MEDIUM OPTIMIZATION FOR CEPHAMYCIN C OVERPRODUCTION AND COMPARISON OF ANTIBIOTIC PRODUCTION BY *ASK, HOM* AND *ASK+HOM* RECOMBINANTS OF *STREPTOMYCES CLAVULIGERUS*

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ABSTRACT

MEDIUM OPTIMIZATION FOR CEPHAMYCIN C OVERPRODUCTION AND COMPARISON OF ANTIBIOTIC PRODUCTION BY *ASK, HOM* AND *ASK+HOM* RECOMBINANTS OF *STREPTOMYCES CLAVULIGERUS*

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Streptomyces clavuligerus is well-known for synthesizing several β-lactam antibiotics like cephamycin C which is produced through aspartic acid pathway initiated by aspartokinase (Ask) enzyme encoded by *ask*.

Four different strains were constructed in our laboratory to increase cephamycin C production by *S. clavuligerus*. TB3585 and BA39 contained extra copies of *ask* gene on a multicopy plasmid, control strains TBV and BAV contained vector only in wild type strain NRRL3585 and *hom*-minus background, AK39, respectively.

In this study, the effects of carbon and nitrogen sources incorporated into chemically defined medium were investigated for optimum growth and cephamycin C production by AK39. A modified-chemically defined medium (mCDM) was obtained by increasing the asparagine concentration two-fold and replacing glycerol with sucrose. Subsequently, growth and cephamycin C

production by recombinant *S. clavuligerus* strains (TB3585, AK39, BA39, BAV, TBV) in Tryptic Soy Broth (TSB) and mCDM were compared. The specific antibiotic production in mCDM by TB3585 was 3.3- and 3.2-fold higher than TBV at 72h and 96h, respectively.

Aspartokinase activity of *S. clavuligerus* recombinants was measured to verify the *ask* overexpression. TB3585 showed the highest activity at 48h.

Finally, intracellular amino acid pools of the strains were measured to relate the Ask activity and antibiotic production to the amino acid content within the cells. AK39 was shown to have the highest intracellular levels of lysine, leading to cephamycin C precursor synthesis; lysine plus threonine, exerting concerted feedback inhibition on Ask enzyme; methionine, which cannot be produced by AK39 like threonine due to *hom* disruption.

Keywords: *Streptomyces clavuligerus,* Cephamycin C, mCDM, TSB

ÖZ

SEFAMİSİN C ÜRETİMİNİN ARTIRILMASI İÇİN BESİYERİ OPTİMİZASYONU VE *STREPTOMYCES CLAVULIGERUS ASK, HOM* VE *ASK+HOM* REKOMBİNANTLARININ ANTİBİYOTİK ÜRETİMLERİNİN KARŞILAŞTIRILMASI

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Eylül 2010, 87 sayfa

Streptomyces clavuligerus, ask geninin kodladığı aspartokinaz (Ask) enzimi tarafından başlatılan aspartik asit biyosentetik yolu sonunda üretilen sefamisin C gibi önemli β-lactam antibiyotikleri sentezlemesiyle tanınmaktadır.

Antibiyotik üretimini arttırabilmek için laboratuvarımızda dört farklı *S. clavuligerus* suşu oluşturuldu. TB3585 ve BA39, sırasıyla, yabanıl (NRLL3585) ve *hom* delesyon mutantı *S. clavuligerus* içerisine çoklu kopya plazmitler üzerinde taşınan ekstra *ask* geni aktarılarak, kontrol suşları TBV ve BAV ise bu suşlara gen içermeyen çoklu kopya vektörlerin aktarılmasıyla elde edildi.

Bu çalışmada, kimyasal olarak tanımlanmış besiyerine eklenen karbon ve azot kaynaklarının, AK39 suşunun optimum üreme ve sefamisin C üretimi üzerine etkileri araştırıldı. Asparajin konsantrasyonu 2 katına çıkarılarak ve

gliserol yerine sükroz ilave edilerek kimyasal kompozisyonu değiştirilmiş bir besiyeri (mCDM) elde edildi. Daha sonra, Triptik Soya Besiyeri (TSB) ve mCDM'de rekombinant *S. clavuligerus* suşlarının (TB3585, AK39, BA39, BAV, TBV) üreme ve antibiyotik üretimleri karşılaştırıldı. TB3585'in mCDM'deki spesifik antibiyotik üretimi fermentasyonun 72 ve 96. saatlerinde TBV'ye göre sırasıyla 3.3 ve 3.2 kat daha fazla oldu.

ask ekspresyonunu doğrulamak için, *S. clavuligerus* rekombinantlarının aspartokinaz aktiviteleri ölçüldü. TB3585, 48. saatte en yüksek aktiviteyi gösterdi.

Son olarak, suşların Ask aktivitelerinin ve antibiyotik üretimlerinin hücre içindeki amino asit miktarlarıyla bağlantısını kurmak amacıyla hücre içi amino asit havuzu ölçüldü. AK39'un hücre içi lizin, lizin artı treonin ve metiyonin içeriği bakımından en yüksek değerlere sahip olduğu gösterildi. Lizin, sefamisin C sentezi sırasında gereken bir öncülün oluşumunu sağlamaktadır. Lizin artı treoninin, Ask enzimi üzerine toplu geribildirim inhibisyonu yaptığı bilinmektedir. Metiyonin ise treonin gibi *hom* delesyonu sebebiyle AK39 tarafından sentezlenememektedir.

Anahtar Kelimeler: *Streptomyces clavuligerus,* Sefamisin C, mCDM, TSB

To My Family and Precious Friends For Their Holy Presence

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ABBREVIATIONS

Ask	: Aspartokinase
ask	: Aspartokinase Gene
HSD	: Homoserine Dehydrogenase
hom	: Homoserine Dehydrogenase Gene
TSB	: Tryptic Soya Broth
CDM	: Chemically Defined Medium
mCDM	: Modified-Chemically Defined Medium
suc	: Sucrose
glyc	: Glycerol
asn	: Asparagine

CHAPTER 1

INTRODUCTION

1.1. The genera of actinomycetes

In terms of guanine + cytosine (GC) content of their genome, gram-positive bacteria can be subdivided into low GC organisms and high GC organisms. *Bacillus*, *Clostridium*, *Staphylococcus* and *Streptococcus* are examples for low GC containing gram-positive bacteria, whereas high GC containing gram-positive bacteria are categorized as actinomycetes (Keiser *et al.*, 2000).

Though there have been attempts using taxanomic tools to map out the genera and the subgroups of actinomycetes (Goodfellow, 1989; Embley and Stackbrandt, 1994), the most valuable information for the construction of a concise genera tree of actinomycetes came from partial sequence analyses of 16S rRNA (Keiser *et al.*, 2000) (Figure 1.1).

Actinomycetes can be isolated from terrestrial or marine environments indicating a large distribution of them all over the world (Fenical and Jensen, 2006; Duraipandiyan *et al.*, 2009). They can be found in various forms from the simplest forms like rod or sphere as in corynebacteria to the complex life forms in hyphal filaments, the distinguished example of which is *Streptomyces* spp. (Chater, 2006). Actinomycetes are well-known for being able to decompose plant, fungal and animal materials in soil by releasing a large variety of extracellular enzymes, hence they are important recyclers of nutrients in the environment (Goodfellow and William, 1983; Keiser *et al.*, 2000) Furthermore, the fact that they have a wide range in production of secondary metabolites like antibiotics, antitumor compounds, immunosuppressants, antiviral, antiparasitic agents making them quite

prominent in biotechnological area. Last reports indicate that among 23,000 biologically active secondary metabolites produced by microorganisms, the 10,000 are produced by actinomycetes and the 7,600 alone are produced by *Streptomyces* genus of actinomycetes (Sacramento *et al.*, 2004; Olano *et al.*, 2008).

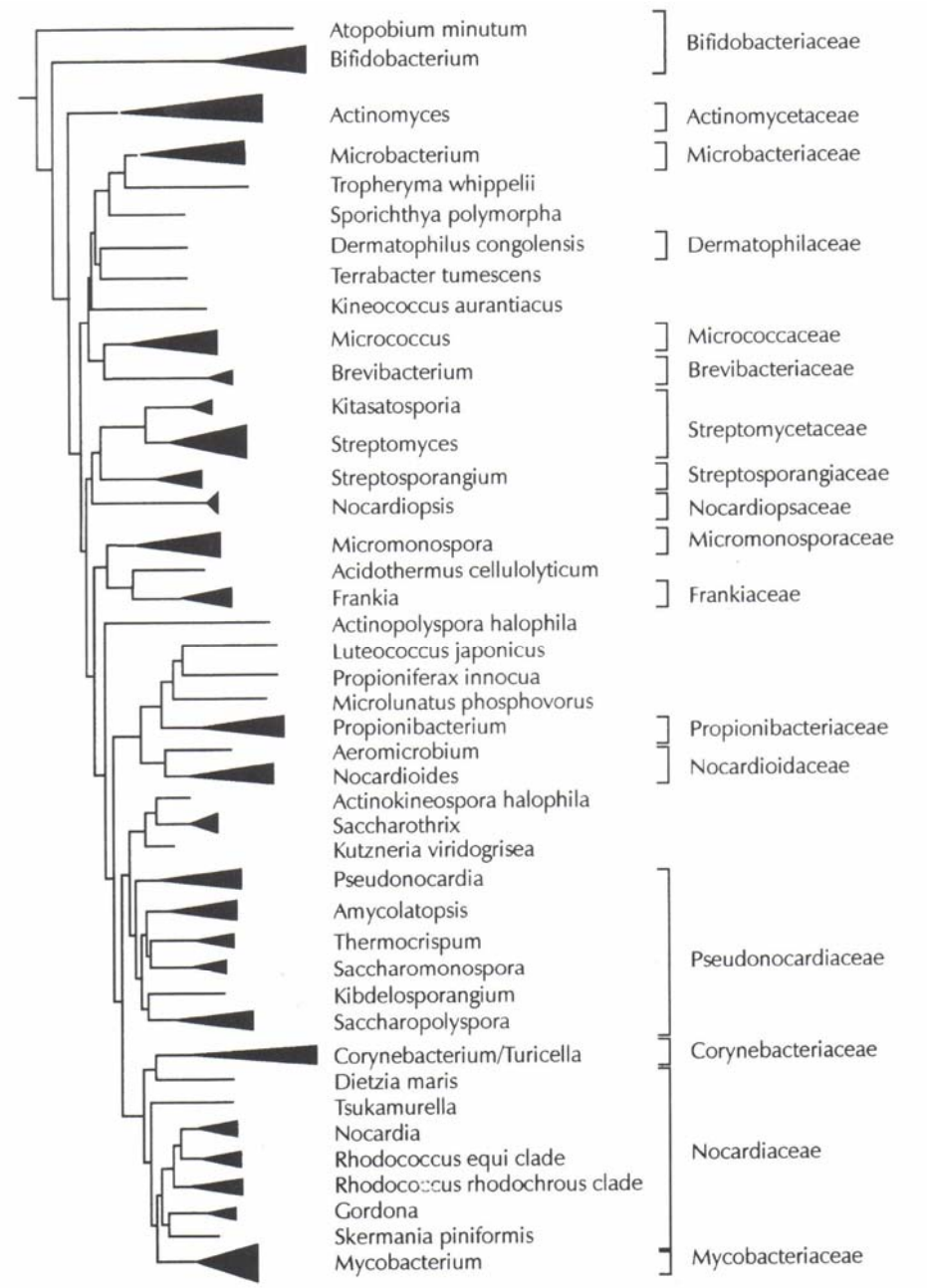

Figure 1. 1. Phylogenetic tree of actinomycetes constructed by using 16S ribosomal RNA sequences (Keiser *et al*., 2000).

1.2. *Streptomyces* genus and life cycle

Streptomyces, a soil bacterium, shows some distinctive properties when compared to other actinomycetes. Their GC content in the chromosomes as well as their ability to produce industrially important secondary metabolite production is the highest (Manteca *et al.*, 2008). *Streptomyces clavuligerus*, for example, is known to produce 21 secondary metabolites, industrially most important of which are the β-lactam antibiotics. (Ortiz *et al.*, 2007). β-lactam antibiotics produced by *S. clavuligerus* are cephamycin C, penicillin N and five clavam compounds which show weak antifungal and antibiotic properties, such as clavulanic acid, a β-lactamase inhibitor (Aharonowitz and Demain, 1978; Tahlan *et al.*, 2007; Mackenzie, 2007) .

Another distinctive feature of *Streptomyces* is their complex life cycle, which begins with the formation of branching hyphal filaments and ends with the formation of spores from the branching hypha of these mycelial structures. With spores representing the semi-dormant stage of the life cycle, it is possible to collect these bacteria from 70 year-old soil samples (Nedal, 2007; Engels, 2009).

There are three separate phases of the developmental life cycle of *Streptomyces* on solid medium (Figure 1.2a and b). First, the spores of the organism germinate to produce the vegetative (substrate) mycelium which is comprised of filamentous multinucleoidal hypha separated into large compartments with thin-layered septa (Ohnishi *et al.*, 2002; Manteca *et al.*, 2008). Vegetative mycelium continues to grow in and on the top of the medium until a brief growth arrest characterized by reduced macromolecular synthesis (Manteca *et al*., 2008). This stage marks the initiation of aerial (reproductive) mycelium formation, which occurs as upright extensions by the branching of vegetative mycelium into air (Wildermuth, 1970; Miguelez *et al.*, 1999; Manteca *et al.,* 2007). Meantime, there occurs a rapid development of mycelium during which the compartments disappear and the surface of

aerial hypha is covered with a characteristic hydrophobic layer, so called rodlet layer (Manteca *et al.*, 2007).

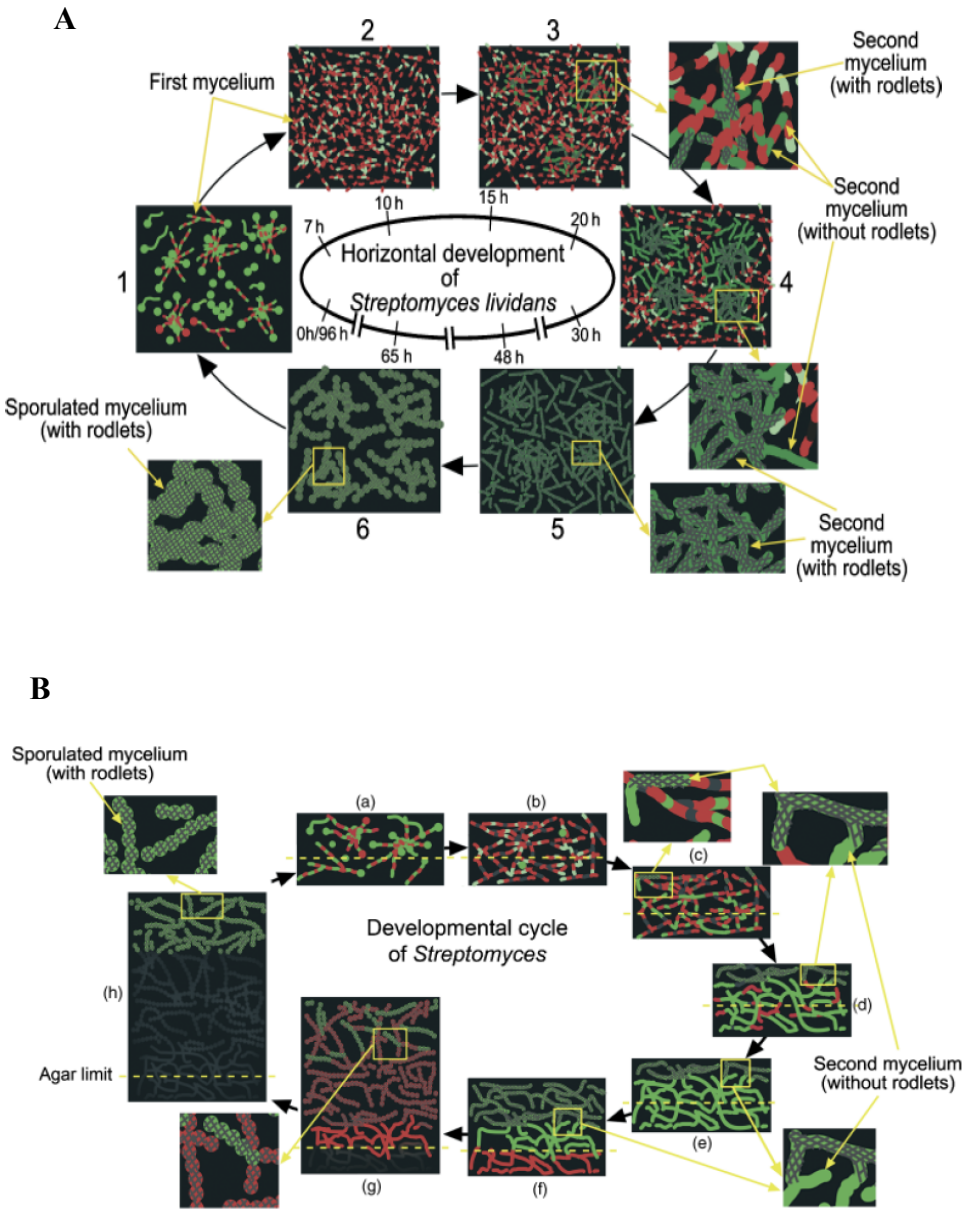

Figure 1. 2. Schematic representation of *Streptomyces* developmental cycle (A) (horizontal sections) with respect to cultivation times. (B) (vertical sections). Red color shows dead cells; green color shows live cells and rodlet proteins are shown as grey lines. Spherical shapes in phase 6 represent mature, differentiated spores. Below the dotted yellow lines is the agar. In (g) and (h) phases, black areas below the presporulated and sporulated mycelium layer occur due to absence of fluorescence since dead hyphae disintegrate and nucleic acids are degraded (Manteca *et al*., 2007).

Afterwards, aerial hypha at the tips undergo extensive compartmentalization separated by double layered sporulation septa, destined to be hydrophobic spore chains (Kwak and Kendrick, 1996; Ohnishi *et al.,* 2002; Manteca *et al.,* 2008). Fibrous sheath wrapping the spore chains breaks out after the spores get mature. Then the cycle begins again upon the germination of released spores. Therefore, it is possible to summarize the life cycle of *Streptomyces* as follows: vegetative hyphae, lysing of vegetative hyphae, aerial hyphae, immature and mature spores, and germinating spores (Kwak and Kendrick, 1996).

It is also a well-known phenomenon that the morphological differentiation of *Streptomyces* spp. correlates well with the antibiotic formation. Antibiotic formation by this genus usually coincides with a time interval that amounts to end of the exponential growth phase and the beginning of the stationary phase, with the contributory effects of physiological and environmental factors in liquid cultures (Rodriguez *et al.,* 2008; Bibb, 2005). On solid cultures, antibiotic production occurs just before or during the aerial hypha formation (Bibb, 2005). Furthermore, the nutritional conditions and especially the presence of carbon and nitrogen sources in growth media were shown to have a considerably important impact on antibiotic production (Aharanowitz and Demain, 1978; Manteca *et al.,* 2008).

1.3. β-lactam antibiotics and their mechanism of action

There are several groups of antibiotics produced by *Streptomyces* spp. and their specificity is determined by the action mechanisms. They can be either bacteriostatic (inhibiting the growth of bacteria) or bacteriocidal (killing the bacteria) (Table 1.1). Furthermore, bacteriostatic and bacteriocidal antibiotics can be differentiated as broad or narrow spectrum ones; the broad activity spectrum means that the antibiotic of concern is effective against a large

variety of microorganisms, whereas the narrow activity spectrum means that it is effective against a species or a genus (Mackenzie, 2007).

Table 1. 1. Classes of antibiotics, their effect and mode of action (Mackenzie, 2007).

Class	*Effect	Mode of Action
Aminocyclitols	bs	Protein synthesis
Aminoglycosides	bc	Protein synthesis
β-lactams	bc	Cell wall
Fluoroquinolones	bc	Genetic replication
Lincosamides	bs	Protein synthesis
Macrolides	bs	Protein synthesis
Sulfonamides	bs	Metabolic processes
Tetracyclines	bs	Protein synthesis
Phenicol	bc	Protein synthesis

*bs=bacteriostatic, bc=bacteriocidal

β-lactam antibiotics inhibit the activity of enzymes involved in bacterial cell wall (peptidoglycan) synthesis. Thus, they show bacteriocidal effect on only growing bacterial cells (Mackenzie, 2007).

β-lactams are categorized according to their chemical structures and differences in their chemistry influence the physical properties of the antibiotics such as solubility, stability and their spectrum of activity (Mackenzie, 2007).

The core of all β-lactam antibiotics is the strained, four-membered, heterocyclic ring, also called β-lactam ring. Merging with the β-lactam ring is

a second ring, thus both forming a bicyclic ring structure. If the second ring is five-membered, it is named as penam/penem or if six-membered, it is called cepham/cephem. The difference in nomenclature in five- and six-membered rings depends on the saturation of the ring (Mackenzie, 2007) (Figure 1.3).

Given the chemistry of second ring, β-lactam antibiotics are subgrouped as penams, ceph-3-ems, clavams, carbapenems and monolactams (Table 1.2). Penicillins, cephalosporins and cephamycins are well-known examples for penams, ceph-3-ems (Brakhage, 1998; Thykaer and Nielsen, 2003). Clavulanic acid belongs to clavams and is a weak antibiotic and but quite a strong β-lactamase inhibitor *per se* (Demain and Elander, 1999).

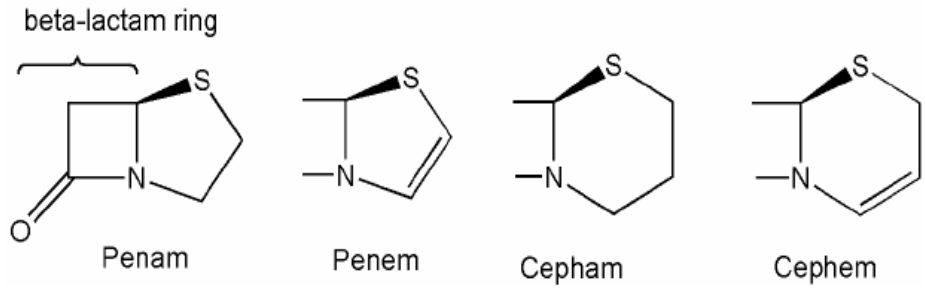

Figure 1. 3. Nomenclature of β-lactam antibiotics according to their second ring (Mackenzie, 2007).

Table 1. 2. Classes of β-lactam antibiotics (Brakhage, 1998).

Classes of naturally occuring β-lactams	Antibiotics	Producing microorganisms (examples) Fungi	Bacteria	
			Gram$^+$	Gram$^-$
Penam	Penicillins	*P. chrysogenum* *P.notatum* *A. nidulans*		
Ceph-3-em	Cephalosporins Cephamycins Cephabacins Chitinovorins	*A. chrysogenum* *(C. acremonium)* *P. persinicus*	*Streptomyces clavuligerus* *Nocardia lactamdurans*	*Flavobacterium sp.* *L. lactamgenus*
Clavam	Clavulanic acid		*Streptomyces clavuligerus*	
Carbapenem	Thienamycin Olivanic acid Epithienamycin		*Streptomyces clavuligerus* *S. olivaceus*	*E.carotovora* *Seratia sp.*
Monolactam	Nocardicines		*Nocardia uniformis subsp. tsuyamanensis*	
	Monobactams			*A. radiobacter* *P. acidophila*

Bacterial cell wall mainly consists of glycan chains crosslinked with small peptide molecules and is known as peptidoglycan layer forming mesh-like structure. The pepytidoglycan layer is essential for the rigidity and the shape of bacterial cell walls as well as serving as attachment sites for virulence factors and adhesins. The basic repeating unit of the glycan chains forming the peptidoglycan layer is a modified disaccharide molecule containing N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues to the carboxyl group of which is attached a short peptide (Figure 1.4). Despite the sequence of the peptide changes depending on gram negative and gram positive species, it universally ends with two D-alanine residues (Schleifer and Kandler, 1972; Rogers and Perkins, 1980; Holtje, 1998; Wilke *et al.*, 2005; van Dam *et al.*, 2009).

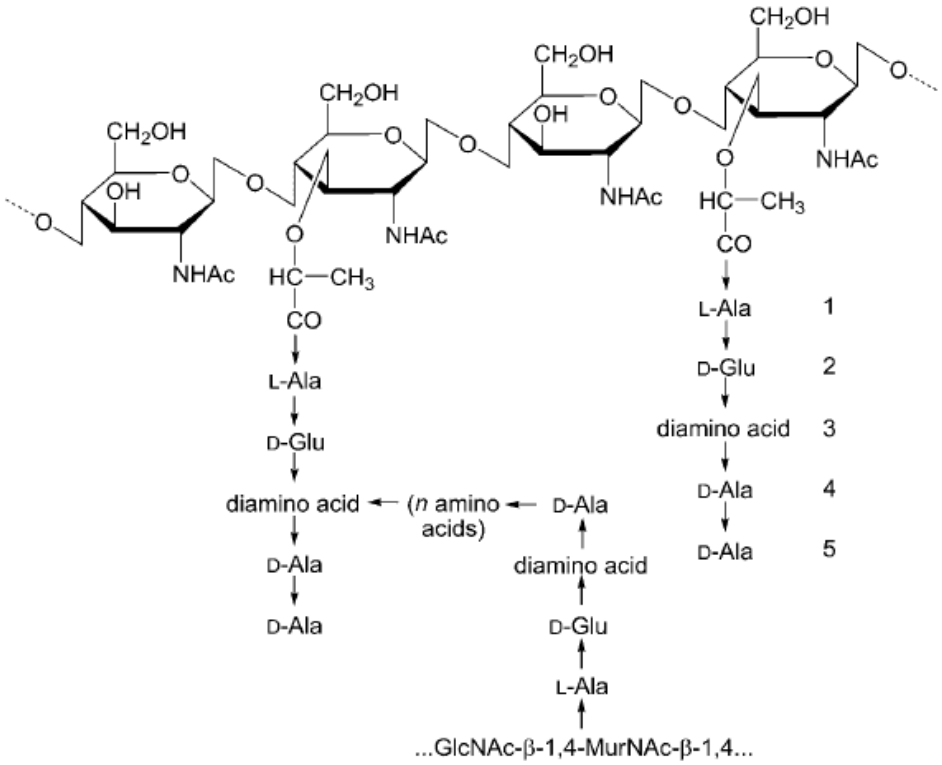

Figure 1. 4. Representation of two peptidoglycan subunits of bacterial cell wall. Numbers indicate the positions of each amino acid in pentapeptide. Apart from amino acid constitution, length and components of interpeptide bridge differs among species (van Dam *et al*., 2009).

The modified disaccharide unit is synthesized in the cytoplasm and transported out to form the peptidoglycan chains. The final step of the peptidoglycan layer synthesis is the crosslinking between the peptide units attached to MurNAc on each peptidoglycan chain, a reaction which is catalyzed by membrane anchored cell-wall transpeptidases. Crosslinking occurs with the formation of a peptide bond between the D-alanine on one glycan chain of the peptidoglycan layer and the free amino end of a diaminopimelic acid (gram-negative) or an L-lysine (gram-positive) on the other chain (Blumberg and Strominger, 1974; Wilke *et al.,* 2005) (Figure 1.4). Serine residue at the active site of the transpeptidases binds to the acyl-D-ala-D-ala moiety on growing peptidoglycan layer and is acylated to complete the crosslinking of glycan chains. However, when there are β-lactams in the

environment, transpeptidases bind to the β-lactam ring structure which very well mimics the 3D structure of the acyl-D-ala-D-ala moiety. Acylated by the β-lactams, transpeptidases are irreversibly inhibited. Bacterial cell walls lyse and cell death occurs as a result of poor crosslinking of the peptidoglycan layer (Lee *et al.*, 2003).

1.4. Biosynthesis of cephamycin C by *S. clavuligerus*

Cephamycin C is an extracellular β-lactam antibiotic produced by actinomycetes such as *S. cattleya, S. clavuligerus* and *Nocardia lactamdurans* (Bussari *et al.*, 2009). Besides being a broad spectrum and low toxic to animals like other β-lactam antibiotics, cephamycin C becomes more prominent by having resistance against β-lactamases produced by penicillin resistant bacteria to hydrolyze the β-lactam ring. This is a result of methoxy substitution at the 7α position of the cepham nucleus in the bicyclic ring structure of β-lactams (Liras, 1999; Kagliwal *et al.*, 2009).

L-α-aminoadipic acid, L-cysteine and L-valine are the common precursors for the synthesis of β-lactam antibiotics. In contrast to L-cysteine and L-valine, L-α-aminoadipic acid is a non-proteinogenic amino acid that must be synthesized by the β-lactam producing actinomycetes through a specific pathway. First, α-aminoadipic acid semialdehyde is produced from lysine by lysine-6-aminotransferase (LAT) encoded by *lat*. α-Aminoadipic semialdehyde is spontaneously turned into piperideine-6-carboxylate (PCD), then a piperideine-6-carboxylate dehydrogenase (P6C-DH) catalyzes the formation of α-aminoadipic acid from PCD. The gene coding for P6C-DH is *pcd* (Liras and Martin, 2006; Martin *et al.*, 2009) (Figure 1.5).

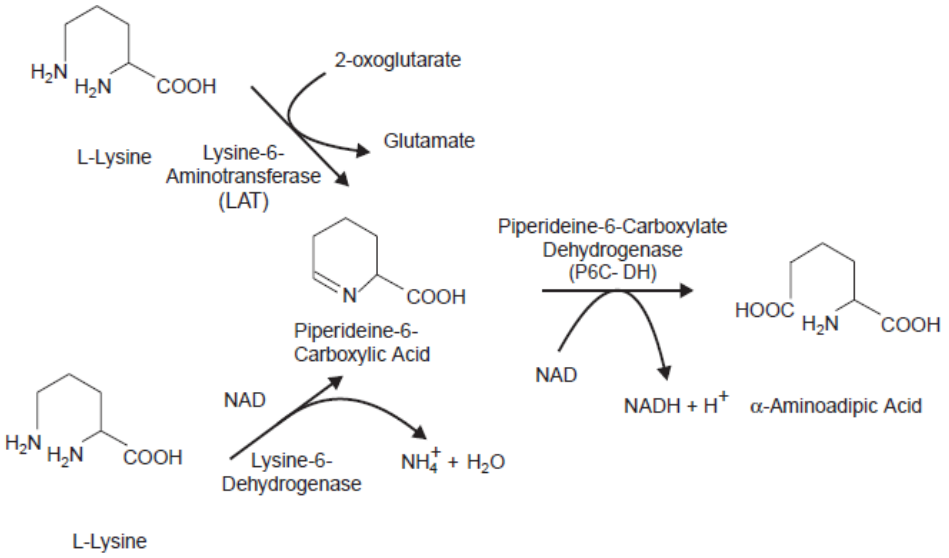

Figure 1. 5. Conversion of lysine into α-aminoadipic acid by lysine-6-aminotransferase and piperideine-6-carboxylic-acid dehydrogenase. (Martin, 1998).

The pathway for cephamycin C biosynthesis begins when the three amino acids L-α-aminoadipic acid, L-cysteine and L-valine are condensed by δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (ACVS), a non-ribosomal peptide synthetase, to form L-δ(α-aminoadipyl)-L-cysteinyl-D-valine. ACVS encoded by *pcbAB* uses ATP to activate the three amino acids and so has three separate domains specific for each amino acid. IPN synthase (IPNS, ACV cyclase) converts ACV tripeptide to isopenicillin N, the bicyclic structure also called penam nucleus by eliminating four hydrogen atoms from its structure. In the third step, isopenicillin N is epimerized to the D-isomer, penicillin N by pyridoxal phosphate-dependent enzyme IPN epimerase encoded by *cefD* (Liras and Martin, 2006; Martin *et al.*, 2009). Penicillin N is the precursor for antibiotic products containing cephem nucleus like cephamycins or cephalosporins produced by bacteria or fungi, respectively. First, the five-membered thiazolidine ring of penicillin N is converted into a six-membered dihydrothiazine ring by the deacetoxy-cephalosporin C (DAOC) synthase (expandase) which is encoded by *cefE*, and the second

methyl group at carbon atom 3 of six-membered dihydrothiazine ring (deacetoxycephalosporin C; DAOC) is hydroxylated/oxidized resulting in deacetylcephalosporin C (DAC). The gene responsible for this hydroxylation/oxidation step is encoded by *cefF*. 3-hydroxymethyl ceph-3-em O-carbamoyltransferase encoded by *cmcH* catalyzes the conversion from DAC to O-carbamoyl-DAC (OCDAC). OCDAC undergoes hydroxylation at C-7 position by OCDAC hydroxylase encoded by *cmcI*. The final step includes the action of cephamycin C synthetase encoded by *cmcJ* to methylate the hydroxy group at C-7 in order to give the cephamycin C as the final product (Liras, 1999; Brakhage *et al.*, 2005) (Figure 1.6).

1.5. Gene clusters for cephamycin C biosynthesis in S. *clavuligerus*

As already mentioned, *S. clavuligerus* is a prolific producer of β-lactam antibiotics such as cephamycin C, clavulanic acid and other clavams as well as penicillin N (Romero *et al.,* 1984; Alexander and Jensen, 1998) among which the cephamycin C and clavulanic acid are of special concern. It has been reported that the genes for the cephamycin C and clavulanic acid are clustered (Mackenzie, 2007) (Figure 1.7). Arrangement of antibiotic biosynthetic genes into clusters is a common property of bacteria. The possible explanation for this phenomenon is its ecological advantage to the bacteria; furthermore, the regulation of the genes which is more efficient when they are controlled by a common promoter (Brakhage *et al.*, 2005).

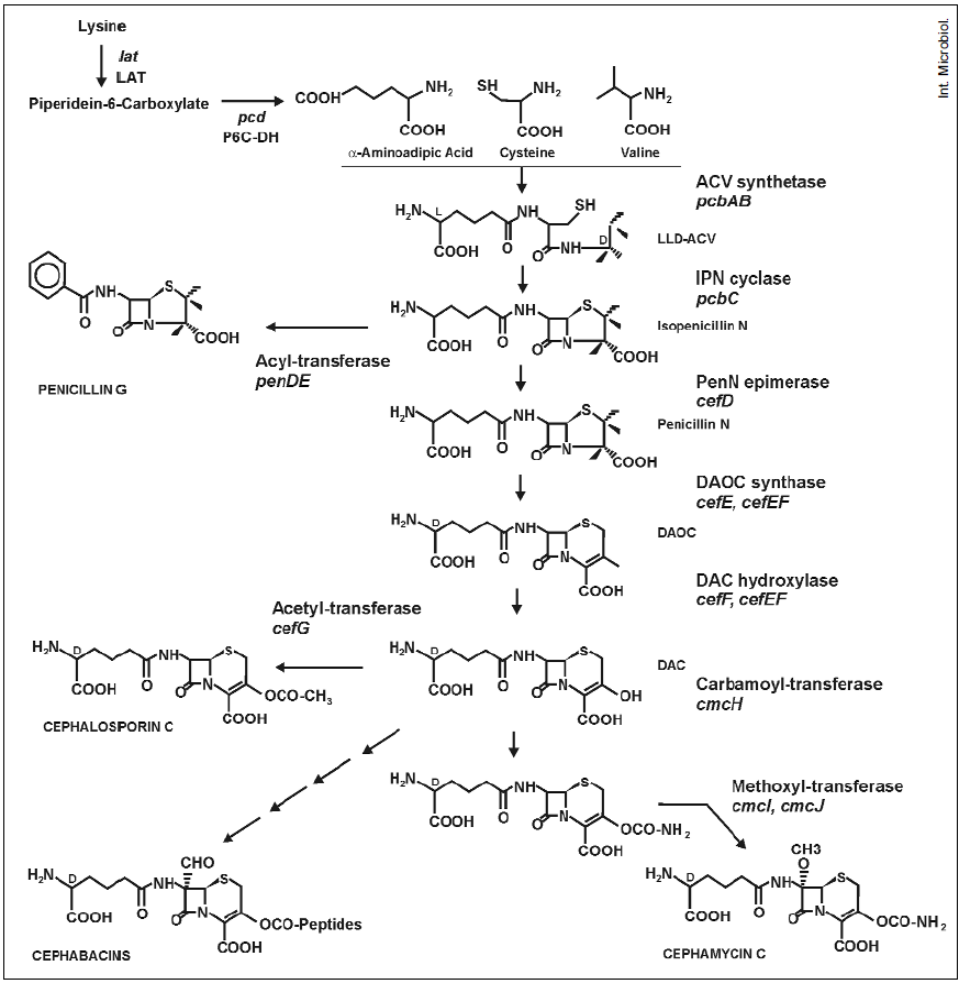

Figure 1. 6. Biosynthetic pathway of penicillins, cephalosporins, cephamycins, and cephabacins (Liras and Martin, 2006).

The genes forming the supercluster of cephamycin C and clavulanic acid cover approximately 60 kb-length of the genome. The genes that constitute the cephamycin C gene cluster are *pcbAB, pcbC, cefD, cefE, cefF, cmcI, cmcJ, cmcH* coding for biosynthetic enzymes (described in the former section); *lat* and *pcd* coding for α-aminoadipic precursor production; *bla, pcbR a*nd *pbp74* coding for β-lactam resistance; *cmcT* encoding a putative cephamycin transport protein, *ccaR* coding for a SARP regulatory protein (*Streptomyces*-Activator Regulatory Protein). The clavulanic acid cluster is comprised of *ceaS2, bls2, cas2, pah2, gcaS, car* genes for the synthesis of biosynthetic enzymes*, pbpA* and *pbp2* genes coding for penicillin binding

proteins and some genes like *cyp*, *oppA1* and *oppA2* whose functions are not known but involved in clavulanic acid biosynthesis, and *claR* gene for a LysR-type regulatory protein synthesis (Liras *et al.*, 2008).

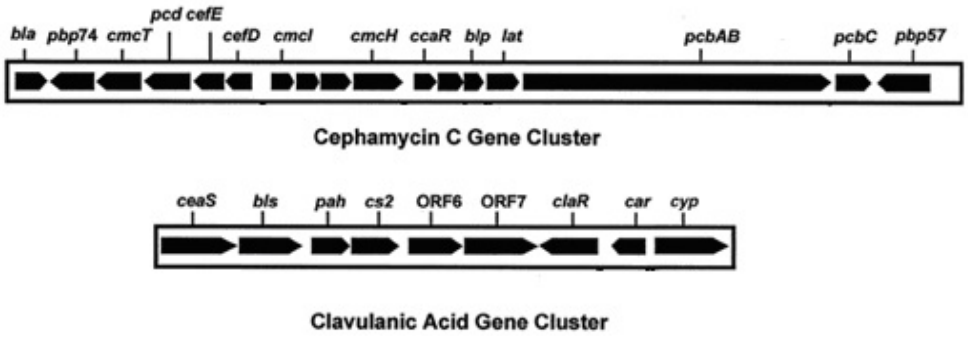

Figure 1. 7. Schematic representation of cephamycin C-clavulanic acid gene cluster (Santamarta *et al*., 2002).

As shown in Figure 1.7, biosynthetic gene clusters contain several genes of different functions; that is, the biosynthetic genes are neighbored by the genes responsible for resistance, transport and regulatory functions (Alexander and Jensen, 1998).

1.6. Regulation of cephamycin C biosynthesis by *S. clavuligerus*

Besides physiological and environmental factors, gene expression level is quite important in controlling the antibiotic production concurring with morphological differentiation in *Streptomyces* (Rodriguez, 2008; Liras, 2008).

Cephamycin C production is strongly dependent on the presence of CcaR protein. Its gene is located in the cephamycin C gene cluster and codes for a positive autoregulatory protein (Santamarta *et al.*, 2007), being a member of SARP family of transcriptional activators (Bignell *et al.*, 2005; Santamarta *et al.*, 2007). The mechanism of action of the CcaR protein could not be identified at molecular level (Wang *et al.*, 2004). However, *ccaR* gene was

shown to interact with the promoter regions of *lat* and bidirectional *cefD-cmcI* genes (Santamarta *et al.*, 2002), in addition CcaR protein was shown to control lysine aminotransferase (LAT), isopenicillin-*N* synthase (IPNS), isopenicillin-*N* epimerase (IPNE) and deacetoxycephalosporin-*C* synthase (DAOCS) (Alexander and Jensen, 1998). Furthermore, it was shown that overexpression of *ccaR* increases the antibiotic production whereas the disruption of the gene inhibited the cephamycin C production (Bignell *et al.*, 2005). *bld* genes, first characterized in *S. coelicolor,* code for various Bld proteins and mutants of these genes could neither form aerial hypha nor sporulate. Thus, the phenotype of these mutants is described as "bald" phenotype (Bignell *et al.*, 2000; Liras, 2008 *et al.*). Mutants of *bldA* and *bldG* genes defined in *S. clavuligerus* also show a bald phenotype (Trepanier *et al*, 2002; Bignell *et al*, 2005) and the *bldG* mutants were shown to synthesize neither cephamycin C nor clavulanic acid or 5*S*-clavams (Mackenzie, 2007). BldG was anticipated to be an anti-anti sigma factor (Bignell *et al.*, 2000) affecting the activity of a sigma-factor. In *Bacillus*, the function of anti-sigma factors is based on inhibition of specific sigma factors like sporulation-specific (σF) and stress response-specific (σB) sigma factors, which otherwise would bind to core RNA polymerase to carry out the transcription. Anti-anti sigma factors reverse the effect of anti-sigma factors on sigma factors by binding to them and thus releasing the sigma factors to associate with the RNA polymerase. The BldG of *S. clavuligerus*, the putative anti-anti sigma factor, shows resemblance to anti-anti-sigma factor proteins of *Bacillus* spp. (Bignell *et al.*, 2005).

1.7. Aspartate pathway and cephamycin C production

Aspartate pathway in *S. clavuligerus* begins with the aspartate as substrate that is converted to lysine through several steps and ends with the formation of cephamycin C. Meanwhile, it gives rise to threonine, methionine, meso-diaminopimelate (DAP, peptidoglycan precursor of bacteria) (Malumbres and

Martin, 1996; Tunca *et al.*, 2004). Two enzymes, aspartokinase (Ask) and aspartate semialdehyde dehydrogenase (Asd) catalyze the initial reactions in aspartic acid pathway forming the aspartate β-semialdehyde, from which two branch points emerge. One is the lysine-specific branch and the other one is leading the homoserine formation (Mendelovitz, and Aharonowitz, 1982) (Figure 1.8). Multiple forms of the Ask enzymes exist in the same organism as can be exemplified by *E. coli* having lysine, methionine or threonine as regulators for three Ask isoenzymes, respectively (Theze *et al.*, 1974) and *Bacilli*, having meso-diaminopimelate, lysine or both lysine and threonine as regulators for three Ask enzymes (Zhang *et al.*, 1990). On the other hand, *Pseudomonas* (Cohen *et al.,* 1969), *Corynebacterium glutamicum* (Cremer *et al.,* 1988), *Amycolatopsis mediterranei* (Zhang *et al.*, 1999) and *A. lactamdurans* (Hernando-Rico *et al.*, 2001) have only one aspartokinase. Ask of *S. clavuligerus* was reported to be concerted-feedback inhibited by lysine and threonine (Mendelovitz and Aharonowitz, 1982). Unlike Ask usually found in multiple forms, Asd shows itself in one form in many microorganisms excluding some such as *Vibrio cholera* (Moore *et al.*, 2002).

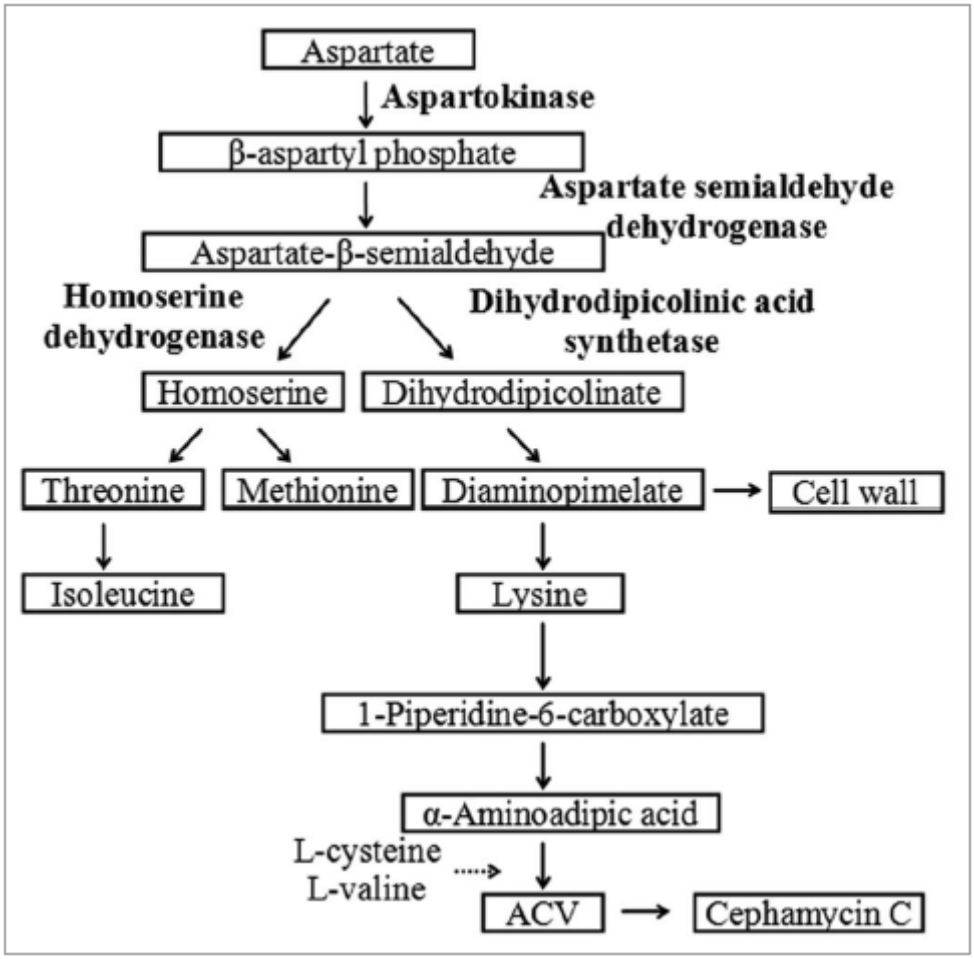

Figure 1. 8. Aspartate pathway in *S. clavuligerus* (from Özcengiz *et al*., 2010).

α-AAA (α-aminoadipic acid) is one of the three precursors of cephamycin C and it is derived from lysine, which is in turn derived from aspartate at the beginning of aspartic acid pathway. Given that aspartokinase, first enzyme of the pathway, is inhibited by the lysine and threonine, it is proposed to be the first rate limiting enzyme (Mendelovitz and Aharonowitz, 1982). Furthermore, lysine specific branch of aspartate pathway is also shown to be a rate limiting step in terms of carbon flow to form cephamycin C (Mendelovitz and Aharonowitz, 1982; Malmberg *et al.,* 1993).

The branch that leads to the formation of homoserine from aspartate β-semialdehyde is catalyzed by homoserine dehydrogenase (HSD) enzyme. The catabolic products of the homoserine are methionine and threonine, which is then converted to isoleucine. Mendelovitz and Aharonowitz (1982) showed that there was an increase in HSD synthesis in the presence of excess L-lysine; however, L-isoleucine, L-threonine, homoserine, L-methionine decreased the HSD activity.

ask-asd gene cluster of *S. clavuligerus* was cloned and characterized for the first time by Tunca *et al*. (2004) in our laboratory. Later *ask* gene was subcloned into a multicopy plasmid and introduced to *S. clavuligerus* NRRL 3585 (Taskin, 2005), the new recombinant, given the name TB3585, exhibited a cephamycin C production increased 1.1 fold in Trypticase Soy Broth (TSBS) medium. As furthered, Yılmaz *et al*. (2008) disrupted homoserine dehydrogenase gene (*hom*) of *S. clavuligerus* NRLL 3585, thereby channeling the carbon flow through aspartic acid pathway to the production of cephamycin C and preventing the concerted-feedback inhibition by lysine and threonine on aspartokinase. By this means, 2- fold increase in cephamycin C production was achieved in chemically defined medium.

1.8. Effects of nutritonal conditions on cephamycin production by *S. clavuligerus*

Nutritional conditions of the environment are strong determinants of the secondary metabolite production by microorganisms. For example, penicillin N, cephalosporin and cephamycin C having the common pathway in *S. clavuligerus* are affected considerably by carbon and nitrogen sources (Aharanowitz and Demain 1978). On the other hand, inorganic phosphate has adverse effect on antibiotic production by the repression of aminoadipylcysteinylvaline synthase, cyclase, epimerase and expandase and

by inhibition of the cyclase and expandase activities (Lübbe et al. 1985; Lebrihi *et al.,* 1987; Jhang *et al.,* 1989a; Rius and Demain *et al.,* 1997)

Ammonium source strongly affects the production of β-lactam antibiotics in microorganisms including *S. clavuligerus*, but the molecular mechanism operating in each species can be different. It was shown that in a chemically defined medium containing 120 mM of ammonium, the formation of ACVS decreased by 75 %, cyclase by 70 %, and expandase by 50 % in *S. clavuligerus* (Zhang *et al.,* 1989; Demain and Vaishnav, 2006).

Romero *et al*. (1984) showed that sulphur source is an indispensible component for cephamycin C production and that sulphate, sulphite and thiosulphate were efficiently used by *S. clavuligerus* as sulphur sources while L-methionine and L-cysteine did not affect cephamycin production considerably. The sulphonic acid part of the MOPS buffer was also utilized as sulphur source. Furthermore, in the absence of sulphur in the medium, *S. clavuligerus* could produce clavulanic acid, but no cephamycin C and the addition of only 1 mM sulphur could give a considerable increase in cephamycin C production.

Lysine was shown to increase the aspartokinase activity and stimulate cephamycin C biosynthesis in *S. clavuligerus* (Mendelovitz and Aharonowitz 1982). Furthermore, Fang *et al*. (1996) and Rius *et al*. (1996) proposed that L-lysine increased antibiotic production by behaving both as a substrate and an inducer of the LAT enzyme involved in the synthesis of α-aminoadipic acid, one of the three precursors for the cephamycin C production (Liras and Martin, 2006). On the other hand, lysine plus threonine addition into chemically defined medium exerted a concerted feedback inhibiton on Ask enzyme of *S. clavuligerus* as well as threonine which would alone repress cephamycin C production (Mendelovitz and Aharonowitz 1982).

Alanine and histidine are reported to have inhibitory effects on cephamycin production. Alanine stimulates alanine dehydrogenase which in turn causes an increase in alanine intracellularly which acts as an inhibitor to ACVS, cyclase and expandase (Brana *et al.,* 1986; Kasarenini and Demain, 1994; Demain and Vaishnav, 2006).

L-asparagine was used both as a nitrogen and a carbon source (Aharonowitz & Demain, 1978). Accordingly, asparagine, glutamine, arginine and urea serve as efficent nitrogen sources, hence increasing the production of cephamycin (Aharonowitz and Demain, 1979).

S. clavuligerus can utilize various carbon sources. Although starch, maltose, glycerol, organic acids, and amino acids are favorable carbon sources, it surprisingly cannot utilize glucose (Aharonowitz and Demain, 1978) due to lack of a putative active transport system to take glucose into the cell (Garcia-Dominguez *et al.*, 1989). Each carbon source has different effects on antibiotic production by *S. clavuligerus*. Glycerol and maltose turned out to be unfavorable carbon sources for the production of cephamycin C while both increased growth rate of *S. clavuligerus*. On the other hand, starch or organic acids like α-ketoglutarate and succinate were shown to increase cephamycin production despite being poor carbon sources (Aharonowitz and Demain, 1978). It has been proposed that expandase is repressed when rapidly utilized carbon sources are available in the environment. Another study demonstrated that the presence of high concentrations of maltose decreased the synthesis of penicillin N pointing that another enzyme earlier than the expandase is inhibited, thereby showing that the carbon regulation of cephamycin C pathway might begin at earlier steps (Rius and Demain, 1997**)**. As found by Romero *et al.* (1984), *S. clavuligerus* grown in GSPG medium containing glycerol, sucrose, proline and glutamic acid as carbon and nitrogen sources utilizes sucrose rather slowly (Romero *et al.*, 1984). However, in another study by Garcia-Dominguez *et al.*(1989), sucrose utilization by *S. clavuligerus* was not detectable**.**

1.9. The present study

In the present study, the effects of carbon and nitrogen sources incorporated into chemically defined medium (CDM) were investigated for growth and cephamycin C overproduction by a *hom* mutant *Streptomyces clavuligerus* constructed in our laboratory (Yılmaz *et al*., 2008), hence a modified CDM (mCDM) was defined. Thereafter, the growth and cephamycin C production by recombinant and vector only strains of *S. clavuligerus* (TB3585, BA39, BAV, TBV, AK39) were investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains

Bacterial strains used in this study and their characteristics are described in Table 2.1. *Escherichia coli* strains were grown in Nutrient broth or Luria broth at 37 °C and stored in 25 % glycerol at -80 °C. Mycelium stocks of *S. clavuligerus* were stored in 25 % glycerol and 25 % TSB at -80 °C.

2.2. Culture media and conditions

S. clavuligerus strains were grown at 28 °C at 220 rpm in baffled flasks. For recombinant and transformant strains of *S. clavuligerus* except for AK39, the media were supplemented with 50 μg/mL thiostrepton. CDM variants were supplemented with 150 μg/mL methionine and 50 μg/mL threonine, amino acids of auxotrophy, for AK39, BA39 and BAV strains. CDM (Malmberg *et al.*, 1993) variants and TSB were used for growth and antibiotic biosynthesis. The composition (Table 2.2) and preparation of culture media were described in Appendix A.

Table 2. 1. Bacterial strains used in this study.

Stains	Description	Source or reference
S. clavuligerus		
NRRL 3585	Wild type, cephamycin C and clavulanic acid producer	Prof. J. Piret, Northeastern University, USA
AK39	Null mutant of *S. clavuligerus* NRRL 3585, *hom::kan*	Yılmaz *et al.* (2008)
TB3585	*S. clavuligerus* NRRL 3585 carrying pTB486, $Thio^R$	Özcengiz *et al.* (2010)
BA39	*S. clavuligerus* AK39 carrying pTB486, $Thio^R$, Kan^R	Özcengiz *et al.* (2010)
TBV	*S. clavuligerus* NRRL 3585 carrying pIJ486, $Thio^R$	Özcengiz *et al.* (2010)
BAV	*S. clavuligerus* AK39 carrying pIJ486, $Thio^R$, Kan^R	Özcengiz *et al.* (2010)
***E.coli* ESS**	β-lactam supersensitive *E. coli* strain	Prof. J. Piret, Northeastern University, USA

Table 2. 2. CDM variants in regard to their carbon and nitrogen composition.

CDM variants	Nitrogen source	Carbon sources	
	Asparagine (g/L)	Glycerol (g/L)	Sucrose (g/L)
CDM	10	25	0
CDM+asn	20	25	0
CDM+suc	10	25	25
CDM+asn+suc	20	25	25
CDM+suc-glyc	10	0	25
CDM+asn+suc-glyc	20	0	25
CDM+asn-glyc	20	0	0

2.3. Growth of seed cultures

For the preparation of seed cultures, 200-600 µL of *S. clavuligerus* stock cultures (due to different growth rates of each strain to reach the OD_{600} 0.7-0.9) were added into 50 mL of TSB medium and incubated for 24-48 h at 28 °C at 220 rpm. Optical density of seed cultures was measured according to the procedure by Malmberg *et al*. (1993). 0.5 mL of sample from seed culture was mixed with 3 mL of distilled water and 0.5 mL of 2.5 M HCl and then the mixture was homogenized via sonication (Ultrasonic Processor, Cole Parmer) for 3x30 seconds at 50 % amplititude. When the OD_{600} of homogenized mixture reached at 0.7-0.9, 25-30 mL of seed culture was inoculated into the fresh media.

30 mL of seed culture, the OD_{600} of which already reached 0.7-0.9, was centrifuged at 3200 rpm for 10 min at 4 °C and the pellet was washed with 10 mL of fresh medium (CDM or TSB). The cells were resuspended in 50 mL of

inoculation medium and the cultures were grown at 28 °C at 220 rpm for 5 days in TSB or 6 days in CDM variants.

2.4. Growth determination via DNA quantification

Growth measurement of cultures via DNA quantification was performed according to Burton (1968). 2 mL of a culture was taken into an Eppendorf tube at each 24h interval 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 (physiological saline) solution. 500 µL of the diluted samples and standards were distributed into 2 mL Eppendorf tubes and 500 µL of 1 N $HClO_4$ was added to each. Tubes were mixed by inversion and incubated at 70 °C for 20 min. 1 mL of diphenylamine reagent (Appendix B) was added into each tube. Samples were mixed by inversion and incubated at 30 °C for 15-17h after which the 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 in samples was calculated according to a standard curve drawn using herring sperm and expressed as µg of DNA per mL of culture.

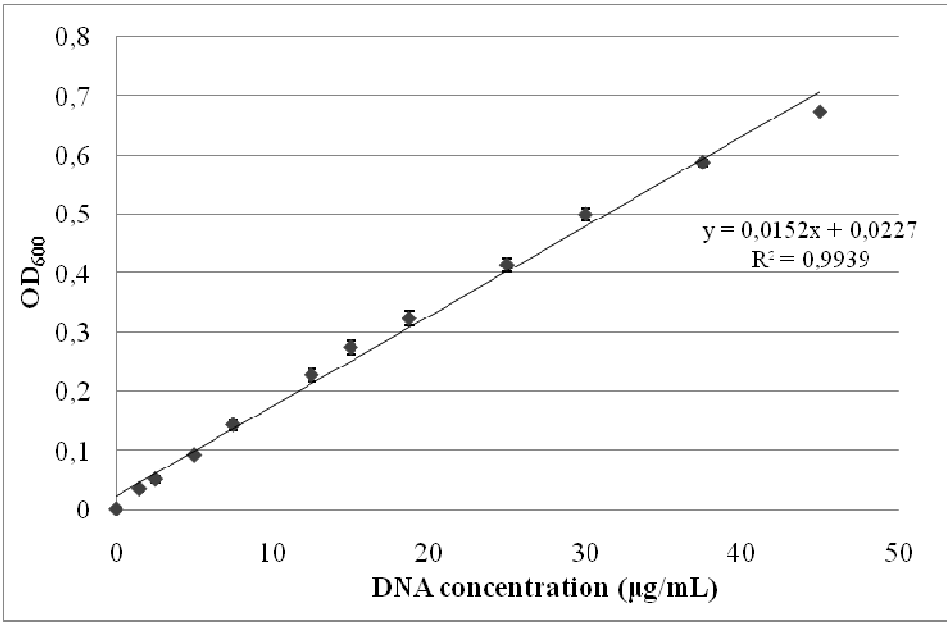

Figure 2. 1. Standard curve for the determination of DNA concentration.

2.5. Bioassay of β-lactam antibiotics

Supernatants of 2 mL of cultures collected at 24h intervals were used for the determination of cephamycin C production by the cultures. Samples which were kept at -20 °C were thawed on ice. *E. coli* ESS cells were grown in TSB up to an OD_{600} of 0.9-1.0. When OD_{600} reached to 1.0, 3 mL of cell culture was mixed with 100 mL of TSA, poured into petri plates and left for solidifying. 5mm-diameter holes were punched into TSA petri plates and 80 µL of sample supernatant and also cephalosporin C standards prepared in 0.01 M phosphate buffer, pH 3.5 (Appendix B) were filled into the holes. The plates were first incubated at 4 °C for 2h and then at 30 °C for 12-15h. The results were expressed as either volumetric cephamycin C concentration, µg of cephamycin C per mL of culture, or specific cephamycin C concentration, µg of cephamycin C per mg of total bacterial DNA. Cephamycin C amount was calculated by constructing a calibration curve with cephalosporin C standards.

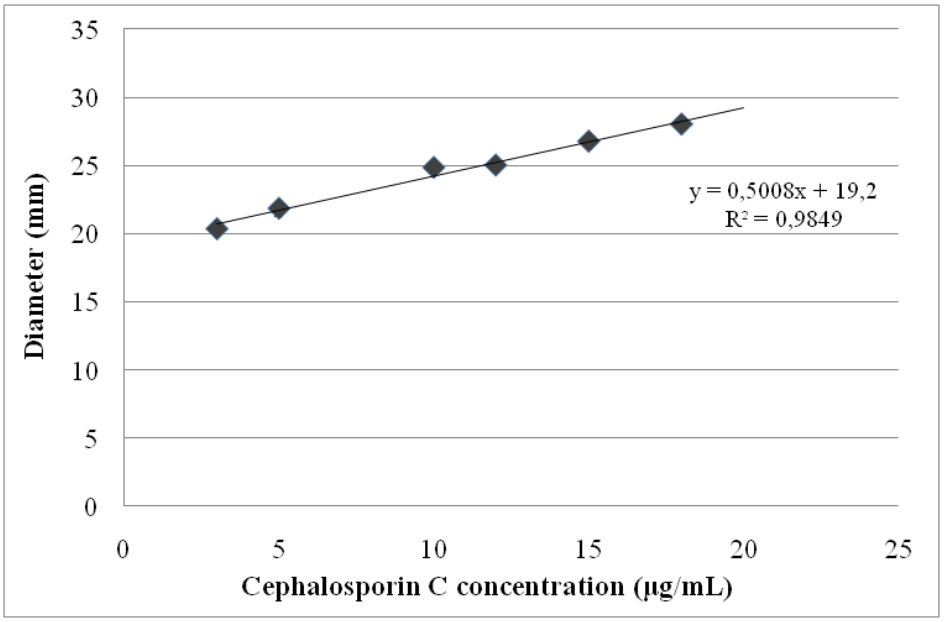

Figure 2. 2. Standard curve for the determination of cephamycin C concentrations.

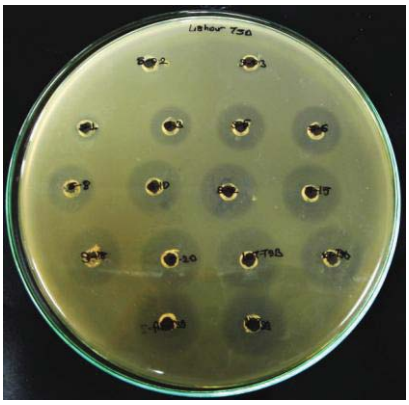

Figure 2. 3. TSA petri plate in which standards and samples produced zones proportional to antibiotic concentration.

2.6. Measurement of aspartokinase (Ask) activity

Aspartokinase (Ask) activity was measured according to Follettie *et al*. (1993) with slight modifications. *S. clavuligerus* cultures were grown in CDM+asparagine+sucrose-glycerol medium (Appendix A) at 28 °C at 220

rpm. At 24, 48, 60 and 72h fermentation, 2 mL of samples were centrifuged at 8000 rpm for 15 min. Pellets were washed with 1 mL of 50 mM cold phosphate buffer (Appendix B). Cells were then resuspended in 300 µL of 10 mM sonication buffer (Appendix B). Resuspended cells were sonicated at 50 % amplitude for 1 min for the disruption of the cells. The cell debris was removed by centrifugation (13200 rpm, 10 min). Determination of Ask activity depends on the formation of the stable aspartate-hydroxymate. The protein extract obtained was added into a separate Eppendorf tube containing 0.5 mL of assay mixture (Appendix B). The mixture was incubated at 30 °C for 60 min. The reaction was stopped by adding 0.75 mL of ferric chloride solution (Appendix B) and the protein debris was removed by centrifugation at 13200 rpm for 5 min. Optical density of supernatant was measured at 540 nm. The results were expressed as mM of aspartate-hydroxymate by using the calibration curve constructed with aspartate-hydroxymate standards. After subtracting the background activity (when no substrate is present in the medium), Ask activity was defined as nanomoles of aspartate-hydroxymate formed per milligram protein per minute.

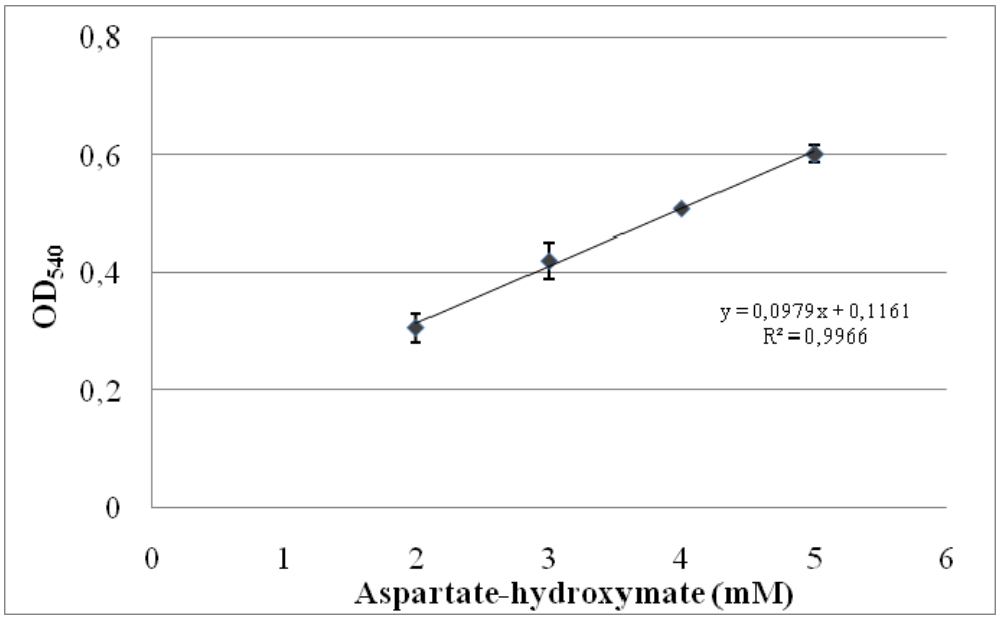

Figure 2. 4. Absorbance (OD_{540}) versus aspartyl-hydroxymate concentration (mM) calibration curve.

2.7. Determination of protein concentration

Protein concentrations were measured by the Bradford quantification method (1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 at 595 nm is obtained when its binding to protein occurs. Assay reagent was made by dissolving 100 mg of Coomassie Blue G250 in 50 mL of 95 % ethanol. The solution was then mixed with 100 mL of 85 % phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Watman No. 1 filter paper. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 2, 5, 10, 20, 25, 30, 40 µL of 1 mg/mL of BSA were added into Eppendorf tubes and and the volume of each was adjusted to 500 µL with distilled water. 500 µL of distilled water was added into a tube as reagent blank. 1.5 mL of assay reagent was added into each tube and mixed gently, but thorougly. Samples were kept in dark for a few minutes. A standard curve of absorbance versus microgram protein was prepared and the amount of proteins was determined from the slope of the curve.

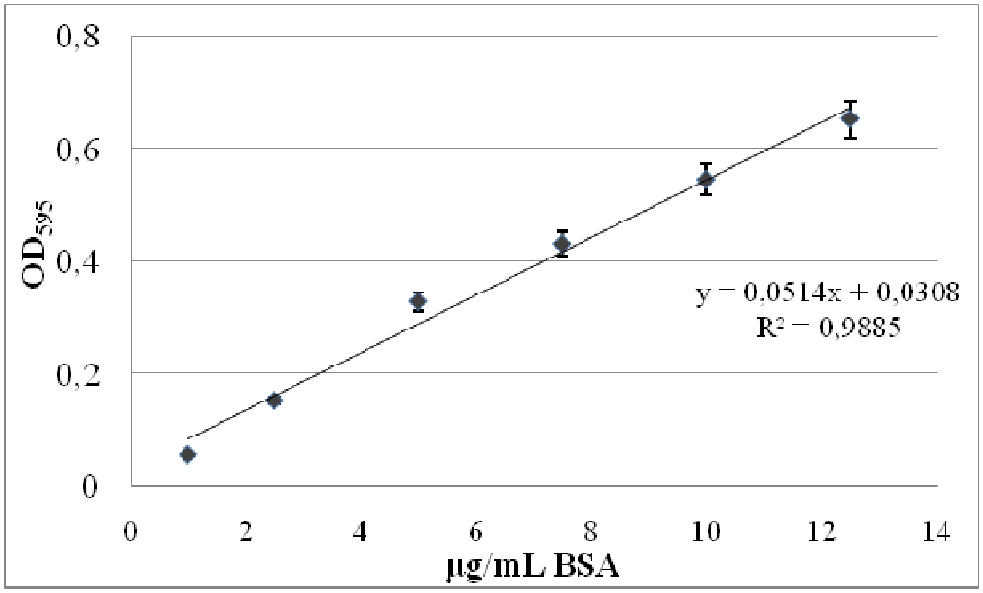

Figure 2. 5. Protein determination calibration curve.

2.8. Determination of intracellular free amino acid concentrations

Cell pellets kept at -20 °C were thawed, weighed and washed twice with cold 0.05 M potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl. Each pellet was suspended in distilled water as 4 mL per g cell wet weight. Cell suspension was incubated in boiling water bath for 30 min. Suspension was centrifuged at 13200 rpm for 10 min and supernatant was saved. The pellet was washed twice with hot water and centrifuged at 13200 rpm for 10 min. Supernatant fractions were combined and filtered through a membrane filter of 0.45 μm along with an equal volume of cold acetone (-20 °C). Samples were kept in cold for 30 min. The supernatant-acetone mixture was centrifuged at 13200 rpm for 10 min and then dried by vacuum evaporation at 40 °C. Samples were dissolved in fixed volumes of water from which 10 to 50 μL of portions were mixed with 0.02 M sodium citrate pH 2.2 (Appendix B). After centrifugation at 13200 rpm for 10 min samples were injected into a Durum-550 amino acid analyzer at Düzen Laboratories, Ankara, Turkey. Chromatography was performed with Dovex AG-50V as the ion-exchange resin. Amino acid concentrations were expressed as micromoles of amino acid extracted from 1 g wet weight of bacteria.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Optimization of chemically defined medium for an increased bacterial growth and cephamycin C production by *Streptomyces clavuligerus*

Production and regulation of secondary metabolites are strictly dependent on the primary metabolites that are produced by the catabolism of the energy and carbon sources utilized by the microorganisms inasmuch as the precursors emerging during primary metabolism can be the suppliers of the secondary metabolism. Nutrient shortage results in depression of the growth which in turn boosts the secondary metabolite production. Secondary metabolite production was observed late in growth phase occurring as a result of nutrient deficiency (Martin and Demain, 1980) and each strain has unique control mechanisms for secondary metabolite production under nutrient limitation (Bushell and Fryday, 1983; Hobbs *et al*., 1990; Sánchez and Brańa, 1990).

In our previous studies, a chemically defined medium (CDM; Aharonowitz, 1978) was used for cephamycin C production in parental (WT) and recombinant strains of *S. clavuligerus* since it is possible to observe the individual effects of carbon and nitrogen sources only in defined medium. *S. clavuligerus* AK39 was constructed in our laboratory (Çaydaşı, 2006) in order to direct primary metabolism to cephamycin C production by eliminating the branchpoint enzyme homoserine dehydrogenase activity which leads to production of L-threonine, L-methionine and L-isoleucine (Figure 1.8). AK39 and WT strains were compared to observe the effect of *hom* deletion in *S. clavuligerus* on the production of cephamycin C. Although AK39 produced

1.7- to 2-fold higher specific cephamycin C yields, it was not as much as expected due to much poorer growth of this recombinant in CDM (Çaydaşı, 2006). Therefore, CDM was subjected to six modifications in terms of carbon sources and/or their concentration in order to overcome the growth problem of *hom* minus strain, AK39.

Asparagine, glutamine, arginine, and urea can be utilized by *S. clavuligerus* quite efficiently as nitrogen sources (Aharanowitz and Demain, 1979). Aharanowitz and Demain (1978) showed that asparagine can serve as both a carbon and nitrogen source in the absence of another carbon source in a defined medium and when glycerol was added in increasing concentrations into the defined medium containing 0.4 % asparagine as a nitrogen source, bacterial growth was improved; however, there was a corresponding decrease in both the volumetric and specific cephamycin C concentrations. In another study, it was observed that glycerol at 50-110 mM was optimal for the production of cephamycin production by *S. clavuligerus* in GSPG (defined medium) but higher concentrations resulted in a small decrease (Romero *et al.*, 1984). The concentration of glycerol in CDM is 272 mM, over the optimal amount reported. This might be a reason for the lack of expected increment in antibiotic production. Romero *et al*. (1984) showed that sucrose was utilized slowly in GSPG. However, sucrose was utilized by neither *S. clavuligerus* nor its mutant capable of using glucose as carbon source in minimal media (MM) (Garcia-Dominguez *et al.*, 1989). Ozcengiz *et al.* (2010) suggested that sucrose is not utilized as a carbon source by *S. clavuligerus,* but it provides a homogeneous and disperse growth.

By playing with three components, sucrose, glycerol, and asparagine, we expected to design an optimal media for growth and antibiotic production by *hom*-mutant of *S. clavuligerus*, AK39. CDM, which contains K_2HPO_4, MOPS, L-Asparagine, $MgSO_4$, glycerol and trace metals, was used as the control medium. The medium was also supplemented with L-threonine and L-

methionine, i.e., amino acids of auxotrophy, as well as thiostrepton. Asparagine content was increased 2-fold in "CDM+asn" (increased from 10 to 20 g/L), 25g/L sucrose was added to "CDM+suc", "CDM+asn+suc" contained both 2-fold more asparagine and 25g/L sucrose. Glycerol replaced sucrose in "CDM+suc-glyc". "CDM+asn+suc-glyc" contained 2-fold more asparagine, 25g/L sucrose, but no glycerol. "CDM+asn-glyc" contained 2-fold more asparagine but no glycerol (Table 2.2).

The growth of bacteria was determined by performing DNA quantification (Burton, 1968). Dry cell weight measurements are not much reliable in filamentous microorganisms since the accumulation of lipids and secondary metabolites contribute to dry cell weight (Martin and McDaniel, 1975).

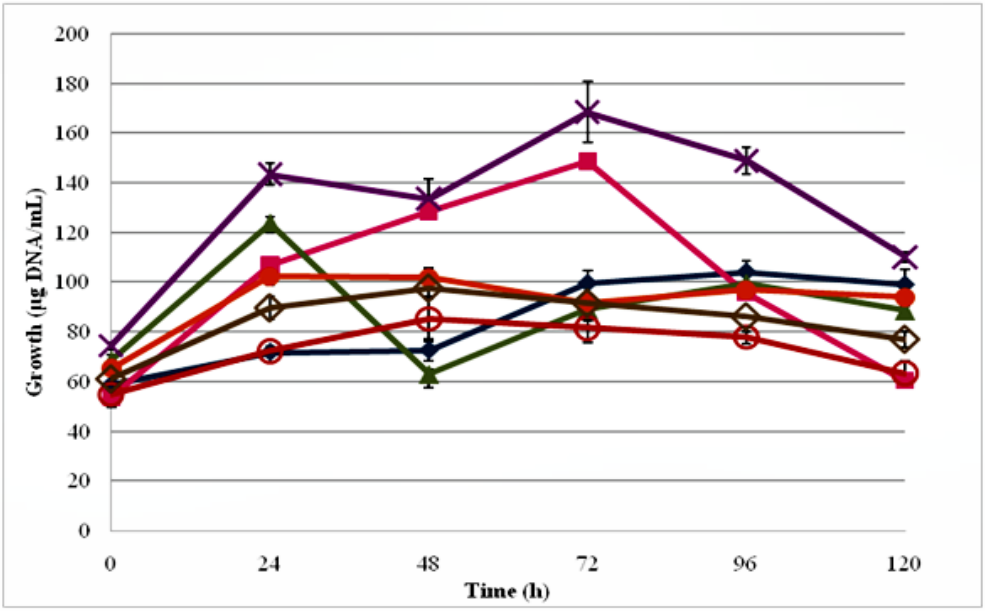

Figure 3. 1. Growth of WT in CDM variants. (◆) CDM, (■) CDM+asn, (▲) CDM+suc, (✖) CDM+asn+suc, (○) CDM+suc-glyc, (●) CDM+asn+suc-glyc, (◊) CDM+asn-glyc.

The growth of WT strain in CDM+asn+suc medium was the highest amongst the six media (Figure 3.1). The organism also grew well in CDM+asn.

Bacterial growth was fast in CDM+suc up to 24h then showed a sharp decrease till 48h of incubation, and a slight increase in growth was observed thereafter. The growth in CDM+asn+suc-glyc increased no further after an increase till 24h of fermentation. The growth in CDM+asn-glyc was slightly higher than that in CDM+suc-glyc, but in both media, first small increase in growth till 48h was followed by a slight decrease. The growth of WT strain in CDM was rather poor till 48h incubation, then an increase between 48h and 72h and then a constant phase till 120h fermentation were seen. Glycerol and asparagine had been shown to be favorable nutrient sources for the growth of *S. clavuligerus* in defined medium (Aharonowitz and Demain, 1978). Our results indicated that WT strain grown in the media containing 2-fold more asparagine as well as glycerol (CDM+asn, CDM+asn+suc) exhibited higher growth than those in others. Growth in the media lacking glycerol was more stable without significant fluctuations during the fermentation; however, it was not as high as in their counterparts containing glycerol. Addition of sucrose seemed to support the growth slightly. For example, a higher growth was observed in CDM+asn+suc when compared to that in CDM+asn or in CDM+asn+suc-glyc than CDM+asn-glyc.

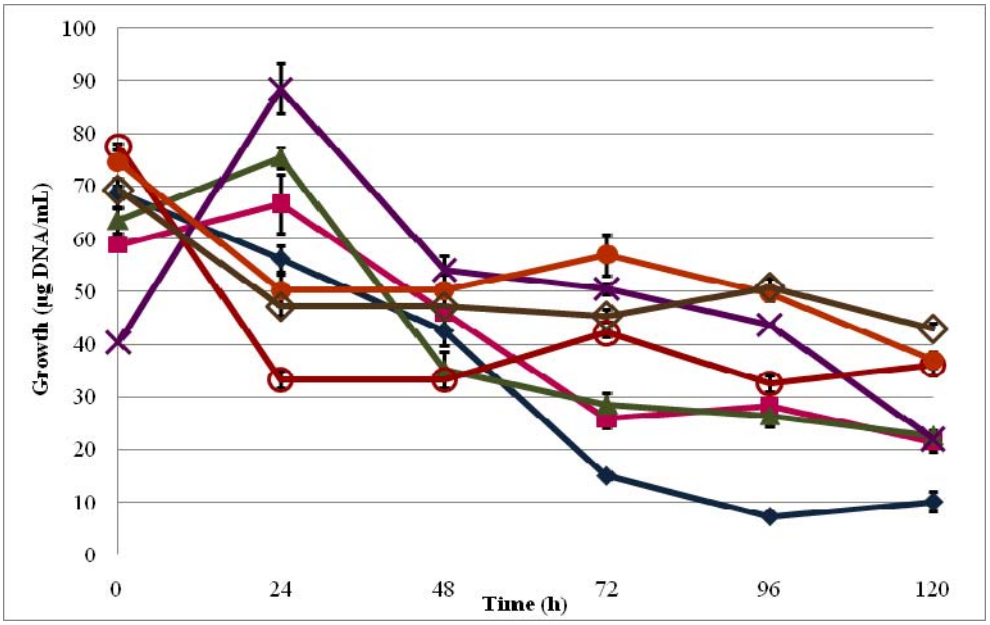

Figure 3. 2. Growth of AK39 in CDM variants (◆) CDM, (■) CDM+asn, (▲) CDM+suc, (✖) CDM+asn+suc, (○) CDM+suc-glyc, (●) CDM+asn+suc-glyc, (◊) CDM+asn-glyc.

When the growth of AK39 was compared in modified CDMs, the highest growth was observed in CDM+asn+suc as was shown for the WT strain (Figure 3.2). There was a steep increase in growth till 24h and a sharp decrease till 48h and then moderate decrease till 120h incubation. The growth in both CDM+asn and CDM+suc showed an increase till 24h fermentation and then a decrease thereafter. In the media lacking glycerol (CDM+suc-glyc, CDM+asn+suc-glyc), a decrease in growth till 24h incubation and then a relatively stable pattern with slight increases and decreases were observed. AK39 strain showed a constant decrease in growth in CDM.

As to the growth patterns of AK39 and WT strains in modified CDMs, it was observed that there was a faster increase and then a sharp decrease in growth in the media having glycerol, but the growth did not exhibit such dramatic fluctuations in the media lacking glycerol. Furthermore, 2-fold increment of asparagine concentration resulted in an increase in growth. Addition of

sucrose with 2-fold increase in asparagine content and/or glycerol favored the growth of both AK39 and WT.

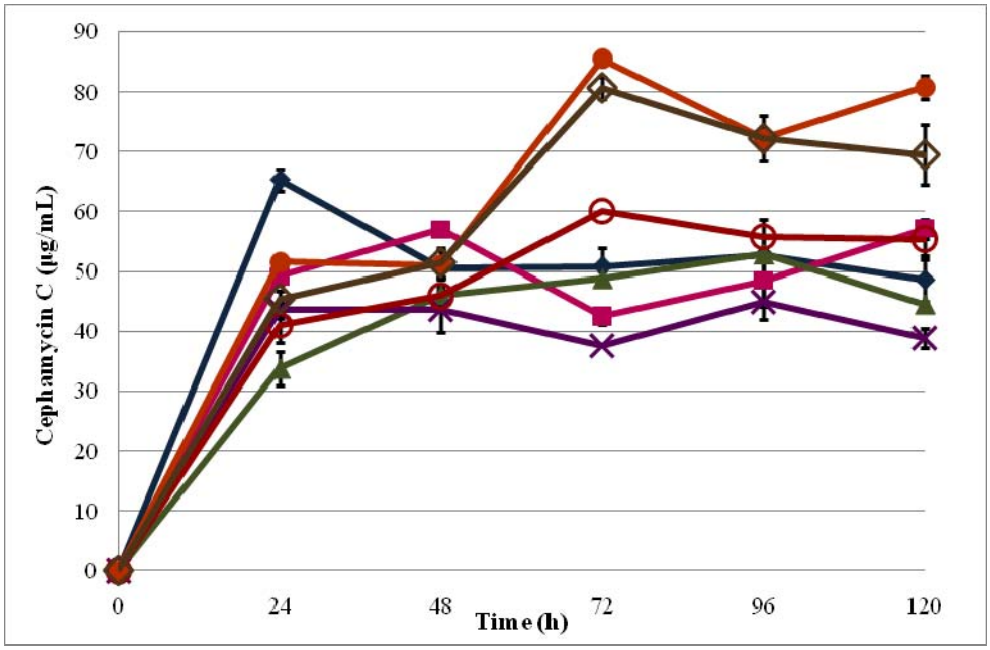

Figure 3. 3. Volumetric cephamycin C production profile of *S. clavuligerus* WT strain in CDM variants. (◆) CDM, (■) CDM+asn, (▲) CDM+suc, (✖) CDM+asn+suc, (○) CDM+suc-glyc, (●) CDM+asn+suc-glyc, (◊) CDM+asn-glyc.

Volumetric cephamycin C production was defined as micrograms of cephamycin C formed per mL of culture. The highest production by WT strain (85 µg/mL) was observed in CDM+asn+suc-glyc at 72h incubation which was 1.7-fold higher than that in the control medium, CDM. The increment in CDM+asn-glyc was 1.1- and 1.2-fold at 72h and 120h fermentation, respectively. The production in CDM+suc-glyc was also higher than that in CDM after 48h incubation (Figure 3.3). Being consistent with the results found by using WT strain, the highest production by AK39 was observed in CDM+asn+suc-glyc which was followed by CDM+asn-glyc. There was 50- and 15-fold increase in antibiotic production in CDM+asn+suc-glyc as compared to that in CDM at 72 h and 120h cultivation, respectively (Figure 3.4).

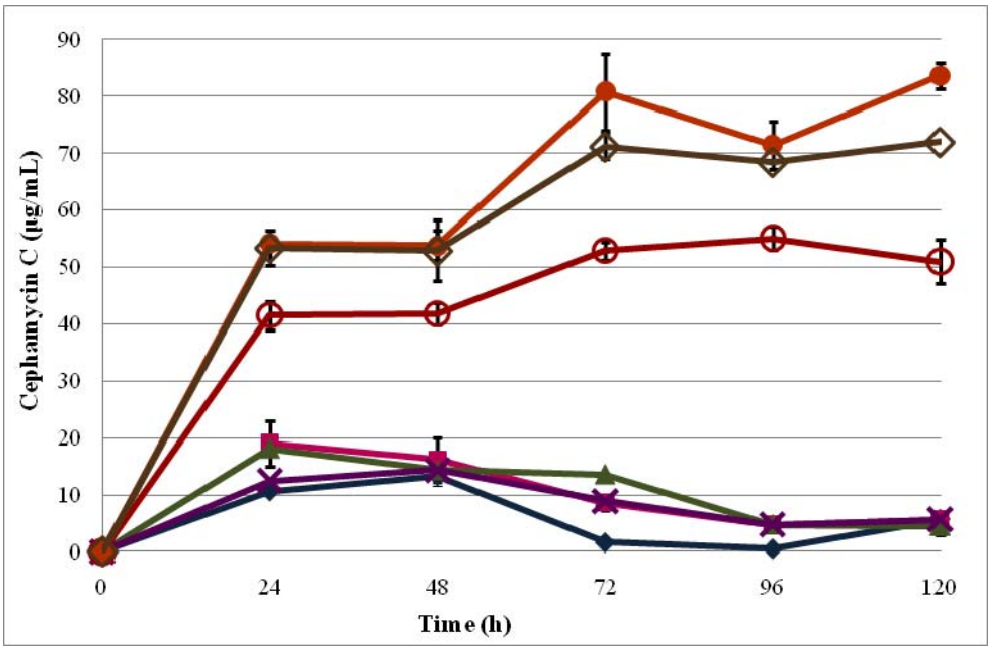

Figure 3. 4. Volumetric cephamycin C production profile of *S. clavuligerus* AK39 strain in CDM variants. . (◆) CDM, (■) CDM+asn, (▲) CDM+suc, (✖) CDM+asn+suc, (○) CDM+suc-glyc, (●) CDM+asn+suc-glyc, (◊) CDM+asn-glyc.

Taken together, CDM+asn+suc-glyc and CDM+asn-glyc were the best regarding volumetric antibiotic production by both AK39 and WT strains. Moreover, the cephamycin production by both strains in CDM+suc-glyc was also higher than that in respective medium containing glycerol. Therefore, it is plausible to generalize that glycerol had a negative effect on the antibiotic production. When the absence of glycerol was combined with the doubled asparagine concentration, there was also a considerable increase. The addition of sucrose to these media slightly favored production (Figure 3.3 and 3.4). Given the fact that sucrose is not a favorable carbon source for the organism, the observed positive effect of sucrose might be a result of the homogenous growth in its presence.

Specific cephamycin C production was described as micrograms of cephamycin C formed per mg of DNA and it is more accurate to use the specific production for the comparison since it correlates the cell's capacity to

produce antibiotic to the extent of cultural growth more effectively (Çaydaşı, 2006).

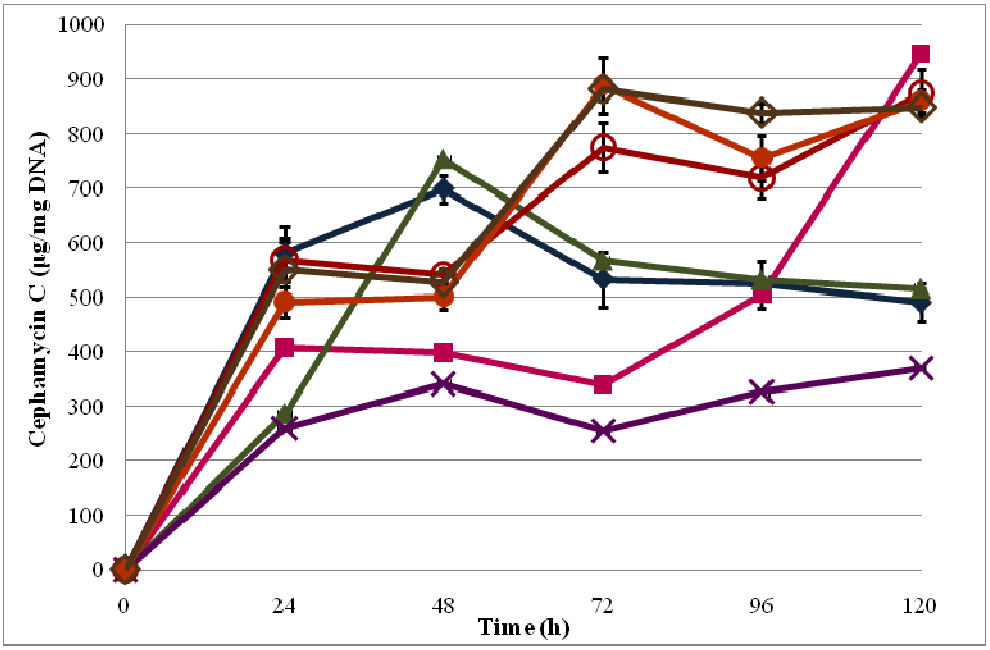

Figure 3. 5. Specific cephamycin C production profile of *S. clavuligerus* WT strain in CDM variants. (◆) CDM, (■) CDM+asn, (▲) CDM+suc, (✖) CDM+asn+suc, (○) CDM+suc-glyc, (●) CDM+asn+suc-glyc, (◊) CDM+asn-glyc.

Specific antibiotic production by WT strain was next measured when grown in CDM variants. As compared to the production in CDM, specific cephamycin production was the highest at 72h fermentation in CDM+asn-glyc (1.7-fold) was observed in two media. In CDM+asn+suc-glyc and CDM+asn-glyc media, the increment was 1.4- and 1.6-fold, respectively at 96h fermentation. The third medium in which the production was quite close to those in the first two media was CDM+suc-glyc, with a 1.5- and 1.4-fold increased production than that in CDM after 72h and 96h fermentation, respectively. The lowest production was observed in CDM+asn+suc. There was quite a similar production pattern in CDM+suc and CDM after 48h fermentation and the antibiotic production in CDM was 2-fold higher than that of CDM+suc at 24h cultivation. Specific cephamycin C production by WT strain in CDM+asn was lower than that in CDM till 96h fermentation;

however, it increased sharply after 72h becoming one of the highest among all at 120h incubation. Although the volumetric production at this time interval was not that high, the boost in specific production resulted from sharply decreased growth (Figure 3.2).

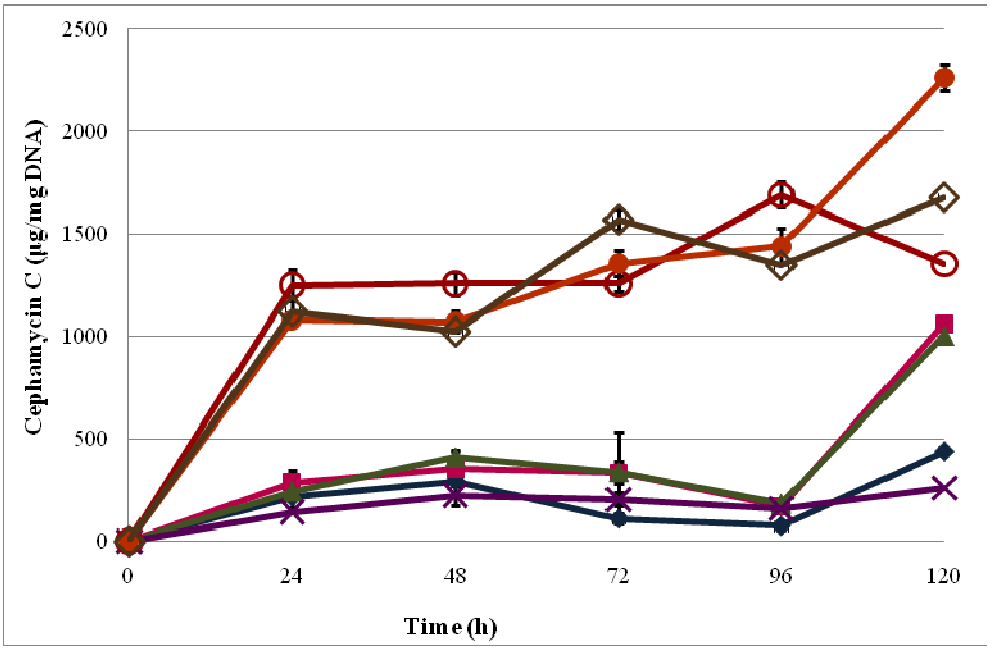

Figure 3. 6. Specific cephamycin C production profile of *S.clavuligerus* AK39 strain in CDM variants. (◆) CDM, (■) CDM+asn, (▲) CDM+suc, (✖) CDM+asn+suc, (○) CDM+suc-glyc, (●) CDM+asn+suc-glyc, (◊) CDM+asn-glyc.

Specific cephamycin C production profile of AK39 was similar in the media lacking glycerol; i.e., CDM+asn+suc-glyc, CDM+suc-glyc and CDM+suc-glyc. The highest antibiotic titer (2258 µg/mg DNA) was found in CDM+asn+suc-glyc at 120h fermentation. Production was 13-, 20-, 5- fold higher in CDM+suc+asn-glyc than that in CDM at 72 h, 96 h, 120 h, respectively. On the other hand, specific antibiotic production by AK39 in the media containing glycerol was consistently lower than those lacking glycerol.

Because our goal is to optimize growth and cephamycin C production by *hom*-minus *S. clavuligerus*, CDM+asn+suc-glyc was determined as the optimum medium for the subsequent studies.

3.2. Comparison of recombinant *S. clavuligerus* strains with multicopy *ask* gene with or without *hom* disruption with respect to growth and cephamycin C production

CDM+asn+suc-glyc medium chosen as the principal medium for further analyses was named as mCDM (modified chemically defined medium) for the ease of nomenclature throughout this thesis.

Three recombinants of *S. clavuligerus* have already been constructed in our lab to increase its cephamycin C yield; i) TB3585 containing multicopy *ask* gene in wild type strain (Taşkın, 2005), ii) AK39, *hom*-deleted mutant of *S. clavuligerus* (Çaydaşı, 2006), iii) BA39 containing multicopy *ask* gene in the *hom* mutant *S. clavuligerus* AK39 (Özcengiz *et al*. 2010). In order to observe the effect of multicopy *ask* on cephamycin C production, any impact of expression vector without gene insert (pIJ486) had to be considered. For this purpose, TBV and BAV strains were generated by transformation of WT and AK39 with pIJ486, respectively (Table 2.2). *ask* gene codes for aspartokinase enzyme which initiates aspartic acid pahway. *hom* gene codes for homoserine dehydrogenase enzyme which catalyzes the branch point in aspartic acid pathway that leads to homoserine production. In *hom*-deleted mutants, carbon flow occurs from aspartate to directly cephamycin C production through lysine specific branch point and multicopy *ask* increases the level of intracellular aspartokinase enzyme; therefore, recombinants were expected to exhibit higher cephamycin C yield than that of wild type.

Cephamycin C production by TB3585 was compared with that of TBV and WT, and the antibiotic production of BA39 was compared with that of BAV and AK39. Six strains of *S. clavuligerus* were grown in both TSB, the rich medium, and mCDM for a solid comparison of the effect of nutritional conditions.

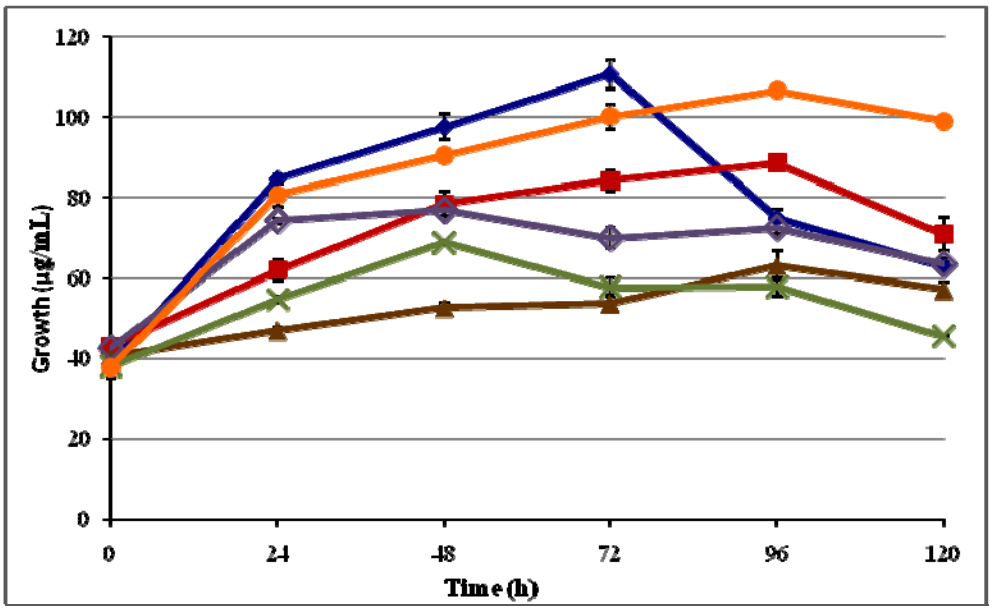

Figure 3. 7. Growth of *S. clavuligerus* strains in TSB. (◆) WT, (■) AK39, (▲) BAV, (✖) TB3585, (◊) BA39, (●) TBV.

Being rich in carbon and nitrogen sources, TSB is quite a favorable medium for growth of a large variety of bacteria (Hall *et al.*, 1974). TSB was the most favorable for the growth of WT strain. The respective patterns from higher to lower growth in TSB medium were WT>TBV>AK39>BA39>TB3585>BAV (Figure 3.7).

Microorganisms transformed by plasmids were shown to undergo physiological stress and this phenomenon is described as "metabolic burden" (Glick, 1995; Hoffman *et al.*, 2002). The most prominent effect of the "metabolic burden" is the decreasing growth rate of the microorganisms, the level of which varies according to plasmid size (Cheah *et al.*, 1987; Ryan *et al.*, 1989; Khosravi *et al.*, 1990; Kiefer *et al.*, 2009) and copy number (Seo and Bailey, 1985; Birnbaum and Bailey, 1991). Different copy numbers and size of plasmids demand different amount of host energy and resources because the plasmid burdens the cell by its own replication (Seo and Bailey,

1985) and/or the expression of the genes on the plasmid (Hejazi *et al.*, 2009). Furthermore, plasmids carrying antibiotic resistance gene for selection purposes contribute more metabolic load to host organism as a result of constitutive expression of the gene (Panayotatos, 1988; Rozkov *et al.*, 2004, Mairhofer *et al.*, 2010). Hejazi *et al*. (2009) compared the wild type *E. coli* with two *E. coli* strains, one transformed with an empty plasmid and the other with the same plasmid carrying a non-toxic protein gene to observe the effect of foreign DNA and expression of recombinant protein gene in the host cell. They observed that plasmid DNA alone imposed stress on host cell and that the expression of the foreign gene for recombinant protein imposed more stress than the plasmid DNA *per se*. In our study, having no mutations or multicopy plasmid, WT grew better in TSB than other strains till 72h incubation. TB3585 grew less than TBV, indicating the extra metabolic burden imposed by multicopy *ask* plasmid but this was not valid for BA39 which grew better than BAV. Multicopy *ask* might have somehow reduced the negative effect of *hom* mutation on growth of BA39.

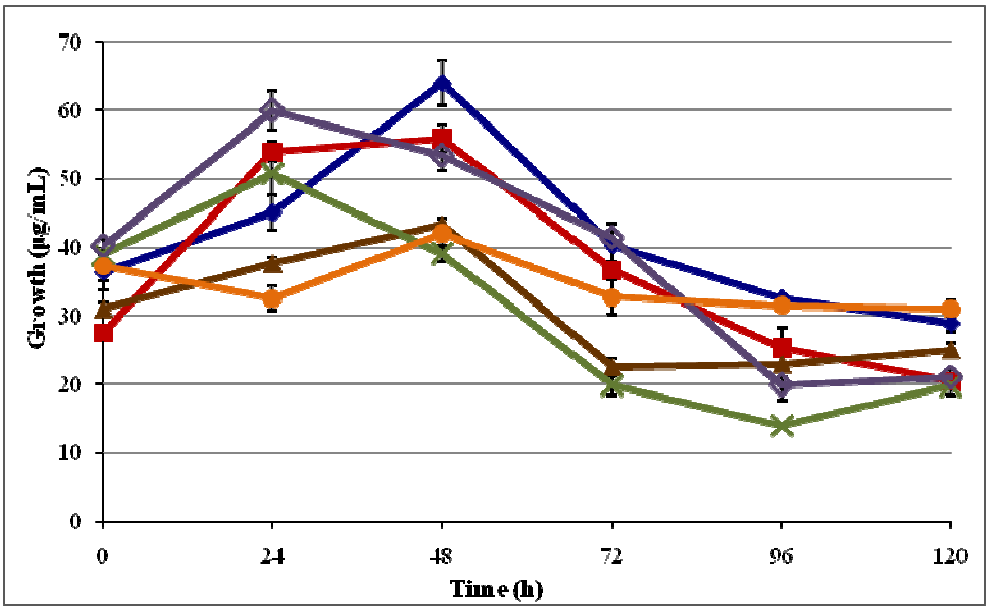

Figure 3. 8. Growth of *S. clavuligerus* strains in mCDM. (◆) WT, (■) AK39, (▲) BAV, (✖) TB3585, (◊) BA39, (●) TBV.

WT, AK39, TBV, and BAV in mCDM showed increased growth till 48h incubation followed by a decrease till 120h (Figure 3.8). On the other hand, the growth of BA39 and TB3585 in mCDM exhibited a fast increase till 24h, and then a gradual decrease. Although the strains displayed maximum growth at different times of cultivation, WT, AK39 and BA39 could grow much better in this medium. mCDM was less favorable for the growth of BAV and TBV strains (Figure 3.8).

When the growth in TSB and mCDM was compared, *S. clavuligerus* strains exhibited quite different patterns. The death phase of the bacteria in TSB occured after 72h to 96 h fermentation, whereas it was observed after 24h to 48h incubation in mCDM. The amount of bacteria after 120h fermentation was not less than the inoculum in TSB; however, in mCDM, it was less, even half for BA39 and TB3585, than the inoculum. Furthermore, WT strain grew better than the others, while BAV carrying *hom* mutation and multicopy plasmid without insert had the poorest growth in both TSB and mCDM. AK39 showed a better growth profile than BA39 and TB3585. However, an exceptional case was observed for TBV as its growth was the highest among the recombinants in TSB, but the poorest in mCDM till 48h fermentation.

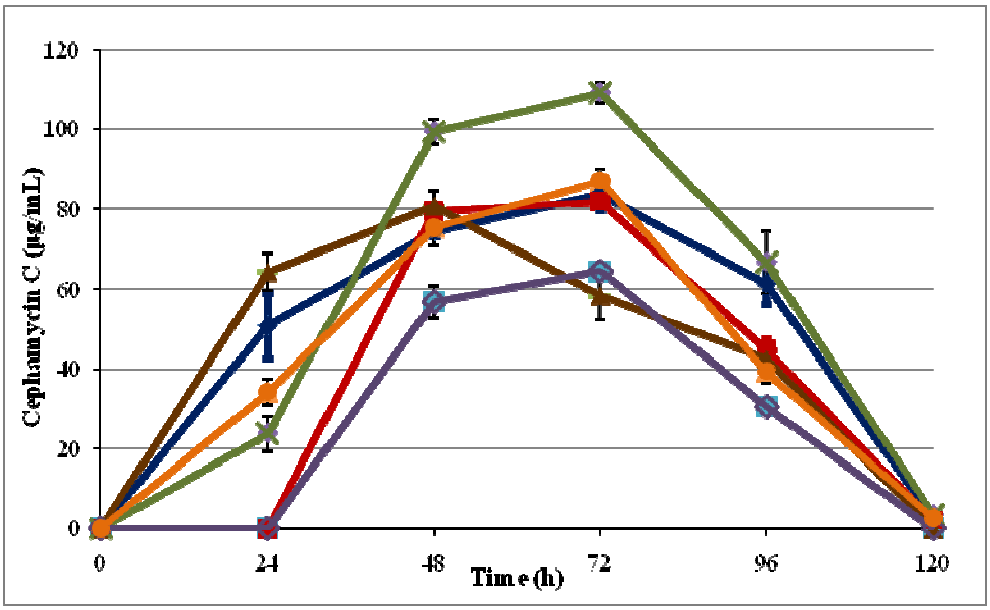

Figure 3. 9. Volumetric cephamycin C production profiles of *S. clavuligerus* strains in TSB. (◆) WT, (■) AK39, (▲) BAV, (✕) TB3585, (◊) BA39, (●) TBV.

Volumetric antibiotic production by the strains was next compared in TSB medium and mCDM. The highest volumetric cephamycin C production in TSB was observed in TB3585 cultures, being 1.3-, 1.3- and 1.7-fold higher than that of TBV at 48h, 72h and 96h incubation, respectively. The antibiotic production by BA39 was the lowest and had a 24h delay as compared to BAV (Figure 3.9).

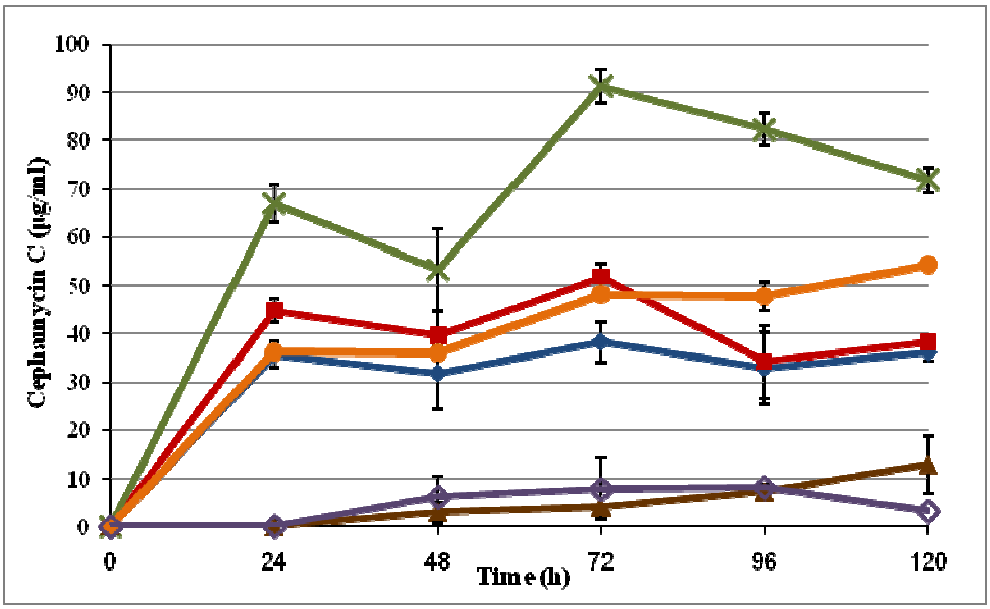

Figure 3. 10. Volumetric cephamycin C production profiles of *S. clavuligerus* strains in mCDM. (◆) WT, (■) AK39, (▲) BAV, (✕) TB3585, (◊) BA39, (●) TBV.

In mCDM, the highest volumetric cephamycin C production was obtained by using TB3585. When compared to its control, TBV, the production was 1.9- and 1.7-fold higher at 72h and 96h fermentation, respectively. Both BAV and BA39 showed the poorest antibiotic production in this medium. The antibiotic production by AK39 was higher than that of WT with 1.2- and 1.4-fold increase observed at 48h and 72h fermentation, respectively (Figure 3.10). Multicopy *ask* expressed in a WT background exerted the most beneficial effect on volumetric production. *hom* deletion itself did also have a positive effect if not as beneficial as multicopy ask expression. However, it was apparent that *hom*-disrupted mutant was not a suitable host for either multicopy vector only or recombinant multicopy vector expressing *ask.*

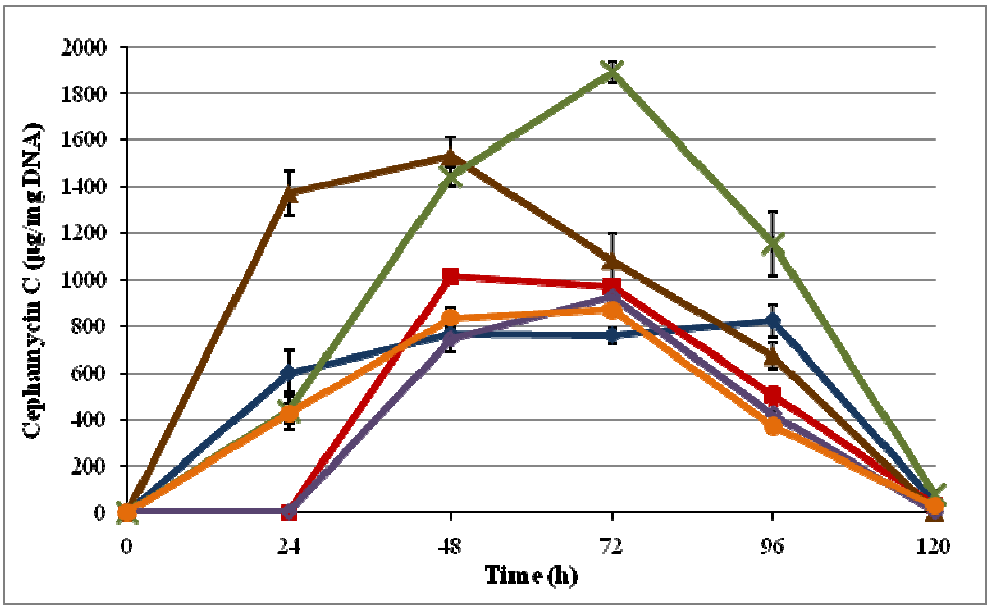

Figure 3. 11. Specific cephamycin C production profiles of *S. clavuligerus* strains in TSB. (◆) WT, (■) AK39, (▲) BAV, (✕) TB3585, (◊) BA39, (●) TBV.

Specific antibiotic productions by each strain was also determined in both TSB and mCDM (Figure 3.11, 3.12.). Specific antibiotic production profiles were quite similar to those of volumetric production.

In TSB medium, the highest specific production was made by TB3585 which was 1.7-, 2.2- and 3.1-fold higher as compared to its TBV control at 48h, 72h and 96h cultivation, respectively. TBV and WT showed approximately similar production profiles over time. AK39 showed 1.3-fold higher production than WT at 48h and 72h fermentation. Interestingly, second highest specific antibiotic production was attained by BAV which showed 2-, 1.2-, and 1.6-fold higher production than that of BA39 at 48h, 72h, and 96h fermentation, respectively (Figure 3.11).

Since *ask* gene codes for aspartokinase enzyme which initiates aspartic acid pathway, the finding that TB3585, which contained multiple copies of *ask,* produced significantly more antibiotic than both WT and TBV was as expected. Furthermore, although AK39 was expected to produce an increased

amount of cephamycin C as compared to WT, there was only a slight increment. *hom*-disruption in AK39 eliminates the pathway from the second branch point and channels all intermediates towards cephamycin C production (Çaydaşı, 2006). Considering that the TSB medium is rich in carbon and nitrogen sources, and thus there was not a strict nutrient limitation in the environment, elimination of second branch point might not have greatly affected cephamycin C production. Surprisingly, BA39 produced the lowest amount of antibiotic production in TSB, in contrast to our expectation that a combination of the multicopy *ask* gene and *hom*-disruption in the same strain would boost the antibiotic production. Furthermore, BAV, the control strain of BA39, yielded higher amounts of cephamycin C than WT, AK39 and BA39. As discussed earlier, the expression of *ask* gene in BA39 should have favored its growth in rich medium, thus alleviating the metabolic burden on this strain while the growth of BAV was not supported in this way. The more metabolic burden on BAV might have resulted in a higher volumetric antibiotic production as compared to BA39. The lower growth it displayed, on the other hand, must have increased specific production values.

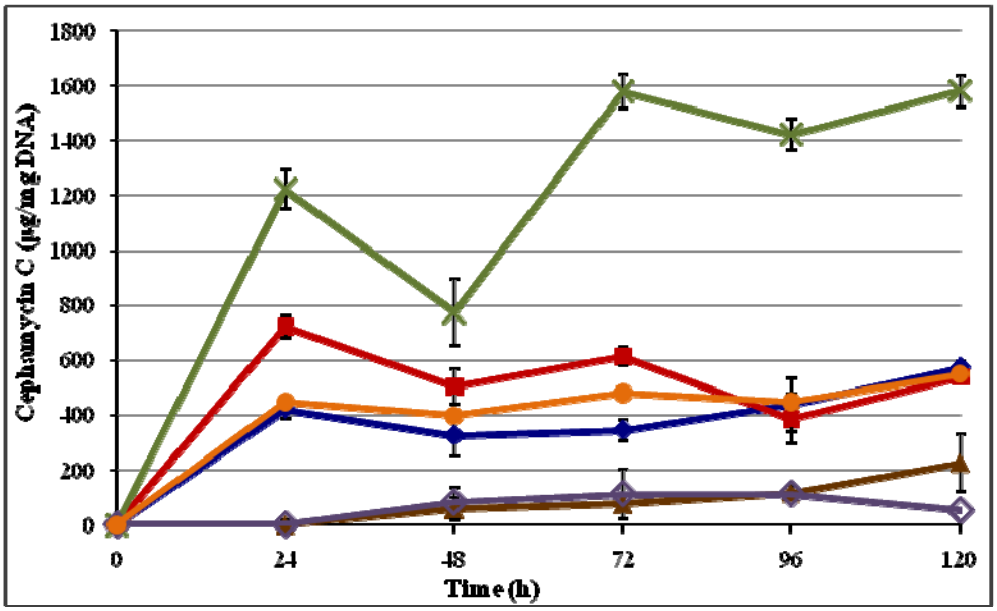

Figure 3. 12. Specific cephamycin C production profiles of *S. clavuligerus* strains in mCDM. (◆) WT, (■) AK39, (▲) BAV, (✕) TB3585, (◊) BA39, (●) TBV.

Specific and volumetric antibiotic production by the strains in mCDM also showed very similar patterns. Specific cephamycin C production by TB3585 was 3.3-, and 3.2-fold higher than that of TBV at 72h and 96h incubation, respectively. AK39 displayed 1.6- and 1.8-fold higher production than that of WT at 48h and 72h fermentation, respectively. BAV and BA39 showed the poorest production among all (Figure 3.12). Furthermore, TB3585 produced more cephamycin C than AK39, which designates that multicopy *ask* expression has more favorable effect than *hom*-deletion on cephamycin C production; however, BA39 did not give rise to a super-producer despite the combination of *hom*-disruption and *ask* overexpression, yet significantly decreased antibiotic yield.

In our study, TBV carrying expression vector without any gene insert produced higher amounts of antibiotic production than WT resulting from multicopy plasmid as a stress factor on the host. Additionally, higher cephamycin production by TB3585 than that of AK39 was a result of its having pTB486 in multiple copies hence expressing *ask* gene and thiostrepton resistance gene which added more burden to the host organism. It was shown that AK39 could not produce spores on minimal media (MM) supplemented with L-threonine and L-methionine but could produce on sporulation medium (Çaydaşı, 2006), indicating that *hom*-disruption might have resulted in some pleiotropic effects responsible for morphological differentiation. This might also explain the decreased growth of AK39 as compared to its parental strain, WT. Hence, probably not only the elimination of *hom* gene *per se* but also other kinds of stress imposed on the strain by the gene disruption contributed to the reduced growth and thus to the increased antibiotic production when compared to WT. In contrast to our expectation, antibiotic production by BA39 was one of the lowest in mCDM, probably due to physiological disturbance resulting from high copy number plasmid burden in an auxotrophic host (Özcengiz *et al.,* 2010).

3.3. Aspartokinase specific activity in *S. clavuligerus* strains

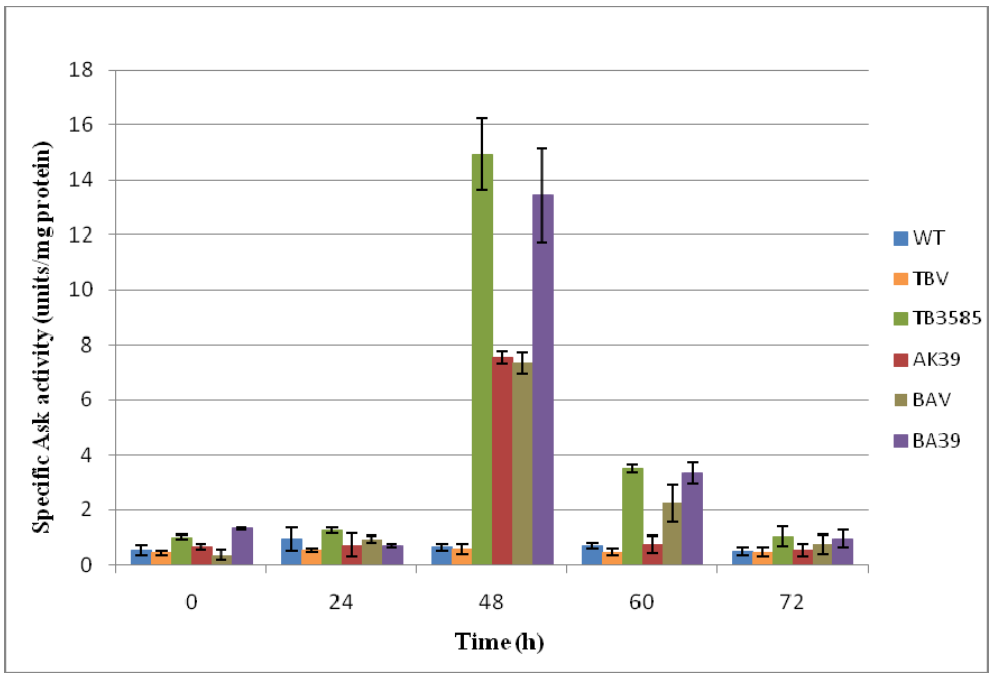

Figure 3. 13. Ask specific activities of *S. clavuligerus* strains grown in mCDM. Blue bars represent WT, orange bars TBV, green bars TB3585, red bars AK39, brown bars BAV, purple bars BA39.

The highest specific aspartokinase (Ask) activities for all strains in mCDM were observed at 48h fermentation (Figure 3.13). There was 12-fold increase in Ask specific activity in TB3585 at 48h as compared to the activity at 24h incubation. Furthermore, it showed 25-fold higher activity than that of TBV at 48h. Ask specific activity in BA39 was 19-fold and 1.8-fold higher than that of BAV at 24h and 48h fermentation, respectively. AK39, on the other had, was found to have 11-fold more activity than that of WT at 48h fermentation.

Increased Ask activities of TB3585 and BA39 as compared to TBV and BAV, respectively confirmed the expression of multicopy *ask* gene in both strains. Additionally, by *hom* disruption, the concerted feedback inhibition of

aspartokinase by lysine and threonine was eliminated in both BAV and BA39 (Mendelovitz and Aharonowitz, 1982).

Higher growth of BA39 as compared to BAV in both TSB and mCDM might be due to increased Ask activity in combination with *hom*-disruption by means of which intermediates will be channeled towards cephamycin C and through this flux, some important intermediates like diaminopimelic acid for bacterial cell wall synthesis (Viola, 2001) and L-lysine for protein biosynthesis, hence supporting the growth (Khetan *et al.*, 1999). As to AK39, Ask activity was quite high when compared to that of WT but not as much as that of TB3585. This should be the result of *hom* disruption that prevents lysine plus threonine concerted feedback inhibition over Ask.

3.4. Intracellular free amino acid concentrations in the wild type and the recombinants when grown in mCDM

In order to find out the relation between aspartokinase activity and amino acid biosynthesis in WT and the recombinant strains, intracellular free amino acid content of WT, TB3585, AK39 and BA39 was identified at intervals when grown in mCDM.

Total amino acid pool (Figure 3.14) as well as the concentration of individual amino acids (Figure 3.15A, B, C, D) varied considerably among the four strains.

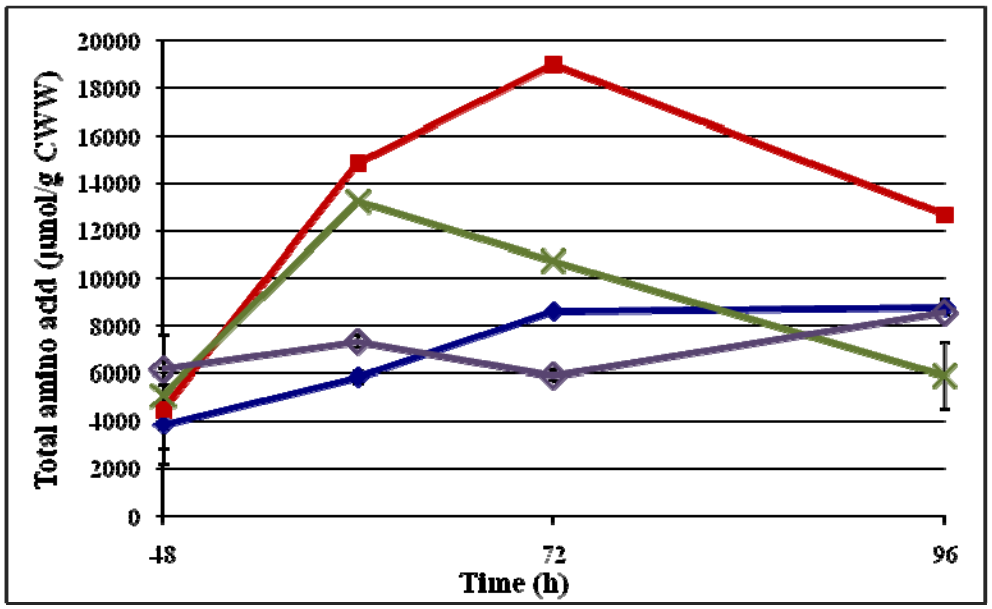

Figure 3. 14. Intracellular total free amino acid pool in (◆) WT, (■) AK39, (×) TB3585 and (◊) BA39 grown in mCDM.

The highest amino acid pool was observed in AK39 throughout the incubation. It had 2.5-, 2.2-, 1.4-fold higher total amino acid content as compared to WT, at 60h, 72h and 96h incubation, respectively. The second highest amino acid pool was observed in TB3585, having 1.6- and 1.2-fold higher total amino acid pool than that of WT at 60h and 72h incubation, respectively. BA39 had relatively lower amounts of amino acids compared to WT.

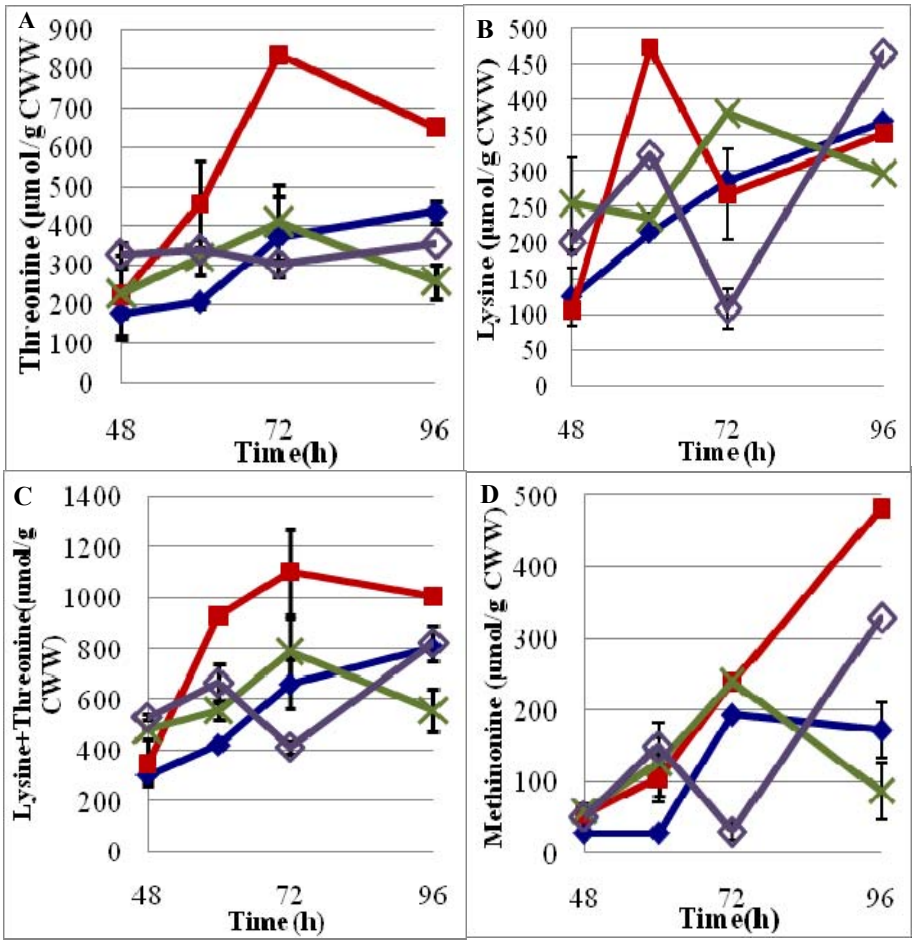

Figure 3. 15. Intracellular pool of (A) threonine, (B) lysine, (C) lysine plus threonine (D) methionine in (◆) WT, (■) AK39, (×) TB3585 and (◊) BA39 grown in mCDM.

Intracellular threonine, methionine and lysine levels are important parameters for the evaluation of metabolic flux through aspartic acid pathway in bacteria (Zhang *et al.,* 1999). In the present study, the highest intracellular threonine level was observed in AK39 which was 2.2-fold higher than that of WT at 72h incubation (Figure 3.15A). Threonine levels in the other strains did not differ significantly; it showed an increase after 60h in WT, an increase till 72h and then a decrease in TB3585. As to the intracellular lysine levels, again AK39 showed 2.2-fold higher levels than WT at 60h incubation (Figure 3.15B). Except for a fluctuation till 72h, BA39 showed an increase in lysine levels till 96h incubation. In WT, intracellular lysine levels stably increased by time,

whereas TB3585 showed an increase till 72h and a decrease thereafter. The intracellular methionine levels exhibited very similar patterns to those of lysine for each strain except that there was a constant increase of this amino acid in AK39 till the end of fermentation (Figure 3.15D).

Given that AK39 and BA39 were *hom* mutant strains, their threonine and methionine levels were expected to be very low. High levels of these amino acids in *hom* minus strains can be considered as accumulation of threonine and methionine supplemented to the medium into the cells (Özcengiz *et al*. 2010). The decrease in aspartokinase activity (Figure 3.13) and the increase in lysine plus threonine levels (Figure 3.15C) after 48h fermentation might be due to concerted feedback inhibition of Ask by lysine and threonine (Aharonowitz and Demain, 1982).

Intracellular levels of aspartic acid, the precursor for initiation of aspartic acid pathway that leads to the production of L-lysine, L-methionie, L-threonine, L-isoleucine and cephamycin C (Mendelovitz and Aharonowitz, 1982), were also identified. AK39 was again the one with the highest level of this amino acid within the cell (Figure 3.16). Aspartic acid concentration increased till 72h and then decreased till the end of incubation. The pattern was nearly the same for WT and BA39 except that relatively lower levels of aspartic acid were observed within the cells. As to TB3585, the increase in this amino acid level till 60h was followed by a stable but mild decrease. Lower aspartic acid levels in BA39 and TB3585 might be due to high levels of Ask activity in these strains increasing the consumption of intracellular aspartate.

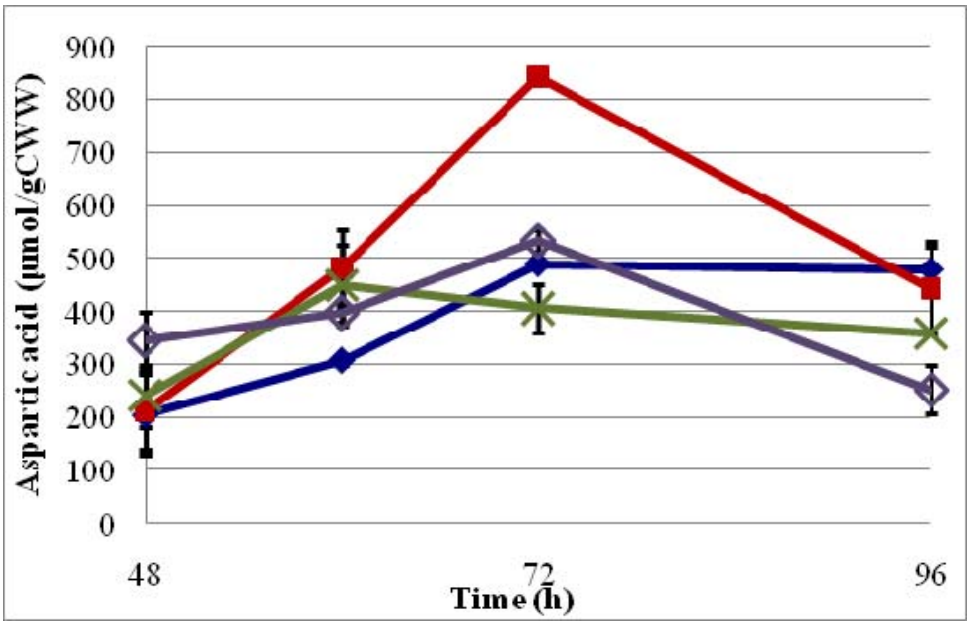

Figure 3. 16. Intracellular aspartic acid pool in (◆) WT, (■) AK39, (×) TB3585 and (◊) BA39 grown in mCDM.

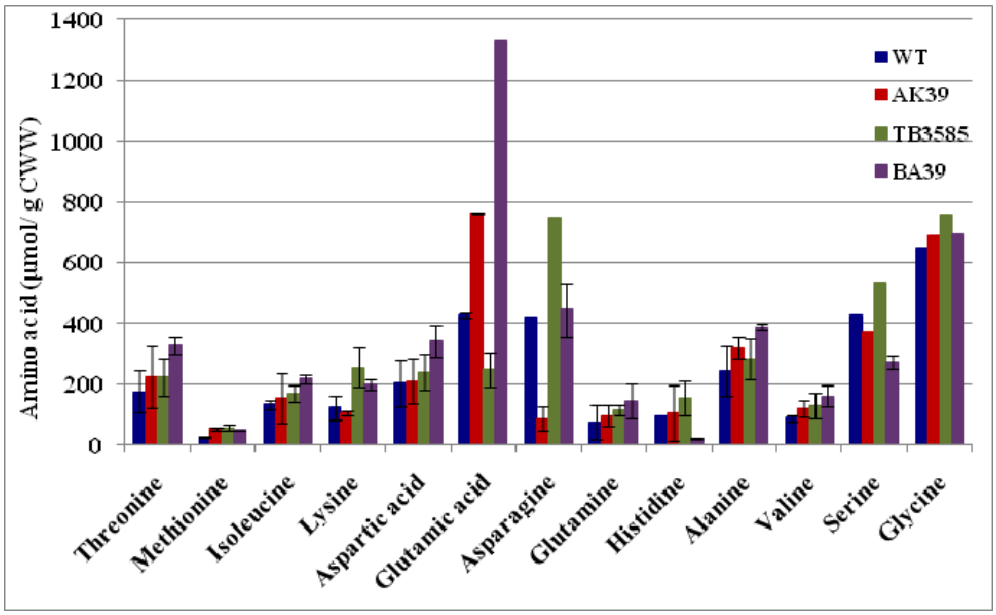

Figure 3. 17. Intracellular free amino acid pools of WT, TB3585, AK39, and BAV after 48h fermentation in mCDM. Blue bars represent WT, red bars AK39, green bars TB3585, purple bars BA39.

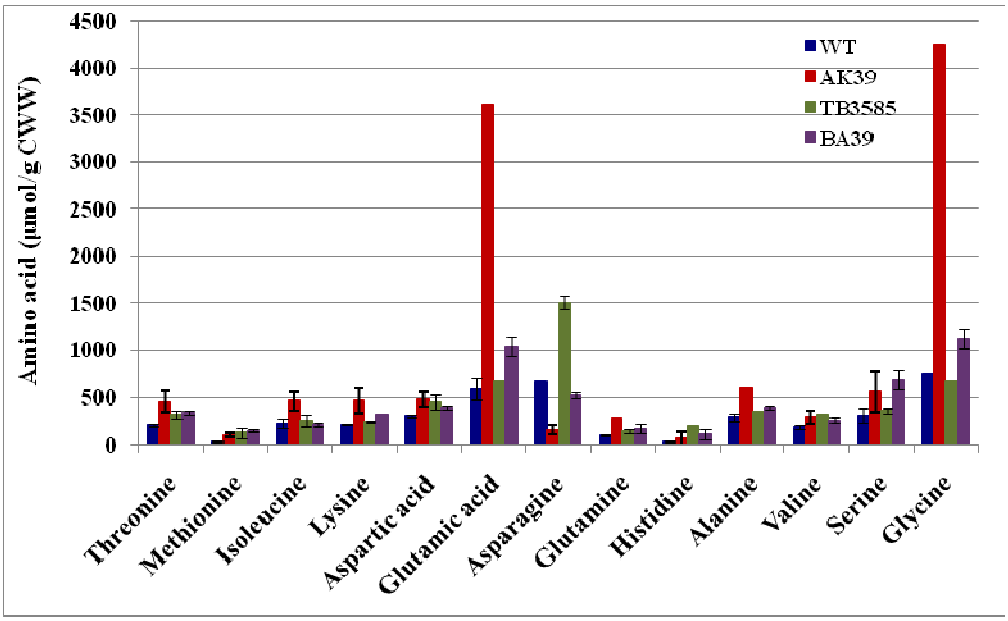

Figure 3. 18. Intracellular free amino acid pools of WT, TB3585, AK39, and BAV after 60h fermentation in mCDM. Blue bars represent WT, red bars AK39, green bars TB3585, purple bars BA39.

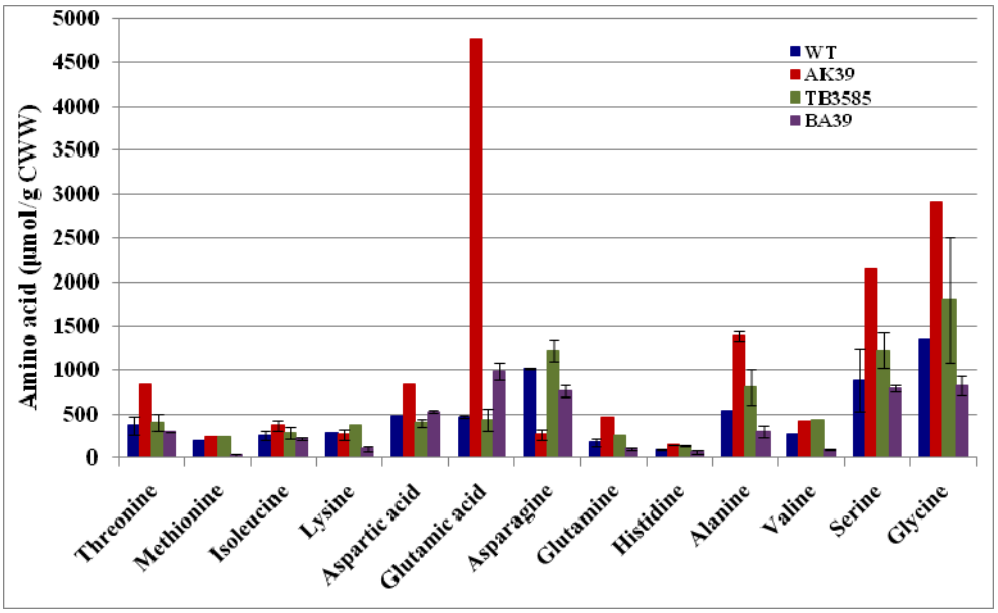

Figure 3. 19. Intracellular free amino acid pools of WT, TB3585, AK39, and BAV after 72h fermentation in mCDM. Blue bars represent WT, red bars AK39, green bars TB3585, purple bars BA39.

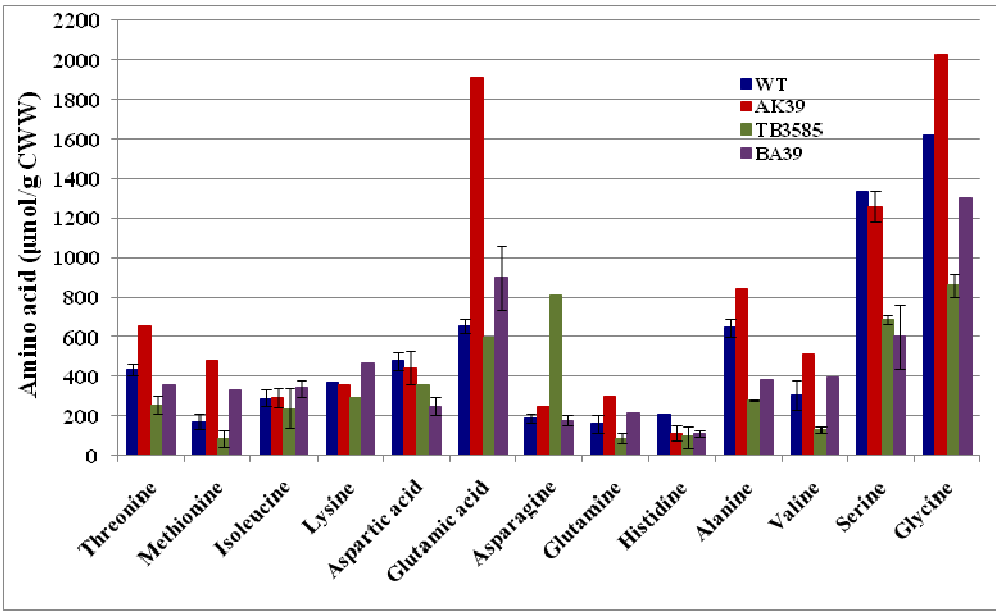

Figure 3. 20. Intracellular free amino acid pools of WT, TB3585, AK39, and BAV after 96h fermentation in mCDM. Blue bars represent WT, red bars AK39, green bars TB3585, purple bars BA39.

Using asparagine as the sole carbon and nitrogen source, the predominating amino acid was found to be glycine followed by asparagine, glutamic acid and serine in WT (Figure 3.17-3.20). Furthermore, there were strong differences in the total pool content as well as in individual amino acids between strains. The glutamic acid and glycine contents of AK39 were 6 to 10 times higher compared to those of the wild type and TB3585 strains, and it always contained much less asparagine than the other strains. The TB3585 strain contained 2- to 4-fold more asparagine than the other strains. The highest intracellular amino acid content (2.2-fold) was found in AK39 (Figure3.14). This strain synthesized appreciable amounts of lysine till 60h and extensively accumulated externally added threonine. Thus, its intracellular lysine plus threonine contents were almost always much higher than that of the other strains. The intracellular pool levels of aspartate pathway amino acids accorded well with the Ask activity levels in TB3585 and AK39 in that the

drastic increment of Ask specific activity at 48h was followed by a sharp increase in the intracellular pool levels of lysine and threonine till 72h during which Ask activity gradually decreased. Cephamycin C levels also increased in this interval, particularly in TB3585. Thus, the initial production of antibiotic within first 24h cultivation in both wild-type and recombinant strains seemed to be independent of Ask expression level and intracellular pools of amino acids

CHAPTER 4

CONCLUSIONS

- When the growth of AK39 and WT strains were compared in CDM, CDM+asn, CDM+suc, CDM+asn+suc, CDM+asn-glyc, CDM+suc-glyc and CDM+asn+suc-glyc, the highest growth was observed in CDM+asn+suc for both strains. A 2-fold increase in asparagine concentration with 25 g/L sucrose addition with or without glycerol resulted in an improved growth.

- On the other hand, the highest volumetric cephamycin C production by WT and AK39 strain was observed in CDM+asn+suc-glyc at 72h incubation which was 1.7- and 50-fold higher than that in CDM, respectively.

- The highest specific antibiotic production by WT strain was found to occur in CDM+asn+suc-glyc and CDM+asn-glyc and the increment was 1.4- and 1.6-fold, respectively, at 96h fermentation. The highest production by AK39 strain was in CDM+asn+suc-glyc at 120h fermentation. Production was 13-, 20-, 5- fold higher in this CDM variant than that in CDM at 72h, 96h and 120h, respectively.

- Among the CDM variants, antibiotic production was higher in the media lacking glycerol than those having glycerol providing further evidence for previously reported negative effect of glycerol on cephamycin C production.

- After selecting CDM+asn+suc-glyc (mCDM) as the optimal CDM variant supporting cephamycin C biosynthesis and fairly good levels of

growth, the growth of WT, TB3585, AK39, BA39, TBV and BAV were compared in TSB and mCDM. In TSB, the best growth was performed by WT strain having no mutation and/or multicopy plasmids. In mCDM, AK39 and BA39 could grow much faster than WT.

- The highest volumetric cephamycin C production was displayed by TB3585 as compared to TBV with 1.3- and 1.9-fold increment in TSB and mCDM, respectively at 72h incubation.

- When compared to TBV, TB3585 gave the highest specific antibiotic production with 2.2- and 3.2-fold increased levels in both TSB and mCDM, respectively, at 72h fermentation. The positive effect of multicopy *ask* expression rather than *hom*-disruption on cephamycin C production was the major finding of this study since *ask* overexpression coupled to *hom*-disruption did not give rise to a super producer, but significantly decreased cephamycin C production in BA39.

- TB3585 exhibited the maximum specific aspartokinase activity among all strains. The increments of Ask activity at 48h fermentation in TB3585 and BA39 were 25- and 1.8-fold as compared to those of TBV and BAV, respectively. Increased Ask activities of TB3585 and BA39 as compared to TBV and BAV, respectively. All these findings confirmed the expression of multicopy *ask* gene in both strains, and provided further proof for the beneficial effect of homologous *ask* overexpression in industrial cephamycin C producers.

- The total as well as individual amino acid pools were found to be contained in AK39. High levels of threonine plus lysine pool in *hom* minus strain can be considered as accumulation of these amino acids supplemented

to the medium into the cells. The decrease in aspartokinase activity and the increase in lysine plus threonine levels after 48h fermentation might be due to concerted feedback inhibition of Ask by lysine and threonine.

- Industrial and renewable raw materials as well as wastes can be investigated for cephamycin C production by TB3585 to decrease overall cost.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Media

1.1. Liquid Media

Luria Broth (LB)

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

Sterilized at 121 °C for 15 minutes

Nutrient Broth (NB)

	$g.L^{-1}$
Nutrient Broth	8

Sterilized at 121 °C for 15 minutes

Tryptone Soy Broth (TSB)

	$g.L^{-1}$
Tryptic Soy Broth	30

Sterilized at 121 °C for 15 minutes

Chemically Defined Medium (CDM) variants(Malmberg et al., 1993; modified):

CDM

	$g.L^{-1}$
K_2HPO_4	4.49
MOPS	26.8

pH is adjusted to 6.9 and volume is completed to 780 mL.

Sterilized at 121°C for 15 min

After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (10 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
Glycerol (50 %)	20
*L-threonine (50 mg/mL)	1
*L- methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50mg/mL)	1

CDM +asparagine (CDM+asn)

	$g.L^{-1}$
K_2HPO_4	4.49
MOPS	26.8

pH is adjusted to 6.9 and volume is completed to 780 mL.

Sterilized at 121°C for 15 min

After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (20 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
Glycerol (50 %)	20
*L-threonine (50 mg/mL)	1
*L-methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50 mg/mL)	1

CDM +sucrose (CDM+suc)

	$g.L^{-1}$
K_2HPO_4	4.79
MOPS	28.6

pH is adjusted to 6.9 and volume is completed to 780 mL.
Sterilized at 121^{o}C for 15 min
After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (10 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
Glycerol (50 %)	20
*L-threonine (50 mg/mL)	1
*L-methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50 mg/mL)	1
Sucrose (0.5 g/mL)	50

CDM+ asparagine+sucrose (CDM+asn+suc)

	$g.L^{-1}$
K_2HPO_4	4.79
MOPS	28.6

pH is adjusted to 6.9 and volume is completed to 780 mL.

Sterilized at 121°C for 15 min

After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (20 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
Glycerol (50 %)	20
*L-threonine (50 mg/mL)	1
*L-methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50 mg/mL)	1
Sucrose (0.5 g/mL)	50

CDM+sucrose-glycerol (CDM+suc-glyc)

	$g.L^{-1}$
K_2HPO_4	4.67
MOPS	27.87

pH is adjusted to 6.9 and volume is completed to 780 mL.

Sterilized at 121°C for 15 min

After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (10 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
*L-threonine (50 mg/mL)	1
*L-methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50 mg/mL)	1
Sucrose (0.5 g/mL)	50

CDM+asparagine+sucrose-glycerol (CDM+asn+suc-glyc)

	$g.L^{-1}$
K_2HPO_4	4.67
MOPS	27.87

pH is adjusted to 6.9 and volume is completed to 780 mL.
Sterilized at 121 °C for 15 min
After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (20 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
*L-threonine (50 mg/mL)	1
*L-methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50 mg/mL)	1
Sucrose (0.5 g/mL)	50

CDM+asparagine-glycerol (CDM+asn-glyc)

	$g.L^{-1}$
K_2HPO_4	4.38
MOPS	26.13

pH is adjusted to 6.9 and volume is completed to 780 mL.

Sterilized at 121^{o}C for 15 min

After sterilization following sterile components were added to the medium:

	$mL.L^{-1}$
L-Asparagine (20 g/L)	200
$MgSO_4 \cdot 7H_2O$ (0.615 g/mL)	2
*L-threonine (50 mg/mL)	1
*L-methionine (50 mg/mL)	3
*trace salt solution	1
*thiostrepton (50 mg/mL)	1

* L-threonine (50 mg/mL) and L-methionine (50 mg/mL) was only added when BA39, BAV and AK39 strains were grown.

* Thiostrepton was added into BAV, TB3585, BA39, TBV cultures that contain pIJ486 plasmid with thiostrepton gene marker.

*trace salt solution

	$g.100\ mL^{-1}$
$MnCl_2 \cdot 4H_2O$	0.1
$FeSO_4 \cdot 7H_2O$	0.1
$ZnSO_4 \cdot 7H_2O$	0.1
$CaCl_2.2H_2O$	0.133

1.2. Solid Media

LB Agar

	$g.L^{-1}$
Luria Broth	25
Agar	15

Sterilized at 121 °C for 15 minutes

NB Agar

	$g.L^{-1}$
Nutrient Broth	8
Agar	15

Sterilized at 121 °C for 15 minutes

APPENDIX B

BUFFERS AND SOLUTIONS

Diphenylamine Reagent

Diphenylamine (15 g/L)

H_2SO_4 (15 mL/L)

1.6 % acetaldehyde (5 mL/L)

0.01 M Phosphate Buffer

	$g.L^{-1}$
KH_2PO_4	1.36

pH was adjusted to 3.5 by dropwise addition of acetic acid

50 mM cold Phosphate Buffer

	$g.L^{-1}$
KH_2PO_4	6.8
KOH	1.9

pH adjustment was done to with 50 mM KH_2PO_4 solution (without added KOH) to lower the pH 0.1 M KOH, added dropwise, to raise the pH.

0.02 M Sodium Citrate Buffer

	$g.L^{-1}$
Trisodium citrate dihydrate	5.8

pH was adjusted to 2.2 by dropwise addition of HCl

10 mM Sonication Buffer

150 mM KCl
5 % Glycerol (v/v)
1 mM EDTA
1 mM PMSF in isopropanol
Completed to 100 mL with 50 mM phosphate buffer (pH 7.5)

Ferric Chloride Solution

10 % (w/v) $FeCl_3.6H_2O$
3.3 % (w/v) TCA
0.7 N HCl
Completed to 100 mL with dH_2O and protected from light

Assay Mixture (pH 7.6)

100 mM HEPES (pH 7.8)
400 mM KCl
12 mM $MgCl_2$
500 mM hydroxylamine
10 mM ATP
15 mM L-aspartate

APPENDIX C

CHEMICALS AND SUPPLIERS

Chemicals

Acetaldehyde	Sigma
Agar	Merck
L-Asparagine	Sigma
L-Aspartate	Sigma
L-Aspartate-β-Hydroxymate	Sigma
ATP	Sigma
BSA (Bovine Serum Albumin)	Sigma
$CaCl_2.2H_2O$	Merck
Cephalosporin C	Sigma
Coomassie Brilliant Blue G-250	Merck
Diphenylamine	Sigma
EDTA	Sigma
Ethanol	Botafarma
$FeCl_3.\ 6H_2O$	Merck
$FeSO_4.7H_2O$	Merck

Glacial Acetic Acid	Merck
Glycerol	Amresco
Herring Sperm	Sigma
HCl	Fluka
HEPES	Sigma
Hydroxylamine	Fisher
Isopropanol	Merck
KCl	Merck
K_2HPO_4	Meck
KH_2PO_4	Merck
KOH	Merck
Luria Broth	Q-Biogene
L- Methionine	Sigma
MOPS	AppliChem
$MgCl_2.6H_2O$	Merck
$MnCl_2.4H_2O$	Merck
$MgSO_4.7H_2O$	Merck
NaCl	Merck
Nutrient Broth	Merck
PMSF (Phenylmethylsulfonylfluoride)	Sigma
Tri-Sodium Citrate Dihydrate	Merck

Sucrose	Merck
TCA (Trichloroacetic acid)	Merck
Thiostrepton	Sigma
L-Threonine	Sigma
Tris-HCl	Merck
Tryptic Soy Broth	Oxoid
$ZnSO_4.7H_2O$	Merck