway of serine β-lactamases (Livermore, 1995).

Mutants of the TEM and SHV β-lactamases have evolved; however, they are not susceptible to the action of these inhibitors. Therefore, there is still a need for novel inhibitors of these enzymes (Bradford, 2001).

1.3. The β-lactamase inhibitor clavulanic acid

Clavulanic acid is a very powerful β-lactamase inhibitor and was discovered independently by Napier *et al*. (1981) and Brown *et al*. (1986). Clavulanic acid is active against a wide spectrum of bacteria; however it possesses a weak antibiotic activity with minimum inhibitory concentration values of 25–125 μg/ml (Brown *et al*., 1986; Lee *et al*., 2002). Thus, it could not been convenient to administer clavulanic acid alone as an antibacterial product. Instead, it is co-formulated in conjunction with conventional β-lactam antibiotics. Clavulanic acid is now used as its potassium salt and prescribed clinically in combination with amoxycillin, as Augmentin™ and with ticarcillin as Timentin™ (Santos *et al*., 2009).

In vitro studies indicate that more than 50% of the penicillin-resistant *Staphylococcus aureus* strains became susceptible to 0.2 μg of benzylpenicillin in the presence of 5 μg of clavulanic acid per ml (Dumon *et al*., 1979). In addition, clavulanic acid has been reported to be 93 times more active than other β-lactamase inhibitor sulbactam and 8 times more active than tazobactam against the *S. aureus* enzyme (Payne *et al*., 1994).

1.4. Mechanism of β-lactamase inhibition by clavulanic acid

The X-ray structures reveal that clavulanic acid is a bicyclic β-lactam with 3*R*, 5*R* stereochemistry across the strained bicyclic ring, this feature is also common to penicillins (Howarth *et al.*, 1976; Brown *et al.*, 1984). In this respect, clavulanic acid acts as a very slow substrate for β-lactamases.

Clavulanic acid binds irreversibly to the serine hydroxyl group of the catalytic serine (Ser70) of β-lactamases by hydrogen bonding of its own C7 carboxyl group (Figure 1.5). This subsequently leaves the carbonyl carbon vulnerable to nucleophillic attack by Ser70, and yields an acyl-intermediate. The rupture of the β-lactam ring produces a stable acylated intermediate. This is followed by the opening of the five-membered oxazolidinic ring, resulting in the linearization of the inhibitor, as an imine intermediate. The imine intermediate then undergoes isomerization, and results in the formation of a *cis*-enamine. *cis*-enamine further isomerises to the more stable *trans*-enamine. After several hours, the β-lactamase enzyme is irreversibly inhibited via the covalent acylation (Chen and Herzberg, 1992; Padayatti *et al.*, 2005).

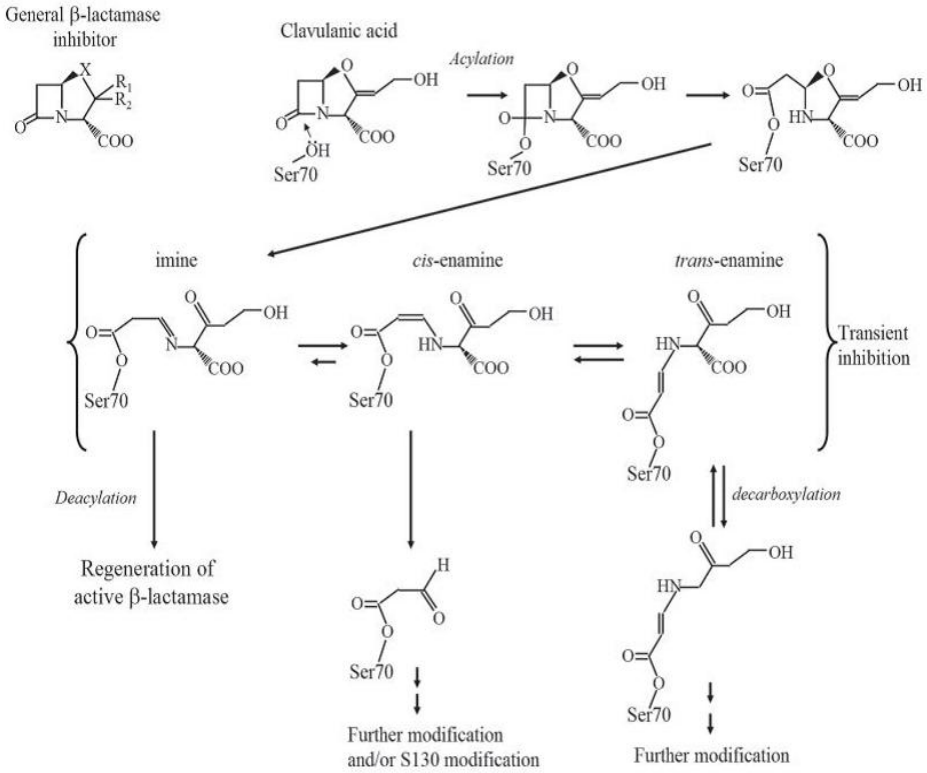


Figure 1.5. Inhibition of β-lactamases by clavulanic acid (Padayatti *et al.*, 2005).

1.5. Clavulanic acid gene cluster

Clavulanic acid gene cluster was initially characterized after purification of CAS1 and CAS2 (Marsh *et al*., 1992). Eventhough, cephamycin C and clavulanic acid pathways do not share any biosynthetic enzymes, the genes for these two pathways are adjacent in the genome and arranged in a supercluster about 60 kb (Ward and Hodgson, 1993) (Figures 1.6 and 1.7).

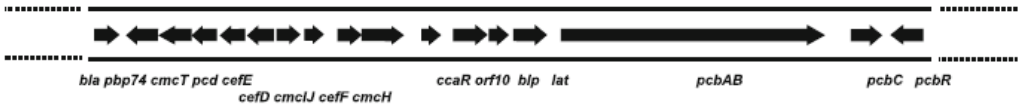


Figure 1.6. Cephamycin C gene cluster of *S. clavuligerus* (Liras *et al*., 2008).

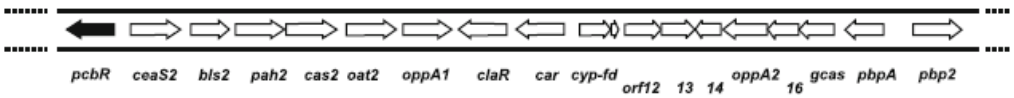


Figure 1.7. Clavulanic acid gene cluster of *S. clavuligerus* (Liras *et al*., 2008).

Several genes which are responsible in the early biosynthetic steps of clavulanic acid are duplicated. A gene cluster containing paralogs to at least four genes *ceaS, bls*, *pah* and *oat* is located elsewhere in the genome (Jensen *et al.*, 2004; Tahlan *et al.*, 2004b) (Figure 1.8). This paralogue gene cluster is differentially regulated, and only expressed in soy growth medium (Tahlan *et al.*, 2004a). In addition, a homologue to *cas1* is located separately in clavam gene cluster (Mosher *et al.*, 1999). There are approximately eighteen genes (*orf* 2-19) (Table 1.1) that have been reported to be responsible for the biosynthesis, transport, and regulation of clavulanic acid

(Hodgson *et al*., 1995; Jensen *et al.*, 2000; Jensen *et al.*, 2004a; Li *et al*., 2000; Mellado *et al.*, 2002), however only half of these genes have been defined yet.

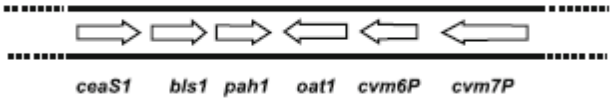


Figure 1.8. Gene cluster containing the genes paralogous to those of clavulanic acid cluster (Liras *et al*., 2008).

Table 1.1. Open reading frames of clavulanic acid gene cluster and their functions.

Gene	Gene Product/ Putative Function	Reference
ceaS	N2-(2-carboxyethyl) arginine synthase	(Khaleeli *et al*., 1999)
bls	β-lactam synthase	(Bachman *et al*., 1998; McNaughton *et al*., 1998)
pah2	proclavaminate amidinohydrolase	(Wu *et al*., 1995)
cas2	clavaminate synthase	(Elson *et al*., 1987; Baldwin *et al*., 1990; Marsh *et al*., 1992; Salowe *et al*., 1990)
oat	ornithine acetyltransferase	(Kershaw *et al*., 2002)
oppA1	oligopeptide binding protein	(Hodgson *et al*., 1995; Jensen *et al*., 2000; Jensen *et al*., 2004a; Mellado *et al*., 2002; Lorenzana *et al*., 2004)

Table 1.1. (continued)

claR	ClaR regulatory protein (DNA binding protein)	(Paradkar *et al*., 1998; Pérez-Redondo *et al*., 1998)
cad	clavulanic acid dehydrogenase	(Nicholson *et al.*, 1994)
cyp	P450 mono-oxygenase	(Jensen *et al.*, 2000; Li *et al*., 2000; Jensen *et al*., 2004a; Mellado *et al*., 2002)
fd	ferredoxin	(Li *et al.*, 2000; Jensen *et al*., 2004a)
orf12	acetyl transferase	(Jensen *et al*., 2004a; Li *et al*., 2000; Mellado *et al.*, 2002)
orf13	efflux pump	(Jensen *et al*., 2004a; Mellado *et al.*, 2002)
orf14	acetyltransferase	(Jensen *et al*., 2004a, Mellado *et al.*, 2002)
oppA2	oligopeptide binding protein	(Hodgson *et al*., 1995; Jensen *et al*., 2000; Jensen *et al*., 2004a; Mellado *et al*., 2002; Lorenzana *et al.*, 2004)
orf16	hypothetical protein	(Jensen *et al*., 2004a; Mellado *et al*., 2002)
gcas	N-glycyl-clavaminic acid synthetase	(Arulanantham *et al.*, 2006)
pbpA	PBP	(Jensen *et al*., 2004a; Mellado *et al*., 2002)
*pbp*2	PBP	(Jensen *et al*., 2004a; Mellado *et al*., 2002)

1.6. Clavulanic acid biosynthesis

The first reaction in the clavulanic acid biosynthesis starts with the condensation of a three-carbon glycolytic intermediate; 3-phosphoglyceraldehyde with L-arginine catalyzed by the enzyme carboxyethylarginine synthase (CEAS) (Khaleeli *et al*., 1999) (Figure 1.9). This reaction results in an intermediate of N^2-(2-carboxyethyl) arginine (CEA) (Elson *et al*., 1993). Two copies of the gene encoding CEAS are present in *S. clavuligerus*; *ceaS2* resides in the clavulanic acid gene cluster, and *ceaS1* lies in the paralogue gene cluster (Pérez-Redondo *et al*., 1999; Jensen *et al*., 2000; Tahlan *et al*., 2004). This gene was previously called as *pyc* (Pérez-Rodondo *et al*., 1999), *orf2* (Jensen *et al*., 2000) and *ceaS* (Liras and Rodriguez-Garcia, 2000).

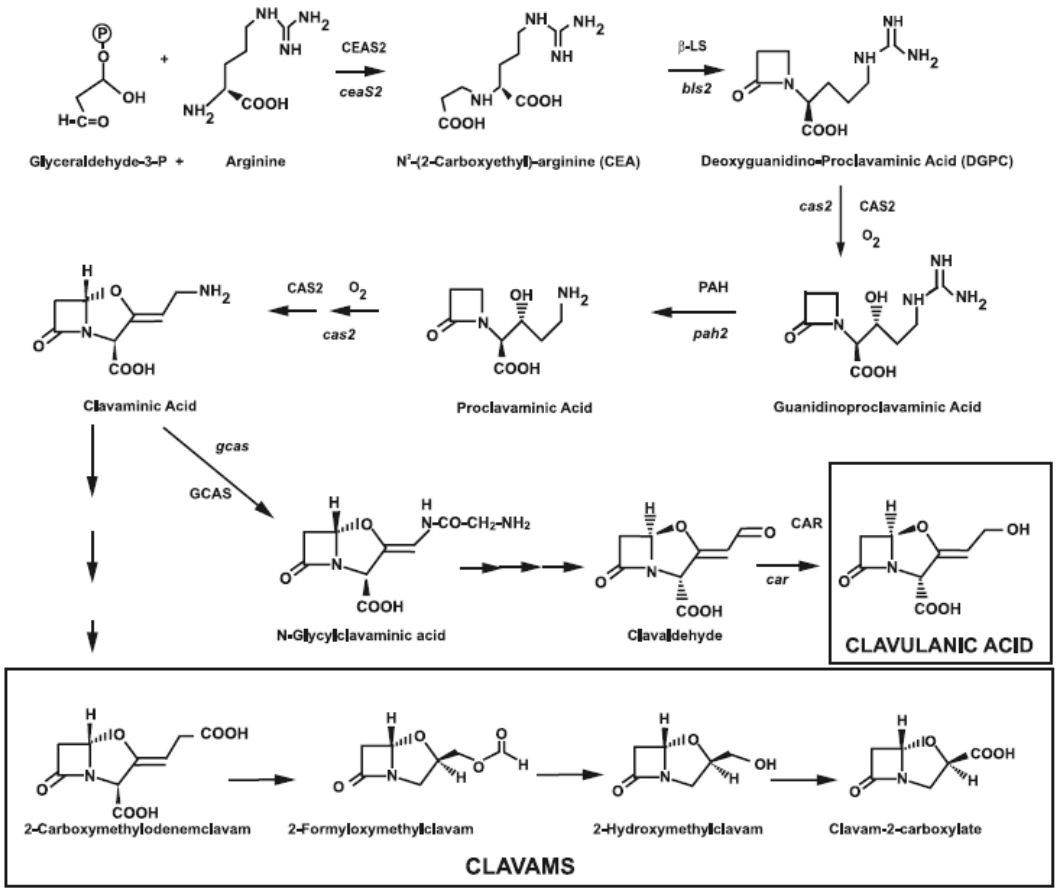


Figure 1.9. The biosynthetic pathway of clavulanic acid (Liras *et al.*, 2008).

The second reaction in the clavulanic acid biosynthesis yields a monocyclic intermediate deoxyguanidino proclavaminic acid and is catalyzed by β-lactam synthetase (BLS). Likewise, BLS is encoded by two distinct genes (*bls1* and *bls2*) (Bachman *et al*., 1998, McNaughton *et al*., 1998). This intermediate is immediately hydroxylated to form guanidinoproclavaminic acid by clavaminate synthetase (CAS) (Baldwin *et al*., 1993; Paradkar *et al*., 1998). Sequence analysis has revealed that CAS exists as two isozymes; CAS1 and CAS2 which are encoded by similar but separate genes, *cas1* and *cas2*, respectively (Marsh *et al*., 1992, Paradkar and Jensen, 1995, Mosher *et al*., 1999). Subsequently, the guanidino group is removed by proclavaminate amidinohydrolyase (PAH) to yield proclavaminic acid (Wu *et al*., 1995). PAH is encoded by *pah* gene (Elson *et al*., 1993a; Aidoo *et al*., 1994). Proclavaminic acid serves as a substrate for clavaminate synthase (CAS) that oxidatively catalyses the formation of the oxazolidine rings, resulting in the formation of clavaminic acid (Elson *et al.*, 1987). This catalysis occurs by a two-step reaction involving the transient intermediate, dihydroclavaminic acid (Baldwin *et al*., 1991; Salowe *et al*., 1990). Both steps are catalyzed by CAS. Clavaminic acid is finally converted to clavulanic acid by a NADPH dependent dehydrogenase called clavulanic acid dehydrogenase (CAD) which is encoded by *car* gene (Nicholson *et al*., 1994, Fulston *et al*., 2001).

1.7. Regulation of secondary metabolism in *S. clavuligerus*

The regulation of a secondary metabolite biosynthesis can be constituted at least by four different levels. The influence of global regulators of secondary biosynthetic gene expression has the highest rank in the hierarchy of this regulation. Eventhough they are unsufficiently described, these genetic elements are able to influence the biosynthetic rates crucially. At the second level of this hierarchy, the structural genes and their protein products specify the biosynthetic pathway of a particular secondary metabolite. At this level, the activities of the enzymes constituting the pathway influence the rate of secondary metabolite production. At the interface between

secondary and primary metabolism there are cofactors and precursors for secondary metabolite biosynthesis, whose supply rate represents a third level of cellular control. Finally, at the fourth level, the rate that an individual cell can effectively transport the metabolites out is yet another controlling point on the production of secondary metabolites (Khetan *et al*., 1999).

Individual antibiotic biosynthesis pathways are controlled by specific regulatory proteins that are frequently encoded by genes linked to the biosynthetic gene clusters. The Streptomyces Antibiotic Regulatory Proteins (SARPs) do not appear to contain the characteristic helix-turn-helix DNA-binding motifs, but rather contain N-terminal DNA binding domains similar to OmpR family of proteins, and are pathway specific regulators controlling the biosynthesis of one or more antibiotics (Wietzorrek and Bibb, 1997).

The transcription activation protein, CcaR (Cephamycin C and Clavulanic Acid Regulator) is also a SARP and coregulates clavulanic acid and cephamycin C pathways (Pérez-Llarena *et al*., 1997; Alexander and Jensen, 1998). The *ccaR*-encoded protein CcaR resembles the ActII-ORF4, RedD, AfsR and DnrI regulatory proteins of other *Streptomyces* species, all of which shares several motifs. However, it also differs from these proteins by having a higher rank in the hierarchy of regulation, since these proteins regulate the biosynthesis of a single antibiotic in their respective organisms. CcaR is a pathway-specific transcriptional regulator for cephamycin and clavulanic acid biosynthesis (Figure 1.10), it specifically binds to the promoter region of *lat* gene (Kyung *et al*., 2001). It also interracts with the bidirectional promoter of *cefD* and *cmcI* in the cephamycin C gene cluster (Santamarta *et al*., 2002). Mutants disrupted in *ccaR* do not transcribe cephamycin C or clavulanic acid structural genes and also lack several enzymes required for cephamycin C synthesis. (Pérez-Llarena *et al*., 1997a; Alexander and Jensen, 1998). Complementation of the *ccaR* disrupted mutant restored production of both secondary metabolites while amplification of *ccaR* gene on a multicopy plasmid resulted in a two to threefold increase in cephamycin C and clavulanic acid productions (Pérez-Llarena *et al*., 1997a; Liras, 1999). In spite of that, the

mechanisms that play role on the regulation of transcription of *ccaR* still needs investigation although there are reports describing autoregulation and regulation by a γ-butyrolactone-based quorum-sensing system (Santamarta *et al*., 2002; Wang *et al*., 2004; Santamarta *et al*., 2005).

In addition, CcaR controls expression of the *claR* (Clavulanic Acid Regulator) gene from the clavulanic acid gene cluster (Paradkar *et al*., 1998; Pérez-Rodondo *et al*., 1998). *claR* is another pathway-specific transcriptional regulatory gene, and is located in the clavulanic acid cluster (Paradkar *et al*., 1998; Pérez-Redondo *et al*., 1998) (Figure 1.10). It has a significant degree of homology to many transcriptional activators of the *lys*R family which positively controls clavulanic acid production and acts negatively on cephamycin C (Pérez-Redondo *et al*., 1998). The ClaR protein has two characteristic helix-turn-helix motifs, corresponding to the DNA binding regions of transcriptional activators. Disruption of *claR* results in a mutant that produces higher levels of cephamycin C, but no clavulanic acid. This may be a mechanism to balance formation of the cephamycin C and clavulanic acid in accordance with the cell's needs (Pérez-Redondo *et al*., 1998). In addition, overexpression of *claR* gene results in two fold increase in clavulanic acid production (Pérez-Redondo *et al*., 1998).

The *car* gene encoding clavulanate-9-aldehyde reductase is linked to the regulatory gene *claR*. Expression of *car* and *claR* did not occur in *ccaR*-disrupted mutants (Pérez-Redondo *et al*., 1998).

As mentioned previously, clavaminate synthase (CAS) has two isozymes; CAS1 and CAS2 which are encoded by *cas1* and *cas2*, respectively (Marsh *et al*., 1992; Paradkar and Jensen, 1995; Mosher *et al*., 1999). The *cas1* and *cas2* are paralogous genes which are known to be nutritionally regulated and can replace each other functionally (Paradkar and Jensen, 1995). Their predicted gene products share 82% identity and 87% similarity at the amino acid level (Marsh *et al*., 1992). *cas1* is expressed only when *S. clavuligerus* is grown on complex soy medium, but not in defined starch asparagine (SA) medium. Unlikely, the *cas2* paralogue is expressed on both complex soy and defined SA media (Paradkar and Jensen, 1995). It was

reported that, *cas2* mutant could still produce clavulanic acid and 5S clavams in soy medium but not in SA medium. This ability of *cas2* mutant to produce clavulanic acid and 5S clavams in soy medium was attributed to replacement of *cas2* with its paralogue *cas1* (Mosher *et al*., 1999).

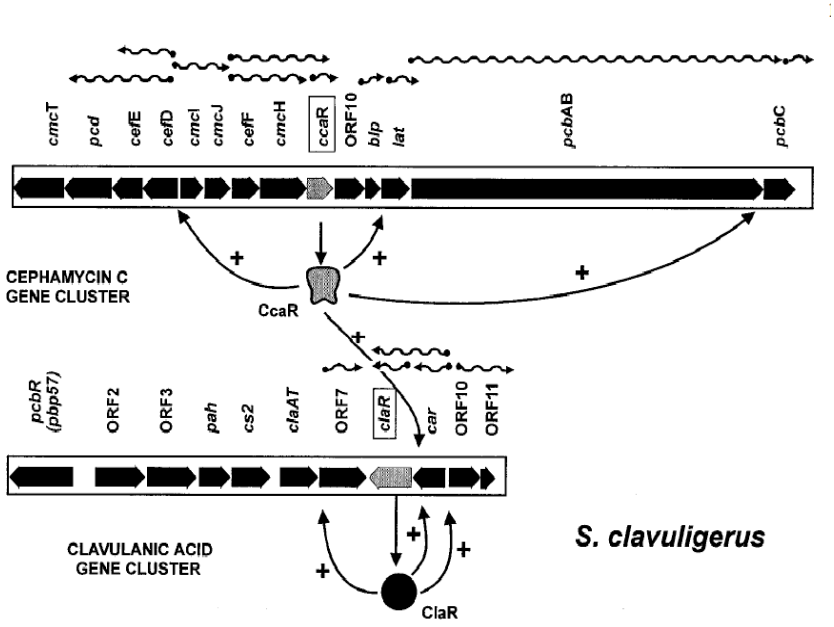


Figure 1.10. Proposed model for the regulation of cephamycin C and clavulanic acid biosynthesis in *S. clavuligerus* (Liras, 1999).

In streptomycetes, the production of secondary metabolites is linked to the metabolism of nitrogen; therefore, nitrogen catabolite regulation has been investigated for streptomycetes for several years (Burkovski, 2003). It has been showen that the use of NH_4Cl as the only nitrogen source decreases antibiotic production in *S. clavuligerus* (Romero *et al*., 1997).

There are some other factors that seem to affect the onset of antibiotic biosynthesis. Some small diffusible signalling molecules (*e.g.* γ-butyrolactone A-factor) which are produced in response to certain physiological or environmental signals, play an important role in triggering antibiotic production in *Streptomyces* (Horinouchi and

Beppu, 1994). Other than these diffusible factors, the highly phosphorylated nucleotide ppGpp has been suggested to play a key role as an intacellular effector in determining the onset of antibiotic biosynthesis (Bibb, 1996).

1.8. Industrial antibiotic fermentation and strain improvement

Most antibiotic fermentation processes are run in two stages. The first stage starts from spores, usually of established strains, because antibiotic production is often an unstable trait that can be lost if the stock is kept growing constantly through serial cultures. Due to its capability of sporulation, it is common to use spore suspensions of *S. clavuligerus* in the first steps of industrial fermentation (Hockenhull, 1980). The organisms are cultured under submerged conditions with sufficient aeration and a generous supply of nutrients so that they will attain near-maximal density in a short time. In the second stage, when the culture reaches the stationary phase or stops growing and begins to reproduce antibiotics, the concentration of the key nutrients such as carbon source, phosphate and nitrogen source must be controlled carefully by continuous-fed batch processes (Glazer and Nikaido, 1998). However, the vegetative phase is also needed and therefore is used in the industry in order to produce enough volume of preculture medium to inoculate the production vessels. One of the main drawbacks in using vegetative inocula is to get an equable standard preculture due to the morphological differentiation appertaining to the growth of *S. clavuligerus* (Neves *et al*., 2001). Moreover, it has been hypothesized that splashing of the liquid medium and wall growth of the cells because of baffled Erlenmeyer flasks in shaken cultures could contribute to this variability (Büchs, 2001; Betts and Baganz, 2006).

In a commercial scale, antibiotic production has been carried out in batch systems; on the other hand, by using immobilized cells, antibiotics can either be produced by continuous or semi-continuous methods (Saudagar *et al*., 2008a).

Traditionally, yield improvements in antibiotic fermentations have been achieved by classical mutagenesis and high-throughput screening of the mutants. The selected

strains are then investigated for genetic stability and optimization of feeding and processing strategies (Lein, 1986). However, these techniques are indirect, time consuming and in most cases, only a little improvement could be achieved (Thykaer and Nielsen, 2003).

For many of the antibiotics commonly used for humans today, the genetics and biochemistry of their biosynthesis are known, so pharmaceutical companies have also been running programs to apply genetic engineering techniques to improve antibiotic yields based on this knowledge (Paradkar, 2001). As in the case of clavulanic acid production in *S. clavuligerus*, empirical approach of random mutagenesis and selection could still remain effective, although time consuming. However, strategies for improving secondary metabolite yields by using metabolic engineering techniques may enable more directed and rational (Olano *et al*., 2008).

As summarized by Saudagar *et al*. (2008b), strain improvement is an essential part of process development for microbial fermentation products since;

- It enables cost reduction by developing strain with increased productivity.
- It confers ability to use inexpensive raw materials.
- It imparts more desirable special characteristics such as improved filtration properties.
- It enables to perform the process under certain conditions of temperature or oxygen tension.

Secondary metabolite genes are weakly expressed or not expressed at all under the given conditions and the yields of the compounds are very low. For the industrial purposes, an appropriate expression host should be optimized for the particular secondary metabolite. One of the options is to develop a superhost from already optimized industrial strains (Smolke, 2010). However, it should be also kept in mind that, the use of industrial strains as host organisms in metabolic engineering studies could be restricted due to their proprietary and lack of information about their sequences (Smolke, 2010).

Many studies have been conducted in order to obtain clavulanic acid overproducers of *S. clavuligerus.* Gene insertion with *orf2* from clavulanic acid biosynthetic pathway together with manipulation of fermentation conditions have been reported to increase the production of clavulanic acid (Townsend *et al*., 2001). In *S. clavuligerus*, multicopy expression of *ceaS2* resulted in recombinant strains with 60% and 100% higher CA production, respectively (Pérez-Redondo *et al*., 1999). Moreover, elimination of the clavams, by means of inactivation of the clavam pathway which shares the common intermediate clavaminic acid with the clavulanic acid pathway, has been shown to give an increased yield of clavulanic acid. Paradkar *et al*. (2008) demonstrated 10% increase in CA production by double distruptant mutants by means of blocking cephamycin C and clavam biosynthesis both in wild type and industrial strain of *S. clavuligerus*. Targeted disruption of *lat* and *cvm1* genes blocked the productions of cephamycin C and non-clavulanic acid clavams in both strains, while resulting in an increased production of CA.

Other studies involved disruption of negative regulatory gene(s) or increased expression of positive regulatory gene(s). For instance, disruption of *claR* yielded a mutant producing higher levels of cephamycin C, but unable to produce clavulanic acid. However, multicopy expression of *claR* gene resulted in 2 fold increase in CA production by wild type strain (Pérez-Redondo *et al*., 1998). Pérez-Llarena *et al*. (1997) cloned *ccaR* gene into a multicopy plasmid and showed 2-3 fold increase in cephamycin C and clavulanic acid productions by wild type *S. clavuligerus* ATCC 27064. In a recent study, Hung and his co-workers (2006) constructed three recombinant plasmids carrying *ccaR*, *claR*, *afsR-p* (a global regulatory gene from *Streptomyces peucetius*) alone and one with *ccaR-claR-afsR-p* along with the *ermE* * promoter to enhance the production of CA in *S. clavuligerus* NRRL 3585. Overexpression of these genes resulted 2.5, 1.5, 1.6 and 1.5 fold enhanced CA production, respectively.

Hung and his co-workers (2007) reported that overexpression and chromosomal integration of *cas2* in wild type *S. clavuligerus* NRLL 3585 resulted in 5.06 and 2.93 fold increase in the production of CA, respectively. In the study, the effect of *ccaR*-

cas2 gene combination on CA production of the wild type was also analysed, and found to be 9.79 fold higher in integration, and 23.8 fold higher in overexpression of *ccaR-cas2*. The choromosomal integration was carried out by means of pSET152 *ermE** vector. pSET152 is a site-specific, nonreplicative plasmid that contains φC31 *int*, *attP*, *oriT* of RK2, and aac(3)IV. φC31 integrase catalyses the recombination and pSET152 is a suitable conjugative vector for intergeneric conjugation between *Streptomyces* and *E. coli* (Bierman *et al*., 1992, Sioud *et al*., 2009).

Another approach to enhance CA production was to channel precursor flux of glycolitic pathway to favour CA biosynthesis. Li and Townsend (2006) inactivated *gap1* gene which encodes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in *S. clavuligerus* by targeted gene disruption. Two fold increase in CA production was achieved and reversed by complementation. Addition of arginine to the culture further improved CA production by this mutant strain.

In a very recent study, by the combination of strategies including the manipulation of the primary metabolism and overexpression of regulatory genes together with media optimization, an improvement in CA production was obtained by *S. clavuligerus* NRRL3585 in a fed-batch culture (Nath *et al*., 2010). According to this, *ccaR-claR* was cloned both in a replicative plasmid and a integrative pSET152 plasmid along with a *ermE** and these recombinant plasmids were transformed into Δ*gap*1 mutant of *S. clavuligerus* NRRL 3585 strain. Overexpression and chromosomal integration of *ccaR-claR* in Δ*gap*1 mutant resulted 2.95 and 5.85 fold increase in CA production, respectively. The supplemetation of ornithine and glycerol to the culture medium was further increased CA production by the recombinant strains up to 7.02 fold.

As to the other *Streptomyces* spp, the industrial strains of *Streptomyces fradiae* and *Saccharopolyspora erythraea* have been engineered for enhanced erythromycin and tylosin production, respectively (Rodriguez *et al*., 2003).

There are many studies in which multicopy plasmids were used for strain improvement purposes. However, there have been reports that multicopy vectors reduce antibiotic production; therefore it is advisable to use integrating vectors

whenever possible. High producing strains seem to be particularly sensitive to the metabolic load imposed by multicopy plasmids and vectors are therefore lost in the absence of selection pressure (Keiser *et al*., 2000). Due the inheritance stability it provides, and unnecessity for antibiotic use in the fermentation tanks, strain improvement by means of choromosomal integration is more preferred for the industrial processes (Nath *et al.*, 2010).

1.9. The present study

As stated earlier *S. clavuligerus* is a well-known and biotechnologically very important species used for the industrial production of clavulanic acid. The aim of the present study was to integrate the biosynthetic gene *cas2* into the chromosome of a clavulanic acid overproducer industrial strain of *S. clavuligerus* by means of a *Streptomyces* integration vector, called pSET152 in order to further enhance its clavulanic acid yields. Overexpression of the biosynthetic *cas2* gene via chromosomal integration would be more a direct and rational approach of strain improvement when compared to random mutagenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, media and culture conditions

Characteristics and sources of bacterial strains and plasmid vectors used in this study are listed in Table 2.1. *Escherichia coli* DH5α cultures were grown in either Luria broth liquid medium (LB) or on agar plates (LA) (Appendix A) at 37 ℃. *E. coli* was maintained on agar plates or kept in 20% glycerol at -80 ℃. For fermentation process, *S. clavuligerus* was maintained on CC1 sporulation medium (Appendix A). *S. clavuligerus* spore suspension was prepared by using the protocol described by Hopwood *et al*. (1985) and stored in 20% glycerol at -80 ℃. Liquid cultures of *S. clavuligerus* were grown in CC2 vegetation and CC3 fermentation media (Appendix A) by incubating on a rotary shaker (240 rpm) in baffled flasks at 23.5 ℃. *S. clavuligerus* was also grown in Trypticase soy broth (TSB) (Appendix A) and Trypticase soy agar (TSA) (Appendix A) when required.

E. coli vectors were pGEM-T Easy (Promega) and pBluescript II KS (+) (Stratagene) (Figure 2.1). *E. coli*/*Streptomyces* shuttle vector pSET152 (Figure 2.2) was used for integration of *cas2* gene to the chromosome of *S. clavuligerus*.

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

Strains and plasmids	**Description**	**Source or reference**
Strains		
S. clavuligerus		
Industrial strain	Clavulanic acid overproducer	DEPA Pharmaceuticals, İzmit, Turkey
ATCC 27064	Wild type, cephamycin C and clavulanic acid producer	Prof. P. Liras, INBIOTEC, Leon, Spain
Klebsiella pneumoniae		
ATCC 29665	Indicator organism	Prof. P. Liras, INBIOTEC, Leon, Spain
E. coli		
DH5α	Cloning host; 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

Table 2.1 (continued)

ET12567	dam$^-$ 13::Tn9 dcm-6 hsdM hsdR, *lac*YI	Prof. Keith Chater, John Innes Centre, Colney, Norwich, UK
Plasmids		
pGEM-T Easy	AmpR, *lacZ*'	Promega
pSET152	*lacZ*, rep^{puc}, $att^{\Phi C31}$, *oriT*	Prof. Paloma Liras, INBIOTEC, Leon, Spain
pBluescript II KS (+)	(+) AmpR, *lacZ*'	Stratagene
pGVAK01	pGEM-T with 1016 bp *S. clavuligerus cas2* gene	This study
pGVAK02	pBluescript KS (+) with 1016 bp *S. clavuligerus cas2* gene at its *EcoR*I site	This study
pGVAK03	pSET152 with 1016 bp *S. clavuligerus cas2* gene at its *Xba*I site	This study

2.2. Culture media

The composition and preparation of culture media are listed in Appendix A.

2.3. Buffers and solutions

Buffers, solutions, their compositions and preparations are listed in Appendix B.

2.4. Chemicals and enzymes

The chemicals and the enzymes used as well as their suppliers are listed in Appendix C.

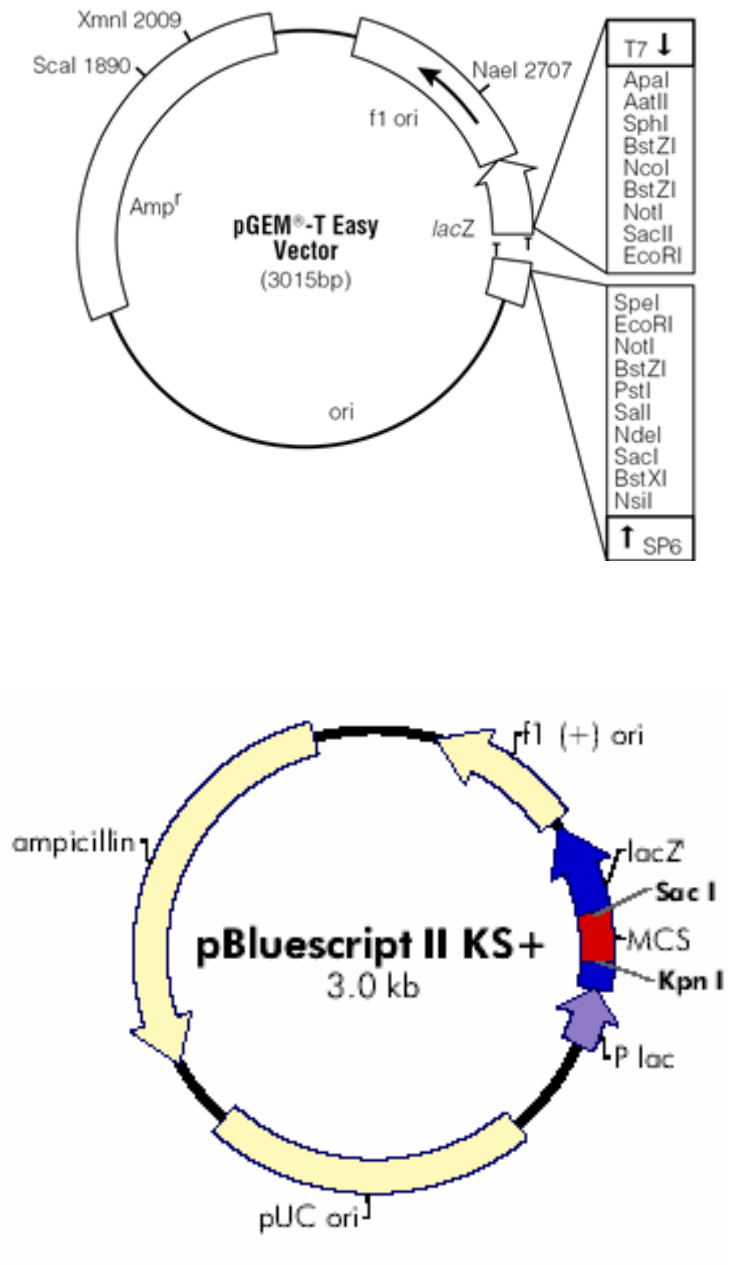


Figure 2.1. Maps of the pGEM-T Easy and pBluescript II KS (+) plasmids.

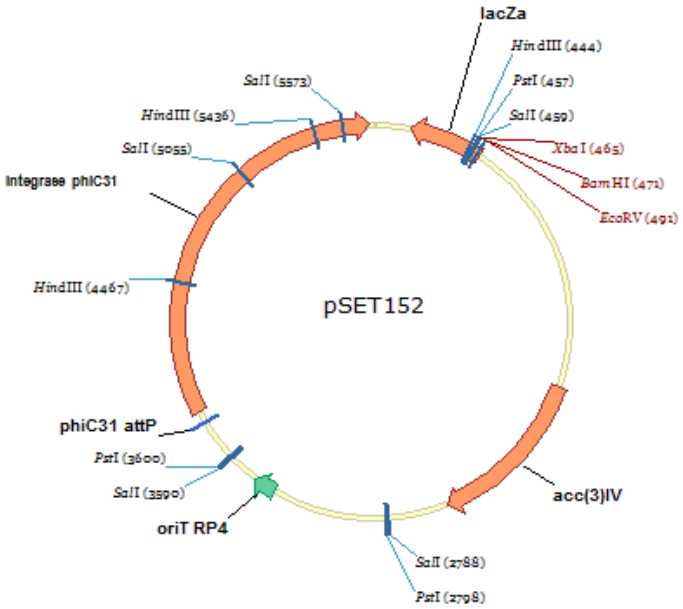


Figure 2.2. Map of the *Streptomyces* integration vector pSET152.

2.5. Isolation of genomic DNA from *S. clavuligerus*

Salting out method described by Pospiech and Neumann (1995) was used for chromosomal DNA isolation from *S. clavuligerus*. For isolation of genomic DNA, 50 ml of TSB (Appendix A) was inoculated with 50 µl of mycelium stock of *S. clavuligerus* and the culture was incubated at 220 rpm and at 28 ℃ for 48-60 h. 30 ml of this seed culture was harvested by centrifugation at 2000 rpm for 15 min, and it was resuspended in 5 ml of SET buffer (Appendix B). 100 µl of lysozyme (Appendix C) was added to the resuspension and incubated at 37 ℃ for 30-60 min. 140 µl of proteinase K addition to the mixture (Appendix C) was followed by SDS treatment. 600 µl of 10% SDS (Appendix C) was added and the solution was mixed by inversion. Next, 2 ml of 5M NaCl (Appendix C) was mixed thoroughly. This step was followed by addition of 5 ml of choloroform (Appendix C) to the mixture. After mixing of the solution by inversion for 30 min at 20 ℃, the mixture was centrifuged

at 6000 rpm and at 20 ℃ for 15 min. The supernatant was transferred to fresh test tube and 0.6 vol isopropanol was added and mixed again by inversion. After 3 min, DNA was spooled onto a sealed Pasteur pipette, and rinsed in 5 ml of 70% ethanol, air dried and finally dissolved in 1-2 ml TE (Appendix B) at 55 ℃ (Kieser *et al*., 2000).

2.6. Plasmid isolation from *E. coli*

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were used for isolation of *E. coli* plasmid DNA as specified by the manufacturers. *E. coli* plasmid DNA was also prepared by using the plasmid miniprep method described by Hopwood *et al*. (1985). Each strain was grown as patches on selective medium, LB agar containing the selective antibiotic in required concentration. About 1 square cm of cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µl cold TSE buffer (Appendix B). Each tube was mixed by vortexing to disperse the cells and the toothpick was discarded. Then, the tubes were incubated on ice for 20 min. 3/5 volume of lysis solution (Appendix B) was added to each tube and vortexed immediately. The mixture was incubated at room temperature (RT) for 10 min to lyse the cells and then at 70 ℃ for 10 min to denature DNA. Then, tubes were cooled rapidly in cold water. An equal amount of phenol-chloroform solution was added (water-saturated, Appendix B), vortexed hard until homogeneous and milky white mixture was obtained. Finally, the samples were spun for 5 min at 13000 rpm to separate phases. 10 µl of supernatant was loaded directly on an agarose gel for electrophoresis.

2.7. Preparation of *E. coli* competent cells

E. coli competent cells were prepared according to the protocol described by Sambrook *et al*. (1989) with some modifications. 5- 50 ml of LB broth (Appendix A)

was inoculated with *E. coli* DH5α from a fresh LB agar plate in a 250 ml flask and incubated on a rotary shaker at 37 ℃ to obtain a stationary phase culture. 300 µL from this seed culture was inoculated into a fresh flask containing 50 ml LB and incubated for 2- 2.5 h at 37 ℃ with vigorous shaking around (300 cycles per min) in an orbital shaker to obtain an exponentially-growing culture. Then the culture was aseptically split into two sterile pre-chilled 40 ml screw cap centrifuge tubes and stored on ice for 10 min. Next, the cells were spun at 4000 rpm for 10 min at 4 ℃ in a Sigma 3-16 PK rotor. After centrifugation, supernatants were decanted and each pellet was resuspended in 5 ml ice-cold 10 mM $CaCl_2$ by vortexing. The cells were centrifuged at 3000 rpm for 10 min at 4 ℃ and the supernatant was discarded. Finally, each pellet was resuspended very gently in 1 ml ice-cold 75 mM $CaCl_2$. The competent cells were diluted in 1:1 ratio with 40% glycerol and were stored at -80 ℃ as 100 µl aliquots.

2.8. Transformation of *E. coli* competent cells

Competent *E. coli* cells were transformed as described by Sambrook *et al*. (1989) with slight modifications. *E. coli* competent cells kept at -80 ℃ were slowly thawed on ice. Plasmid DNA 1-10 µl (1- 50 ng) or ligation product (in a maximum volume of 10 µl) was added to 100 µl of *E. coli* competent cells and mixed gently by pipetting. The tubes were left on ice for 30 min. Then, the tubes were placed in a 42 ℃ water bath and heat-pulsed for 90 sec. Next, the tubes were immediately put on ice for 2 min. 0.9 ml of LB was added to each tube and the cultures were incubated for 1.5 h at 37 ℃ with gentle shaking (100 rpm). Cells were centrifuged at low speed (3000 rpm) for 8 min and most of the supernatant was removed while 100-200 µl of the supernatant was left in the tube in which the pellet was resuspended. Finally, cells (100 µl) were spread onto a selective LA plate and incubated at 37 ℃ for o/n. The single colonies of transformants were selected and plasmids from these colonies were isolated for further analysis.

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

The procedure applied for conjugation from the non-methylating *E. coli* donor ET12567 is a modification (Flett *et al*., 1997) of the method described by Mazodier *et al*. (1989). Competent cells of *E. coli* ET12567/pUZ8002 were grown in the presence of kanamycin (Km) (25 μg/ml) and chloramphenicol (Cm) (25 μg/ml) (Appendix C) to maintain selection for pUZ8002 and the *dam* mutation, respectively. The competent cells were transformed with the *ori*T-containing vector, and were selected with the appropriate antibiotics. A colony from recombinant *E.coli* ET12567/pUZ8002 containing the desired plasmid was inoculated into 10 ml of LB containing antibiotic (Cm, Km, and Apramycin to select *ori*T-integration vector) (Appendix A) and grown over night (o/n) at 37 ℃ and 200 rpm. The o/n culture was diluted 1:100 in fresh LB plus three antibiotics and grown at 37 ℃ to reach an OD_{600} value of 0.4-0.6. The cells were washed twice with an equal volume of LB and resuspended in 0.1 volume of LB. For each conjugation, approximately 10^8 industrial *S. clavuligerus* spores were added to 500 μl YT broth (Appendix A). The spore cells were treated with heat shock at 50 ℃ for 10 min, and then allowed to cool down. 500 μl of *E. coli* cells were added to 0.5 ml heat shocked spores. After mixing thoroughly, the *E.coli*-spore suspension was centrifuged at 3000 rpm for 10 min and three quarters of the supernatant was poured off and the pellet was resuspended in the residual liquid. The cells were plated out on MS agar containing 10 mM MgCl and incubated at 30 ℃ for 16-20 hours. Next, the MS agar plates were overlaid with 1 ml of water containing 0.5 mg of nalidixic acid (Appendix C) and 1 mg of apramycin by using a spreader. Streptomycetes are naturally resistant to nalidixic acid, however, *E. coli* is sensitive (Keiser *et al*., 2000). The incubation was continued at 30 ℃ for 3-4 days. The potential exconjugants were picked off to a selective TSA media containing nalidixic acid and apramycin as final concentrations of 25 μg/ml and 50 μg/ml, respectively (Keiser *et al*., 2000).

2.10. Manipulation of DNA

2.10.1. Digestion with restriction endonucleases

Restriction enzyme digestions were performed under the conditions specified by the manufacturers.

2.10.2. Agarose gel electrophoresis

Agarose gels were used for DNA analysis generally at a concentration of 0.9%. Gels were run for 1-1.5 h at 90 V. The electrophoresis buffer used was 1xTAE (Appendix B). Gels were stained in ethidium bromide solution (Appendix C) with a final concentration of 0.4 ng/ml for 15-20 minutes at RT. The DNA bands were visualized on a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. Generally, Lambda (λ)/*Pst*I DNA size marker (Appendix C) was used to determine the molecular weights of DNA bands.

2.10.3. Extraction of DNA fragments from agarose gels

The desired fragments were extracted from the gel by using a Genemark Gel Extraction kit (http//www.genemark.com.tv). The gel slice containing the DNA band was excised from the gel and weighed. Gel extraction was performed according to the GeneMark's instructions. After recovery, an aliquot was run on agarose gel to assess the yield.

2.10.4. Ligations

Ligation reaction of PCR products with pGEM-T Easy vector was performed as follows: 1 µl T4 ligase (Promega), 5 µl of 2X ligation buffer (supplied by the manufacturer), 1 µl (55 ng/µl) pGEM-T Easy vector, 3 µl insert DNA were mixed and volume was completed to 10 µl with dH_2O. Ligation was carried out at 4 ℃ for 16 h.

Ligation reactions for *cas2* insertion to pBlueskript II KS+ (pKS+) and pSET152 vectors were performed as follows: 1 µl T4 DNA ligase (Promega), 1 µl 10X ligation buffer (supplied by the manufacturer), 5 µl of insert DNA, 1 µl of vector DNA were mixed and volume was completed to 10 µl with dH_2O. Reaction mixtures were incubated at RT for 2 h or at 4 ℃ for 16 h.

2.11. Primer design

The nucleotide sequence of the primers used in this study is given in Table 2.2. The underlined letters indicate *Xba*I site in cas2XF primer, and *Hind*III site in cas2HR primer. pSET152F primer together with cas2XF were used to amplify 1558 bp PCR product including 1016 bp *cas2* gene and 542 bp vector sequence to confirm integration of pGVAK03 to the chromosome of industrial strain of *S. clavuligerus*. The designed primers were synthesized by the Iontek Company (İstanbul, Turkey). The sequence of *cas2* gene and the positions of cas2XF, cas2HR (red arrows) and pSET152R (black arrow) primers are shown in Figure 2.3.

Figure 2.3. Nucleotide sequence of *cas2* (GenBank accession number U87786.2) and location of the primers.

Table 2.2. Primers used in this study.

Primer	Primer Sequence	Tm (℃)
cas2XF forward	tctagaaggagacatcgtgtcatgg	Tm= 63.2
cas2HR reverse	aagcttatgagccgggctcagc	Tm= 64
pSET152R	cgccgaccgacgacgacgaa	Tm= 66

2.12. Polymerase chain reaction

PCR was performed in a total volume of 50 µl. Reaction mixture components were as follows: Promega 5X Go-Taq PCR buffer 10 µl; Promega 25 mM $MgCl_2$ solution 2 µl; primers (10 µmol of each), 2.5 µl for each; 10 mM dNTP mix 1 µl; DMSO, 2.5 µl; 2.5 µl of template DNA (chromosomal DNA or plasmid DNA) and 1 unite Go-Taq DNA Polymerase (5 u/µl). PCR mixture was completed to 50 µl with dH_2O. The Touchdown PCR method was used to clone *cas2* gene. PCR amplification conditions were as follows: 95 ℃ for 5 min (initial denaturation), 94 ℃ for 45 s (denaturation), 60 ℃ for 45 s (annealing), 72 ℃ for 80 s (extension), as a total touchdown step of 10 cycles, then these three steps were repeated for 30 cycles by keeping annealing temperature constant at 50 ℃. PCR was ended with a final extension step at 72 ℃ for 10 min. At the end of the reaction, the PCR products were run in a 0.9% agarose gel, DNA bands were visualized, and were extracted from the gel, if necessary.

2.13. Growth determination via DNA quantification

Growth measurement of cultures by means of DNA quantification was performed according to Burton *et al.* (1968). 1 ml aliquots of cell cultures was taken into Eppendorf tubes at each 24 h intervals and centrifuged at 13200 rpm for 10 min. The supernatants and pellets were kept at -20 °C. Pellet samples were thawed on ice and resuspended in 1 ml of 0.85% NaCl (saline) solution. 400 µl of diluted samples and standards were distributed into 2 ml eppendorf tubes into which 400 µl of 1 N $HClO_4$ was added. Tubes were mixed by inversion and incubated at 70°C for 20 min. 800 µl of diphenylamine reagent (Appendix B) was added into each tube. Samples were mixed by inversion and incubated at 30 °C for 15-17 h. Samples taken out of the incubator were mixed and mycelium was precipitated by centrifugation at 13200 rpm for 10 min. The absorbance of the supernatant was measured at 600 nm. The amount of DNA samples were calculated according to a standard curve drawn using herring sperm and expressed as µg of DNA per ml of culture.

2.14. Clavulanic acid fermentation

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

Since growth phase is an important parameter during antibiotic production in streptomycetes (Demain and Fang, 1995), mycelia of the parental and recombinant strains at the same growth phase were used for sporulation. Initially, mycelia of all the strains grown on TSA were inoculated into fresh TSB by using ear sticks and incubated at 28 ℃ and 220 rpm to reach an OD_{600} of 3 to 4. Then, 400 µl of these mycelia from each culture was spread on CC1 sporulation media and they were incubated for about 7 days at 25 ℃. After incubation, a thick greenish layer of mature spores on top of the petri plates was observed as a result of successful sporulation. The matured spores were harvested from CC1 according to the protocol described by Hopwood *et al.* (1985). Accordingly, 400 µl of TT-X 100 (Appendix B) suspended spores of parental and recombinant strains were inoculated into 50 ml of CC2 in 500 ml single buffled flasks and incubated at 23.5 ℃ for 40 h to allow spores enough time for germination and transition to vegetative growth phase. At the end of 40 h of incubation, optical density of the cultures ranged between 6-7 at 600 nm. 400 µl of cultures from each flasks were then transferred into CC3 fermentation medium, and incubated at 23.5 ℃ and 240 rpm up to 168 hours. All fermentations for each strain were carried out in duplicate. 0.5 g samples collected from cultures at 24 h intervals by starting from 72 h of incubation were prepared for injection to the column.

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

At each given specific hour of fermentation, 0.5 g of sample from each flask was taken. The samples in test tubes were suspended in sodium acetate solution (Appendix B) as a final volume of 25 ml. The samples were kept at 4 ℃ prior to filtration process. The test tubes were shaken vigorously to avoid any clumps of culture medium. The suspension was filtered through a Millipore membrane (pore diameter 0.4 μm) to remove any mycelia and other insoluble ingredients. Bondapak C18 (300×3.9 mm, 5-10 m) column was used in HPLC. Samples were eluted with a mobile phase consisting of aqueous solution of sodium dihydrogen phosphate (Appendix B) which was adjusted to a pH 4 with phosphoric acid (Appendix C) and mixed with HPLC grade methanol (Appendix C) in a 95:5 ratio. The HPLC column was washed and saturated with the mobile phase after and prior to the injections of the samples. The column eluant was monitored by Waters 2487 Dual λ Absorbance detector at 220 nm. The flow-rate was 1.0 ml/min. Total run time for each injection was 7 min. All injections were performed at RT.

In this study, Waters Alliance (2695+2448) HPLC separation module was used and the data was processed by Waters Empower™ software. CA concentration in the cultivation media was assayed by using HPLC analysis based on the method of the European Pharmacopoeia (1993).

2.16. Construction of CA calibration curve

For the construction of a CA calibration curve, pure CA (potassium clavulanate salt) was used as the standard. The standards were kindly provided by DEPA Pharmaceuticals, İzmit, Turkey. Stock solution of CA was prepared as 50 mg/ml in sodium acetate buffer. Serial dilution with sodium acetate buffer was performed to yield standard CA solutions with final concentrations of 0.4, 0.8, 1, 1.5, 2, 3.5, 5, 7.5,

10, 15 mg/ml. 10 µl of sample was taken for each injection and two injections were performed for each sample. Standard solutions were freshly prepared for each run. The injections were done according to the method of European Pharmacopia (1993).

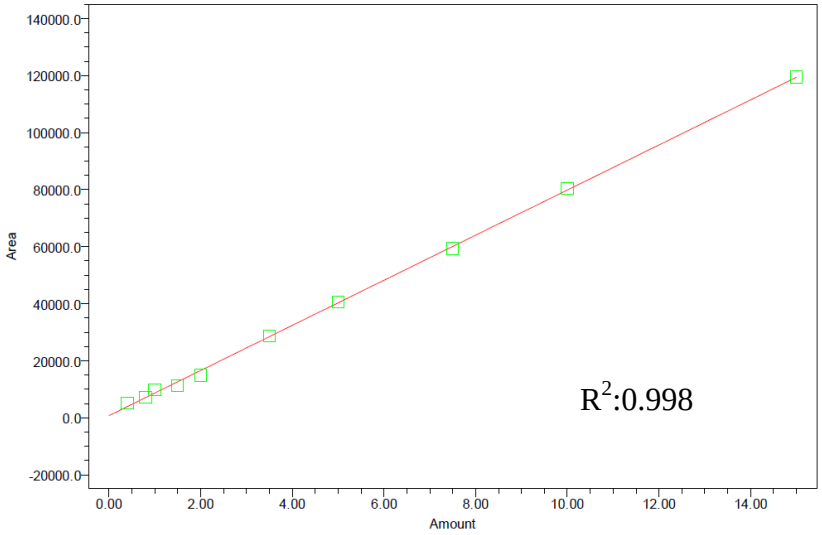


Figure 2.4. CA amount versus peak area calibration curve.

2.17. Bioassay of clavulanic acid

The procedure for clavulanic acid bioassay was adopted form Romero *et al*., (1984). Supernatant of 1 ml cell culture collected at each 24 h intervals was used for the determination of clavulanic acid production in the cultures. Sample kept at -80 ℃ was thawed on the ice on the assay day. *K. pneumoniae* cells were grown in TSB up to an OD_{600} value ranged between 0.9-1.0. 3.3 ml of cell culture with an OD_{600} value of 1.0 was mixed with 100 ml of melted TSA when it is cooled down to 47 ℃. Then, 100µl of penicillin G (Appendix B) was added and mixed immediately. 100 ml of TSA mixture was poured into each petri plate. Holes were introduced into TSA in petri plates and 60 µl of sample supernatant and also clavulanic acid standards diluted with 1 M (pH: 6.8) MOPS (Appendix B) were added into the holes. After

adding the samples, plates were first incubated at 4 ℃ for two hours and then at 30 ℃ for 12-15 h.

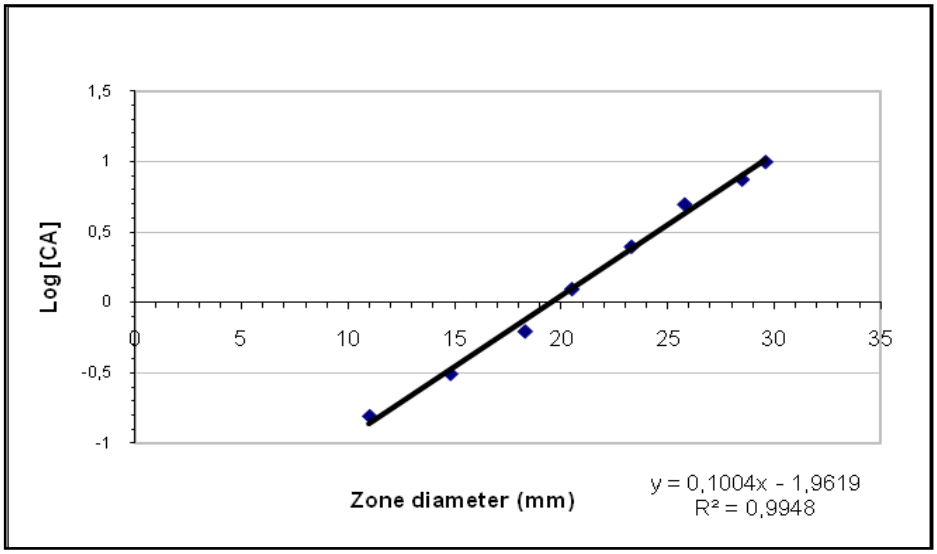


Figure 2.5. CA bioassay calibration curve.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Chromosomal integration of *cas2* gene into *S. clavuligerus*

3.1.1. Cloning of *cas2* gene into pGEM-T

The sequence of *cas2* gene with a GenBank accession number of U87786.2 was used for designing primers to amplify the gene.

cas2 gene was PCR amplified from *S. clavuligerus* chromosomal DNA by using cas2F and cas2R primers flanked with *Xba*I and *Hind*III restriction sites, respectively. Amplification of *cas2* gene resulted in a 1016 bp PCR product (Figure 3.1).

The resulting PCR product was then eluted from the gel by using Qiagen gel elution kit, and ligated to pGEM-T cloning vector. The ligation product was introduced into *E. coli* DH5α competent cells via transformation. The putative recombinant colonies were selected on X-Gal-IPTG (40 µg/ml) and ampicillin (100 µg/ml) containing Luria agar (LA) plates. Agarose gel electrophoresis was performed to visualize the plasmids isolated from white colonies (Figure 3.2).

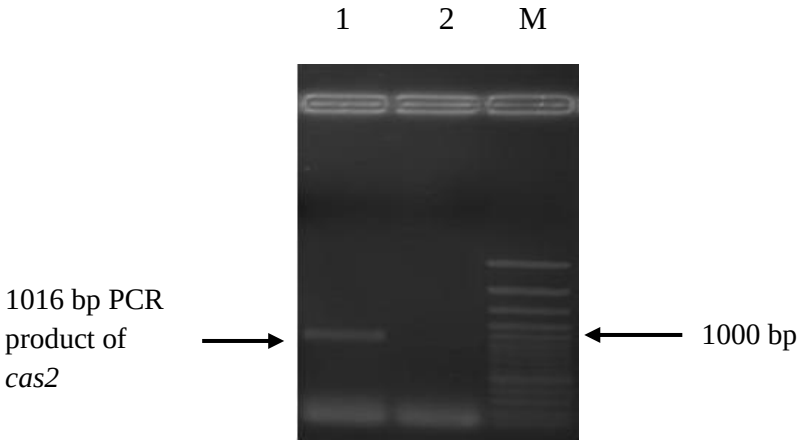


Figure 3.1. PCR amplification of *cas2* gene by using cas2F and cas2R primers. 1: PCR product of *cas2* gene, 2: Negative control with no template DNA, M: O'GeneRuler 100 bp DNA ladder plus.

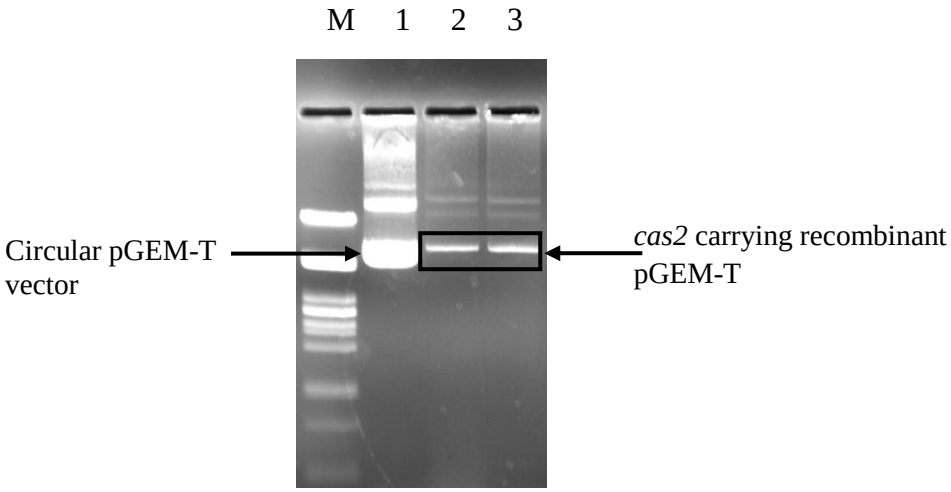


Figure 3.2. Plasmid isolation from two recombinant colonies. M: λ *Pst*I DNA marker, 1: pGEM-T vector isolated from a blue colony, 2-3: Recombinant pGEM-T plasmids containing *cas2* gene.

The recombination was also verified by PCR, restriction enzyme digestion (Figure 3.3), and DNA sequencing. This new recombinant was designated as pGVAK01.

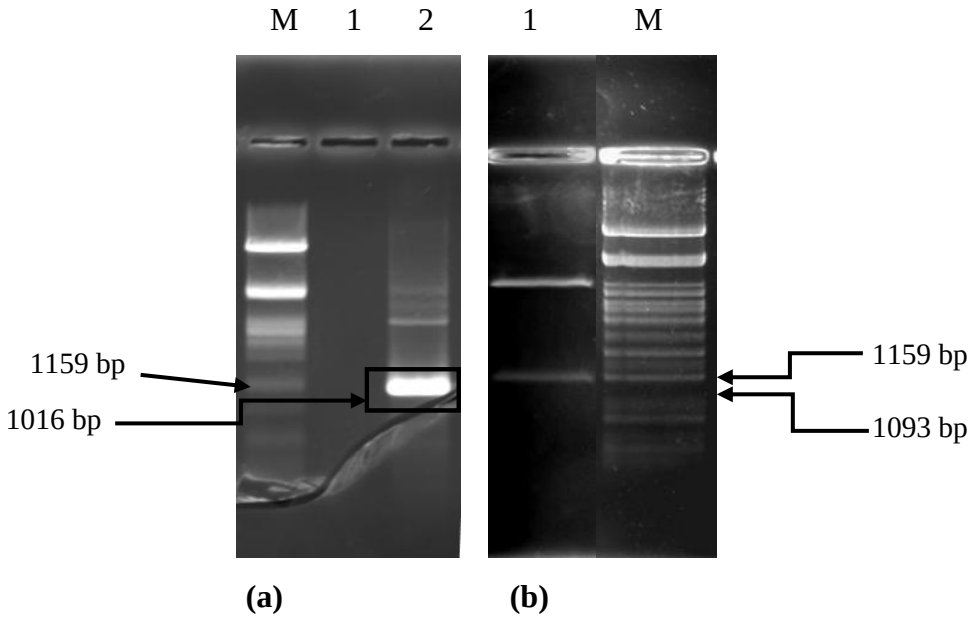


Figure 3.3. Verification of pGVAK01 by PCR (a) and restriction enzyme digestion (b). (a) M: λ *Pst*I DNA marker, 1: Negative control of PCR with no template DNA, 2: PCR amplified *cas2* by using pGVAK01 as the template DNA. (b) 1: *Eco*RI digested pGVAK01, M: λ *Pst*I DNA marker.

3.1.2. Cloning of *cas2* gene in pBluescript II KS+ (pKS+)

After obtaining the recombinant pGVAK01 plasmid carrying *cas2* gene, the next step was the excision of the gene with with *Xba*I and *Hind*III restriction enzymes and cloning it to the linearized pSET152 integration vector. However, it was examined that *Hind*III has an additional cut site inside the pSET152 and it was impossible to get an intact pSET152 after *Xba*I-*Hind*III digestion of the vector itself. Therefore, it was decided to clone the *cas2* gene to the *Xba*I site of pSET152. Although the flanked region before the atg start codon of *cas2* had a *Xba*I site, still another *Xba*I cut site was needed at the 3' end of the gene. Therefore, pBluescript II KS+ (pKS+) (Stratagene) that has a *Xba*I restriction site in its multiple cloning region (MCS) was

chosen as a subcloning vector to get a *Xba*I digested *cas2* gene before inserting it into the *Xba*I digested pSET152 vector.

Accordingly, pGVAK01 and pKS+ were digested with *Eco*RI enzyme. The resultant *Eco*RI digested *cas2* and linerized pKS+ vector were extracted from the gel and both were used for the ligation reaction. After transformation of *E. coli* DH5α competent cells with the ligation mixture, putative recombinant colonies were selected with blue-white screening and ampicillin resistance. One of the white colonies was selected for plasmid isolation and the isolated plasmid was used for the verification of recombination by means of PCR and restriction enzyme digestion (Figure 3.4). This new recombinant plasmid was designated as pGVAK02.

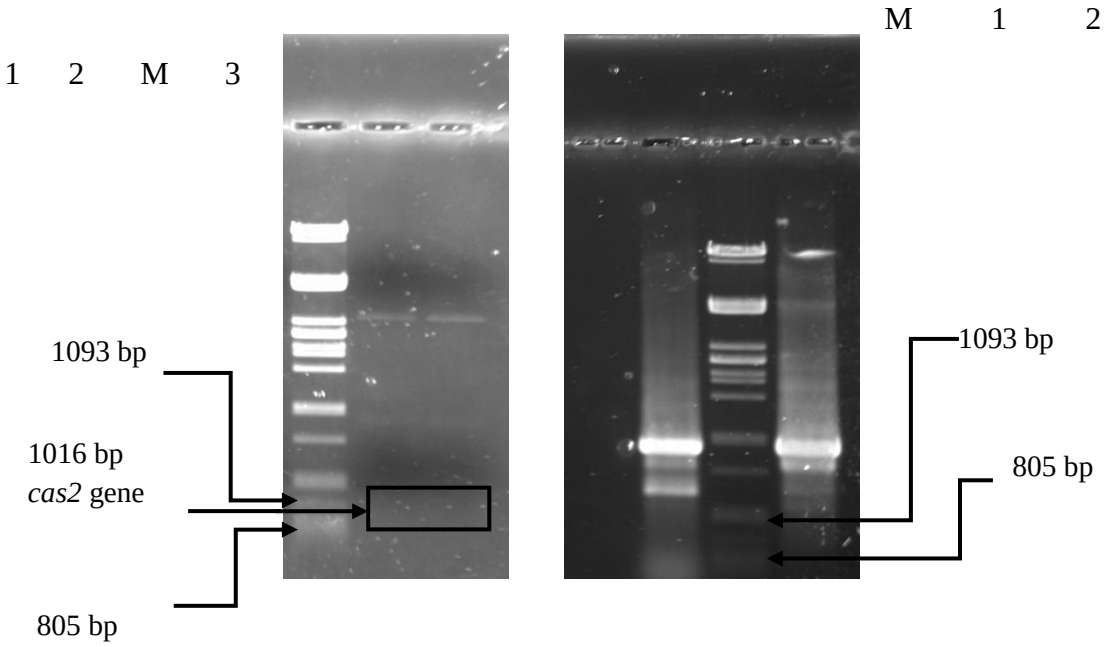


Figure 3.4. Verification of *cas2* containing recombinant pGVAK02 plasmid by restriction enzyme analysis (a) and PCR (b). **(a)** M: λ *Pst*I DNA marker, 1-2: *Xba*I digested pGVAK02, **(b)** M: λ *Pst*I DNA marker, 1: Negative conrol of PCR with no template DNA, 2: PCR amplified *cas2* by using pGVAK02 as the template DNA, 3:

Positive control of PCR using genomic DNA of industrial *S. clavuligerus* strain as the template DNA.

On the other hand, the orientation of *cas2* in pGVAK02 recombinant plasmid was also determined by digestion with *Xba*I, and this digestion resulted in an intact *cas2* gene with a desired orientation (Figure 3.5).

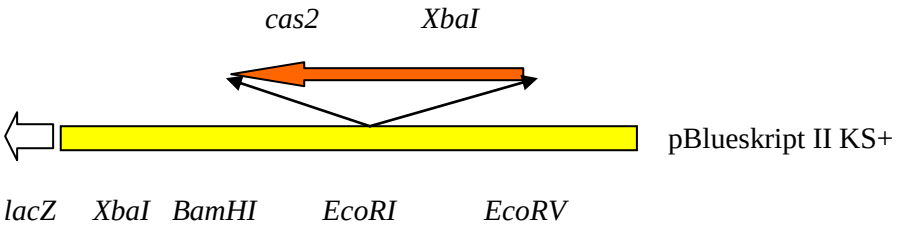


Figure 3.5. Orientation of the *cas2* gene in pGVAK02 recombinant plasmid.

3.1.3. Cloning of *cas2* gene into pSET152 integration vector

For cloning of *cas2* gene to pSET152 integration vector, the recombinant pGVAK02 and pSET152 vectors were isolated from recombinant *E. coli* DH5α cells carrying them and then both vectors were digested with *Xba*I enzyme (Figure 3.6).

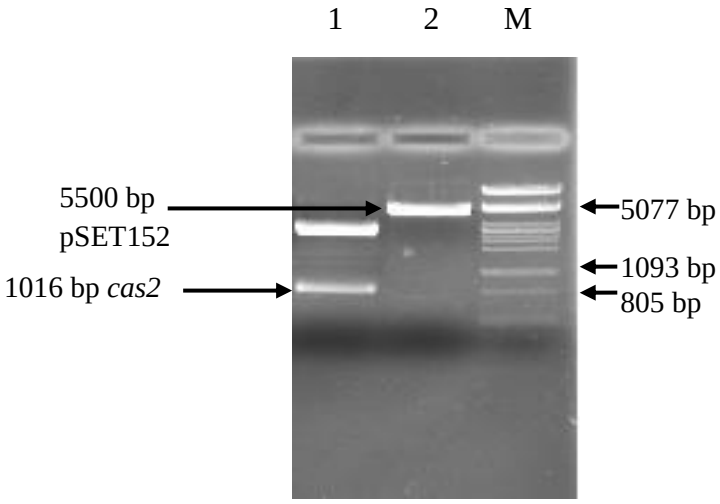


Figure 3.6. *Xba*I digestion of recombinant pGVAK02 carrying *cas2* insert, and empty pSET152. 1: *cas2* excised from pKS+ vector by *Xba*I enzyme, 2: Linearized pSET152 after digestion with *Xba*I, M: λ *Pst*I DNA marker.

The *Xba*I digested *cas2* and pSET152 were extracted from the gel. Then, *Xba*I digested insert was introduced into the linear pSET152 vector with a ligation reaction. This construct was used to transform *E. coli* DH5α competent cells. The transformants were selected from the LA plates containing apramycin (50 μg/ml) as a selective marker. Further verification of the presence of the *cas2* insert in pSET152 was done by performing PCR. The orientation of the gene in the MCS of pSET152 was also determined after digestion of the recombinant plasmid with *Not*I *and Aat*II enzymes (Figure 3.7a,b). The resultant recombinant plasmid was named as pGVAK03.

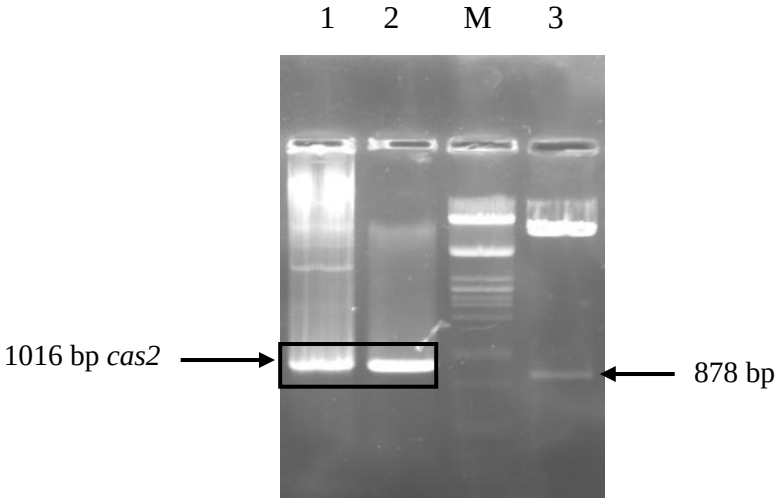


Figure 3.7a. Verification of the cloning of *cas2* gene to pSET152 integration vector and determination of its orientation in the recombinant pGVAK03. M: λ *Pst*I DNA marker, 1: PCR amplification of *cas2* gene by using genomic DNA of industrial *S. clavuligerus* strain as the template, 2: PCR amplified *cas2* gene by using recombinant pGVAK03 plasmid as the template DNA, 3: Digestion of pGVAK03 with *Not*I and *Aat*II enzymes.

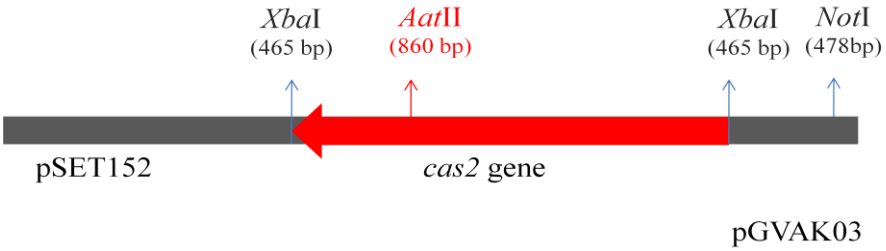


Figure 3.7b. *cas2* gene insterted in pSET152 (pGVAK03).

3.1.4. Conjugal transfer of pGVAK03 into the chromosome of industrial strain of *S. clavuligerus*

There are several ways in which DNA can be introduced into streptomycetes, including transformation, transfection, electroporation, and conjugation between streptomycetes and intergeneric conjugation with *E. coli*. PEG-assisted protoplast transformation or transfection by phages can be applied to some *Streptomyces* spp. due to the need for the development of appropriate protoplast formation and regeneration conditions. However, electroporation by-passes these requirements mentioned above for transformation and transfection, but still, the conditions for electroporation may also be strain specific. On the other hand, it is common to use conjugation with *E. coli* to deliver DNA into *Streptomyces* for nearly all species (Keiser *et al*., 2000). Introducing DNA into a streptomycetes from *E. coli* via conjugation has several advantages over electroporation and protoplast transformation. First of all, (i) it is simple and does not rely on the development of procedures for protoplast formation and regeneration, (ii) restriction barriers may be by-passed or severly reduced by the transfer of single-stranded concatemers of plasmid DNA (Matsushima *et al*., 1994), (iii) a variety of versatile *ori*T vectors are available that permits site-specific or insert-directed choromosomal integration, (iv)

since these vectors replicate in *E. coli*, the production of required constructs is considerably faciliated (Keiser *et al*., 2000).

It was reported that *Streptomyces* are hard to be transformed because of their restriction endonuclease systems which constitute a barrier to transfer DNA from non streptomycetes origins. To avoid methylation, the plasmids have to be passed through a nonmethylating *E. coli* host or through *Streptomyces lividans* before introduction to the actual host (Matsushima *et al*., 1987; MacNeil *et al*., 1987; Flett *et al*., 1997; Keiser *et al*., 2000).

For these reasons, the chromosomal integration of *cas2* gene to *S. clavuligerus* industrial strain was followed by the introduction of pGVAK03 vector to methylation-deficient *E. coli* ET12567/pUZ8002 cells by transformation in order to avoid methyl-specific restriction system in *Streptomyces*. Methylation-deficient *E. coli* ET12567/pUZ8002 donor strain has an *oriT*-RK2 derivative pUZ8002 plasmid. The pUZ8002 plasmid has transacting functions for the mobilization of *ori*T-carrying pSET152 plasmid but the pUZ8002 is not transferable itself due to a mutation in its *oriT* site (Bierman *et al*., 1992; Paget *et al*., 1999).

The *cas2* containing pGVAK03 plasmid was introduced into the chromosome of industrial *S. clavuligerus* strain via conjugation by using a slightly modified procedure described by Flett *et al*. (1997). Since the integration vector pSET152 cannot replicate in *Streptomyces* spp., the resulting exconjugants could be maintained in apramycin containing TSA only if the plasmid is integrated into the choromosome of *S. clavuligerus*. The orientation of pGVAK03 in *S. clavuligerus* chromosome is shown in Figure 3.8.

Figure 3.8. The orientation of pGVAK03 in *S. clavuligerus*.

Following conjugation, 70 exconjugant colonies grown on MS agar plates were streaked to nalidixic acid and apramycin containing TSA plates and incubated for about 48 h at 30 ℃ for propagation. In order to verify the integration of pGVAK03 into the chromosome of *S. clavuligerus* industrial strain, a PCR reaction was performed (Figure 3.9). A pair of primers was designed in such an order that the forward primer included the first 20 nucleotides starting from 5' end of the *cas2* gene and the reverse primer started at 542 nucleotides downstream from the 3' end of the gene. These primers were used to amplify 1016 bp *cas2* plus 542 bp from pSET152 to give a 1558 bp amplicon. Genomic DNAs of arbitrarily selected four exconjugants among 70 were isolated to use as template DNAs for PCR reaction. As shown in Figure 3.8., the 1558 bp PCR product was obtained after PCR from the samples containing genomic DNA's of four exconjugants used as the template DNA, confirming the chromosomal integration of the *cas2* gene into the industrial strain of *S. clavuligerus*.

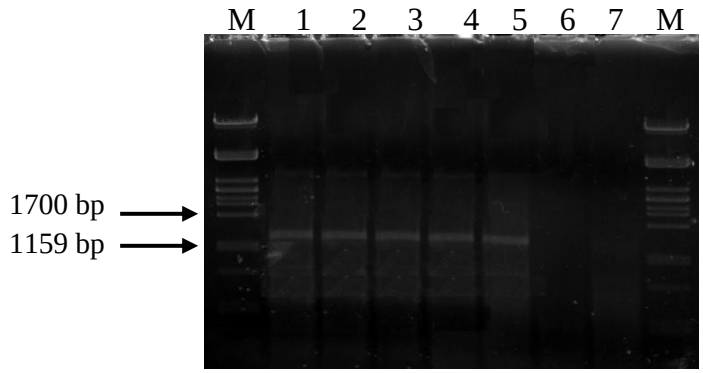


Figure 3.9. Verification of pGVAK03 integration into the chromosome of industrial strain of *S. clavuligerus* by PCR. M: λ *Pst*I DNA marker, 1-2-3-4: PCR amplified 1558 bp amplicon by using genomic DNA of GV8, GV12, GV31 and GV32 respectively, 5: Positive control of PCR by using pGVAK03 as the template DNA, 6: Negative control of PCR obtained with no template DNA, 7: Negative control of PCR obtained by using genomic DNA of parental industrial strain of *S. clavuligerus*.

3.2. HPLC identification of clavulanic acid

Nowadays, secondary metabolites are identified by using advanced techniques such as high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS). Routine quantification of a given secondary metabolite in a given culture is commonly performed via determination of biological activities of the compound by microbiological assays with an appropriate indicator organism. HPLC separation and quantification by comparison with pure standards has also became a routine technique for many components as well as clavulanic acid since it is highly sensitive to heat and light, therefore being an unstable metabolite by making it very hard to detect (Liras and Martin, 2005).

Figure 3.10. shows a chromatogram of 1 mg/ml CA standard run in HPLC column giving a retention time of 5.547 min. Quantified clavulanic acid yields were tabulated as µg/g.

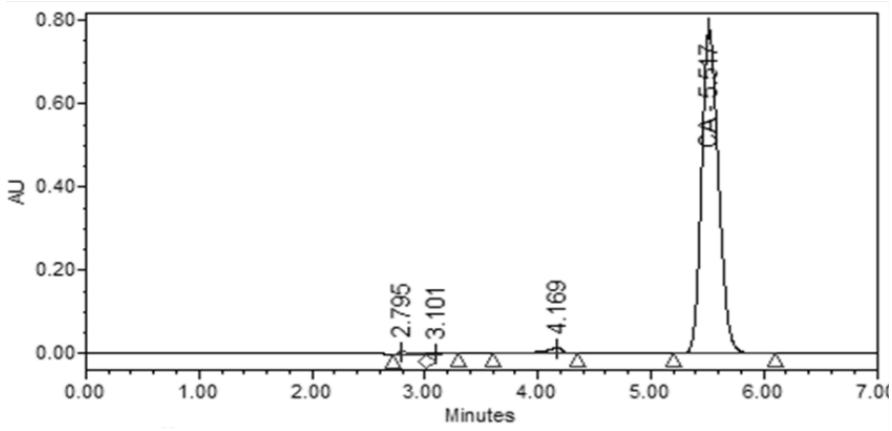


Figure 3.10. HPLC chromatogram of CA standard.

3.3. Comparison of clavulanic acid production capacities of recombinant and industrial parental *S. clavuligerus* strains

CC2 vegetation and CC3 fermentation media were used to compare CA productions of the recombinants (GV1-70) and the parental strain of *S. clavuligerus*. These media contain soybean flour (20 g/l) as the sole nitrogen source, and dextrin (10g/l) as the carbon source. Soybean flour includes arginine, which is the precursor of CA and therefore was shown to be the most important nutrient favoring clavulanic acid biosynthesis in previous studies (Butterworth 1984; Mayer and Deckwer, 1996). In a recent study, Viana and his co-workers (2010) also reported that soybean flour and glycerol as final concentrations of 20 g/l and 5 g/l, respectively, are preferred nutrients to yield considerable amounts of CA in a bench scaled fermenter.

In our preliminary studies, the growth and CA titers of industrial *S. clavuligerus* strain and the wild type *S. clavuligerus* ATCC 27064 were compared by microbiological assay and HPLC analyses (Figure 3.11a,b,c). Both strains were grown in CC3 for 168 h at 23.5 ℃ with shaking at 240 rpm. As shown in Figure 3.11a, the growth of industrial strain was better than that of ATCC 27064 strain during the course of fermentation. However specific CA production of the industrial strain was found approximately 5 fold higher than that of the wild type *S. clavuligerus* strain as determined by bioassay (Figure 3.11b). The marked difference in the CA titers of two strains was also seen by comparing the CA peaks in HPLC chromatograms which revealed at least 7 fold difference (Figure 3.11c).

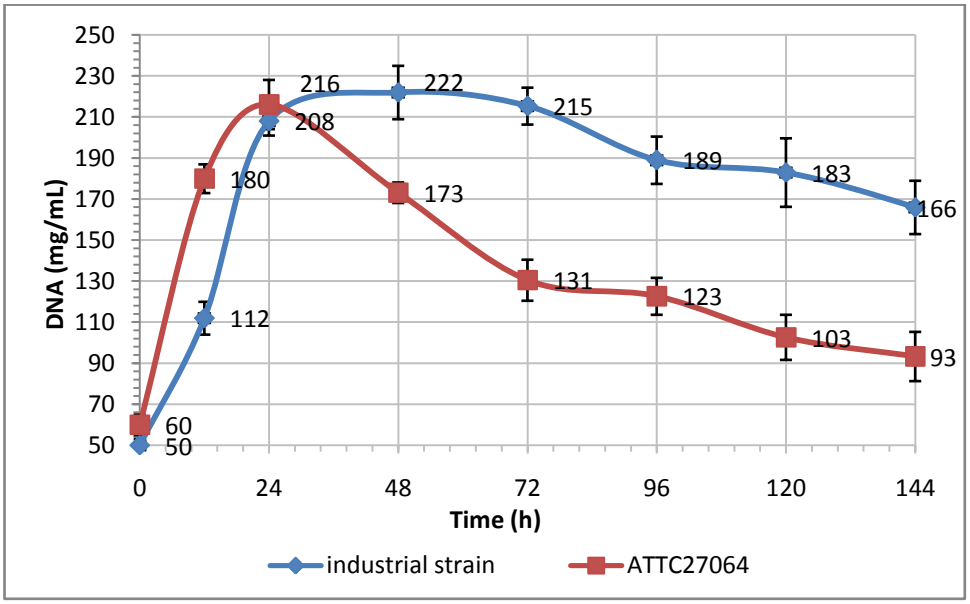


Figure 3.11a. Time dependent growth of wild type *S. clavuligerus* ATTC 27064 and industrial *S. clavuligerus* strain.

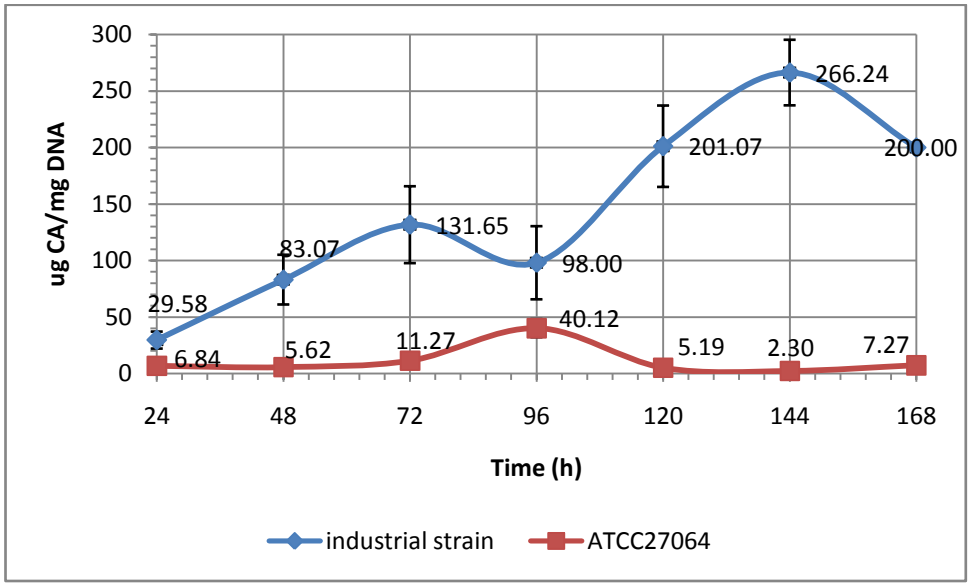
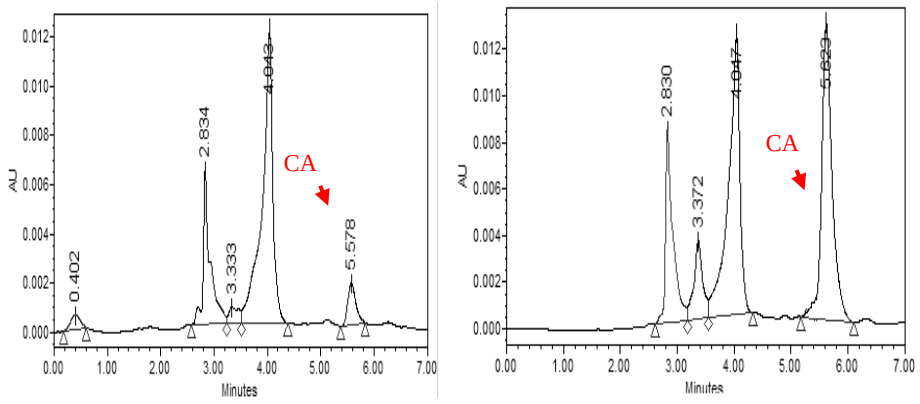


Figure 3.11b. Specific CA production by wild type and industrial *S. clavuligerus* strain, as determined by bioassay.



(a) **(b)**

Figure 3.11c. HPLC analysis of the culture broths taken at 144 h fermentation showing CA biosynthesis by wild type (a) and industrial *S. clavuligerus* strain (b).

Fermentation experiments were started right after the determination of CA titers from the cultures as a function of time during the course of fermentstion which lasts for 144 to 168 h in DEPA Clavulanic Acid Production Plant. Industrial *S. clavuligerus* strain was grown in CC3 for 168 h at 23.5 ℃ by shaking at 240 rpm. Samples were taken from the cultures at 24 h intervals and prepared for HPLC injections. Time dependent CA production by this strain was presented in Figure 3.12.

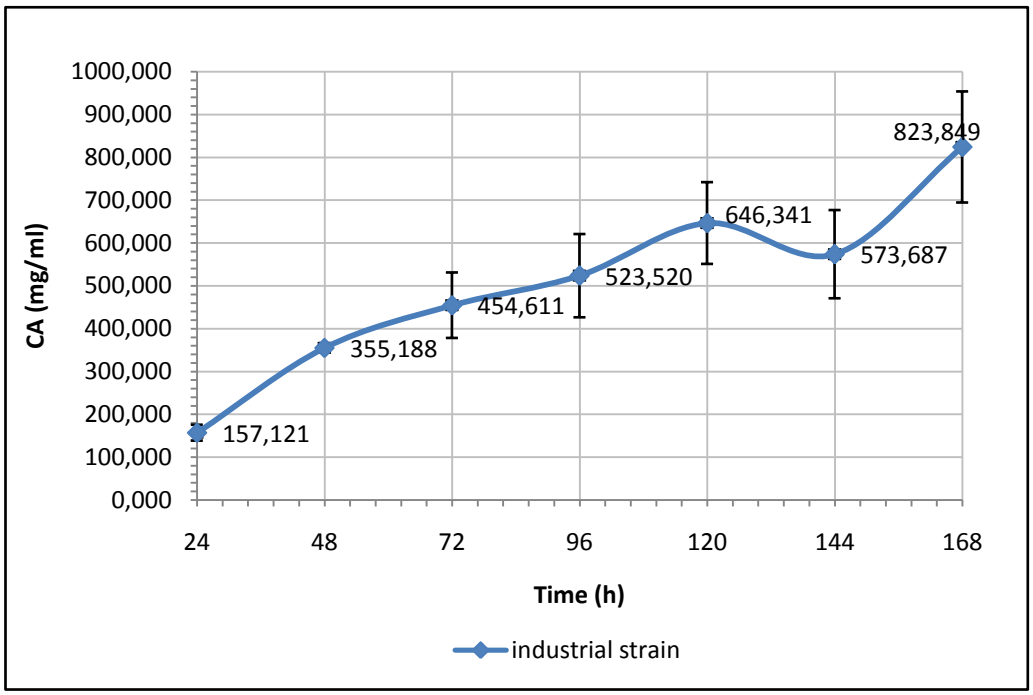


Figure 3.12. Time dependent CA production by industrial *S. clavuligerus* strain as determined by HPLC analysis.

Considering that the cultures are harvested in between 144 to 168 h in DEPA manufacturing plant, the samples for HPLC analyses were withdrawn from the fermentation broths at 24 h intervals, starting form 72 h and ending at 168 h fermentation. 5 sets of fermentation experiments for a total of 70 recombinant strains along with the parental *S. clavuligerus* industrial strain in each set were undertaken. In each fermentation set, culture samples were run in HPLC to compare the CA titers of the parental strain and 13 to 14 different recombinant strains.

In the 1st set of the fermentation experiment, CA production by 13 recombinants (from GV1 to GV13) were analysed by HPLC. Time dependent CA production by those strains was tabulated in Table 3.1. In the chromatograms, the CA peaks of some of the recombinant strains were appeared as overlapping peaks around the expected retention time of the CA standard (Figure 3.13). Due to the absence of a clear CA peak in the chromatograms, the software of HPLC was unable to quantify the CA amount present in the samples. Therefore, such peaks were not evaluated in the average CA productions, but considered as nd (not determined) in the tables.

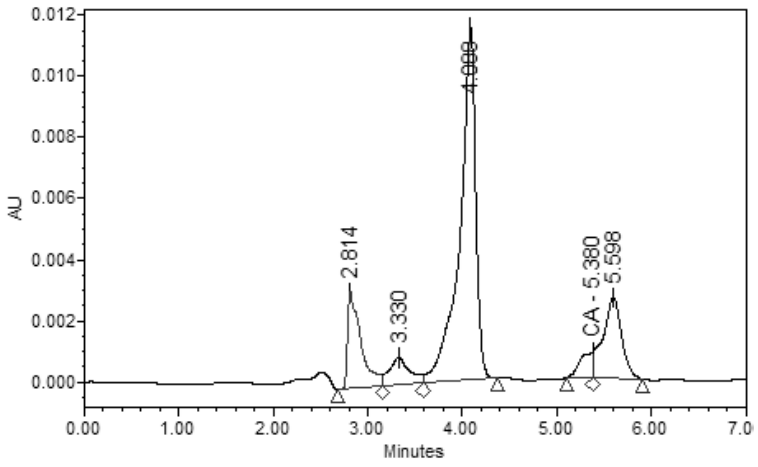


Figure 3.13. HPLC chromatogram of an overlapping CA peak.

Table 3.1. Average CA production by recombinant *S. clavuligerus* GV1-GV13 strains and their parental counterpart.

Average CA production (µg/g)					
Recombinant strain	Hour				
	72	96	120	144	168
S. clavuligerus GV1	592.560	481.984	546.121	1049.451	402.289
S. clavuligerus GV2	504.02	0.00	37.97	39.70	42.28
S. clavuligerus GV3	319.89	471.62	256.28	170.43	215.87
S. clavuligerus GV4	nd*	46.34	52.16	54.19	nd
S. clavuligerus GV5	355.879	458.339	519.561	664.971	682.454
S. clavuligerus GV6	291.407	410.353	461.285	546.622	642.326
S. clavuligerus GV7	497.958	767.993	657.22	963.409	964.009
S. clavuligerus GV8	399.249	548.267	349.51	286.604	95.204
S. clavuligerus GV9	259.182	368.739	343.825	434.296	622.135
S. clavuligerus GV10	389.583	443.615	485.042	670.235	726.216
S. clavuligerus GV11	515.052	552.415	675.367	1003.173	954.432
S. clavuligerus GV12	352.87	428.28	385.17	545.54	469.27
S. clavuligerus GV13	nd	370.47	414.69	466.82	416.37
Parental Strain	393.83	746.39	434.11	588.86	449.07

*nd: not determined

Accordingly, the highest CA production by parental *S. clavuligerus* industrial strain corresponded to 96th h of fermentation. The subsequent fluctuations in its CA production pattern may be due to species-specific characteristics of this strain as commonly experienced in our previous studies (Taskin, 2005; Çaydaşı, 2006; Özcengiz *et al.*, 2010) as well as in other laboratories (Minas *et al.*, 2000; Büchs, 2001). At 72th h of fermentation, *S. clavuligerus* GV1, GV2, GV7, GV8, GV10 and GV11 produced higher amount of CA than the parental strain. Among these strains, only GV7 continued to produce higher amount of CA then the parental strain (Figure 3.14). Recombinant GV7, GV10 and GV12 strains produced higher amounts of CA at the end of 120th, 144th and 168th h of fermentation, respectively as compared to the parental strain. The rest of the recombinant strains produced either comparable or lesser amounts of CA (Table 3.1).

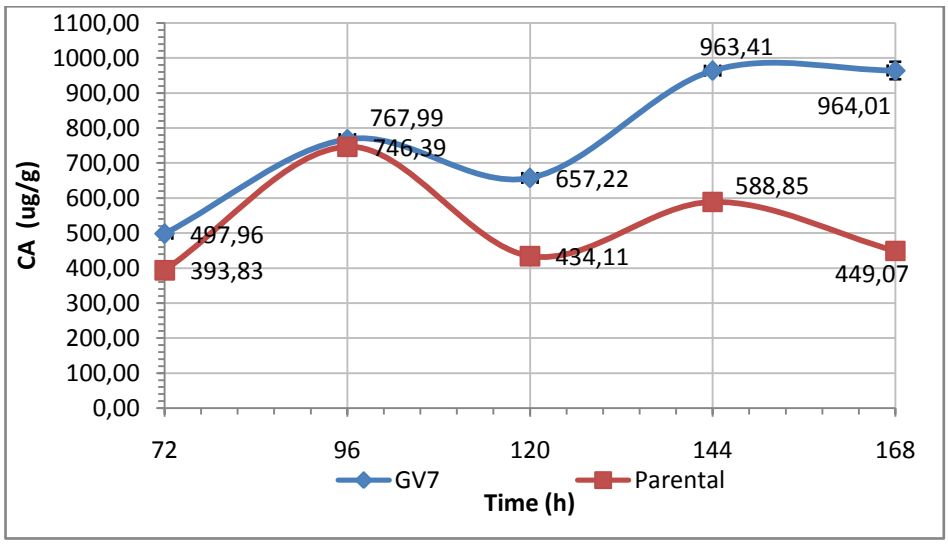


Figure 3.14. Comparison of time dependent CA production by industrial *S. clavuligerus* strain and its recombinant GV7 as determined HPLC.

The recombinant strains which produced higher amounts of CA, and the percentage increase they provide are shown in Figure 3.15. The percentage CA increase among GV1 to GV13 recombinants ranged between 3.4% and 114.7%. The most suitable hours for obtaining high CA yields by these recombinants were found in between 120 to 168 h confirming the results of our preliminary experiment. When compared CA production profiles of all strains, *S. clavuligerus* GV7 and GV11 seemed to be better CA producers with respect to the parental industrial strain especially at 168^{th} h of fermentation.

Figure 3.16, 3.17 and 3.18 show HPLC chromatograms of the parental strain, GV7 and GV11 recombinant strains from 72^{nd} to 168^{th} h of fermentation. HPLC chromatograms revealed a well separated CA peak in each fermentation broth. The source of peaks having earlier retention times in the chromatograms were sodium acetate solution used for buffering of samples prior to HPLC injections. In the aliquots sampled, CA gave its peak at a retention time ranging form 5.1 to 5.5, probably minor changes in pH of daily prepared sodium acetate buffer and mobile phase account for such differences in the retention time of CA. However, retention times of the same days' samples were in a compatible retention range with that of the CA standard.

As mentioned earlier, in the 72^{nd} and the 96^{th} h of the fermentation, GV7 and GV11 recombinant strains produced nearly the same amounts of CA, however, as the fermentation proceeded, CA production of both recombinant strains increased dramatically as compared to the parental strain (Figure 3.16, 17, 18).

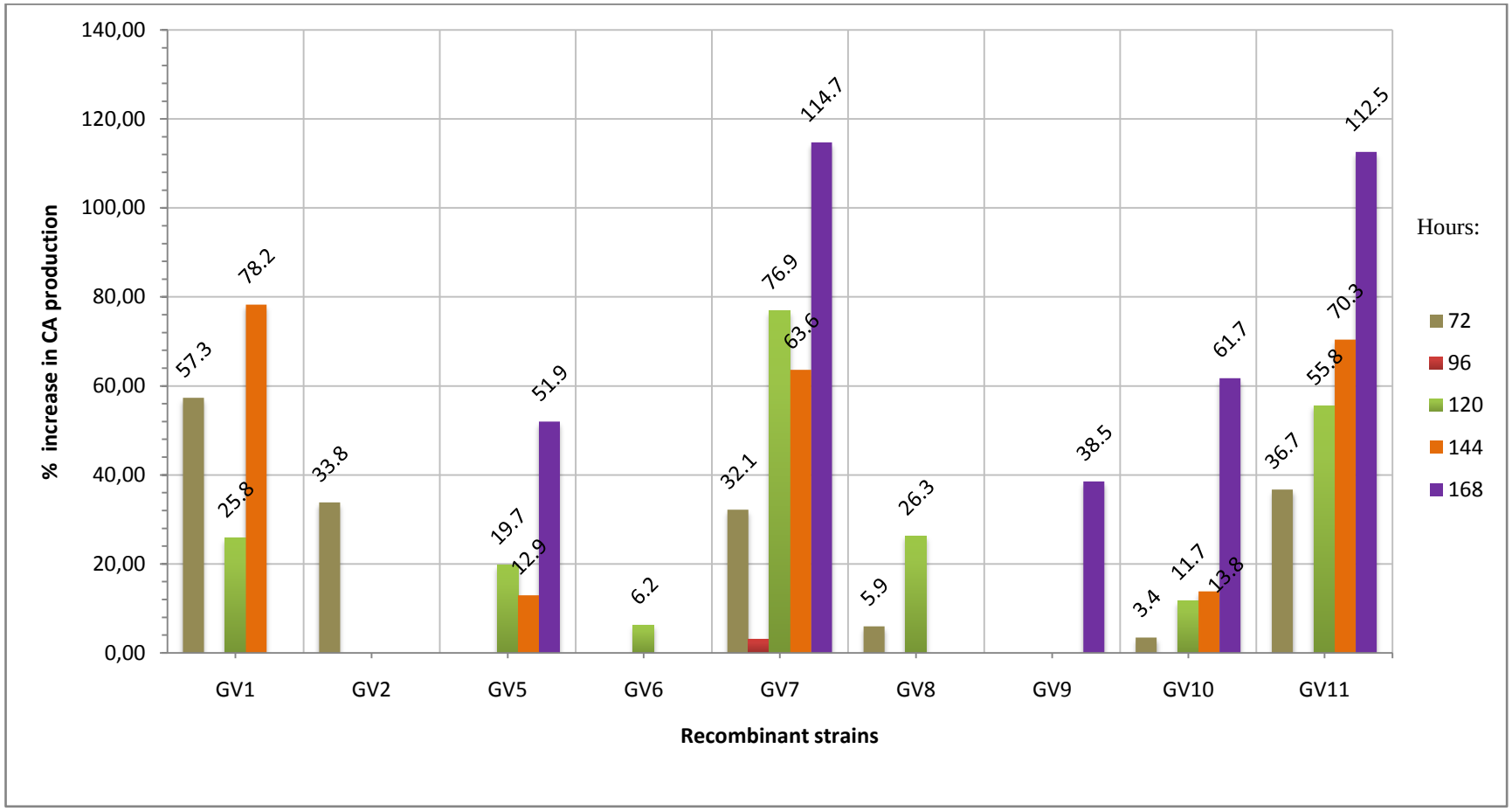
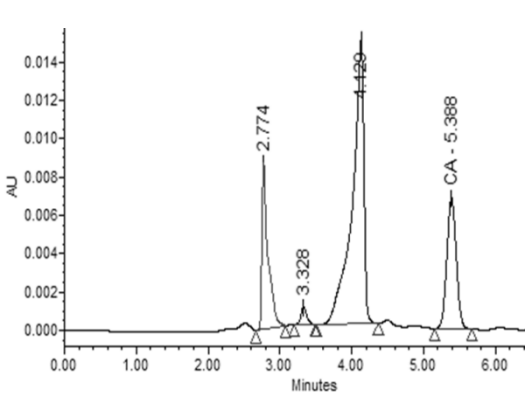
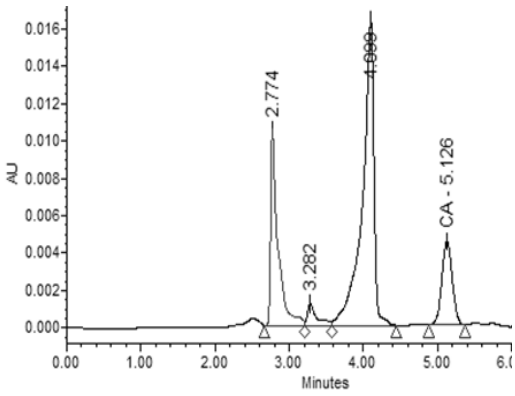


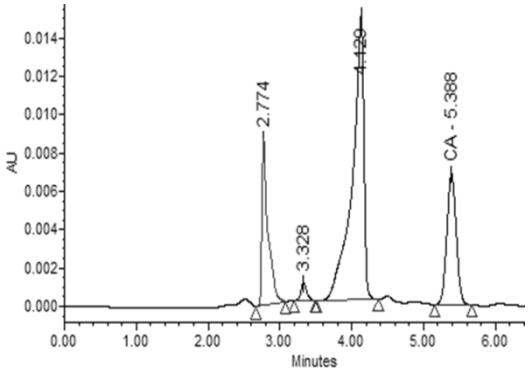
Figure 3.15. The percentage increase in CA production by recombinant *S. clavuligerus* strains in Fermentation Set 1.



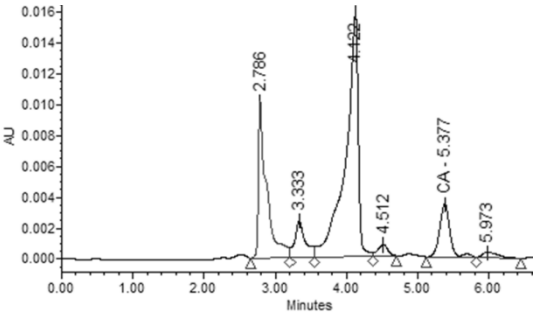
(a)



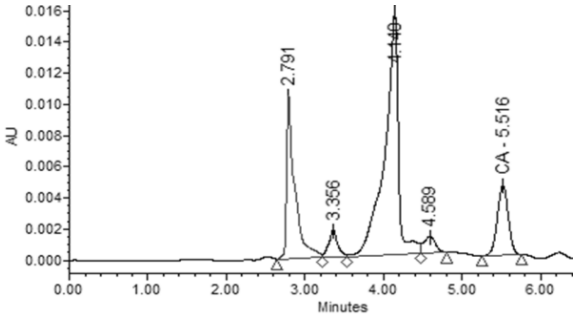
(b)



(c)



(d)



(e)

Figure 3.16. HPLC analysis of the culture broths of industrial parental *S. clavuligerus* at 72 h (a), 96 h (b), 120 h (c), (d) 144 h, and 168 h (e) of the fermentation.

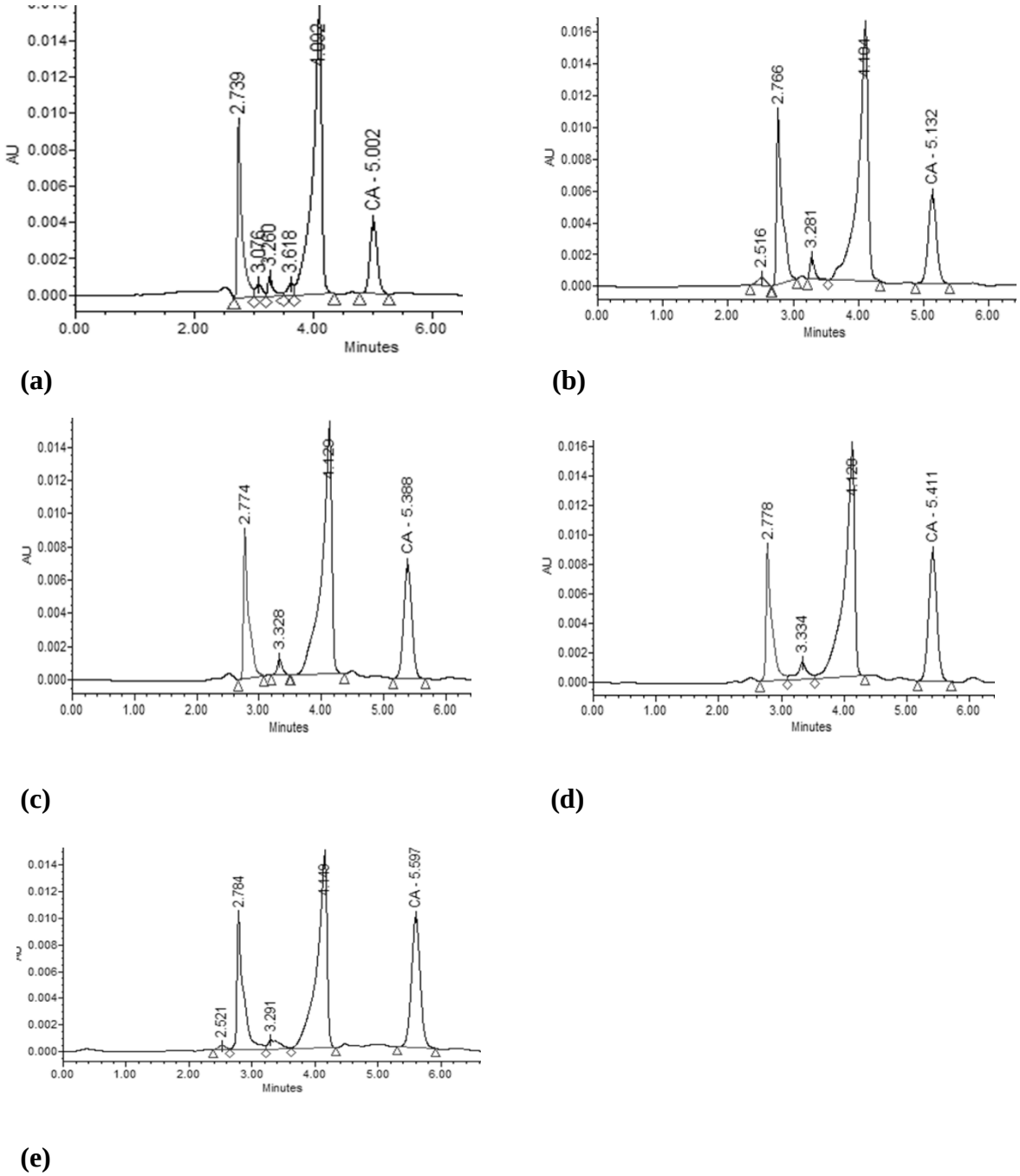
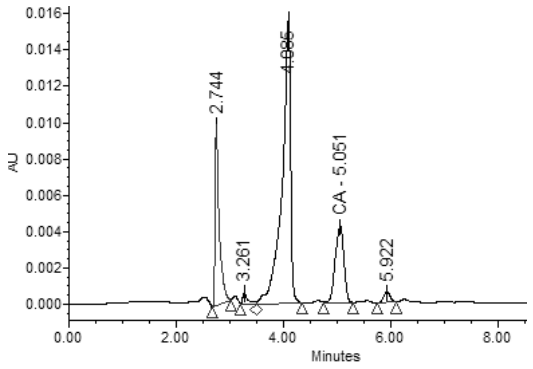
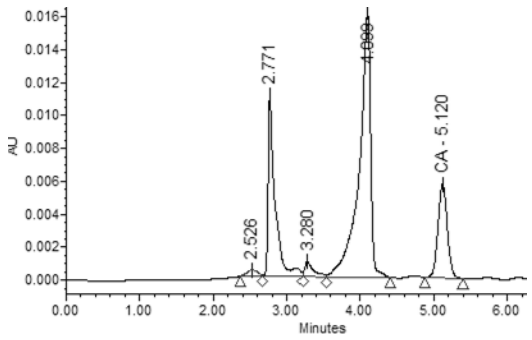


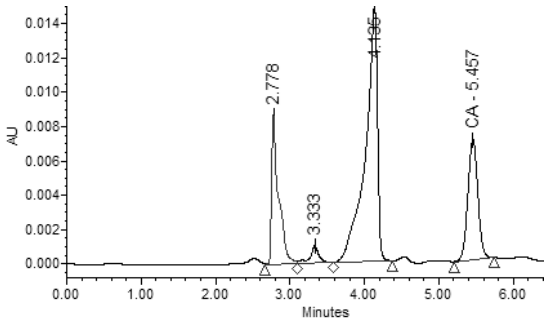
Figure 3.17. HPLC analysis of the culture broths of *S. clavuligerus* GV7 at 72 h (a), 96 h (b), 120 h (c), (d) 144 h, and 168 h (e) of the fermentation.



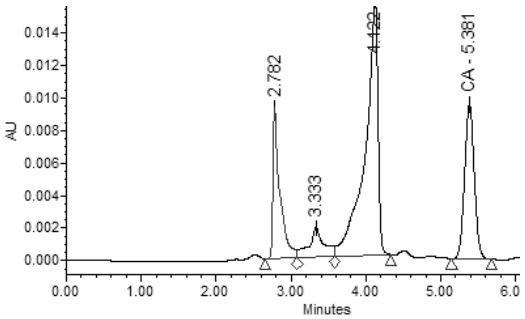
(a)



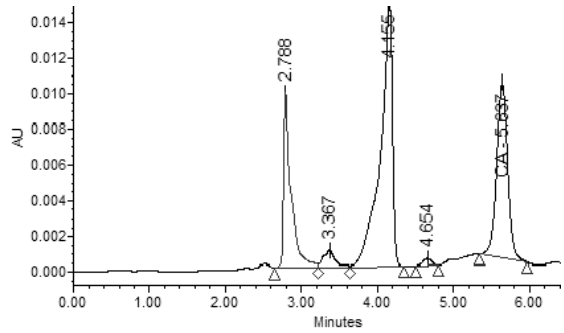
(b)



(c)



(d)



(e)

Figure 3.18. HPLC analysis of the culture broths of *S. clavuligerus* GV11 at 72 h (a), 96 h (b), 120 h (c), (d) 144 h, and 168 h (e) of the fermentation.

In the 2nd set of the fermentation experiments, 14 different recombinant strains were tested for their CA yields in comparison to the CA production by the parental strain. The average CA production by the recombinants and the parental strain are listed in Table 3.2.

The CA production performance of the industrial *S. clavuligerus* strain was found to be lower than that shown in the previous fermentation set. Additionally, all strains in this fermentation set yielded relatively lower amounts CA. However, the number of recombinant strains better than the parental strain was higher than those found in the first experiment. For instance, GV17, GV19, GV20, GV22 and GV24 showed stably increasing CA production throughout the fermentation. The maximum CA yield was obtained from GV27 (845.7 µg/g). At 168th h, CA yield of GV27 dropped drastically (Figure 3.19).

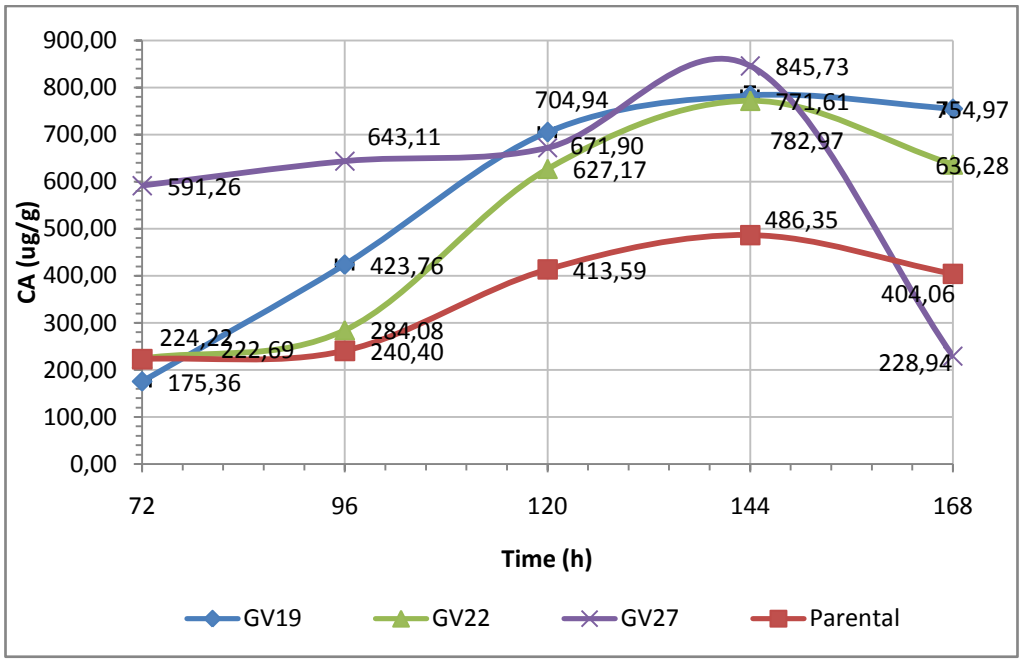


Figure 3.19. Comparison of time dependent CA production by industrial *S. clavuligerus* and its recombinants GV19, GV22 and GV27.

Table 3.2. Average CA production by recombinant *S. clavuligerus* GV14-GV27 strains and their parental counterpart.

Average CA production (µg/g)					
Recombinant strain	Hour				
	72	96	120	144	168
S. clavuligerus GV14	186.50	190.42	259.20	274.79	313.29
S. clavuligerus GV15	276.25	299.05	308.14	349.40	183.61
S. clavuligerus GV16	299.59	445.76	379.26	528.34	429.02
S. clavuligerus GV17	355.02	430.05	527.70	565.62	556.41
S. clavuligerus GV18	448.69	415.94	532.68	606.08	397.15
S. clavuligerus GV19	175.36	423.76	704.94	782.97	754.97
S. clavuligerus GV20	401.68	402.19	511.77	534.29	528.01
S. clavuligerus GV21	161.91	240.28	245.11	347.42	227.08
S. clavuligerus GV22	224.22	284.08	627.17	771.61	636.28
S. clavuligerus GV23	261.61	219.10	212.64	380.57	413.81
S. clavuligerus GV24	372.13	318.23	421.30	622.99	623.61
S. clavuligerus GV25	200.43	231.10	231.10	440.51	384.33
S. clavuligerus GV26	164.12	202.42	188.65	303.16	233.60
S. clavuligerus GV27	591.26	643.11	671.90	845.73	228.94
Parental Strain	222.69	240.40	413.59	486.35	404.06

The recombinant strains which produced higher amounts of CA and percentage increase they provide at different stages of fermentation are shown in Figure 3.20, as well. The percentage CA increase among the recombinants ranged between 0.7% and 167.5%. In this fermentation set, GV27 was found to be the best CA producer with 165.5 and 167.5% more CA yields as compared to the parental strain at 72 and 96 h fermentations, respectively.

Figure 3.21 to 3.24 show HPLC outputs of the parental strain, GV19, GV22 and GV27 recombinant strains during 72 to 168 h fermentation with well separated CA peaks.

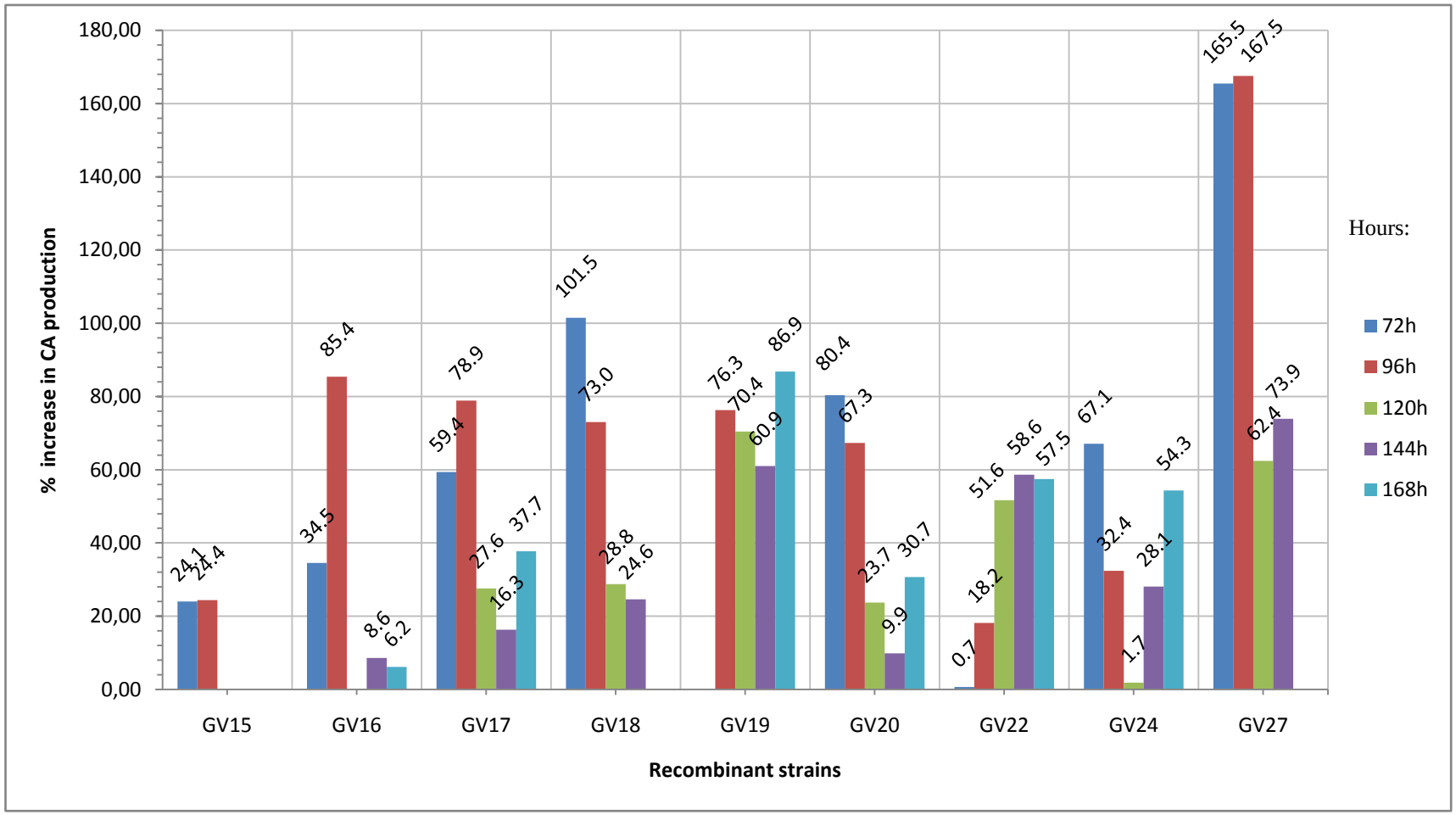


Figure 3.20. The percentage increase in CA production by recombinant *S. clavuligerus* strains in Fermentation Set 2.

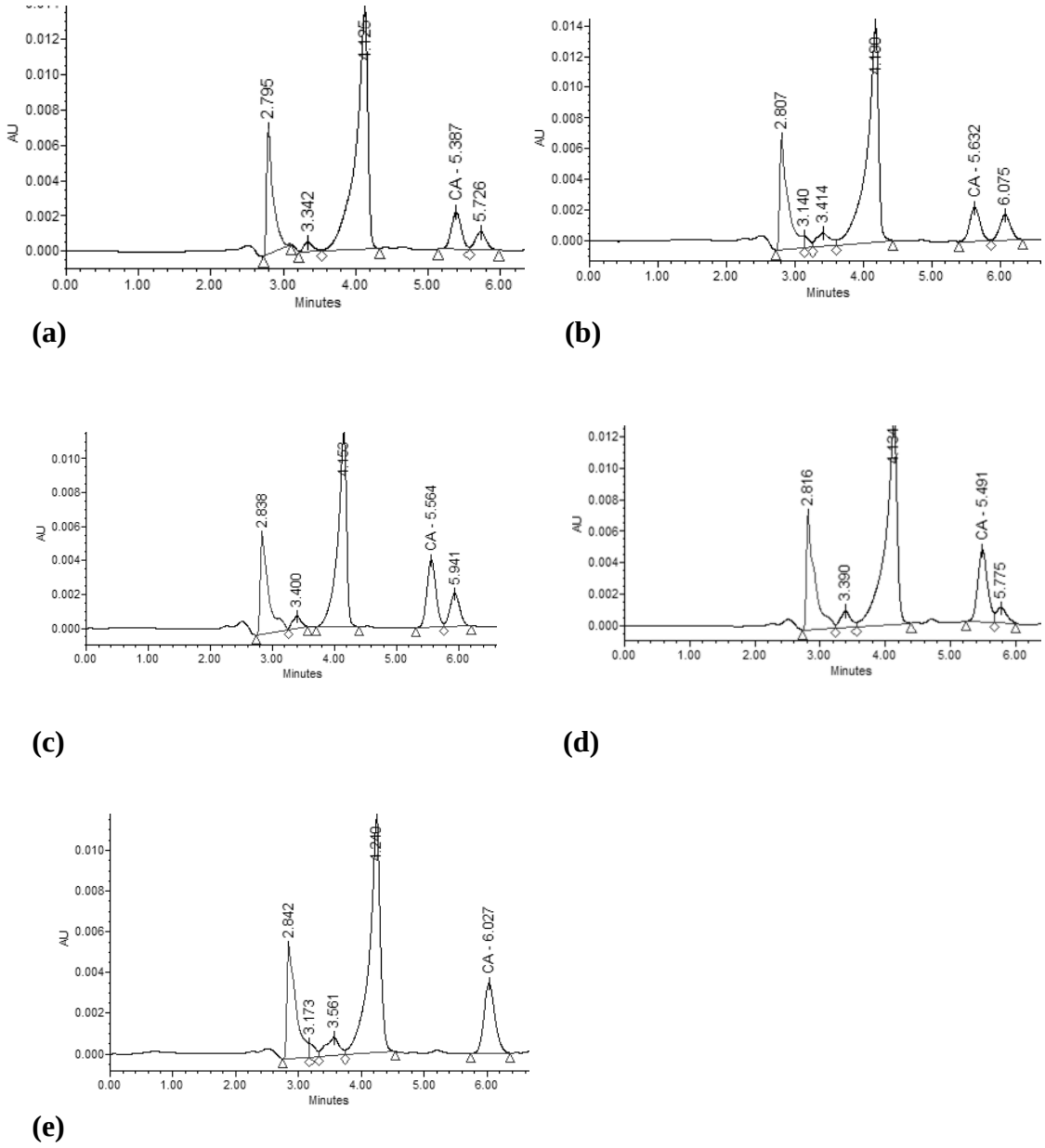
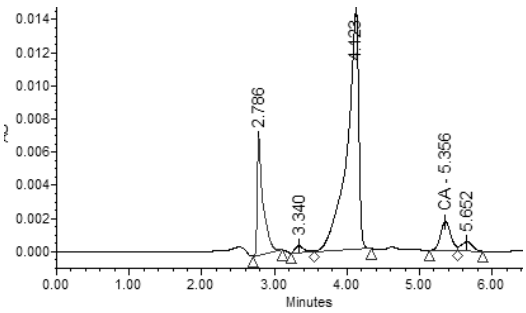


Figure 3.21. HPLC analysis of the culture broths of industrial parental *S. clavuligerus* at 72 h (a), 96 h (b), 120 h (c), 144 h (d), and 168 h (e) of the fermentation.



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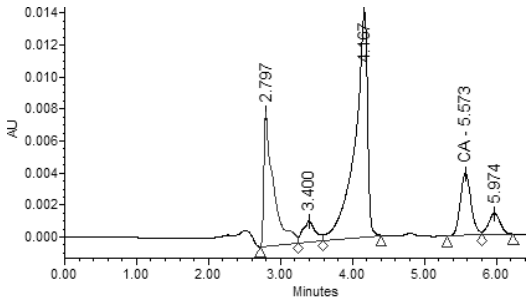

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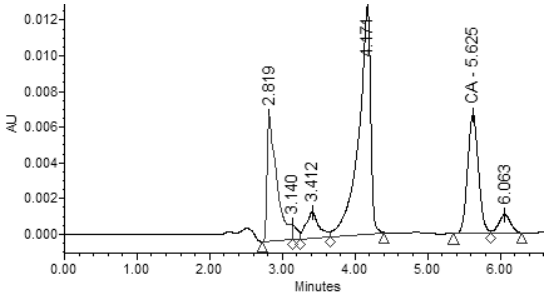
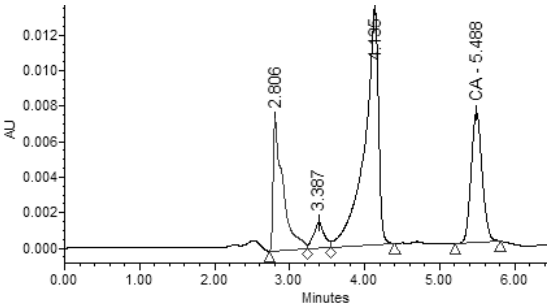


(c)



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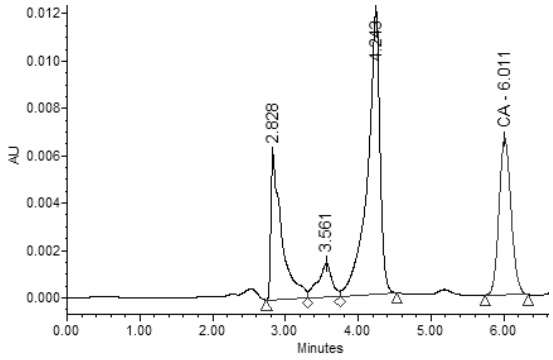
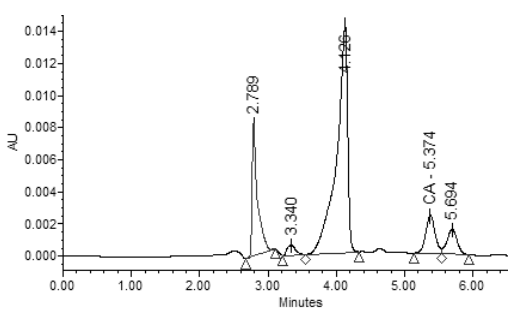


(e)

Figure 3.22. HPLC analysis of the culture broths of *S. clavuligerus* GV19 at 72 h (a), 96 h (b), 120 h (c), 144 h (d), and 168 h (e) of the fermentation.



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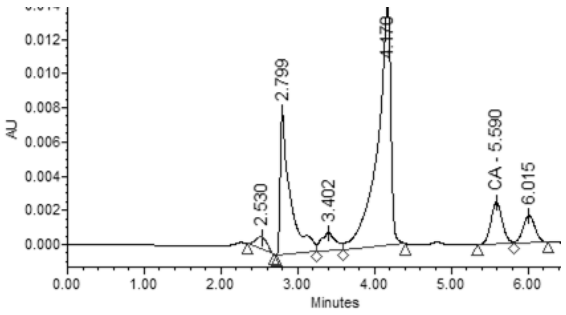

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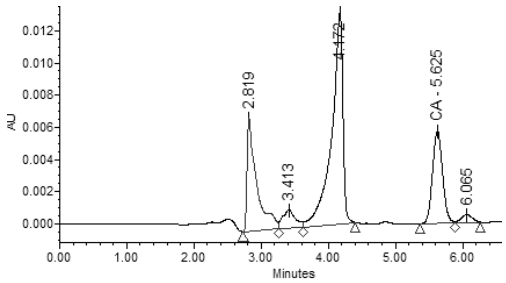

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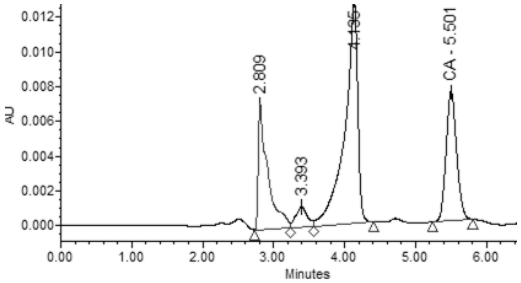

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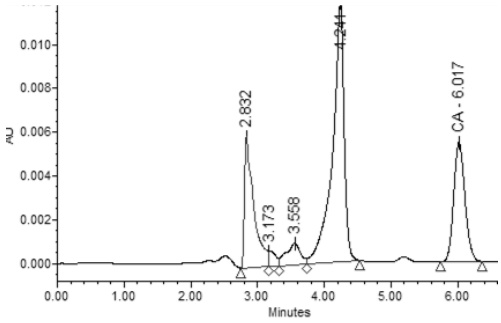
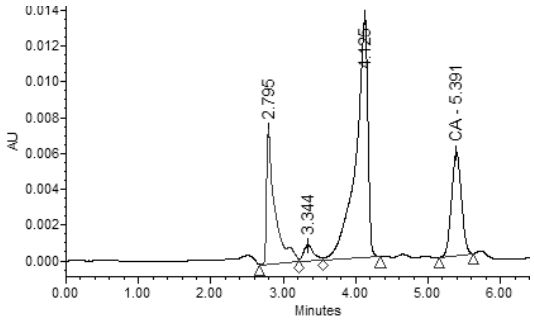
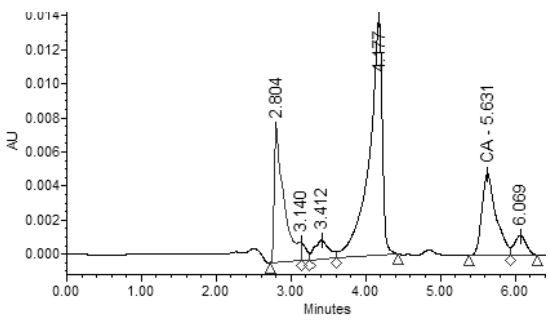


(e)

Figure 3.23. HPLC analysis of the culture broths of *S. clavuligerus* GV22 at 72 h (a), 96 h (b), 120 h (c), 144 h (d), and 168 h (e) of the fermentation.



(a)



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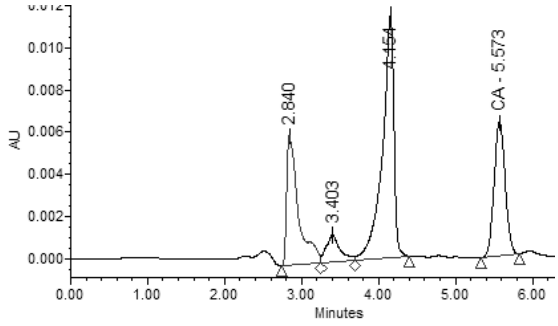

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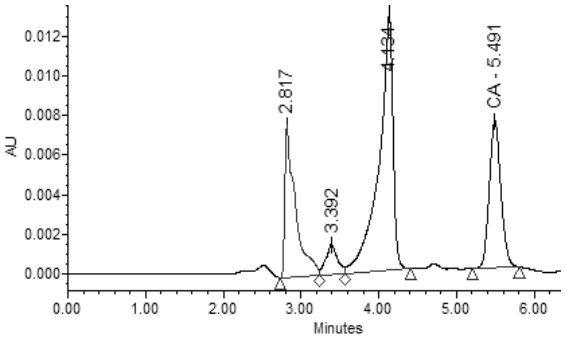

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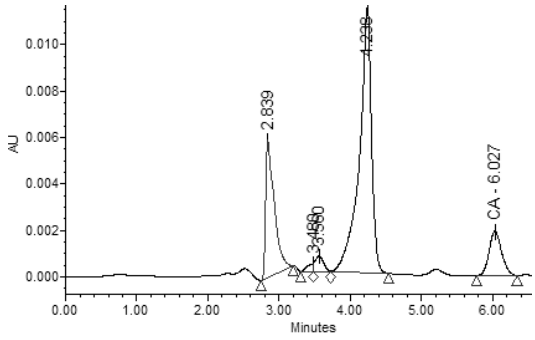

(e)

Figure 3.24. HPLC analysis of the culture broths of *S. clavuligerus* GV27 at 72 h (a), 96 h (b), 120 h (c), 144 h (d), and 168 h (e) of the fermentation.

In the 3rd set of the experiments, another set of 14 recombinant strains were analysed for their clavulanic acid production in comparison to their parental counterpart. The average CA production by the recombinants and the parental strain are listed in Table 3.3. Up to 168 h fermentation, most of the recombinants showed no increase in their CA production with respect to the parental strain. Only a couple of them such as GV37 at 72nd h, GV40 and GV41 at 72nd and 96th h and most remarkably GV38 at 168th h had better CA yields than their parental counterpart. The highest CA yield was attained by the recombinant GV38 (604,6) µg/g at the end of the fermentation which corresponded to 1195.7% increase (12 fold) with respect to the parental strain (Figure 3.25).

Table 3.3. Average CA production by recombinant *S. clavuligerus* GV28-GV41 strains and their parental counterpart.

Average CA production (µg/g)					
Recombinant strain	Hour				
	72	96	120	144	168
S. clavuligerus GV28	64.71	287.93	148.64	141.58	96.04
S. clavuligerus GV29	67.26	320.23	215.70	242.35	252.28
S. clavuligerus GV30	60.25	231.57	114.10	189.19	133.46
S. clavuligerus GV31	56.80	230.39	159.06	128.96	nd
S. clavuligerus GV32	71.06	28.70	52.22	75.24	nd
S. clavuligerus GV33	56.75	42.53	52.94	72.65	nd
S. clavuligerus GV34	79.22	23.88	239.76	272.50	51.13
S. clavuligerus GV35	61.10	26.80	239.36	204.82	87.87
S. clavuligerus GV36	nd	145.29	203.30	258.59	148.55
S. clavuligerus GV37	336.10	300.00	250.25	271.05	78.99
S. clavuligerus GV38	nd	nd	131.62	275.27	604.65
S. clavuligerus GV39	63.60	255.80	156.29	170.44	nd
S. clavuligerus GV40	119.25	424.75	369.04	272.96	164.41
S. clavuligerus GV41	127.68	392.24	324.25	260.96	129.98
Parental Strain	91.37	346.72	385.15	381.22	46.66

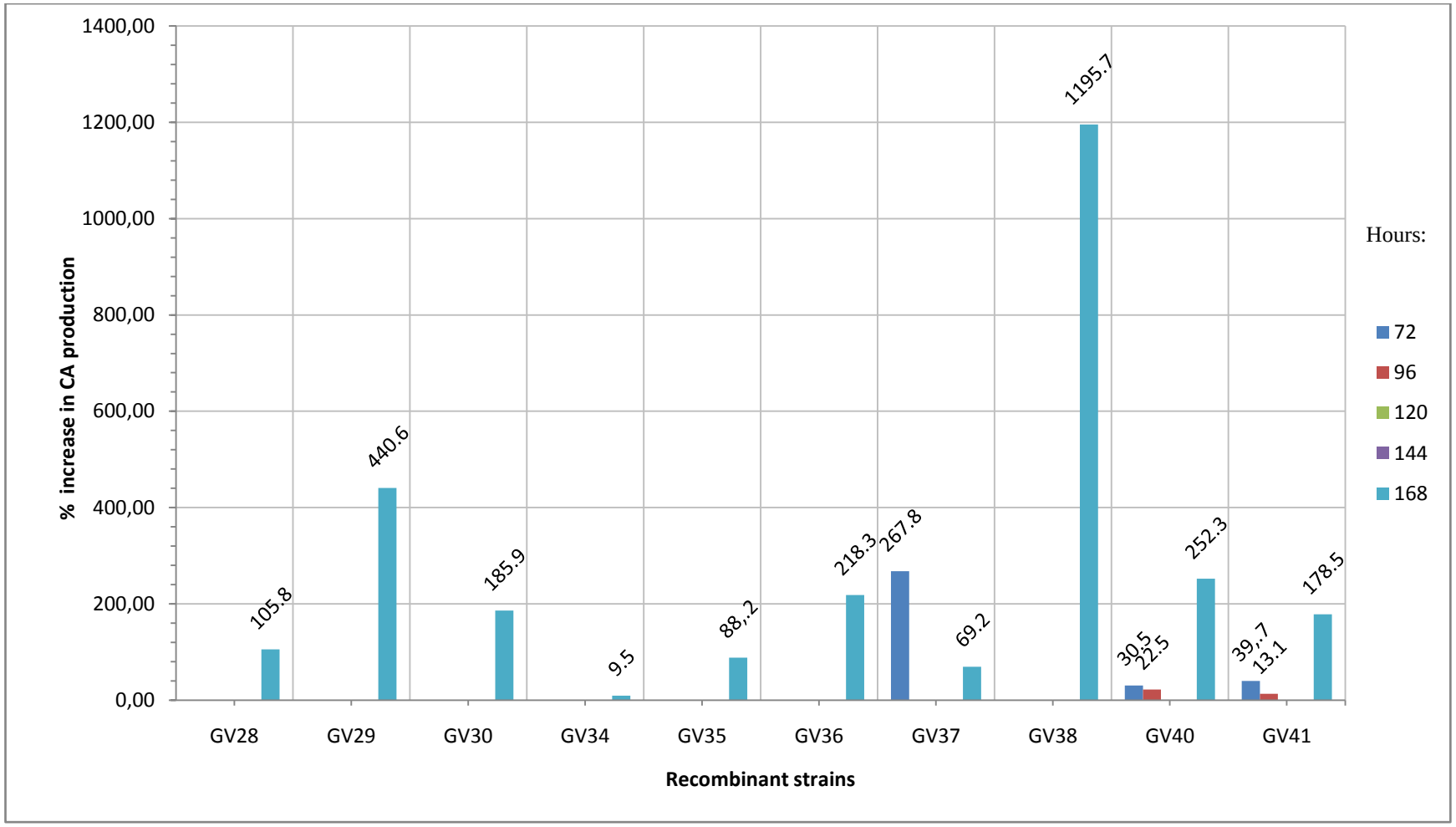

Figure 3.25. The percentage increase in CA production by recombinant *S. clavuligerus* strains in Fermentation Set 3.

In the 4th set of the fermentation experiments, 15 different recombinants were analysed for specific CA production as compared to their parental strain (Table 3.4).

Table 3.4. Average CA production by recombinant *S. clavuligerus* GV42-GV56 strains and their parental counterpart.

Average CA production (µg/g)					
	Hour				
Recombinant strain	72	96	120	144	168
S. clavuligerus GV42	40.020	266.637	376.980	568.429	465.181
S. clavuligerus GV43	57.158	327.581	590.177	666.848	183.082
S. clavuligerus GV44	44.087	321.064	515.576	552.593	93.790
S. clavuligerus GV45	49.937	213.415	330.395	590.897	37.280
S. clavuligerus GV46	242.522	260.637	247.432	244.983	167.411
S. clavuligerus GV47	36.838	220.517	307.637	341.547	40.235
S. clavuligerus GV48	49.211	256.377	350.304	228.106	43.952
S. clavuligerus GV49	35.098	218.372	316.219	351.469	126.908
S. clavuligerus GV50	38.759	296.469	397.989	455.973	324.837
S. clavuligerus GV51	401.325	317.888	441.158	593.428	117.207
S. clavuligerus GV52	48.577	257.923	333.082	376.467	134.503
S. clavuligerus GV53	61.647	262.831	330.969	261.446	143.603
S. clavuligerus GV54	323.420	255.106	380.998	451.723	143.834
S. clavuligerus GV55	192.733	312.100	294.752	308.906	43.572
S. clavuligerus GV56	35.534	466.593	440.339	640.768	194.938
Parental strain	171.445	220.429	446.514	643.275	262.048

In this set, the parental strain performed its maximum CA production (643.275 μg/g) at 144 h fermentation. In the 72nd h, recombinant GV46, GV51, GV54 and GV55 produced higher amounts of CA than the parental strain. In the 96th h, most of the strains produced higher amounts of CA; however, they failed to compete with the parental strain's CA titer in the 120th h except for GV42, GV43 and GV44. The highest CA yield during the whole fermentation was provided by GV43 at a concentration of 666.848 μg/g CA at the 144th h. GV42 and GV49 strains were better CA producers just before harvest. They produced CA at was 77.5 and 24% higher yields with respect to the parental strain, respectively, at this time (Figure 3.26).

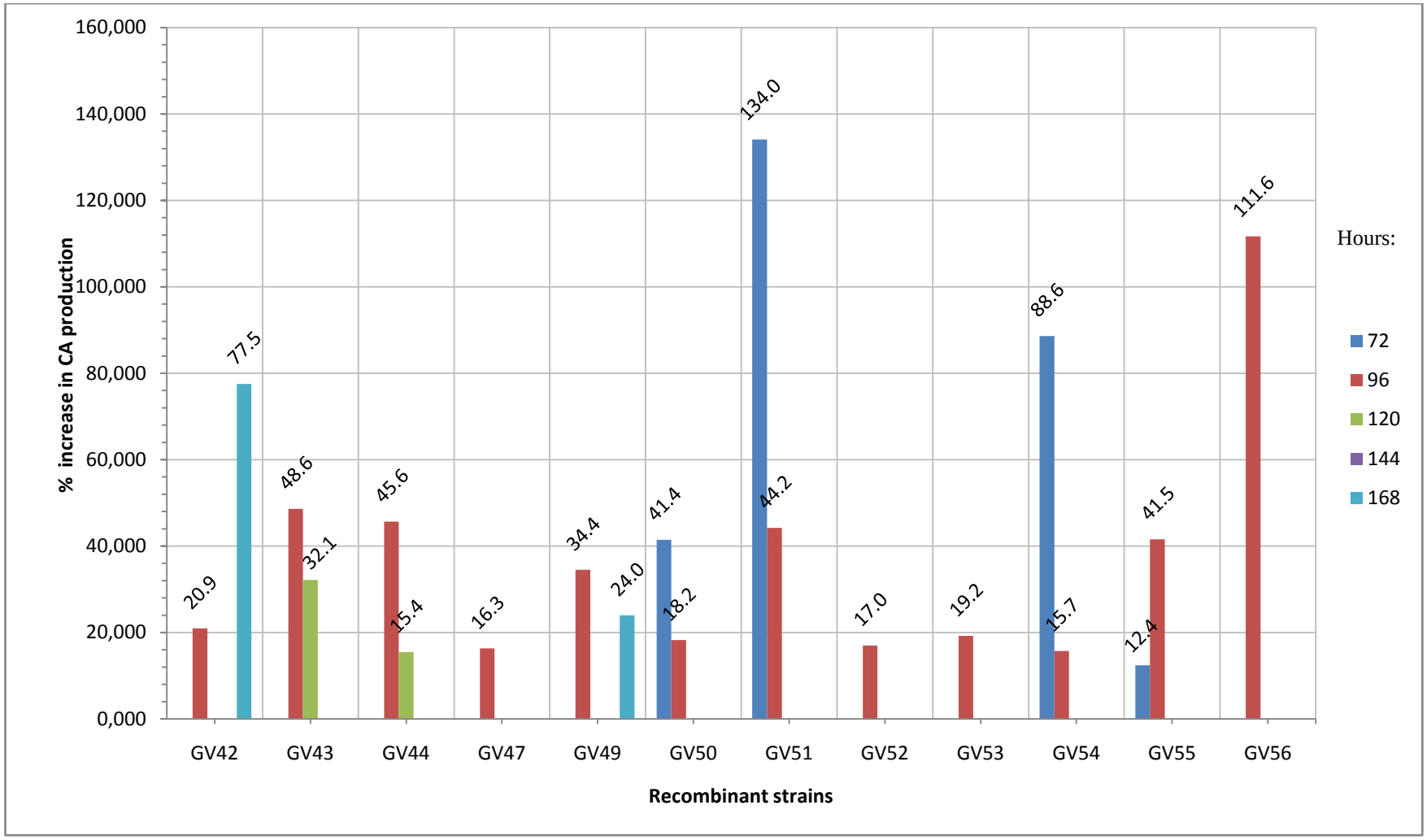

Figure 3.26. The percentage increase in CA production by recombinant *S. clavuligerus* strains in Fermentation Set 4.

In the final fermentation set, the remaining 14 recombinant strains were tested by HPLC for their CA production. The average CA production by the strains is listed in Table 3.5.

Table 3.5. Average CA production by recombinant *S. clavuligerus* GV57-GV70 strains and their parental counterpart.

Average CA production (μg/g)					
Recombinant strain	Hour				
	72	96	120	144	168
S. clavuligerus GV57	nd	24.27	551.26	201.04	297.18
S. clavuligerus GV58	38.24	39.47	236.94	194.90	60.91
S. clavuligerus GV59	nd	53.10	345.18	116.68	nd
S. clavuligerus GV60	nd	93.74	356.07	81.83	158.37
S. clavuligerus GV61	1583.27	1370.45	1492.12	1095.15	459.97
S. clavuligerus GV62	nd	51.48	359.52	291.70	9.51
S. clavuligerus GV63	nd	nd	750.12	710.40	271.90
S. clavuligerus GV64	nd	206.31	390.41	374.00	102.83
S. clavuligerus GV65	nd	nd	1228.65	1301.43	1326.08
S. clavuligerus GV66	87.16	119.87	359.74	213.64	42.72
S. clavuligerus GV67	538.92	203.81	437.04	251.71	35.91
S. clavuligerus GV68	nd	nd	490.76	541.47	473.56
S. clavuligerus GV69	nd	40.81	172.87	47.26	49.02
S. clavuligerus GV70	nd	nd	1123.31	1376.749	1128.85
Parental strain	695.44	515.10	622.62	434.35	30.24

As shown in Table 3.5., the parental strain produced its highest amount of CA (695.44 µg/g) at 72nd h of incubation. Among all strains, the recombinant GV61 produced the highest amount of CA at 72nd, 96th and the 120th h of fermentation (Figure 3.27). GV65 and GV70, on the other hand, were the most successful recombinants with 4284.8 and 3632.6% increasements in CA yields at the end of fermentation (Figure 3.28). The chromatograms of the samples can be examined in detail in Figure 3.29 and 3.30. Although GV63, GV65 and GV70 strains produced fairly higher amounts of CA in the 120th, 144th and 168th h of fermentation, the HPLC outputs at 72nd and 96th h could not reveal a well separated CA, and thus reported as nd.

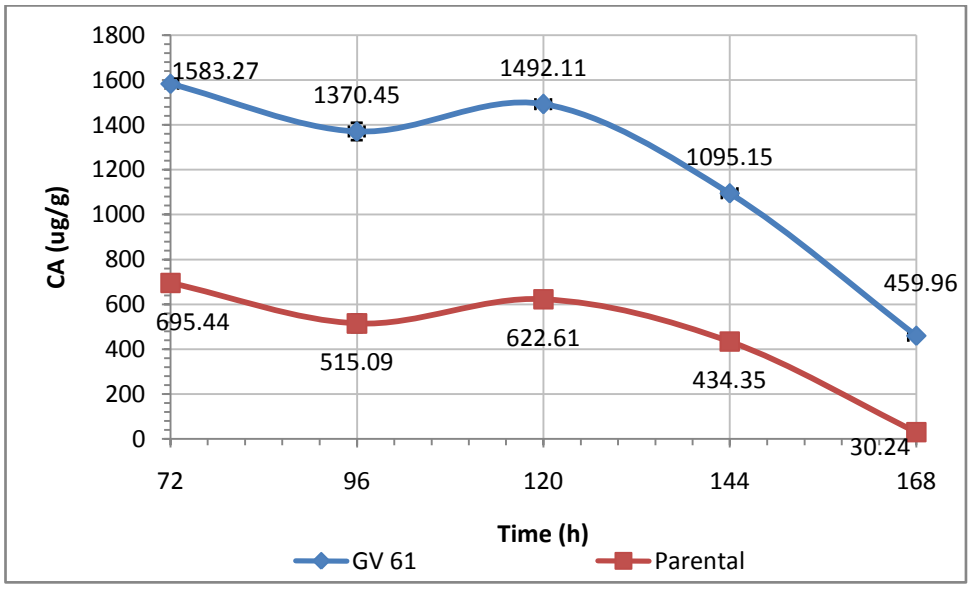

Figure 3.27. Comparison of time dependent CA production by industrial *S. clavuligerus* strain and its recombinant GV61 as determined by HPLC.

Taken together, the recombinant GV61 produced 2.1-2.7 fold more CA as compared to the parental strain during the course of fermentation. Additionally, GV70 strain also produced 3.2 times more CA with respect to the parental strain at 144th h of the fermentation process.

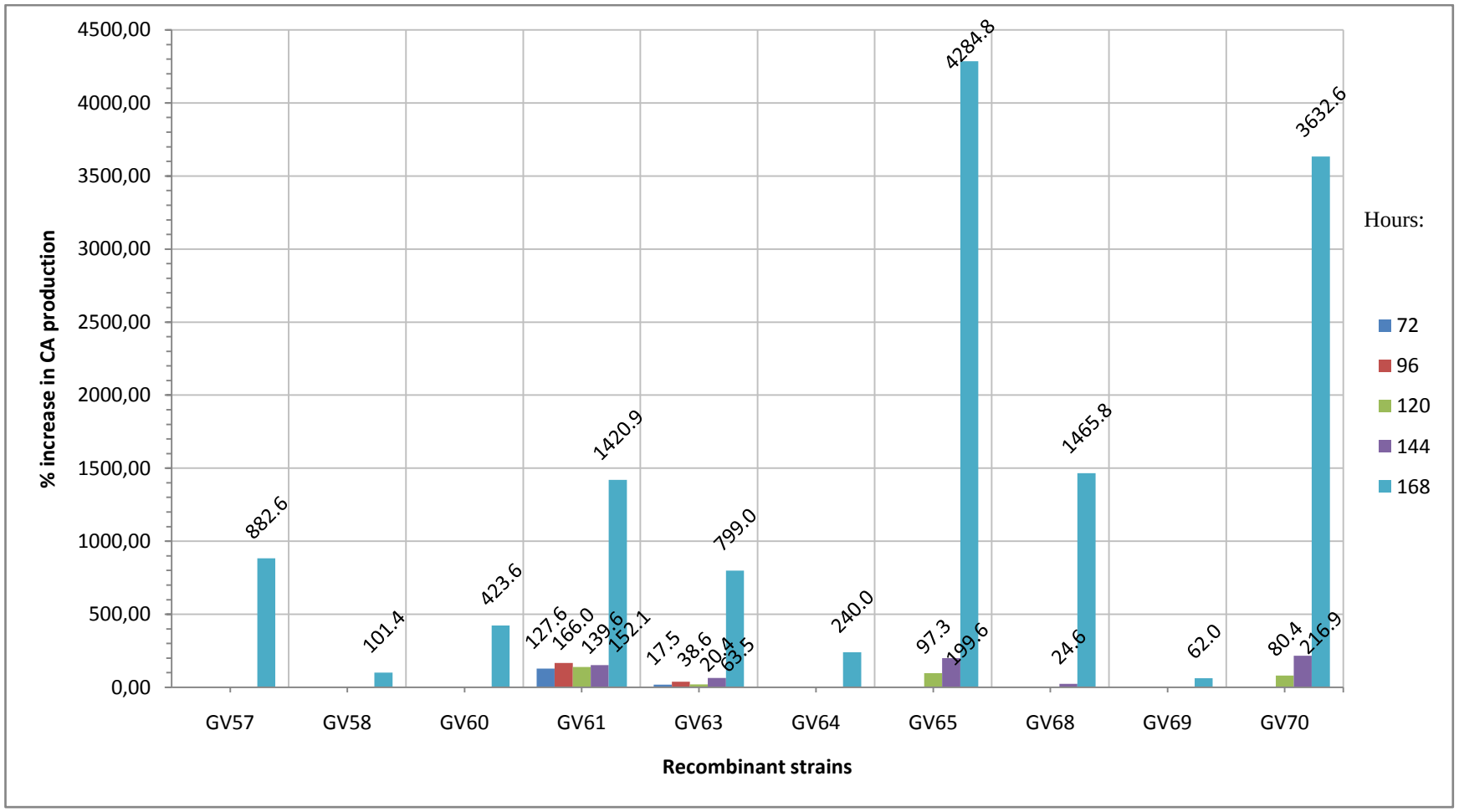

Figure 3.28. The percentage increase in CA production by recombinant *S. clavuligerus* strains in Fermentation Set 5.

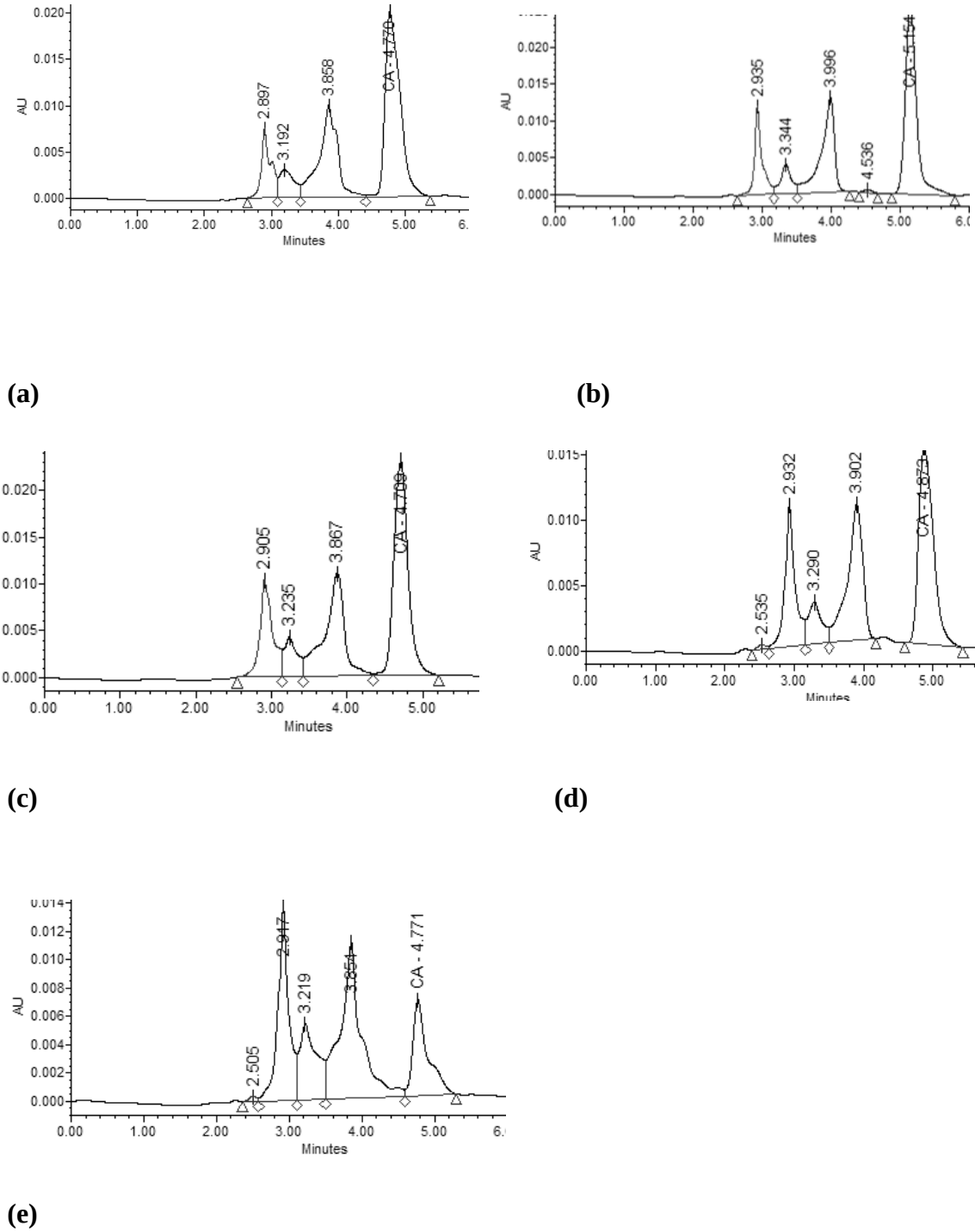

Figure 3.29. HPLC analysis of the culture broths of *S. clavuligerus* GV61 at 72 h (a), 96 h (b), 120 h (c), 144 h (d), and 168 h (e) of the fermentation.

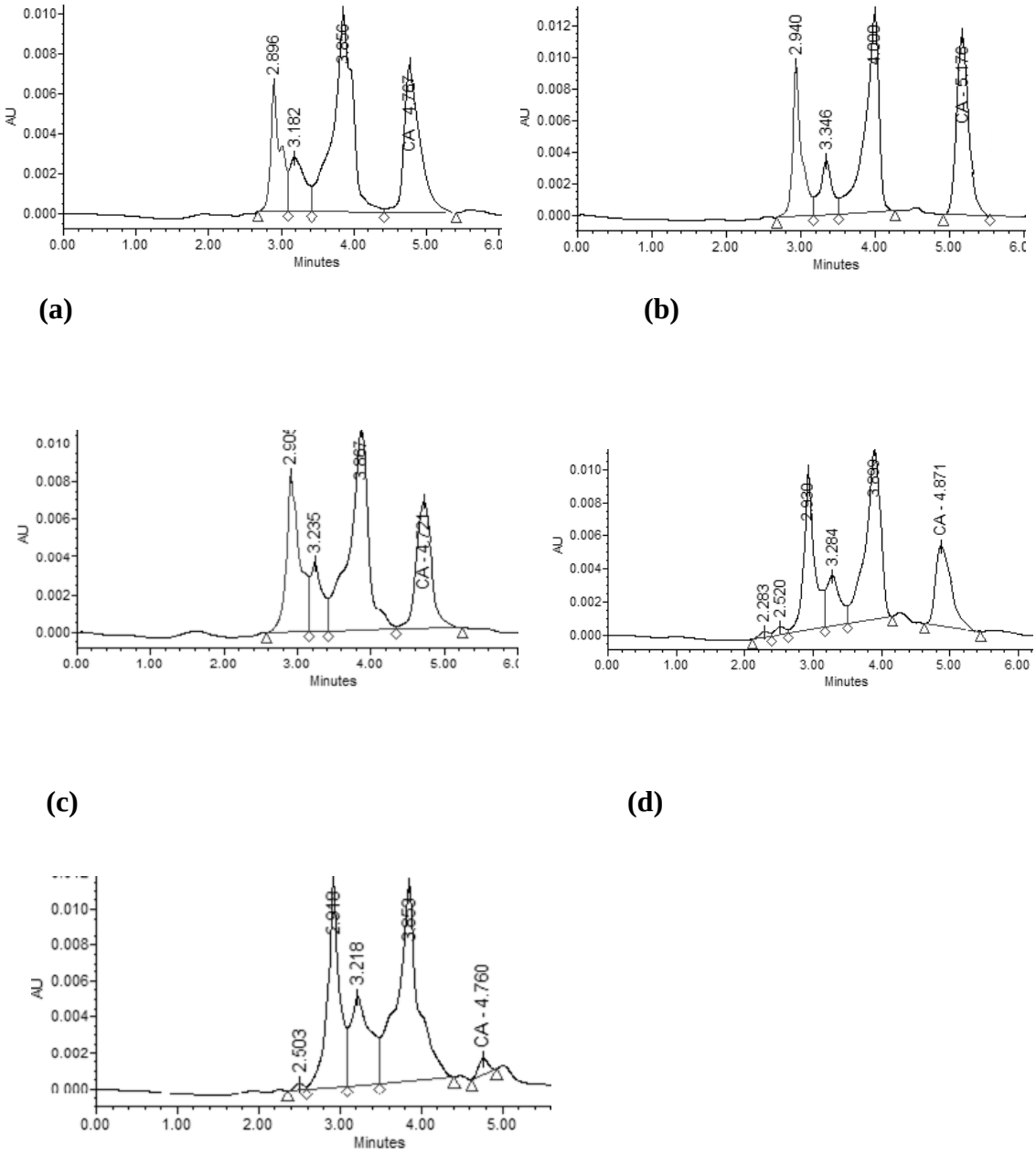

Figure 3.30. HPLC analysis of the culture broths of industrial parental *S. clavuligerus* at 72 h (a), 96 h (b), 120 h (c), 144 h (d), and 168 h (e) of the fermentation.

The CA production pattern by the parental strain showed slight variations in different sets of fermentation experiments despite our attempts to reduce the variability. This is a well know phenomenon for *Streptomyces* spp. and considered to be one of the most challenging part of an investigation on antibiotic production (Minas *et al*., 2000). To sum up, in five distinct experimental sets, 50 out of 70 recombinant strains produced higher amounts of CA in any stage of fermentation than the parental strain. Fouces and his co-workers (2000) were reported that conjugation via pSET152 integration is an efficient and reliable method for introducting foreign DNA into *S. clavuligerus* and has a conjugation frequency of $3x10^{-5}$. Moreover, in a very recent study, it was concluded that chromosomal integration is the preferred method for obtaining high-titer CA producing strains (Nath *et al*., 2010).

The number of plasmid copies with the *attP* site of the φC31 phage integrated into the chromosome depends on the number of *attB* sites in the chromosome and differs in different actinomycete species (Bierman *et al*., 1992). In a different study, analysis of the insertions of pSET152 into both $attB^{+}$ and Δ*attB* strains indicated that this plasmid can integrate at several loci via independent recombination events within a transconjugant due to the presence of multiple pseudo *attB* sites in the genome of most of *Streptomyces* strains such as in *S. coelicolor* and *S. lividans* (Combs *et al*., 2002). These findings might explain the differences in CA titers of the recombinant strains obtained in this study.

CHAPTER 4

CONCLUSION

- The clavulanic acid production by a clavulanic acid overproducer industrial strain of *S. clavuligerus* was further enhanced by chromosomal integration of an additional copy of clavaminate synthase II (*cas2*) gene. The additional copy was inserted into the choromosome by pSET152 integration vector via conjugation. As a result, 70 exconjugants (recombinants) were obtained. Verification of chromosomal integration of pGVAK03 carrying *cas2* gene was performed by using genomic DNAs of four recombinat strains as template DNA in PCR reaction.

- Prior to fermentation experiments with the recombinant strains, CA production capacities of wild type and industrial *S. clavuligerus* strains were compared by bioassays and HPLC analyses. It was found that industrial strain produces at least 5 fold more CA as compared to the wild type, standard *S. clavuligerus*.

- Screening of recombinants for their CA yields was performed by running 5 seperate fermentation sets. Clavulanic acid production by the recombinant strains named GV1–GV70 and the parental strain were compared by growing them in CC3 industrial fermentation medium and by HPLC analyses in fermentation broths at intervals during 72^{nd} to 168^{th} h of fermentations. 50 recombinants out of 70 exhibited higher titers of CA at different stages of fermentation with respect to the parental *S. clavuligerus*. The highest CA production (1583.27 μg/g) which corresponded to more than 2 fold increase in highest CA of yield parental strain was achieved by the recombinant strain namely *S. clavuligerus* GV61.

- The metabolic engineering approach which involved chromosomal integration of *cas2* resulted in stable constructs and obviated antibiotic selection in fermentation media as in the case of use of multicopy plasmids for expression.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Liquid Media

Luria Broth (LB)	$g.L^{-1}$
Luria Broth	25

Sterilized at 121 ℃ for 15 min

Trypticase Soy Broth (TSB)	$g.L^{-1}$
Trypticase Soy Broth	30

Sterilized at 121 ℃ for 15 min

Yeast Extract and Tryptone Broth (YT Broth)	$g.L^{-1}$
Yeast extract and tryptone broth	31

CC2 Vegetation Medium $g.L^{-1}$	
Soy flour	20
Dextrin	10
KH_2PO_4	0.6
GTO	5

pH is adjusted to 7.6 - 8.10 with NaOH, H_2SO_4

Sterilized at 121 ℃ for 20 min

1.2. Solid Media

CC3 Fermentation Medium	$g.L^{-1}$
Soy flour	20
Dextrin	10
KH2PO4	0.6
GTO	5
MOPS	10.5
Oligo elements solution*	10 ml

pH is adjusted to 6.80- 7.20 with NaOH, H2SO4

Sterilized at 121 ℃ for 20 min

*Oligo elements solution	$g.L^{-1}$
CaCl2	10
MgCl2.6 H2O	10
FeCl3	3
ZnCl2	0.5
MnSO4.H2O	0.5
NaCl	10

1.2. Solid Media

LB Agar	g.L-1

Luria Broth	25
Agar	15
Sterilized at 121 ℃ for 15 min	

TSA	$g.L^{-1}$
Tryptic Soy Broth	30
Agar	15
Sterilized at 121 ℃ for 15 min	

CC1 sporulation Medium	$g.L^{-1}$
Dextrin	10
K2HPO4	1
MgSO4.7H2O	1
NaCl	1
(NH4)2SO4	1
CaCO3	4
Oligo elements solution	1ml
Agar	20
pH is adjusted to 6.20-6.90 with NaOH, H2SO4	
Sterilized at 121 ℃ for 20 min	

Manntiol soya flour (MS) Agar	$g.L^{-1}$
Soya flour	2gr
Agar	2gr
Mannitol	20 gr

Sterilized at 121 ℃ for 15 min (autoclaved twice).

APPENDIX B

BUFFERS AND SOLUTIONS

SET buffer

NaCl	75mM
EDTA (pH:8)	25mM
Tris-HCl (pH:7.5)	20mM

TE buffer

Tris-HCl	10mM
Sodium EDTA	1mM

TSE buffer

Sucrose	0.3 M
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM

Tris-Acetate-EDTA Buffer (TAE) (50 X)

Tris Base	242 g
Glacial Acetic Acid	57.1 mL
EDTA (0.4 M, pH 8.0)	125 mL
Distilled water added to	1000 mL

TTX-100 solution	$g.L^{-1}$
Triton X-100	0.5
NaCl	9
Sterilized at 121 ℃ for 20 min	

Sodium acetate solution	$g.L^{-1}$
Sodium acetate	4.10
pH is adjusted to 6.0 with acetic acid, filtered.	

Sodium dihydrogen phospohate solution	$g.L^{-1}$
Sodium phosphate	15
pH is adjusted to 4 with 50% phosphoric acid, filtered.	

Lysis Solution

0.3 M NaOH

2% SDS

Phenol -chloroform solution (water saturated)	
Phenol	500 g
Chloroform	500 mL
Distilled water	400 mL
The solution was stored at RT, protected from light.	

X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)	
X-Gal	20 mg
Dimethylformamide	1 mL

The solution was stored at –20 ° C protected from light.

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 mL

The solution was filter sterilized and stored at –20 ° C.

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

Chemicals	
Acetic acid	Sigma
Agar	Merck
Agarose	Sigma
Ampicillin	Sigma
Apramycin	Sigma
$CaCl_2$	Sigma
Chloroform	Merck
Chloramphenicol	Sigma
DMSO	Sigma
EDTA	AppliChem
Ethanol	Botafarma
Ethidium bromide	Sigma
Glacial Acetic Acid	Merck
Glycerol	Merck
HCl	Fluka
$HClO_4$	Merck
IPTG	MBI Fermentas
Isopropanol	Merck
Kanamycin	Sigma

K_2HPO_4	Meck
KH_2PO_4	Merck
Luria Broth	Sigma
Methanol (HPLC grade)	Sigma
$MgCl_2$	Promega
MOPS	Sigma
NaCl	Merck
Nalidixic acid	Sigma
NaOH	Merck
Nutrient Broth	Merck
Penicillin G	Sigma-Aldrich
Phenol	Merck
Phenolcholoroform	Merck
SDS	Merck
Soybean flour	
TES Buffer	AppliChem
Tris-HCl	Merck
Tryptic Soy Broth	Oxoid
X-Gal	MBI Fermentas

Enzymes

Lysozyme	Q-Biogene
T4 DNA Ligase	Promega
*EcoR*I	Roche
*Hind*III	Roche
*Xba*I	Roche

*Not*I	Roche
*Aat*II	Roche
ProteinaseK	Sigma-Aldrich
Go Taq DNA polymerase	Promega

Size Markers

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

Kits

Plasmid isolation Kit	Qiagen
p-GEM T Vector	Promega
pBluescript II KS (+)	Stratagene