Metabolomic profiling of *Streptomyces coelicolor* wild type and mutant strains lacking glutamine synthetase genes using Orbitrap mass spectrometry

By

Ioannis Daravigkas

Acknowledgments

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Abbreviations

ACP	acyl carrier protein
A-factor	2-isocapryloyl-3Rhydroxymethyl-γ-butyrolactone
a.m.u.	Atomic mass unit
CDA	Calcium-Dependent Antibiotic
c.f.u.	Colony forming units
ESI	Electro Spray Ionisation
FT-ICR	Fourier Transform Ion Cyclotron Resonance
FTMS	Fourier Transform Mass Spectrometer
GS	Glutamine synthetase
HILIC	Hydrophilic interaction chromatography
NIESI	Negative Ion Electrospray Ionisation
PKS	Polyketide syntheses
PIESI	Positive Ion Electrospray ionization
(p)ppGpp	guanosine pentaphosphate
RPC	Reversed -Phase Chromatography
TMS	Trimethylsilyl
WT	Wild type
YEME	yeast/extract-malt/extract

Abstract

A better understanding of the regulatory mechanisms that control the biosynthesis of bioactive secondary metabolites in *Streptomyces spp* is essential in order to increase the production of valuable fermentation products. Nitrogen limitation has a role in promoting antibiotic production. In current study the metabolic effects of limiting nitrogen assimilation in *Streptomyces* mutants were studied.

Streptomyces coelicolor strain M145, and prototrophic derivatives of the A3(2) strain lacking plasmids SCP1 and SCP2 , were used in this study. The bacteria were routinely cultured and maintained following standard procedures. Samples were collected at different time points starting from the 0 hour time point and until the 50-52 hours time point. Twelve batches of samples were analysed in total, four of which were mutants strains since they lacked the glnA and glnII genes which regulate the glutamine synthesis. Sample analysis was carried out on a Thermo Exactive Orbitrap in positive and negative ESI mode with a needle voltage of 4.5kV. The instrument was coupled to a Dionex HPLC. The purpose of this study was to determine whether or not the mutants strains compared to the wild type(WT) would exhibit the same behavior in nitrogen assimilation and how any differences would influence the biosynthesis of nitrogen controlled secondary metabolites. Amino acid behavior was monitored in all samples with a focus on the glutamate/glutamine pair since it is the main nitrogen pool for the *S.coelicolor* and besides they have a direct relationship through the biosynthetic pathway where glutamine is synthesized from glutamate. Purine and pyrimidine biosynthesis was also monitored as these

compounds are rich in nitrogen. Unknown metabolites were also monitored with a molecular formula proposed by the software used . One of them appeared to be an immediate precursor of ergothionine which is a thiourea derivative, whereas another one is appeared to have a similarity to molecular formula of glutathione molecule but fragmentation experiments would need to be done in order to elucidate its structure further.

Over the results obtained from the analyses, gave a good picture of the complexity of the *S.coelicolor* culture. It was obvious that the mutant strains had a serious difficulty in nitrogen assimilation compared to the WT and this might potentially impact on the initiation of antibiotic production.

1. Introduction

1.1 Streptomycetes

The Streptomycetes are members of the bacterial order *Actinomycetales*, bacteria which resemble fungi in their branching filamentous structure. However, they are true bacteria -prokaryotic cells -unlike eukaryotic fungal cells. As Actinomycetes grow (figure1), many of them form branching filaments of cells which become a network of strands called a mycelium, similar in appearance to the mycelium of some fungi.

Actinomycetes are also unique in the way they form spores and in the production of numerous antibiotics. By far the most well studied genus in this group is *Streptomyces* with over 500 species. Few species of *Streptomyces* are pathogenic for animals, although a few species cause plant diseases and although most Streptomycetes are saprophytic, a few species provoke visible lesions on the surface of various root and tuber vegetables. The fact that the genes responsible for pathogenicity can disperse by lateral transfer in different combinations implies that non-pathogenic strains can transform to pathogenic ones (Chater et al 2010).

Streptomyces species are found worldwide in soil and are important in soil ecology. Much of the characteristic earthy smell of soils arises from chemicals called geosmens given off by *Streptomyces* species. In terrestrial habitats, Streptomycetes are important for the initial decomposition of organic material by their large variety of enzymes (Schrempf 2007).

Streptomycetes are metabolically diverse and by producing extracellular hydrolytic enzymes can practically metabolise almost anything, including sugars, alcohols, amino acids, organic acids, and aromatic compounds.

They are also of medical and industrial importance because they synthesize antibiotics. This useful property is due to their ability in producing a sizeable number of diverse natural secondary metabolites of which the best known are the antibiotics currently used worldwide as pharmaceutical and agricultural products (El-Naggar *et al* 2003; Ben-Fguria *et al* 2005). Furthermore they exhibit a broad range of metabolic processes and biotransformations some of which include degradation of the insoluble remains of other organisms, such as lignocellulose and chitin (which is among the world's most abundant biopolymers), making Streptomycetes central organisms in carbon recycling (Bentley *et al* 2002).There are several theories to explain antibiotic production, the most widely accepted one being that antibiotics help the organism compete with other organisms-even with closely related Streptomycetes- in the relatively nutrient-depleted environment of the soil by reducing competition and this is amplified by the fact that production of most antibiotics is more or less species specific.

However, because antibiotic production is usually delayed until most of the growth has been completed, some antibiotics may serve to defend colony biomass against overgrowth by other organisms during the autolysis that accompanies development, rather than to help in competition for the primary biomass accumulation (Chater & Merrick, 1979).

Over 50 different antibiotics have been isolated from Streptomycetes species, including streptomycin, chloramphenicol , tetracyclines and neomycin.(Keiser *et al* 2000).

Fig.1. Actinomycetes isolated from a soil sample growing on agar plates.Linda Cavaletti, Instituto Insubrico di Ricerca per la Vita, Gerenzano, Italy

It already has been previously indicated that the antibiotics produced by Streptomycetes are species specific.

Table 1 shows the therapeutic effects that these compounds have on some diseases, and also implies that different Streptomycete*s* make different antibiotics.

Target disease or organism	**antibiotic**	**Producing organism**
typhoid	chloramphenicol	*Streptomyces venezulae*
TB and leprocy	rifampicin	*Amycolatopsis(formerly Streptomyces) mediterranei*
Methicllin-resistant Staphylococcus aureus(MRSA)	vancomycin	*Amycolatopsis(formerly Streptomyces) orientalis*
river blindness	avermectin	*Streptomyces avermitilis*
cancer	daunomycin	*Streptomyces coeruleorubidus*
pathogens with transmissible penicillin resistance	clavulanic acid	*Streptomyces clavuligerus*

Table 1. Medical importance of some antibiotics from Streptomycetes and their close relatives.(Chater 2006)

The Streptomycetes that have been particularly well studied are the *Streptomyces griseus* which actually was the first to be used for the commercial production of an antibiotic and *S. coelicolor* A3(2) shown in figure 2 the most widely used laboratory strain (Hopwood 2007).

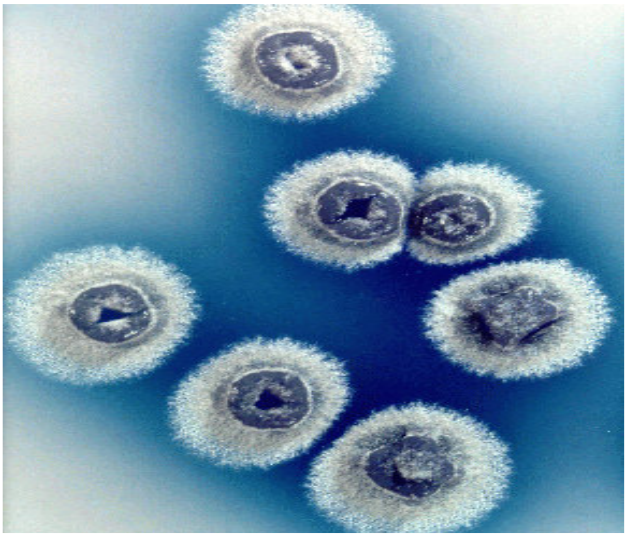


Fig.2. *S. coelicolor* A3(2) (John Innes Molecular Microbiology Center)

1.2 Biosynthesis of antibiotics

An important signal for triggering secondary metabolism which leads to antibiotic biosynthesis is the reduction in growth rate which derives from culture starvation due to the consumption of the available nutrients. It is widely accepted in the that the highly phosphorylated guanosine nucleotide (p)ppGpp has a primary role in triggering antibiotic production in Streptomycetes (Bibb 2005).

ppGpp synthetase is necessary for antibiotic production in *Streptomyces coelicolor* A3(2) (Chakraburtty 1997) under conditions of nitrogen limitation . Modified genes have been used to induce ppGpp synthesis in *Streptomyces coelicolo*r but at the same time without reducing the growth rate resulting in the stimulation of production of actinorhodin by the expression of its pathway-specific regulatory gene (Hesketh *et al* 2001).Although the exact mechanism of how this happens is not known yet, there is strong evidence for a direct role of ppGpp in activating the transcription of antibiotic biosynthetic genes.

In conditions of phosphate limitation the mechanism is different and the ppGpp-synthetase while being necessary in nitrogen limitation is unnecessary under conditions of phosphate limitation where another mechanism triggers the initiation of secondary metabolism (Chakraburtty and Bibb1997).

Phosphate inhibition of antibiotic production is observed at high phosphate levels and it was found that inactivation of the polyphosphate kinase which produces polyphosphates when there is phosphate sufficiency ,resulted in production of actinorhodin and higher levels of transcription of regulatory genes for red and a calcium-dependent antibiotic also (Chouayekh & Virolle 2002).

The reason of the inhibition of production of antibiotic in a high phosphate environment is not yet known. Almost all *Streptomyces* species produce γ-butyrolactone (Horinouchi and Beppu 1992) ,a product that is involved in the secondary metabolism and the best characterized is the A-factor (2-isocapryloyl-3Rhydroxymethyl-γ-butyrolactone).*AdpA* which is necessary for *strR* transcription, which in turn is the pathway – specific regulatory gene for streptomycin production, which is regulated by the A-factor through its binding to its cytoplasmic protein and then with the proper biochemical chain reactions leads to antibiotic production (Natsume *et al* 2004).

In addition to pathway-specific regulatory genes, S.*coelicolor* (the most studied Streptomyces strain)possesses several genes with pleiotropic effects on antibiotic production.*S. coelicolor* produces at least five antibiotics, actinorhodin (figure 3)which is blue pigmented , undecylprodigiosin (figure 4) which is also known as the red antibiotic because of its colour , methylenomycin and a calcium dependantantibiotic(CDA) ,as well as an unusual polyketide, mutactin.Research has shown that some of the pleiotropic

regulatory genes had an effect of stimulating the production of antibiotics in species where normally the presence of a specific regulatory gene was very poorly defined.

For example in the case of actinorhodin production in *Streptomyces lividans* which is closely related with *S. coelicolor* and the actinorhodin and undecylprodigiocin genes are poorly expressed (Horinouchi *et al* 1983).

All these physiological parameters, each in different level ,act as factors for antibiotic biosynthesis ,but it is commonly and widely accepted that many mechanisms are still not yet totally clear or known and there is still a lot of research needed to understand them in depth.

There is a great number of secondary metabolites produced by *Streptomycetes* and these are not only the antibiotics but other products of veterinary and agricultural interest and use also. Many of them are derived from aminoacids. Undecylprodigiocin, previously mentioned as the one of the secondary metabolites produced in *S. coelicolor*, has proline as a precursor.

It should be underlined that although Streptomycetes have an unusual ability of producing such a wide range of secondary metabolites they cannot produce aminoacids themselves (Hood *et al* 1992).

Therefore there is an interaction between primary metabolism i.e. the catabolic and anabolic reactions resulting in the biomass augmentation and secondary metabolism (Drummond *et al* 1995)

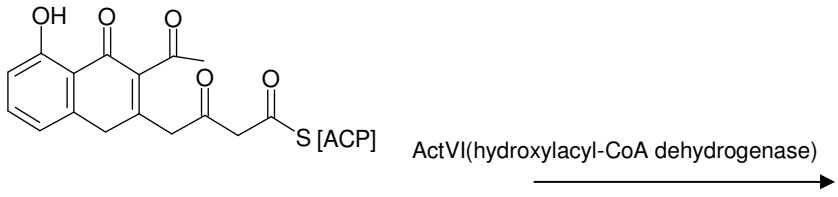
Actinorhodin is an aromatic polyketide antibiotic, whose basic carbon skeleton is derived from type II polyketide synthase. It was the first antibiotic whose whole biosynthetic gene cluster was cloned and has been used as one of the best model compounds for studying type II polyketide synthase (PKS), their ancillary enzymes, and subsequent post-PKS tailoring enzymes (Susumo *et al* 2009).

Its biosynthesis occurs as shown in figure 3.

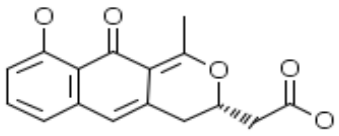
Actinorhodin biosynthesis starts with 8 malonyl-CoAs and the minimal polyketide synthesases (PKS), composed of a ketoacylsynthase and an acyl carrier protein(ACP). The first malonyl group is loaded onto the ACP and once it is loaded it is decarboxylated to the starter acetyl group by the ketosynthase-chain length factor (KS-CLF). As the chain grows into the KS_CLF tunnel, it bends thus initiating the first ring cyclization.In the next step, the double bond between C-14 and C-15 is reduced by ActVI while formation of the chemically favored isomer is assisted by ActVI –OrfIV (Booker *et al* 2005)

Finally, the actinorhodin precursor is oxidized by a two-component NADH:flavin-dependent monooxygenase system composed of a dehydratase and *ActVA-ORF5* thus producing actinorhodin (Fernandez *et al* 1994).

However some biosynthesis phases are still speculative as is the case of the catalysis of the (S)-DNPA conversion to 5-deoxy-dihydrokalafungin by *ActVI-ORF2 and ActVI-ORF4* since both resemble enoyl reductases, but only disruption of *actVI-ORF2* effects actinorhodin production.



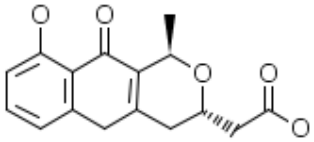
7,9,12 Octaketides [Acyl Carrier Protein]



ActVI-A(dehydratase)

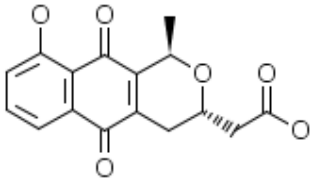
4-Dihydro-9-hydroxy-1-methyl-10-oxo-3-H
naphtho[2,3-c]pyran-3-acetic acid

ActVI-2(secreted protein)



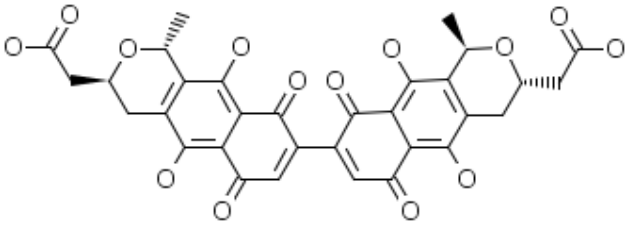
5-Deoxodihydrokalafungin

ActVA(integral membrane protein)



Dihydrokalafungin

ActVA(integral membrane protein)



actinorhodin

Fig 3. Biosynthesis of type II polyketide products – *S. coelicolor* KEGG PATHWAY: sco01057

The red-pigmented antibiotic undecylprodigiocin is the major component of a mixture of at least three prodigionines produced by *S. coelicolor* and it is a highly non-polar compound which contains a triple pyrrolic ring functionality as it is shown in figure 4. Its production occurs in a growth-dependent way. In liquid grown cultures production occurs in the transition to the stationary phase whereas for the surface grown cultures it coincides with the beginning of morphological developments (White and Bibb 1997).In the case of the red antibiotic there are 18 genes necessary for its synthesis whereas for actinorhodin ,the blue pigment, the number of the necessary genes is 22.

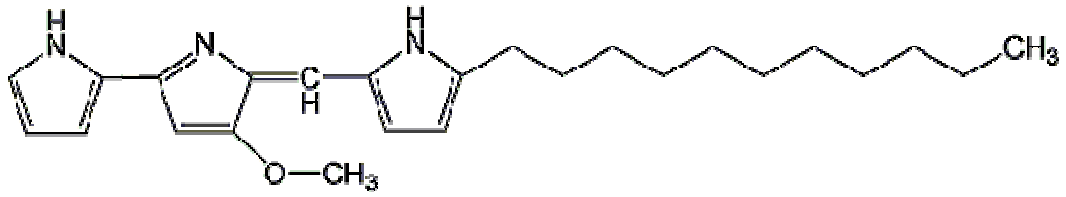


Fig.4 The red antibiotic-undecylprodigiosin

From these 18 genes the red gene appears to be a pathway-specific activator gene. This was concluded due to the inability of redD mutants to co-synthesize red with any of the other red mutants classes (Fietelson *et al* 1985),from the lack of O-methyltransferase activity (redE and/or redF are responsible for the activation in red mutants) and reduced levels of red*E* and redBF transcripts in redD mutants (Narva and Fietelson1990), and the ability of extra cloned copies of redD to evoke overproduction of Red in *S. coelicolor*. (Fietelson *et al* 1985)

Other actinomycetes produce different kind of the prodigionine antibiotics (figure 5) which are all pigmented products and although they are not in use due

to their toxicity ,they have reported to be potent immunosuppressants at non-toxic doses (Lee *et al* 2000 , Azuma *et al* 2000).

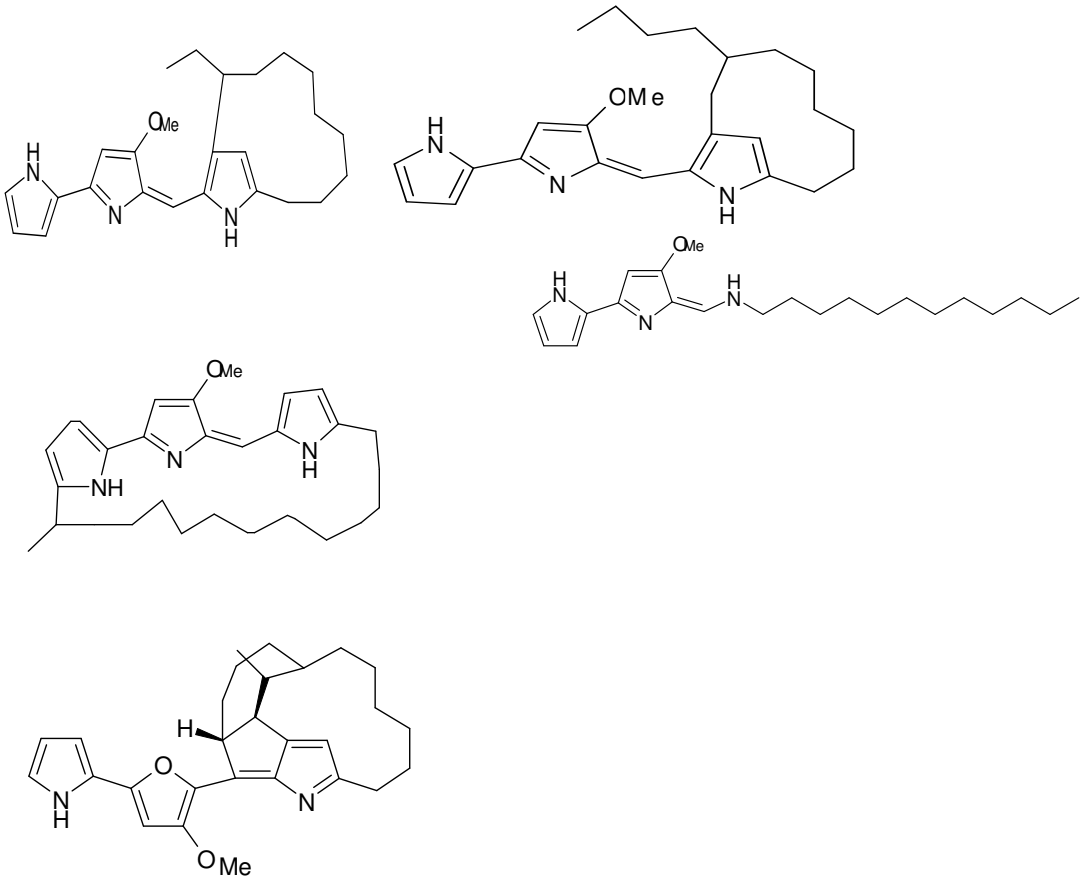


Fig.5 Structures of some prodiginines produced by actinomycetes

The unusual molecular structure of these macrocyclic compounds and their potential use as drugs has attracted interest in carrying out their total chemical synthesis. The main challenge of these studies has been in the efficient construction of the medicinally/chemically unknown, ansa-bridged macrocycles of these metabolites (Kim *et al* 1997)

The biosynthesis of prodigiosin by *Serratia marcescens* and of several actinomycete prodiginines has been examined by the incorporation of

labelled precursors ,thus it has been demonstrated that undecylprodigisine is derived from one unit of proline, one unit of glycine, one unit of serine and several units of acetate, via a merging pathway involving condensation of 4-methoxy- 2,2P-bipyrrole-5-carboxaldehyde and 2- undecylpyrrole at a late stage as shown in figure 6 (Ana *et al* 2001).

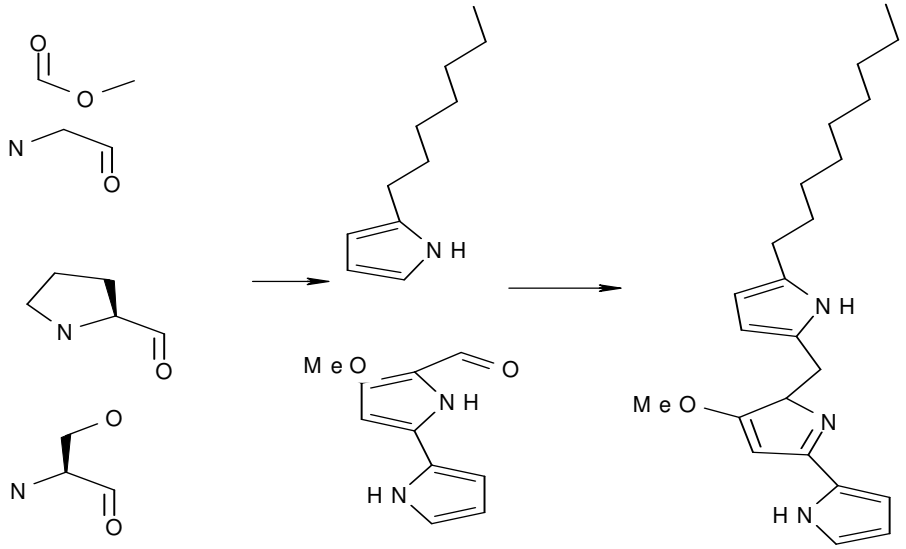


Fig.6 The biosynthetic origin of undecylprodiginine

As already reported the red antibiotic that is produced by *S.coelicolor* is a mixture of predominately undecylprodiginine and butyl-meta-cycloheptylprodiginine in about a 2:1 ratio (Tsao *et al* 1985)due to an oxidative cyclization of the former to the latter (figure 7).

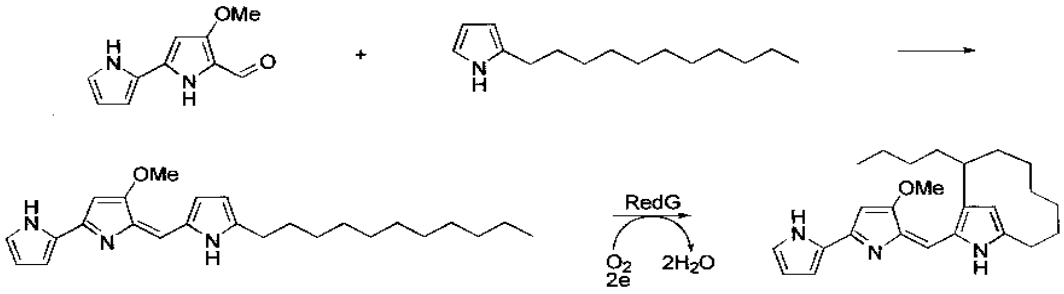


Fig.7 The oxidative cyclization of undecylprodigiosine

So far it has not been possible to determine a gene product for this reaction but there is speculation about the involvement of some unassigned genes (Ana *et al* 2001).

Methylenomycin(figure 8 and 9) is a cyclopentanone antibiotic with antibacterial activity against both Gram-positive and Gram-negative organisms; it is particularly effective against *Proteus* species (Hobbs *et al* 1992). Naturally it is produced in two forms A and the B .Molecular genetic studies have shown that the genes for the synthesis of and resistance to methylenomycin are carried on a plasmid (SCP1) (Kinashi *et al* 1987).

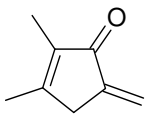


Fig.8 Methylenomycin B

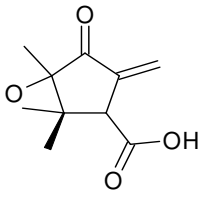


Fig.9 Methylenomycin A

It has been demonstrated that methylenomycin synthesis was achieved by employing an organic nitrogen source, usually alanine, and by using elevated phosphate concentrations to repress actinorhodin biosynthesis.(Hobbs *et al* 1992).The excess presence of phosphate was necessary for the exclusive production of methylenomycin whereas phosphate has the opposite effect on the production of other antibiotics in Streptomycetes as is the case for actinorhodin. The calcium-dependent antibiotic (CDA), from *S.coelicolor*, is an acidic lipopeptide, comprising an N-terminal 2,3-epoxyhexanoyl fatty acid side chain and several non-proteinogenic amino acid residues as well as D-configured ones including D-4-hydroxyphenylglycine,D-3-phosphoxydroxyasparagine and L-3-methylglutamic acid (Kempter *et al* 1997). It is called calcium dependent because its antibacterial activity occurs only in the presence of calcium ions (Hopwood 2007).The amino acids that are required for

CDA biosynthesis are tyrosine, aspartate, asparagine, tryptophan, threonine, glycine, serine, glutamate and oxoglutarate (Hong *et al* 2004).

The CDA biosynthetic gene cluster contains open reading frames encoding nonribosomal peptide synthetases, fatty acid synthases, and enzymes involved in precursor supply and tailoring of the nascent peptide. The structures of some calcium-dependent antibiotics have been determined previously and they differ in the substitution pattern of the aminoacid residue in position 9 and 10 where there is either D-3-hydroxyasparagine or D-3-phosphohydroxyasparagine and either L-glutamic acid or L-3-methylglutamic acid respectively(Hojati *et al* 2002).

CDA resembles in its molecular formula some acidic lipopeptide antibiotics such as daptomycin, friulmicins and amphomycins (Vertesy *et al* 2000).

This resemblance suggests that it may have some of the same activities as well. Some of the aforementioned acidic lipopeptide antibiotics are shown in figure 10:

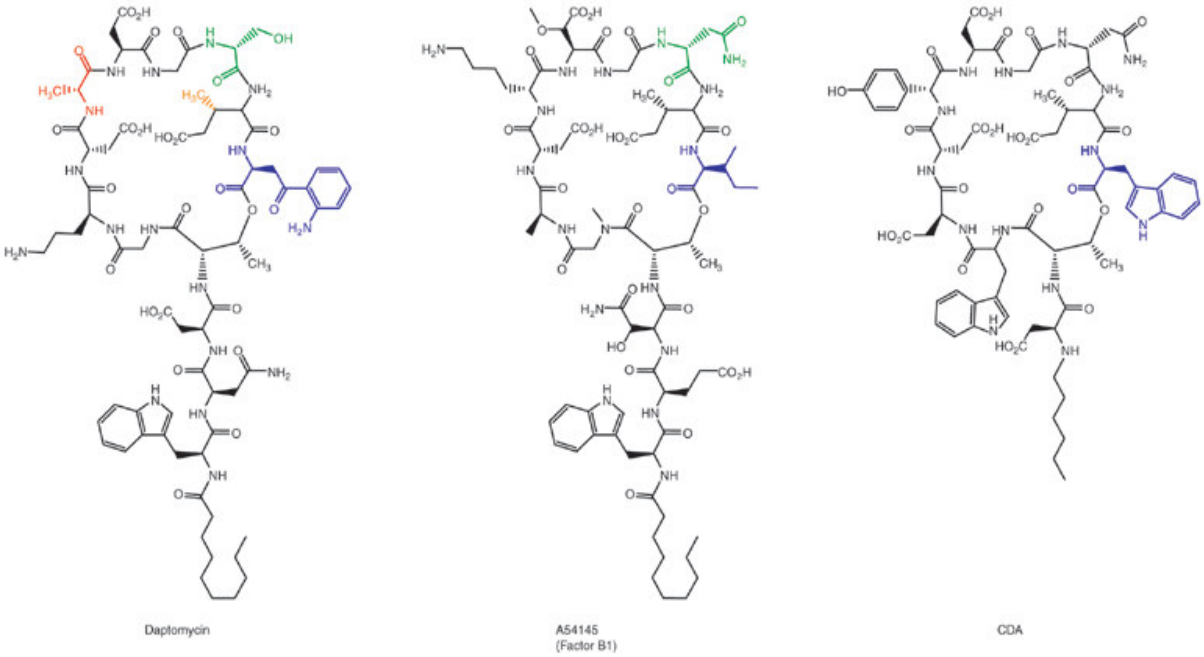


Fig.10 Acidic lipopeptide antibiotics(Nguyen et al 2006)

All these compounds are formed by a decapeptide lactone or lactam ring which derives from the cyclization of L-threonine or L-threo-2,3-diaminobutyrate side chains on the carbon -terminal of the carboxyl group (Nguyen et al 2006).

It is worth mentioning the therapeutic spectrum of the group which is better represented by daptomycin which is active against a wide range of Gram positive bacteria and besides it is used for skin infection treatment caused by Gram positive infections. It is currently undergoing clinical trials for administration to patients with cancer and neutropenia (Rolston *et al* 2010).

One very significant factor for secondary metabolism inception and for the degree of its extension ,is nutrition not only because limitation of any essential nutrient will result in growth restrictions but also because the choice of a limiting nutrient can have specific metabolic and regulatory effects (Doull and Vining 1990).YEME (yeast/extract-malt/extract) is commonly used as nutrient medium for streptomycete cultures.

Yeast extract is rich in amino acids and vitamins and malt extract is a thick syrup which contains vitamins, mineral and sugars.

Addition of peptones to the culture medium provides a nutritional supplement .

Each peptone has a unique amino acid composition which provides distinct properties. They are derived from milk or meat digested by proteolytic enzymes and the final product apart from the amino acids also contains fat, metal, salts vitamins and other biological compounds.

In order to study streptomycetes metabolism in depth and carry out metabolite identification, mass spectrometry based metabolomics can be used.

1.3 Metabolomics

Metabolomics enables the study of the metabolic composition of an organism or biological system, so that all metabolites, both secondary and primary can be observed as far as possible. The term metabolomics , also called metabonomics is part of the “omics” technologies. Genomics is concerned with gene identification. Transcriptomics indicates which genes are being converted into RNA. Proteomics indicates whether or not RNA is translated into protein and which post translational modifications are made to the protein.

Metabolomics which aim to maximize the coverage of a metabolome which is a consequence of protein action so that all metabolites can be seen (Kamleh *et al* 2009).

The metabolome can be defined by quoting Harrigan and Goodacre as “the qualitative and quantitative collection of all low molecular weight molecules which in fact are the metabolites, present in a cell participating in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell” (Harrigan and Goodacre 2003)

Since the interest in this area is of great importance, powerful analytical techniques have been used in order to achieve the best possible result in efforts to produce the most accurate metabolic profile possible.

The major problem that arises when an attempt is made to create the metabolic profile of an organism is to determine which metabolites cannot be detected due to their low concentration or due to an improper analytical technique being used.

The most commonly used techniques are mass spectrometry in conjunction with gas chromatography, liquid chromatography or furthermore combination of these with Nuclear Magnetic Resonance (NMR) spectroscopy.

1.3.1 Gas chromatography MS (GC-MS)

GC-MS is a very versatile technique especially for volatile compounds or for compounds that can become volatile with specific and proper treatment. It uses electron impact as the ionization mode thus promoting efficient ionization of non polar compounds such as fatty acids and hydrocarbons. These latter compounds are not sufficiently ionized by other ionization mode such as the electrospray ionization(ESI) which is used with the liquid chromatography MS thus making the GC-MS more suitable for their detection (Watson 2009).

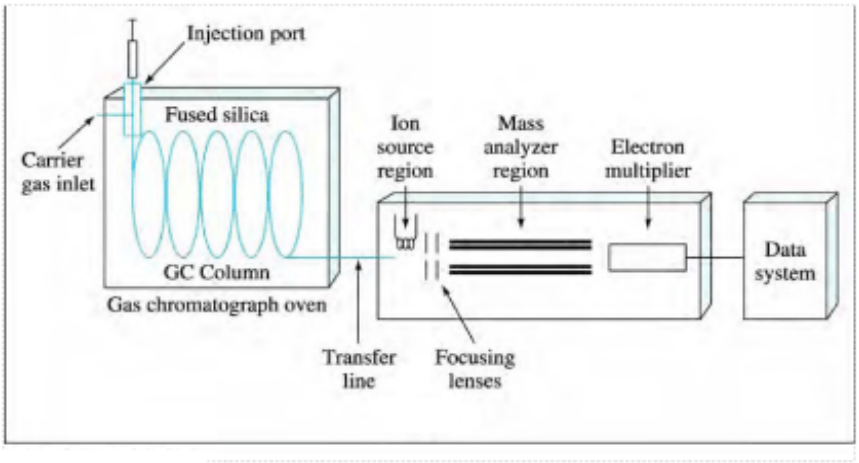


Fig 11. Diagram of a typical quadrupole GCMS.The essential components of a basic mass spectrometer are a sample inlet system, an ion source, a mass analyzer, a detector, a vacuum system, and a data processing device (www.fao.org/UN)

The sensitivity of the above technique tends to be very high since background signal noise from solvent is absent due to the use of helium ,which is an inert gas ,as mobile phase. Furthermore a GC capillary column has around 10 times more resolving power than a typical LCMS column. However usually derivatization of non-volatile compounds such as acids and up to some point amines ,must take place, usually by trimethylsilylating them with a trimethylsilylating (TMS) agent thus producing esters and ethers ,but because the reagent itself generates a signal the analysis become more complex. More

complexity comes from the fact that unstable amine derivatives add more peaks and all these contribute in making the data interpretation harder. In addition there is always the danger that the mass spectrometer might be contaminated by the reagent so frequent cleaning must be performed. With this technique, the quantification of metabolites especially those which are targets and have been monitored with selected ion monitoring mode(SIM), can be very accurate even using the single quadrupole GC–MS instruments which also gives satisfactory results for metabolites of great abundance such as sugars and fatty acids.

1.3.2 Liquid Chromatography MS(LC-MS)

The ionization method most commonly used with the liquid chromatography MS is the electro spray ionisation mode (ESI) which is most sensitive to bases because they give very strong signals in positive ion mode since they are readily protonated. This method can be used both in positive and negative mode and in more advanced instruments there is the possibility of performing the analysis under both modes simultaneously (figure 12).

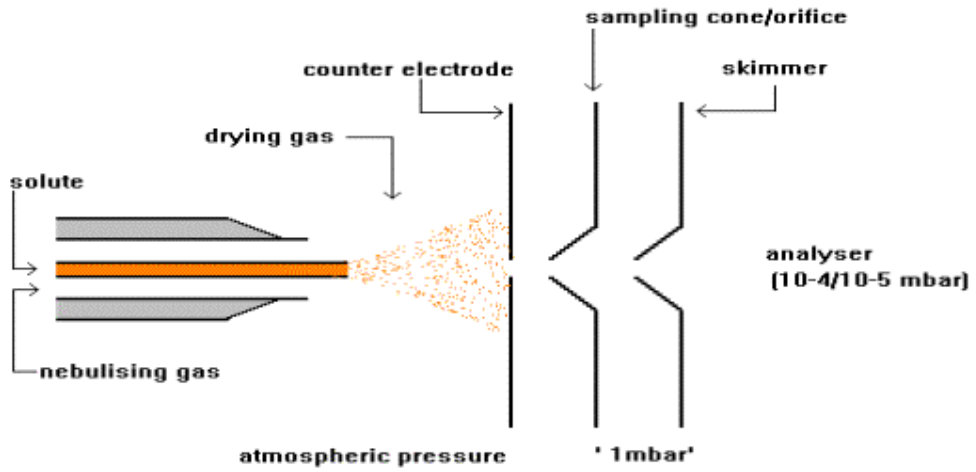


Fig.12 An electrospray ionisation source (Yamashita and Fenn,1984)

During ESI the sample is dissolved in a polar, volatile solvent and pumped through a narrow, stainless steel capillary (75 - 150 µmi.d.) at a flow rate of between 1 µL/min and 1 mL/min. A high voltage of 3 or 4 kV is applied to the tip of the capillary, which is situated within the ionisation source of the mass spectrometer, and as a consequence of this strong electric field, the sample emerging from the tip is dispersed into an aerosol of highly charged droplets, a process that is aided by a co-axially introduced nebulising gas flowing around the outside of the capillary. This gas, usually nitrogen, helps to direct the spray emerging from the capillary tip towards the mass spectrometer as is shown in figure 13. The charged droplets diminish in size by solvent evaporation, assisted by a warm flow of nitrogen known as the drying gas which passes across the front of the ionisation source.

Eventually charged sample ions, free from solvent, are released from the droplets, some of which pass through a sampling cone into an intermediate vacuum region, and from there through a small aperture into the analyser of the mass spectrometer, which is held under high vacuum**.**

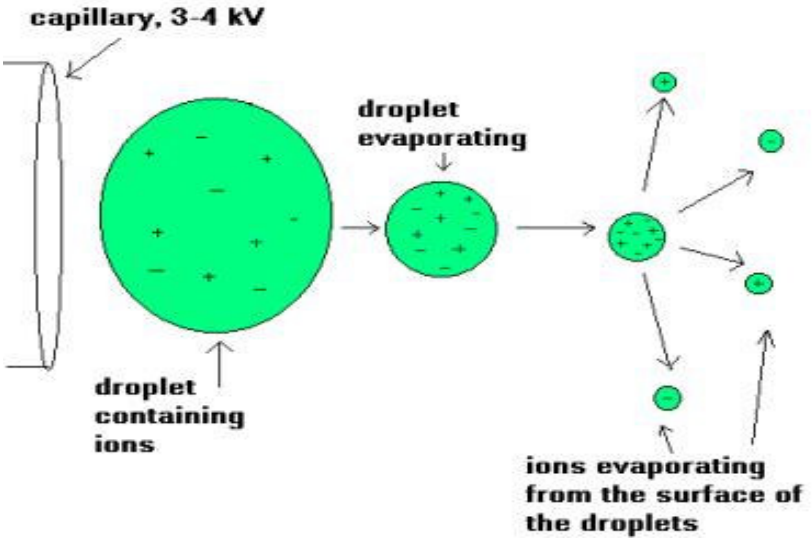


Fig 13. The electrospray ionisation process (The Astbury Centre for Structural Molecular Biology)

Many compounds form adducts with components present in the mobile phase and this produces more data however also makes its interpretation harder. Moreover additional data are also produced by the presence of isotopes peaks and particularly from those of carbon-13, sulphur-34 and nitrogen-15.

A basic LC MS system is based on a single quadrupole since apart from the low cost the can produce a complex information especially when combined with a good chromatography. However their limited resolution which is about 0.5 a.m.u and their poor ability to give fragmentation information of the compounds analysed ,leads to the use of triple quadrupoles tandem mass spectrometers which have the ability to produce much more detailed information of the metabolic profile of the analyte due to their highly sensitive detection limit.

In figure 14 a typical triple quadrupole mass spectrometer is shown.

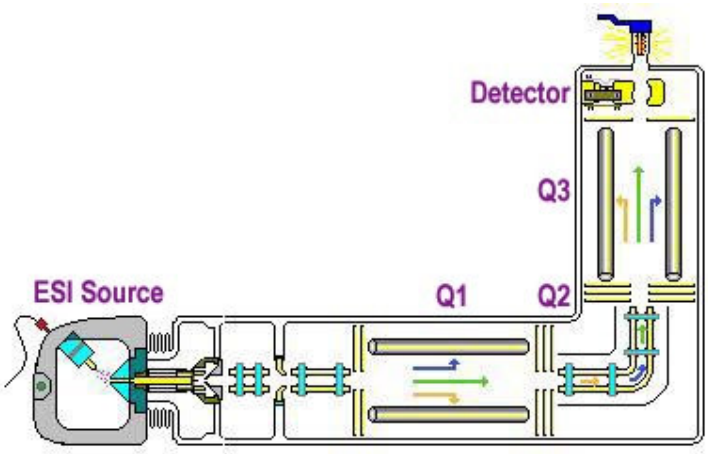


Fig.14 A typical triple quadrupole mass spectrometer(U.S. Enviromental Protection Agency-Ion composition elucidation)

1.3.3 Orbitrap Mass Spectrometry

The Orbitrap is a relatively new type of mass analyzer invented by Makarov (Hu *et al* 2005).The ion separation technology is based on trapping the ions injected into the trap between an outer barrel-like electrode and an inner spindle-like

electrode. The former electrode is coaxial with the latter one and mass/charge values are measured from the frequency of harmonic ion oscillations, along the axis of the electric field, sustained by the orbitally trapped ions. The stable ion trajectories within the Orbitrap combine axial oscillations with rotation around the central electrode and vibrations in the radial direction and these are mass dependant.

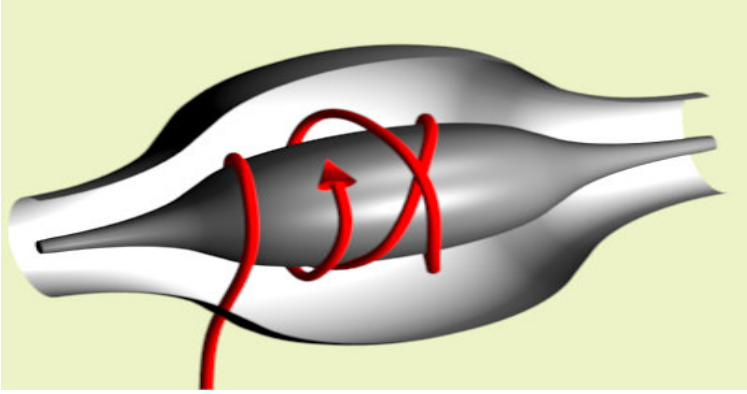


Fig.15 The Orbitrap mass analyser. Ions enter in the direction of the red line with a velocity vertical towards the long axis of the analyser(www.commons.wikimedia.org)

During ion detection, the central electrode and the deflector are maintained at a stable voltage, so that all ions posses the harmonic axial potential in all parts of the space in order to avoid differences to the frequency for ions with the same m/z value which would cause mass shifts. This is important since the frequency of the axial oscillation for them is independent from the initial parameters whereas the radial and rotational frequencies are related with the initial radius and energy. The ions direction after their entrance is shown in figure 15.
The axially oscillating ions produce a periodic signal on the outer electrodes, which is detected as an image current. After amplification, the image current

signals are converted into a frequency spectrum using a Fourier transform algorithm. The image current of the oscillating ions is differentially amplified from each half of the outer electrode and Makarov(2006 a) suggested the addition of an RF to the static voltage of the central electrode thus succeeding in modifying the axial and radial amplitudes of the confined ions as shown in figure 16(Perry *et al* 2008).

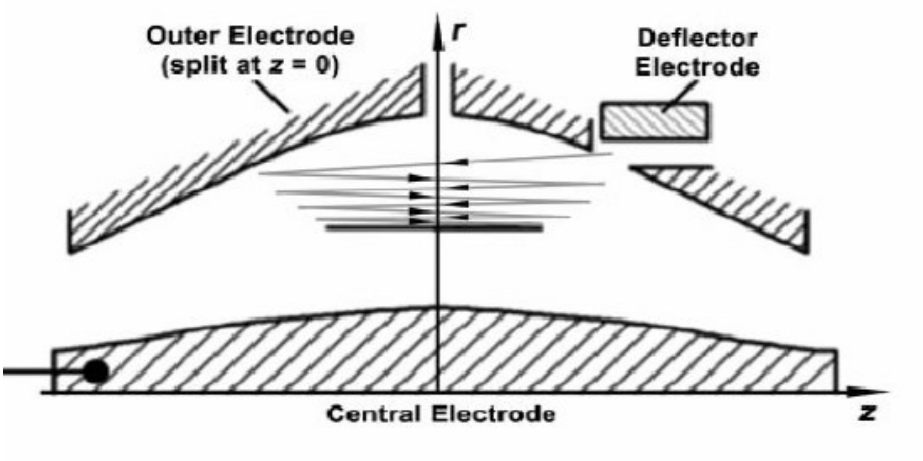


Fig.16 Principle of electrodynamic squeezing(Hu *et al* 2005)

Because the frequency of oscillation is directly related to the mass-to-charge ratio, the frequency spectrum is readily converted into a mass spectrum.
As far as it concerns the radial and rotating trajectories these are not detected due to production of opposite image currents on the outer electrodes that ultimately cancel each other out (Makarov *et al* 2006a).

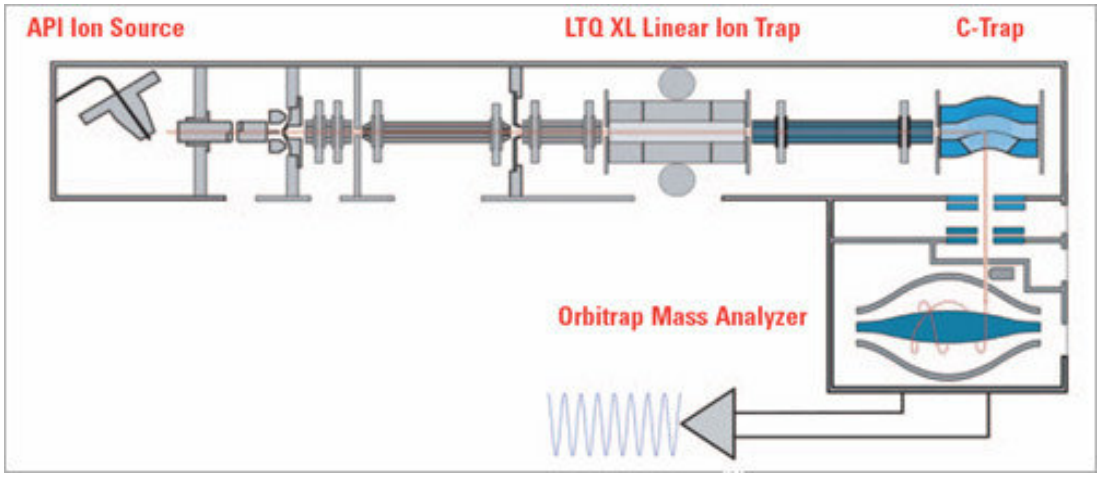


Fig.17 The LTQ Orbitrap mass spectrometer. Ions are transferred from the ion source through three stages of differential pumping using RF guide quadrupoles. The third quadrupole, pressurized to less than 10−3 Torr with collision gas, acts as an ion accumulator; ion/neutral collisions slow the ions and cause them to pool in an axial potential well at the end of the quadrupole. Ion bunches are injected from this pool into the Orbitrap analyzer for mass analysis.(Hu *et al* 2005)

The instrument produces fast measurement of accurate masses and is thus compatible with chromatography. It is also very sensitive and capable of measuring analytes at a concentration of<1 ng/ml (Makarov *et al* 2006a).

The limit of detection of the instrument is remarkable (at pmol/ml) due to its ability in measuring small changes in the transmitted current image(Makarov *et al* 2006a) which also contributes to instrument high resolution.

However the dynamic range is limited due to the FT-ICR instrument limitations since space-charge effects reduce the number of ions that can be delivered to the trap. Moreover time is required to obtain high mass resolution, so scanning rates have to be reduced as the resolution increases.

The Orbitrap is available in three configurations: the Exactive which can measure accurate masses but lacks fragmentation capability, the Discovery which measures accurate masses and produce fragmentation information and the Orbitrap XL which measures accurate masses and deliver both high and low energy fragmentation(Watson 2009).

1.3.4 Reversed Phase Chromatography

Early work in metabolomics used to perform direct sample infusion into ion cyclotron resonance mass spectrometers which is a technique where there is high risk of ion suppression and which does not give the ability to distinguish between isomers. Thus it was realized the chromatographic step prior the mass analysis was important. The method that is often used for analysis of metabolomic samples is the reversed -phase (RP)chromatography shown in figure 18,which is very useful for lipophilic compounds since these latter elute according their lipophilicity and is the technique of choice for the analysis of most drugs in biological matrices (Eschelbach and Jorgenson 2006).

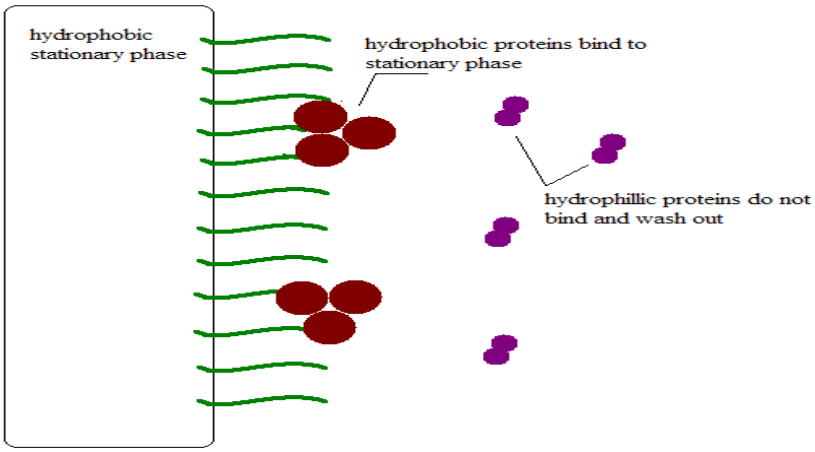


Fig.18. Protein separation using RP Chromatography(Eschelbach and Jorgenson 2006)

Nonetheless some lipophilic compounds such as phospholipids or polymeric residues from plastic-ware and environmental contaminants, tend to accumulate resulting in eluting in subsequent runs, thus causing ion suppression and interfering in the chromatogram. To prevent this case, a wash –out program with high level of organic solvent should be included in the end of the run.

In metabolic profiling, in order to increase the resolving power of RPC it can be used in the form of ultra performance liquid chromatography UPLC (Gika *et al*

2008).This latter offers high chromatographic efficiency: a typical 1.7 μm particle size column delivers about 2.5 times the efficiency of a 5 μm particle column and because of the flat van Deemter plot obtained with low particle size, very high mobile phase flow rates can be used without compromising efficiency. The main disadvantage of this method is that a high back up pressure is required to pump solvent through the long columns that are used. To overcome this it has suggested the use of several 5μm columns in series to produce the maximum number of plates (Desmet 2008) which on the other hand causes run times to be relatively long.

However considering that in biomarker discovery, high throughput analyses are less important than accurate identification, UPLC is perhaps more appropriate for rapid screening post-biomarker discovery (Watson 2009).

Many biomolecules are not well retained in reversed-phase chromatography, and thus polar amino acids such as glycine and alanine will elute in the void volume of most chromatographic columns. The same also happens to sugars, small peptides, pyrimidines and polyamines. The elution in the void volume produces false chromatographic information and in addition there is the risk of ion suppression since the inorganic salts present in the biological matrix also elute in the void (Watson 2009).The above problems might well be overcome by using hydrophilic interaction chromatography (HILIC).

1.3.5 Hydrophilic Interaction Chromatography (HILIC)

This type of separation technique offers an alternative to the RPC and has been used before in the form of the aminopropyl columns ,often used for sugar analysis .It is commonly believed that in HILIC the mobile phase forms a water

rich layer on the surface of the polar stationary phase creating a liquid/liquid extraction system which is shown in figure 19. An appropriate amount of water (usually 5–15%) in the mobile phase maintains a stagnant enriched water layer on the surface of the polar stationary phase into which the analytes partition(Makarov and Scigelova 2010).The analytes are distributed between the water rich stationary layer which schematically is illustrated in figure 20 and the mobile phase with a low water content. More polar compounds have a higher affinity for the stationary aqueous layer than less polar compounds. Thus, separation based on compound polarity will take place.

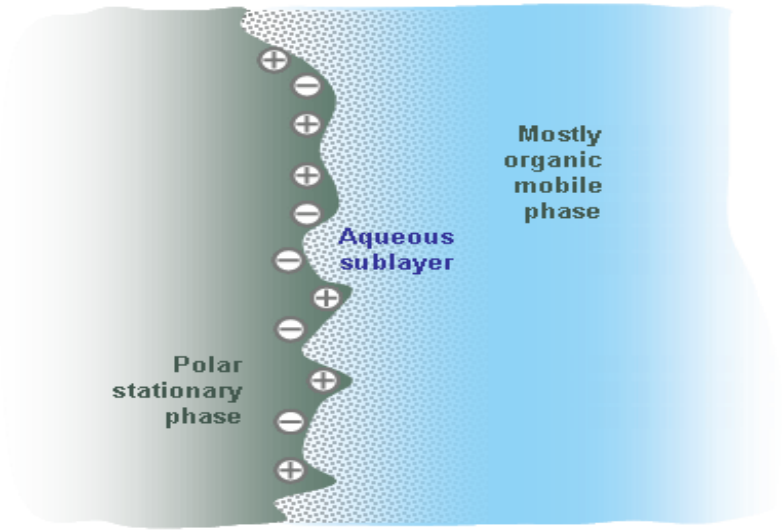


Fig.19 The micro environment around the surface of the stationary phase in HILIC chromatography (http://www.sielc.com/Technology_HILIC.html)

The analytes elution is driven by increasing the water content in the mobile phase. This organic solvent is commonly methanol or acetonitrile and both are highly volatile thus providing low column backpressure and increased API ionization efficiency also (Makarov and Scigelova 2010).

There are several types of columns available for HILIC separation. The column surface itself does not participate in this type of interaction; it just provides a

polar environment which forms a water sub-layer column that can be used for traditional HILIC .An exception is the case when the surface and analytes are engaged in electrostatic interaction as it is shown in figure 21.

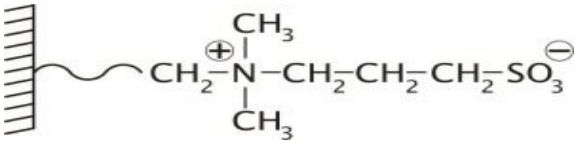


Fig. 20 Schematic illustration of the ZIC-HILIC stationary phase.

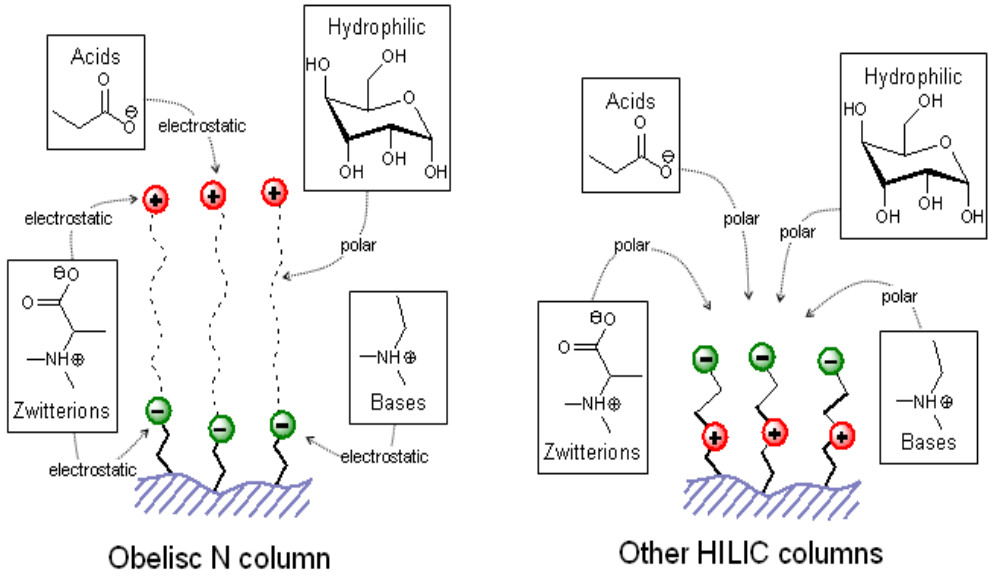


Fig. 21 Electrostatic interaction in a type of HILIC column(www.sielc.com)

The utility of HILIC in retaining hydrophilic compounds while allowing hydrophobic species to flow through rapidly is a significant advantage for metabolomic profiling experiments. Components such as sphingolipids and phosphatidylcholine lipids elute quickly from the column making the separation very robust.

1.4 Aims and objectives

1. To carry out full metabolic profiling of *S.coelicolor* using high resolution mass spectrometry and identify as many metabolites as possible.

2. To observe the changes in the metabolic profile of *S.coelicolor* and its culture medium with time.

3. To observe the effect of deletion of glutamine synthetase genes on metabolic profile.

2. Materials and methods

2.1 Chemicals

HPLC grade acetonitrile was obtained from Thermofischer (Loughborough,UK). Formic Acid 98% AnalaR was obtained from BDH-Merck (Dorset, UK).
HPLC grade water was prepared in house using a MiliQ purification system. All analytical standards for various biomolecules were obtained from Sigma-Aldrich,Poole,Dorset.

2.2 *S. coelicolor* A(3)2 strain

S. coelicolor strain M145(Fink et al 2002), and prototrophic derivative of theA3(2) strain lacking plasmids SCP1 and SCP2 , were used in this study. The bacteria were routinely cultured and maintained following standard procedures (Kieser *et al* 2000).Bacterial spores were stored in frozen 20% v/v glycerol suspension at -80℃. Viable spore count was estimated by plating out a dilution series of the spore suspension on mannitol soya (MS) agar and counting colony forming units (cfu) after 2 days incubation at 30ºC. After determining viability large scale incubations were performed in a two litre Erlenmeyer flasks without any strings. The starting conditions were ca 1 x 10^5 cfu/ml bacterial spores in

400 ml of media. The cultures were incubated at *ca* 30ºC, shaking at 220 rpm, for varying times. At each time point, the pH was measured using standard laboratory pH meter. Biomass concentration was determined in triplicate by vacuum-filtering 5 mL of culture onto pre-weighed, glass microfiber filters (GF/C, 4.7 cm, Whatman, UK). The filters and biomass were washed with 5 mL of distilled water (Nieminen, personal communication).

Samples were collected at different time points starting from the 0 hour time points and reaching until the 50-52 hours time point. The batches were named according to their arithmetical order starting from batch GC3 and reaching until number GC16. At each time point, varying volumes of culture were centrifuged (4000 rpm, 2 mins, +4C). After supernatant(SP) and pellet(lysate form) were separated, 2 ml of ice cold methanol was added to the cell pellet, which was then vortexed briefly and finally samples were stored at -80℃.The mutants were made by D Fink using redirect method for gene deletion which was based on the insertion of a kanamycin resistance cassette at a singular *Bgl*II site in the gene(Fink et al 2002).

The samples were provided by the microbiology laboratory of the Royal College and delivered for metabolomic analysis. The strains were cultured in YEME (yeast extract/malt extract):

Yeast extract 3 g/L

Peptone 5 g/L

Malt extract 3 g/L

D-Glucose 10 g/L

There was no sucrose added to the medium. Its pH initially was ca 6.3 whereas it was not exceeding the value of 7.5 during the growth and samples collection.

2.3 Instrumentation

Prior to analysis the cells extracts were first centrifuged in a Jouan CR3i Multifunction Centrifuge, Thermo Electron Corporation and the supernatant was mixed using a Vortex VELP Scientifica Vortex Mixer.

Sample analysis was carried out on a Thermo Exactive Orbitrap(Thermo Electron Corporation,Hemel,Hempstead U.K) in positive and negative ESI mode with a needle voltage of 4.5kV. The instrument was coupled to a Dionex HPLC with WPS 3000 autosamplerThe column used was a Sequant Zic-HILIC column(150 x 4.6 mm, 5μm,200Å,Hichrom, Reading,UK)The mobile phase used was Acetonitrile / Formic Acid 0.1% v/v as organic solvent(solvent B) and water HPLC graded / Formic Acid 0.1% v/v as aqueous solvent(solvent A) and the following gradient program was used:

Time(min)	Flow(ml/min)	Solvent B%	Solvent A%
0.00	0.300	80	20
12	0.300	80	20
26	0.300	50	50
28	0.300	50	50
36	0.300	20	80
37	0.300	80	20
46	0.300	80	20

Additional mass spectrometry settings are shown below:

Polarity	Microscans	Resolution	Maximum Injection Time	Scan Range	Fragmentation
Positive	3	High	250 ms	75.0-1,200 m/z	None
Negative	3	High	250 ms	75.0-1,200 m/z	None

Run setting segments:1 ,acquisition time:46 min.

2.4 Data processing

The format of the raw data obtained by the Orbitrap analysis were files produced by the Xcalibur software system ,which is the software used by all instruments of

the company. The raw data were then entered in Sieves software (Thermo Electron, Hemel Hempstead, UK) .This latter gave the possibility for data processing by entering the raw files which resulted from the initial analysis using the Xcalibur software, in the mode of control versus treatment. The Sieve software was set to compare extracted ion chromatograms in a 0.02 a.m.u. window. The purpose of this process was to establish the changes that took place in the samples at different time points. The data from each analytical experiment established the presence of known and also unknown compounds It was possible to observe in the reconstructed chromatogram option that the software offered, a comparison of the curves for control versus treatment samples which is illustrated in figure 22.

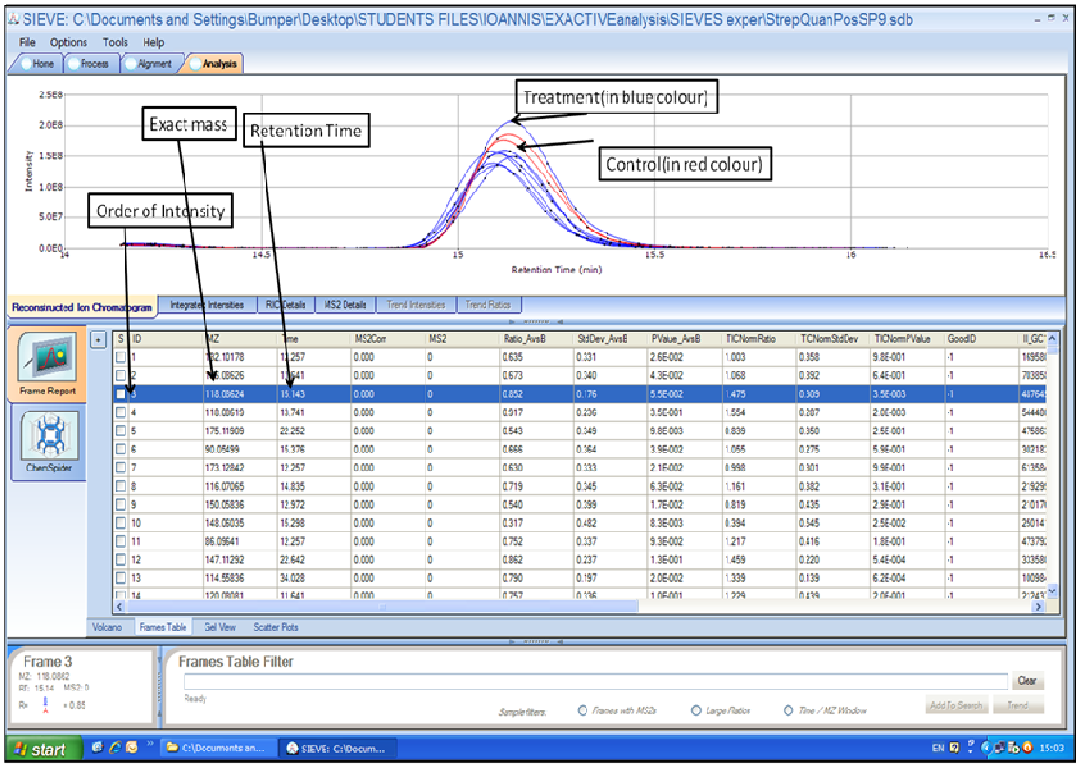


Fig.22 Comparison of L-valine in the sample batches GC9-GC10 in the supernatant layer and how its concentration changes over time with the control vs treatment mode indicating little difference between the batches and not much change with time.

The samples that were used as a control were those of the initial time points i.e. the 0 hours ,the 16 hours and in some case the 20 hours time point whereas the treatment group were the 20,24,28,32,36,45(47),50(52) hours time points. The strain batches that were analyzed are shown in table 2 :

BATCH No	M135 STRAIN	MUTANT STRAIN
GC3-GC4	YES	
GC5-GC6	YES	
GC7-GC8	YES	
GC9-GC10	YES	
GC13-GC14		YES
GC15-GC16		YES

Table 2. Table showing the *S. coelicolor* batches that were analyzed.

The analysis for both pellets (lysate cells) and supernatant layer was carried out at the same time in order to produce information for both intracellular and extracellular metabolic activity of the samples. The results obtained by Sieve software were then entered in the Sieves' Extractor, a program based in Microsoft Excel which was originated in the Strathclyde MS laboratory and which contains a huge library of metabolites which have been identified through their retention time and their exact masses. In order to ensure good chromatographic alignment the analysis of all metabolites was made using the same instrument, Orbitrap and under the same conditions, using the same chromatographic column and the same mobile phase and gradient program. The program utilizes all data that have been produced by Sieve software and using the macro creates an Excel workbook providing information about all compounds that have been identified from the exact masses obtained using Orbitrap MS with the metabolites library.

The Extractor is a powerful tool in data processing since it is programmed in a way to provide information for the exact mass of the analyte molecular ion ,its retention time and metabolic pathway as well. From this latter extra information is obtained for the component of interest since the metabolic pathway for each metabolite is fully detailed on the KEGG pathway database. Thus the data produced from the Extractor, displays also how many compounds are involved in a specific metabolic pathway and from the KEGG database it becomes easier to track a specific analyte of interest and finally gather as much information as possible for it.

3. Results

3.1 The Metabolome of *S. coelicolor* as Detected by the Orbitrap Exactive

All Streptomyces batches that were analysed contained the range of metabolites that are shown in table 3 which were observed in positive ion mode.

These are the major metabolites which can be observed easily, it might be possible by changing the threshold in the Sieve programme to observe further metabolites but the list in tables 3 and 4 provides a sufficient number of metabolites to characterize the growth of *S.coelicolor*. The metabolites are assigned on the basis of their accurate masses in comparison with the data base in Sieve Extractor. To illustrate the identification of metabolites some examples are given below. In figure 23 the extracted ion trace for diaminopimelic acid, which is an important bacterial metabolite involved in cell wall formation, and its mass spectrum are shown. As can be seen in narrow mass window there is one clear peak for the metabolite. The predicted elemental composition $C_7O_4N_2O_4$ for this peak was found with a deviation of 0.3

ppm and this was the only elemental composition found within ± 3ppm corresponding to the mass measured. This composition has only one match in the Kegg data base to diaminopimelic acid. The elements allowed in the search were C30, H60, N10, O15, S 1 and P2. These are the only elements found covalently linked in biomolecules apart from, chlorine which has a characteristic isotope pattern and bromine, iodine and selenium which are very rare.

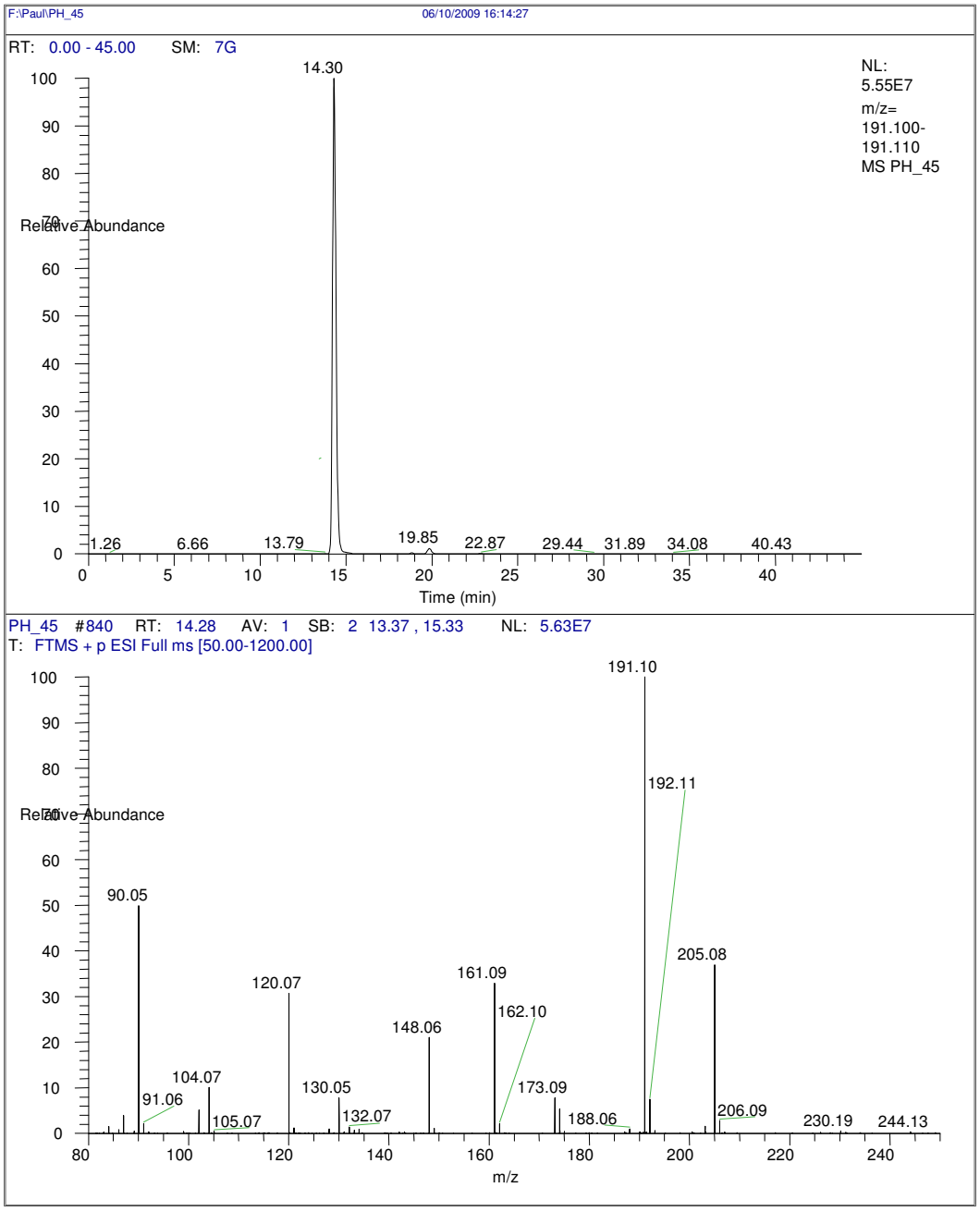


Fig.23 Extracted ion trace for diaminopimelic acid.

Number Order	Compound	M/Z	Retention Time
1	Glycine	76.0394	16.74
2	Piperideine	84.0808	22.6
3	Alanine	90.055	15.41
4	L-Homoserine Lactone Hydrochloride	102.055	15.33
5	GABA	104.071	14.96
6	Choline+	104.107	15.24
7	L-Serine	106.05	16.88
8	Cytosine	112.051	15.41
9	Uracil	113.035	8.37
10	3-Amino-2-piperidone	115.087	22.57
11	L-Proline	116.071	14.88
12	L-Valine	118.086	15.01
13	L-Threonine	120.066	15.86
14	Indoline	120.081	11.58
15	Nicotinamide	123.055	8.93
16	Niacin/Nicotinate	124.039	7.82
17	5,6-Dihydrothymine	129.066	8.93
18	Pyrroline-4-hydroxy-2-carboxylate	130.05	15.33
19	Amino-proline	131.082	15.41
20	Glutamate 5-semialdehyde	132.065	15.32
21	L-Leucine	132.102	12.28
22	L-Asparagine	133.061	16.8
23	L-Ornithine	133.097	23.12
24	L-Aspartate	134.045	16.13
25	Hypoxanthine	137.046	10
26	Ectoine	143.082	14.48
27	guanidino butyric acid	146.093	13.81
28	Glutamine	147.076	16.39
29	L-Lysine	147.113	22.65
30	Glutamic acid	148.06	15.33
31	L-Methionine	150.058	12.91
32	Methyhypoxanthine	151.062	12.91
33	Guanine	152.057	13.32
34	Xanthine	153.041	9.49
35	L-Histidine	156.077	21.59
36	5-Hydroxyectoine	159.076	17.09
37	N-gamma-Acetyldiaminobutyrate	161.092	15.86
38	Tryptamine	161.107	11.6
39	O-Acetyl-L-homoserine	162.076	14.48

40	Carnitine	162.112	14.73
41	Phenylpyruvate	165.055	13.69
42	L-Phenylalanine	166.086	11.58
43	N-Acetyl-L-glutamate 5-semialdehyde	174.076	11
44	oxoarginine	174.087	16.8
45	N-Acetylornithine	175.108	13.86
46	L-Arginine	175.119	22.26
47	L-Citrulline	176.103	17.1
48	N-Carbamoyl-L-aspartate	177.051	11.13
49	D-Galactosamine	180.087	19.69
50	L-Tyrosine	182.081	13.62
51	Iridotrial	183.102	6.2
52	Indole acrylic acid	188.071	12.03
53	N6-Acetyl-L-lysine	189.123	12.62
54	7,8-Diaminononanoate	189.16	23.69
55	N-Acetyl-L-glutamate	190.071	7.71
56	meso-2,6-Diaminoheptanedioate	191.103	15.72
57	Acetylaminocarnitine	203.139	14.03
58	Gly Glu	205.082	15.81
59	L-Tryptophan	205.097	12.23
60	N6-Acetyl-N6-hydroxy-L-lysine	205.118	15.86
61	N-Acetyl-D-phenylalanine	208.097	6.25
62	Acetylarginine	217.13	14.73
63	Glu Ala ,Ala Glu	219.098	14.57
64	Deoxycytidine	228.098	9.4
65	Lue-Pro	229.155	12.38
66	Val-Leu	231.17	10.9
67	ArgGly	232.14	26.07
68	N6-Acetyl-L-2,6-diaminoheptanedioate	233.113	13.38
69	1,1,3-tris(ethoxymethyl)urea	235.165	15.01
70	Cytidine	244.093	16.66
71	Uridine	245.077	9.88
72	Glu Pro ,Pro Glu	245.113	14.4
73	Lys Val ,Val Lys	246.181	23.19
74	Glu Thr ,Thr Glu	249.108	14.29
75	gamma-L-Glutamyl-L-cysteine	251.07	14.06
76	Deoxyadenosine	252.109	11.07
77	Glutamyl-leucine	261.144	11.3
78	Adenosine	268.104	11.68
79	Inosine	269.088	10.81

80	Dimethylallyltryptophan	273.16	9.09
81	Arg Val ,Val Arg	274.187	22.88
82	C11H22N2O6	277.139	18.99
83	Phe Asp ,Asp Phe	281.113	11.25
84	Guanosine	284.099	12.46
85	Glycylprolylhydroxyproline	286.14	16.6
86	5'-Methylthioadenosine	298.097	9.33
87	methylguanosine	298.115	9.82
88	2',3'-Cyclic CMP	306.048	17.5
89	GSH	308.091	14.63
90	2-aminodimethyladenosine	311.146	21.59
91	uridine monophosphate	325.043	14.26
92	2',3'-Cyclic AMP	330.06	14.17
93	cGMP	346.055	14.9
94	AMP isomer 2	348.07	16.19

Table 3. Metabolites detected in *S.coelicolor* in positive ion mode.

Table 3 displays results obtained from the positive ionization mode. This method is much more sensitive for amine compounds such as aminoacids as well as amides and some phosphates too. As can be seen in table 3 the elution time from the Zic-HILIC column is governed by the polarity of the analytes. For example hypoxanthine which is more or less neutral elutes at 10 minutes, valine which has one amine group elutes at 15 min and lysine which has two amine groups elutes at 22.6 min.

In the negative ion electrospray ionization mode it is possible to identify compounds like neutral sugars and carboxylic acids which is not the case when positive ion ESI is used. The table 4 shows metabolites that were identified in negative ion ESI some of which are common to the list generated in positive ion mode e.g. glutamate, which is in this case detected as its anion:

Number Order	Compounds	MZ	Time
1	Pyruvate	87.0087	7.077
2	Alanine	88.0404	16.225
3	D-Glyceraldehyde	89.0244	14.032
4	Sulfate	96.9601	31.584
5	Phosphoric acid	96.9696	17.157
6	GABA	102.056	15.27
7	L-Serine	104.035	16.698
8	D-Glycerate	105.019	10.131
9	2-Hydroxy-2,4-pentadienoate	113.024	14.521
10	N-Methylhydantoin	113.036	16.846
11	L-Valine	116.072	14.576
12	Succinate	117.019	7.541
13	L-Threonine	118.051	15.943
14	Pyrroline-4-hydroxy-2-carboxylate	128.035	8.029
15	L-Leucine	130.087	12.347
16	2-(Hydroxymethyl)-4-oxobutanoate	131.035	14.032
17	L-Asparagine	131.046	16.846
18	L-Ornithine	131.083	16.908
19	L-Aspartate	132.03	16.225
20	Malate	133.014	9.37
21	(R)-2,3-Dihydroxy-3-methylbutanoate	133.051	7.555
22	Adenine	134.047	11.647
23	Threonate	135.03	11.959
24	Hypoxanthine	135.031	9.969
25	3,6-Nonadienal	137.097	5.821
26	2,3-Dimethylmaleate	143.035	14.521
27	guanidino butyric acid	144.078	13.859
28	2-Oxoglutarate	145.014	9.008
29	Glutamine	145.062	16.486
30	Glutamic acid	146.046	15.455
31	2-Methylmalate	147.03	8.127
32	L-Methionine	148.044	12.813
33	Xanthine	151.026	9.514
34	Pinocarveol	151.113	5.821
35	L-Histidine	154.062	21.565
36	N-gamma-Acetyldiaminobutyrate	159.078	15.152
37	O-Acetyl-L-homoserine	160.062	14.576
38	L-Phenylalanine	164.072	11.633
39	D-Xylonate	165.041	13.023
40	Urate	167.021	12.108
41	Glycerolphosphate	171.006	15.923

42	Hydantoin-5-propionate	171.041	10.392
43	N-Acetylornithine	173.093	13.823
44	L-Arginine	173.104	22.305
45	N-Formyl-L-glutamate	174.041	8.284
46	N-Carboxyethyl-gamma-aminobutyric acid	174.077	13.942
47	L-Citrulline	174.088	16.908
48	N-Carbamoyl-L-aspartate	175.036	11.142
49	2-Isopropylmalate	175.061	6.915
50	Pyrophosphate	176.936	17.157
51	D-Glucono-1,5-lactone	177.041	11.584
52	D-Glucose	179.056	14.521
53	Iridotrial	181.087	5.821
54	Homo-cis-aconitate	187.025	11.505
55	N6-Acetyl-L-lysine	187.109	12.658
56	N-Acetyl-L-glutamate	188.057	7.793
57	meso-2,6-Diaminoheptanedioate	189.088	15.796
58	Citrate	191.02	10.396
59	D-Gluconic acid	195.051	14.476
60	L-Tryptophan	203.083	12.193
61	N6-Acetyl-N6-hydroxy-L-lysine	203.104	15.842
62	sn-glycero-3-Phosphoethanolamine	214.049	16.691
63	2-C-Methyl-D-erythritol 4-phosphate	215.033	14.806
64	N2-(D-1-Carboxyethyl)-L-lysine	217.119	14.288
65	L-Cystathionine	221.06	19.494
66	Deoxyuridine	227.067	9.576
67	N6-Acetyl-L-2,6-diaminoheptanedioate	231.099	14.675
68	Uridine	243.062	9.842
69	gamma-L-Glutamyl-L-cysteine	249.055	14.247
70	(1-Ribosylimidazole)-4-acetate	257.078	9.053
71	Naphthyl-2-methyl-succinic acid	257.082	5.941
72	D-Glucosamine 6-phosphate	258.039	20.031
73	Glucose phosphate	259.022	17.8
74	Glutamyl-leucine	259.13	13.047
75	Glutamyl-leucine	259.13	11.322
76	2(alpha-D-Mannosyl)-D-glycerate	267.072	12.877
77	Inosine	267.074	10.88
78	Guanosine	282.085	12.426
79	Sedoheptulose 7-phosphate	289.033	17.073
80	2',3'-Cyclic UMP	305.018	12.9
81	uridine monophosphate	323.029	14.363
82	2',3'-Cyclic AMP	328.046	14.133
83	AMP isomer 2	346.056	16.083

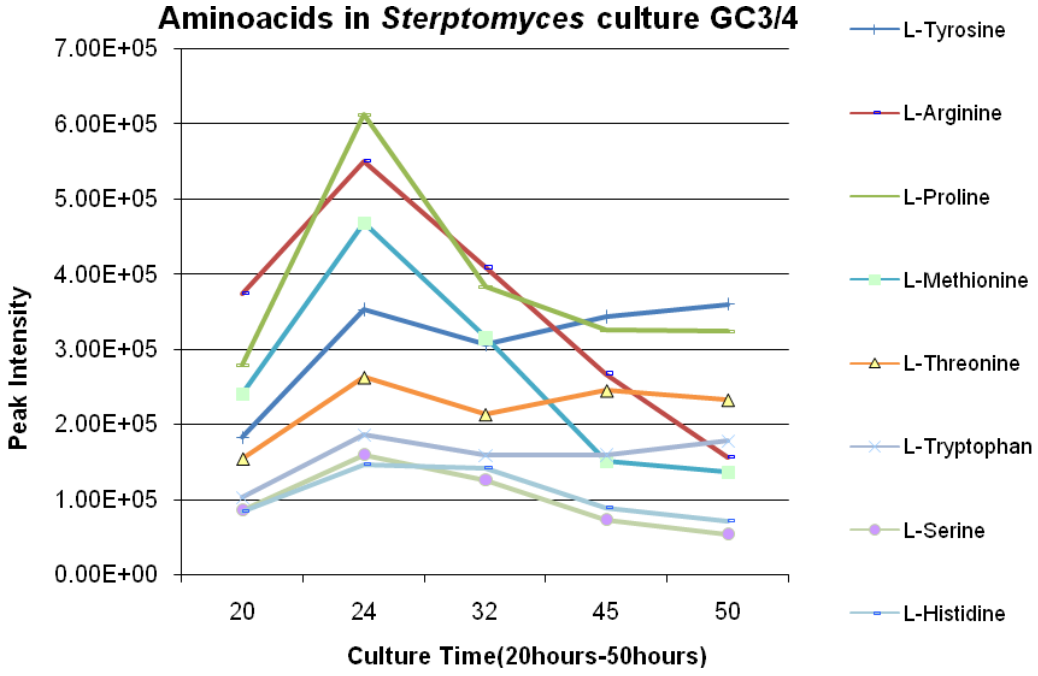
Table 4. Metabolites detected in *S .coelicolor* in negative ion ESI mode

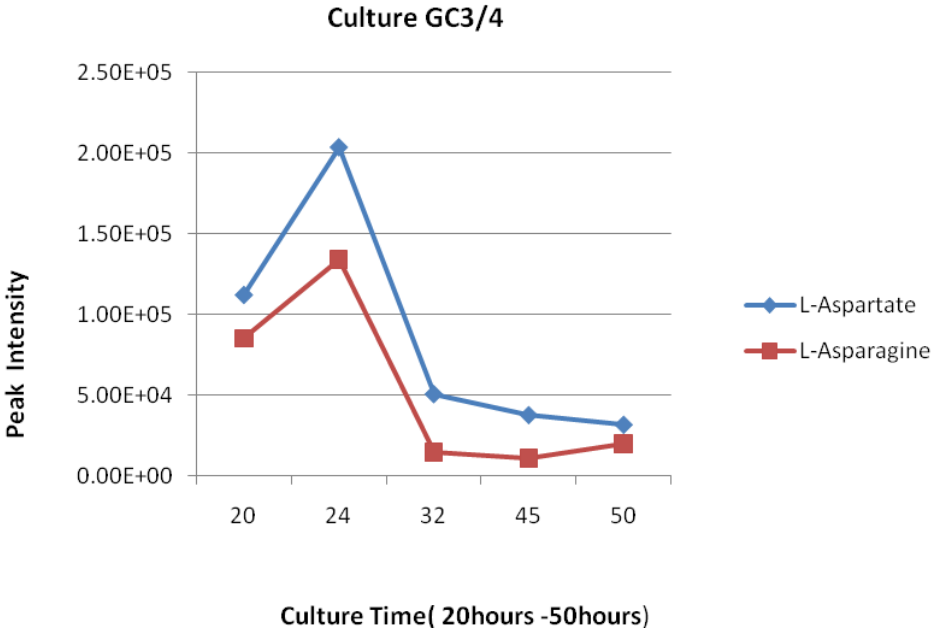
3.2 Results for Amino Acid Metabolism in *S.coelicolor*

There was an interest in the utilization of amino acids in *S.coelicolor* with time in relation to the triggering of antibiotic formation.The amino acids glutamine and glutamate are of particular interest since they are potentially limiting nutrients in the growth cycle and their levels may have a role in triggering off anti-biotic formation which occurs when *Streptomyces* reach a stationary phase. Thus amino acid metabolism was a focus of interest in studying changes in the cultures with time. The first set of culture batches that were analysed were GC3-GC4 which were available only in 5 time points 20, 24, 32, 45 and 50 hours. Table 5 displays the peak areas of most of the aminoacids which correspond to the amount detected and its variation during time. The results were obtained from the pellets (lysates) in positive ion ESI mode. All results reported are semi-quantitative and rely on a degree of instrument stability from run to run. The Orbitrap instrument signal remains quite stable of long periods of time.

AMINOACIDS	**20 h**	**24 h**	**32 h**	**45 h**	**50 h**
Glutamate	9.56E+05	2.59E+06	2.15E+06	1.87E+06	1.56E+06
L-Leucine	2.19E+06	4.02E+06	2.97E+06	2.15E+06	1.87E+06
L-Phenylalanine	8.65E+05	1.56E+06	1.34E+06	1.05E+06	8.71E+05
L-Valine	7.68E+05	1.94E+06	2.29E+06	1.63E+06	1.45E+06
L-Valine	6.19E+05	1.24E+06	1.22E+06	1.20E+06	1.03E+06
Alanine	3.97E+05	8.04E+05	7.58E+05	6.28E+05	8.38E+05
L-Tyrosine	1.83E+05	3.53E+05	3.07E+05	3.43E+05	3.60E+05
L-Arginine	3.74E+05	5.51E+05	4.09E+05	2.68E+05	1.57E+05
L-Proline	2.79E+05	6.13E+05	3.83E+05	3.26E+05	3.24E+05
L-Methionine	2.41E+05	4.68E+05	3.16E+05	1.51E+05	1.37E+05
L-Threonine	1.55E+05	2.63E+05	2.14E+05	2.46E+05	2.33E+05
L-Tryptophan	1.03E+05	1.86E+05	1.59E+05	1.60E+05	1.79E+05
L-Aspartate	1.12E+05	2.03E+05	5.08E+04	3.78E+04	3.18E+04
L-Serine	8.58E+04	1.60E+05	1.26E+05	7.28E+04	5.42E+04
L-Asparagine	8.53E+04	1.34E+05	1.47E+04	1.09E+04	1.98E+04
L-Histidine	8.52E+04	1.47E+05	1.41E+05	8.98E+04	7.22E+04
Glutamine	2.10E+05	1.89E+05	3.09E+05	6.59E+05	1.62E+05

Table 5. Aminoacids from batches GC3-GC4


Aminoacids in *Sterptomyces* culture GC3/4
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
Peak Intensity
20
24
32
45
50
Culture Time(20hours-50hours)
L-Tyrosine
L-Arginine
L-Proline
L-Methionine
L-Threonine
L-Tryptophan
L-Serine
L-Histidine


Culture GC3/4
2.50E+05
2.00E+05
1.50E+05
1.00E+05
5.00E+04
0.00E+00
Peak Intensity
20
24
32
45
50
Culture Time(20hours -50hours)
L-Aspartate
L-Asparagine

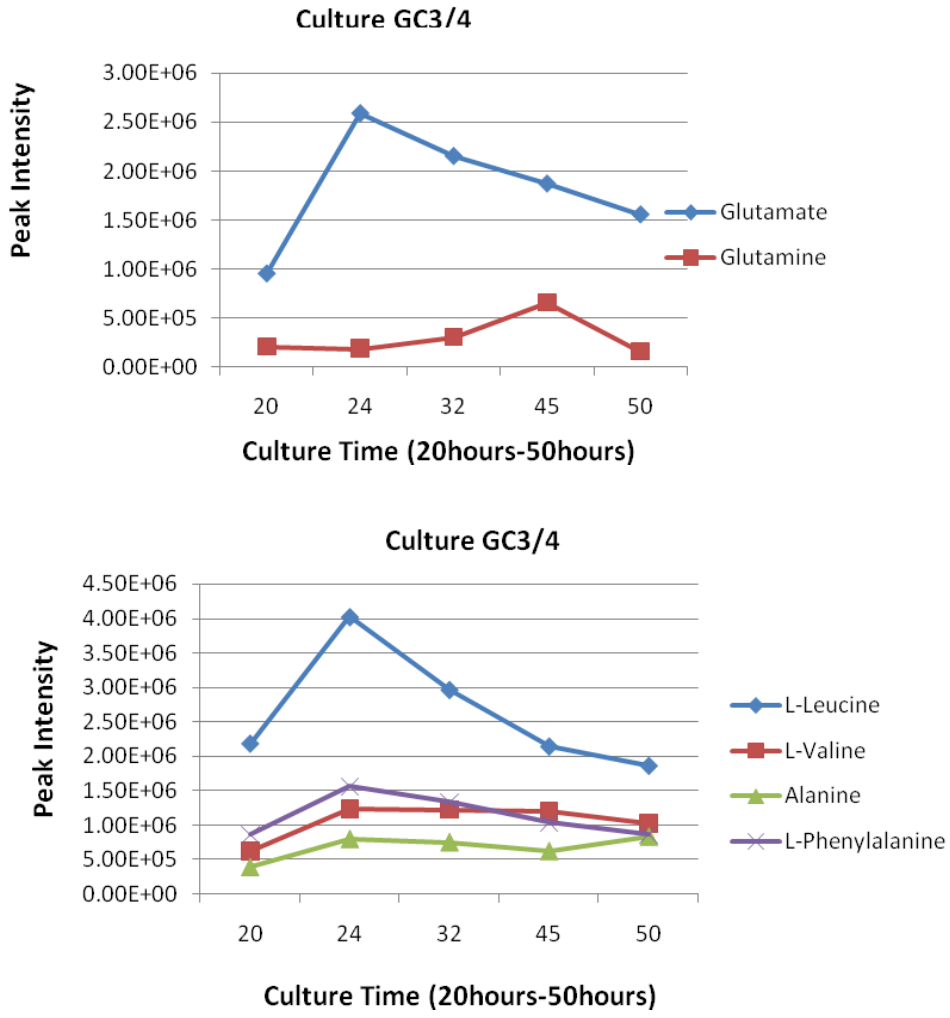


Fig. 24 Graphs of aminoacids from batches GC3,4 showing a marked decline in the levels of glutamate and glutamine and aspartate and asparagine with time.

The results were then plotted for peak area versus time and the graphs shown in figure 24 were obtained. From these plots it would appear as predicted that glutamine and glutamate are limiting nutrients but other amino acids such as aspartate and asparagines also appear limiting. The decline in levels of other amino acids such as valine and phenylalanine with time is less marked. The next set of batches analysed were GC5-GC6 where the time points obtained were at 0, 16, 20, 36, 45 and 50 hours. Both pellet and supernatant layer were analysed ,as this was the case for all batches and the

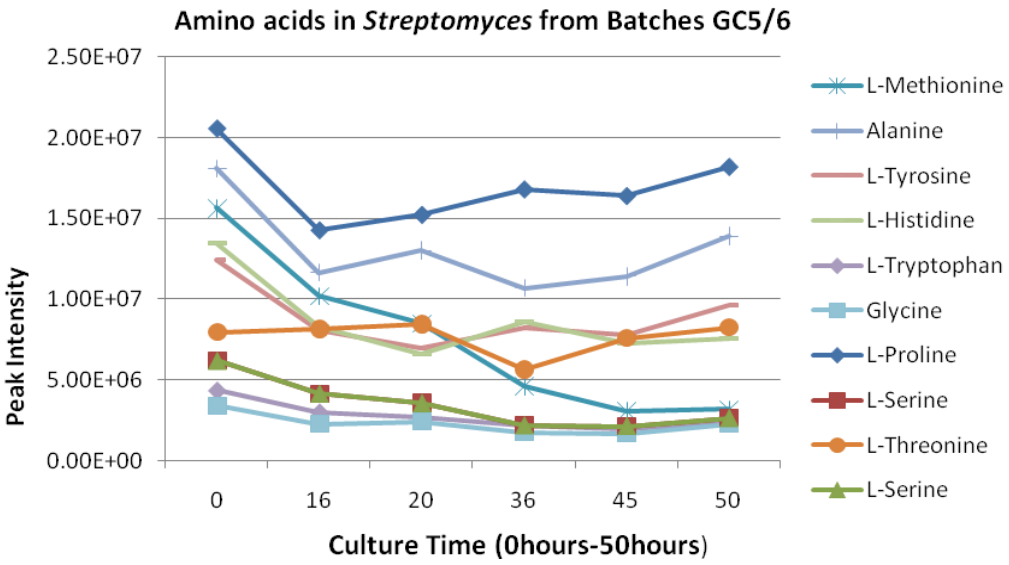
results shown in table 6 were obtained for the aminoacids in positive ion ESI (PIESI) mode :

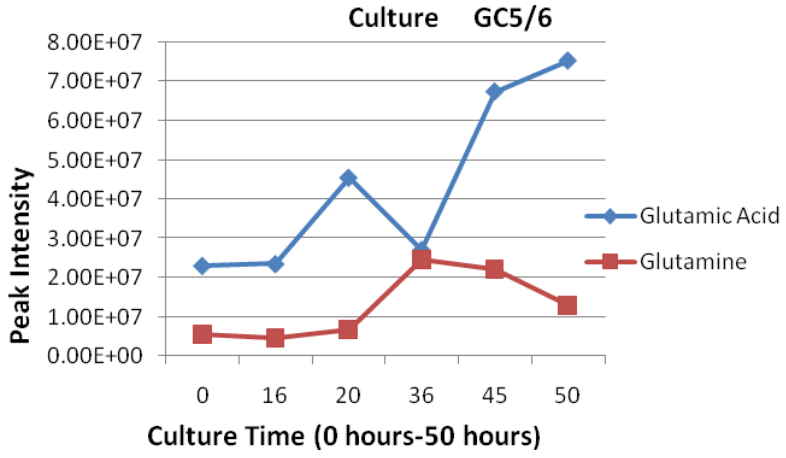
Aminoacids	0 hours	16 hours	20 hours	36 hours	45 hours	50 hours
L-Phenylalanine	6.07E+07	4.15E+07	3.49E+07	2.91E+07	1.81E+07	1.35E+07
L-Arginine	7.07E+07	4.64E+07	3.20E+07	1.85E+07	1.21E+07	7.48E+06
Glutamic Acid	2.30E+07	2.34E+07	4.55E+07	2.71E+07	6.75E+07	7.54E+07
Glutamine	5.52E+06	4.67E+06	6.89E+06	2.45E+07	2.20E+07	1.29E+07
L-Methionine	1.56E+07	1.02E+07	8.50E+06	4.63E+06	3.05E+06	3.19E+06
L-Lysine	4.10E+07	2.49E+07	1.77E+07	1.80E+07	1.54E+07	1.60E+07
Alanine	1.81E+07	1.16E+07	1.30E+07	1.07E+07	1.14E+07	1.39E+07
L-Tyrosine	1.25E+07	8.13E+06	6.99E+06	8.28E+06	7.77E+06	9.65E+06
L-Histidine	1.35E+07	8.27E+06	6.56E+06	8.58E+06	7.24E+06	7.54E+06
L-Tryptophan	4.35E+06	3.01E+06	2.70E+06	2.20E+06	1.96E+06	2.39E+06
Glycine	3.40E+06	2.29E+06	2.41E+06	1.78E+06	1.68E+06	2.26E+06
L-Proline	2.06E+07	1.43E+07	1.52E+07	1.68E+07	1.64E+07	1.82E+07
L-Serine	6.20E+06	4.14E+06	3.55E+06	2.17E+06	2.12E+06	2.62E+06
L-Asparagine	5.17E+06	3.75E+06	3.05E+06	3.41E+05	3.39E+05	4.06E+05
L-Aspartate	6.67E+06	5.55E+06	4.58E+06	1.01E+06	1.34E+06	1.39E+06
L-Threonine	7.95E+06	8.11E+06	8.44E+06	5.60E+06	7.57E+06	8.24E+06

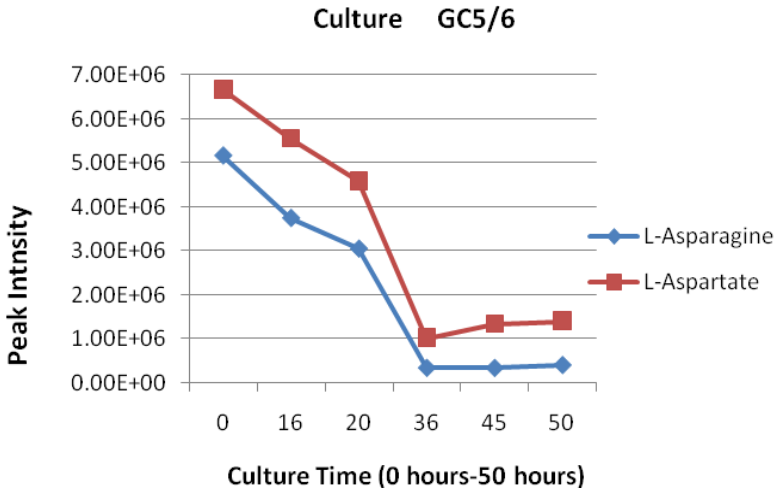
Table 6. Levels of aminoacids detected in the pellet from batches GC5-GC6 with time.

The same analytical procedure was followed since there was an effort to establish the amino acid pattern from batch to batch and follow amino acid consumption during the time of rapid growth until the culture had reached the starvation point which was the initiation of antibiotic production.

The graphs shown in figure 25 were plotted from the results obtained. There was no strong agreement with the previous batch of results since most of the amino acid levels remained constant during the time period studied.


Amino acids in *Streptomyces* from Batches GC5/6
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Peak Intensity
0
16
20
36
45
50
Culture Time (0hours-50hours)
L-Methionine
Alanine
L-Tyrosine
L-Histidine
L-Tryptophan
Glycine
L-Proline
L-Serine
L-Threonine
L-Serine


Culture GC5/6
8.00E+07
7.00E+07
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
Peak Intensity
0
16
20
36
45
50
Culture Time (0 hours-50 hours)
Glutamic Acid
Glutamine


Culture GC5/6
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
Peak Intnsity
0
16
20
36
45
50
Culture Time (0 hours-50 hours)
L-Asparagine
L-Aspartate

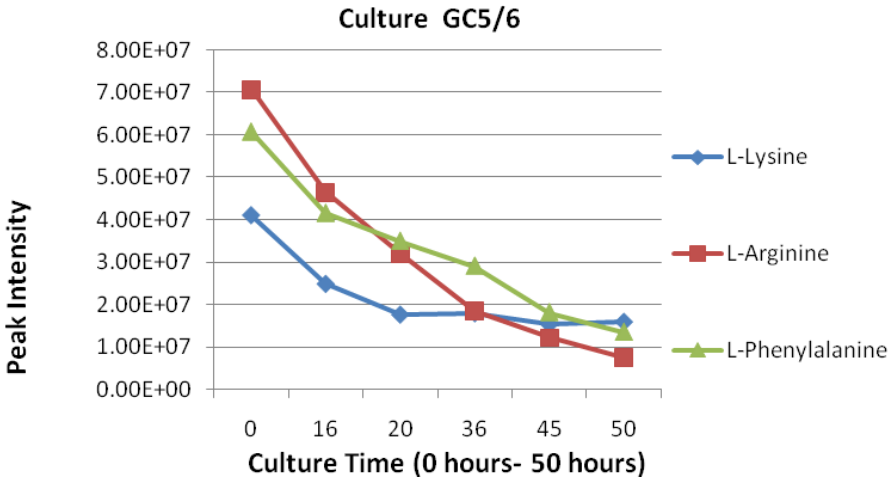


Fig. 25 Graphs of aminoacids from batches GC5 and 6 showing an inconsistent pattern of glutamine/glutamate and asparagine/aspartate utilisation.

Other than observing that amino acid utilization was inconsistent between the two batches the plots shown in figure 25 are difficult to rationalise.

The strains from batch GC7-GC8 were collected at the 0, 16, 20, 24, 28, 32, 45 and 50 hour time points and this was the case for all subsequent samples.

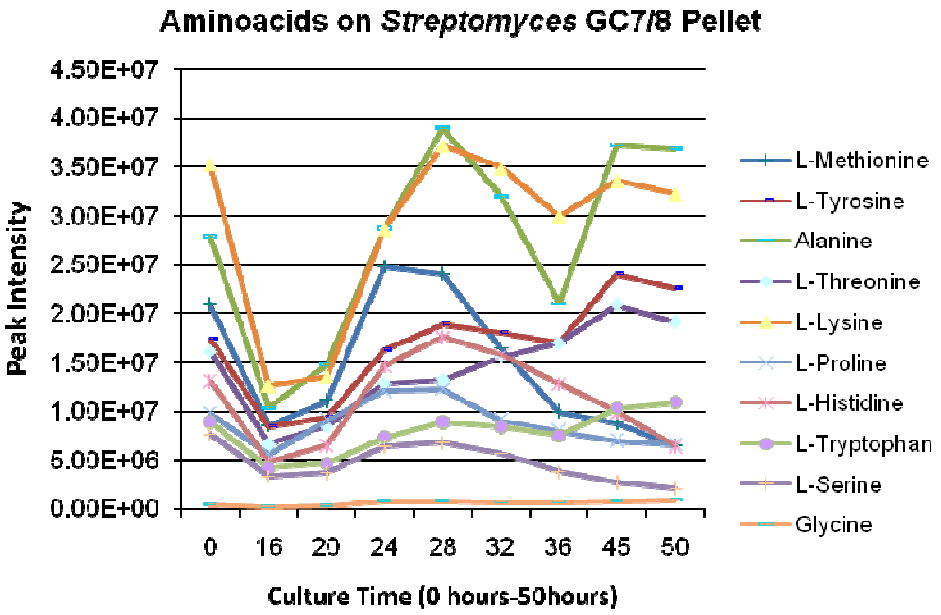
The samples were analysed using both positive and negative ion mode since Orbitrap Exactive provides the facility of switching between positive and negative mode during the analysis run time. Table 7 displays the amino acids that were detected in the samples and how they changed over time.

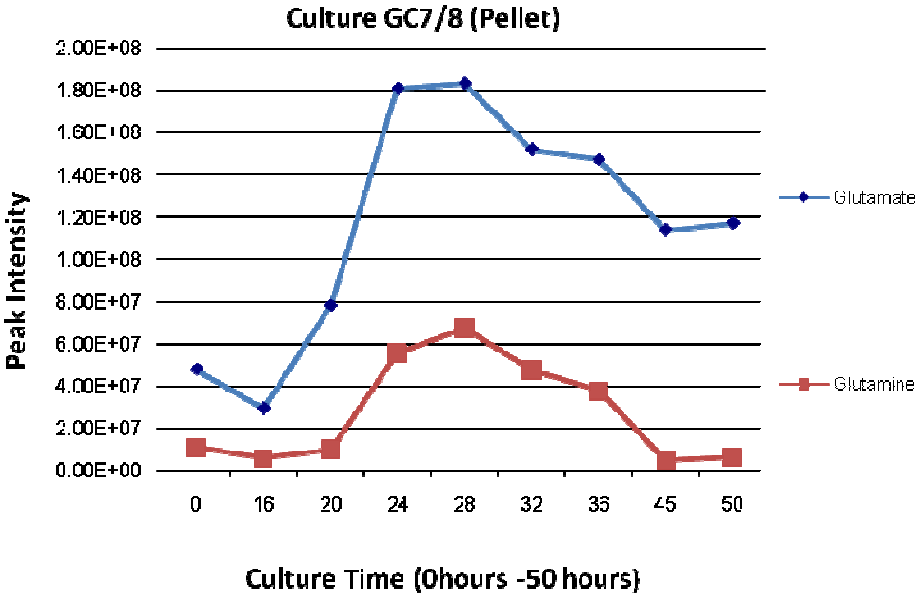
As has already been reported the data processing software is based on the comparison of a set of samples which are set as control versus a set of samples which are set as treatment. In this case the control samples were the 0 and 16 hours time points whereas all the rest were the treatment group. Table 7 shows the results for the amino acids in samples GC7 and GC8.

Amino acid	0	16	20	24	28	32	36	45	50
Glutamate	4.77E+07	2.93E+07	7.81E+07	1.81E+08	1.83E+08	1.52E+08	1.47E+08	1.14E+08	1.17E+08
L-Leucine	2.02E+08	9.46E+07	1.16E+08	1.95E+08	2.06E+08	1.76E+08	1.43E+08	1.33E+08	1.12E+08
Phenylalanine	1.04E+08	4.13E+07	4.99E+07	9.32E+07	1.11E+08	9.51E+07	7.67E+07	5.86E+07	4.44E+07
Glutamine	1.04E+07	5.26E+06	9.59E+06	5.52E+07	6.73E+07	4.71E+07	3.74E+07	4.34E+06	5.85E+06
L-Valine	7.35E+07	3.75E+07	6.32E+07	1.25E+08	1.32E+08	1.13E+08	8.75E+07	7.50E+07	7.80E+07
L-Arginine	6.80E+07	2.62E+07	2.52E+07	4.35E+07	4.50E+07	3.62E+07	2.71E+07	1.54E+07	6.46E+06
L-Methionine	2.09E+07	8.51E+06	1.11E+07	2.48E+07	2.41E+07	1.64E+07	9.94E+06	8.77E+06	6.45E+06
L-Tyrosine	1.74E+07	8.41E+06	9.38E+06	1.63E+07	1.88E+07	1.80E+07	1.70E+07	2.40E+07	2.26E+07
Alanine	2.78E+07	1.03E+07	1.47E+07	2.87E+07	3.89E+07	3.20E+07	2.10E+07	3.72E+07	3.68E+07
L-Threonine	1.61E+07	6.62E+06	8.41E+06	1.28E+07	1.32E+07	1.55E+07	1.69E+07	2.08E+07	1.91E+07
L-Aspartate	1.18E+07	5.31E+06	6.07E+06	1.61E+07	5.41E+06	2.26E+06	1.89E+06	1.56E+06	1.99E+06
L-Lysine	3.52E+07	1.25E+07	1.35E+07	2.86E+07	3.71E+07	3.49E+07	2.99E+07	3.36E+07	3.22E+07
L-Proline	9.74E+06	5.58E+06	9.07E+06	1.21E+07	1.22E+07	9.07E+06	7.96E+06	6.97E+06	6.53E+06
L-Histidine	1.31E+07	4.83E+06	6.54E+06	1.47E+07	1.76E+07	1.58E+07	1.28E+07	9.93E+06	6.42E+06
L-Tryptophan	8.88E+06	4.19E+06	4.66E+06	7.33E+06	8.86E+06	8.38E+06	7.49E+06	1.03E+07	1.08E+07
L-Serine	7.60E+06	3.30E+06	3.71E+06	6.36E+06	6.80E+06	5.62E+06	3.75E+06	2.73E+06	2.06E+06
L-Asparagine	7.52E+06	3.32E+06	3.71E+06	2.77E+06	1.43E+06	5.26E+05	3.75E+05	8.28E+05	1.22E+06
Glycine	4.26E+05	1.36E+05	2.45E+05	7.12E+05	7.40E+05	6.66E+05	5.87E+05	8.34E+05	8.99E+05

Table7. The levels of aminoacids detected in pellet extracts from batches GC7and GC8 at each time point.

When these results were plotted on an axis system of peak area versus time the following graphs shown in figure 26 were obtained:


Aminoacids on Streptomyces GC7/8 Pellet
4.50E+07
4.00E+07
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Peak Intensity
0
16
20
24
28
32
36
45
50
Culture Time (0 hours-50hours)
L-Methionine
L-Tyrosine
Alanine
L-Threonine
L-Lysine
L-Proline
L-Histidine
L-Tryptophan
L-Serine
Glycine


Culture GC7/8 (Pellet)
2.00E+08
1.80E+08
1.60E+08
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
Peak Intensity
0
16
20
24
28
32
35
45
50
Culture Time (0hours -50 hours)
Glutamate
Glutamine

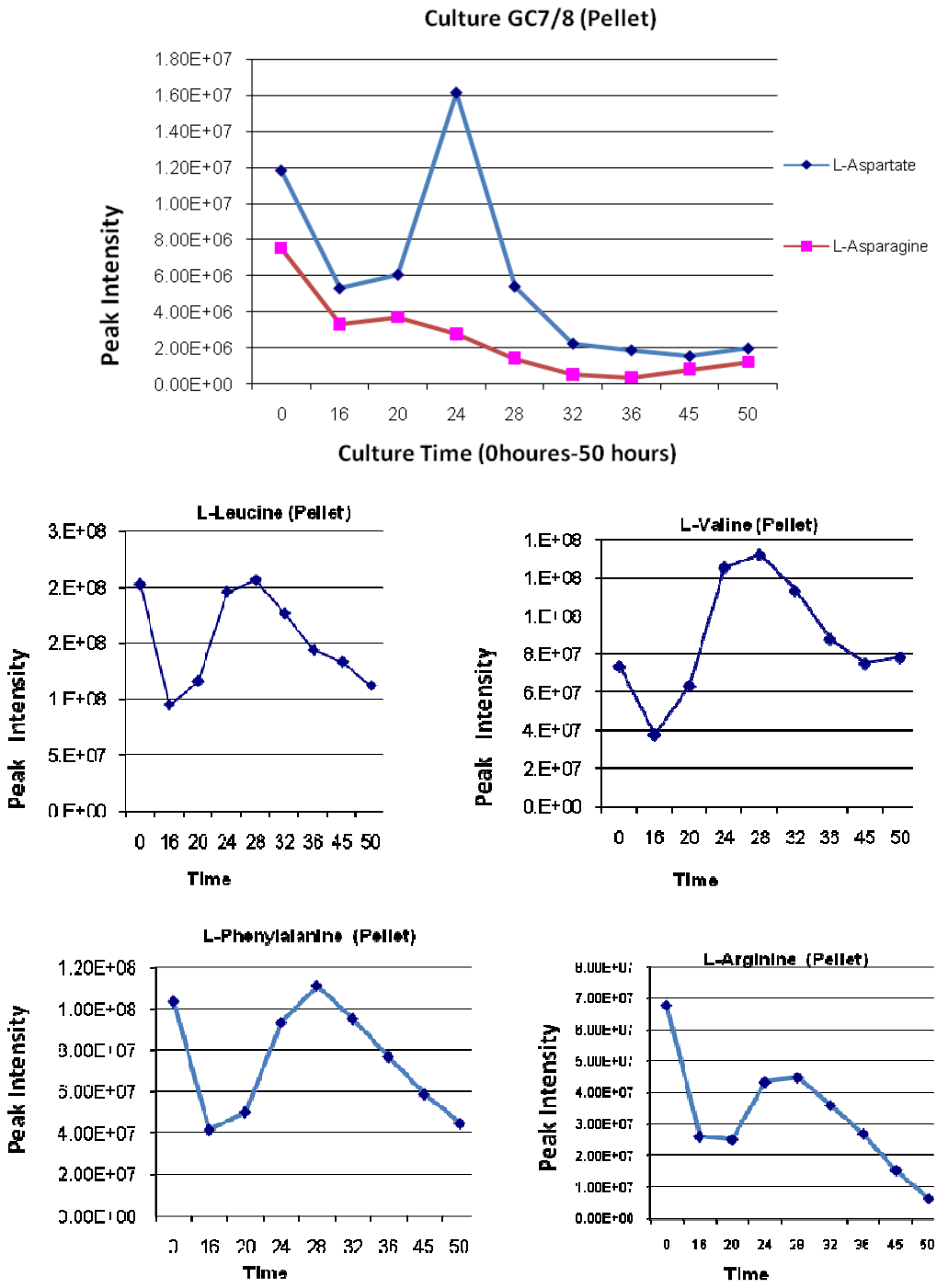


Fig. 26 Graphs of amino acid accumulation in batches GC7and GC8 with the smaller graphs indicating trends of some of the amino acids that show a large variation.

In addition the amino acids in the supernatant were monitored for batches GC7 and 8 in order to follow the consumption of them with time. The results are shown in table 8 and the corresponding plots in figure 27.

Amino acids(SP)	**0h**	**16h**	**20h**	**24h**	**28h**	**32h**	**36h**	**45h**	**50h**
L-Leucine	2.00E+08	2.04E+08	2.31E+08	1.88E+08	1.83E+08	1.55E+08	1.39E+08	1.20E+08	9.66E+07
L-Phenylalanine	1.03E+08	1.05E+08	1.20E+08	9.84E+07	9.96E+07	8.43E+07	7.33E+07	5.26E+07	3.82E+07
L-Valine	5.10E+07	4.97E+07	5.64E+07	5.22E+07	5.39E+07	5.19E+07	5.28E+07	5.58E+07	4.91E+07
Glutamate	4.51E+07	5.02E+07	5.71E+07	4.64E+07	2.34E+07	1.52E+06	8.36E+05	1.76E+06	1.91E+06
L-Methionine	1.96E+07	2.13E+07	2.46E+07	2.01E+07	1.85E+07	1.24E+07	7.92E+06	5.56E+06	4.06E+06
L-Tyrosine	1.74E+07	1.73E+07	1.82E+07	1.83E+07	1.95E+07	1.90E+07	1.87E+07	2.21E+07	2.10E+07
L-Threonine	1.48E+07	1.69E+07	1.93E+07	1.71E+07	1.60E+07	1.74E+07	2.04E+07	2.27E+07	2.04E+07
Alanine	2.65E+07	2.83E+07	3.39E+07	2.69E+07	2.56E+07	2.05E+07	1.78E+07	2.25E+07	2.03E+07
L-Lysine	3.53E+07	3.68E+07	4.23E+07	3.65E+07	3.91E+07	3.60E+07	3.55E+07	3.30E+07	3.09E+07
L-Aspartate	1.09E+07	1.27E+07	1.48E+07	1.14E+07	4.04E+06	1.30E+05	3.62E+04	5.73E+04	5.77E+04
L-Proline	1.13E+07	1.03E+07	1.19E+07	1.02E+07	1.00E+07	8.67E+06	7.77E+06	4.96E+06	4.40E+06
L-Histidine	1.27E+07	1.32E+07	1.61E+07	1.55E+07	1.59E+07	1.34E+07	1.24E+07	8.84E+06	5.06E+06
L-Tryptophan	8.85E+06	8.97E+06	9.57E+06	9.35E+06	9.23E+06	8.95E+06	8.58E+06	1.03E+07	1.03E+07
L-Asparagine	7.41E+06	7.52E+06	8.00E+06	3.89E+06	1.45E+06	3.18E+05	2.47E+05	6.61E+05	1.09E+06
L-Serine	7.39E+06	7.72E+06	8.67E+06	7.75E+06	7.33E+06	5.24E+06	3.26E+06	1.63E+06	1.40E+06
Gluatamine	1.01E+07	1.02E+07	8.99E+06	1.80E+06	4.73E+06	2.20E+06	9.55E+05	5.48E+05	4.03E+05
L-Arginine	4.42E+07	4.30E+07	4.79E+07	5.01E+07	4.39E+07	3.56E+07	3.20E+07	1.53E+07	5.75E+06
Glycine	4.01E+05	4.41E+05	5.64E+05	6.38E+05	6.58E+05	6.05E+05	6.05E+05	7.50E+05	7.28E+05

Table 8. Variation of aminoacids from the supernatant layer of batches GC7 and GC8 with time

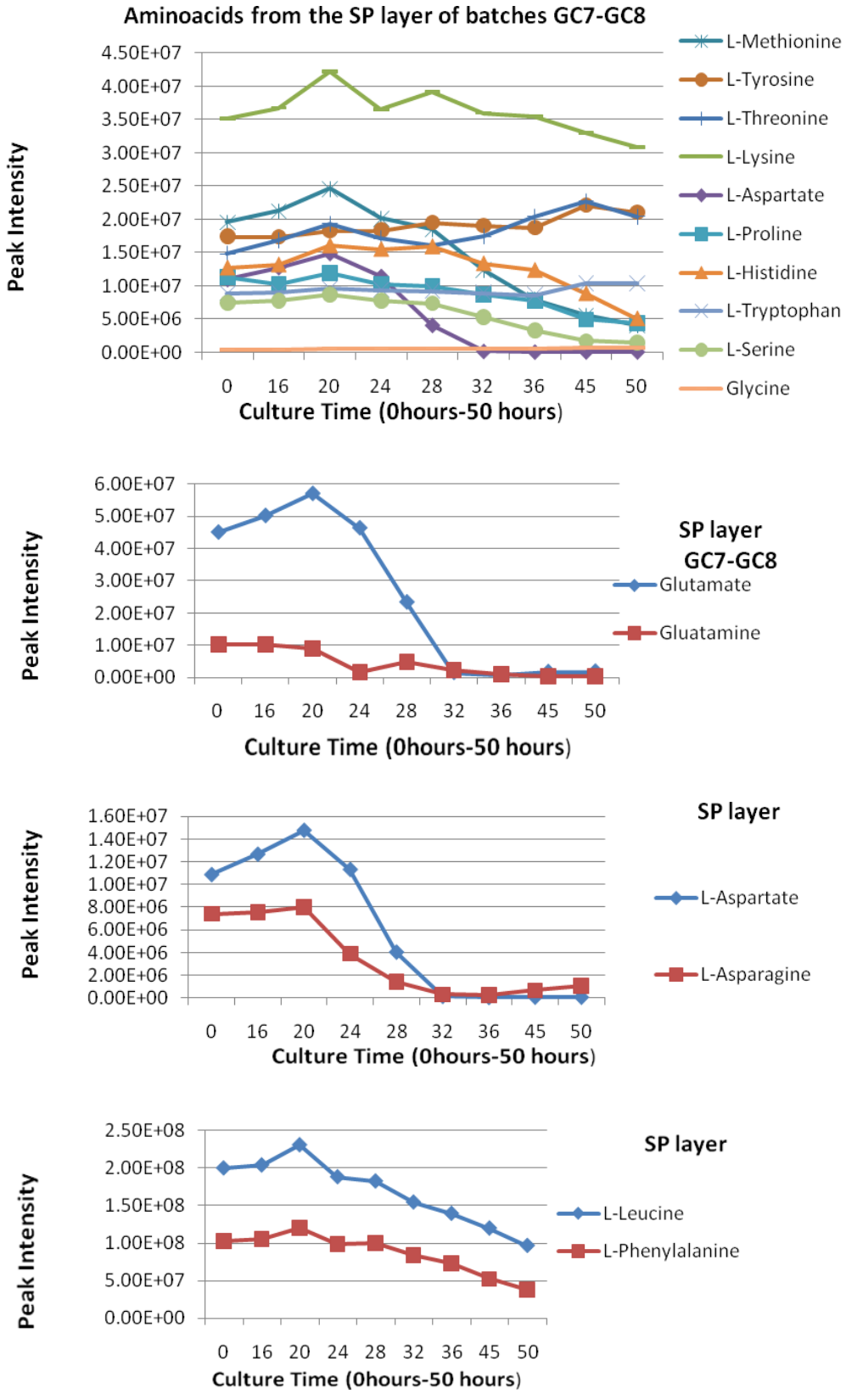


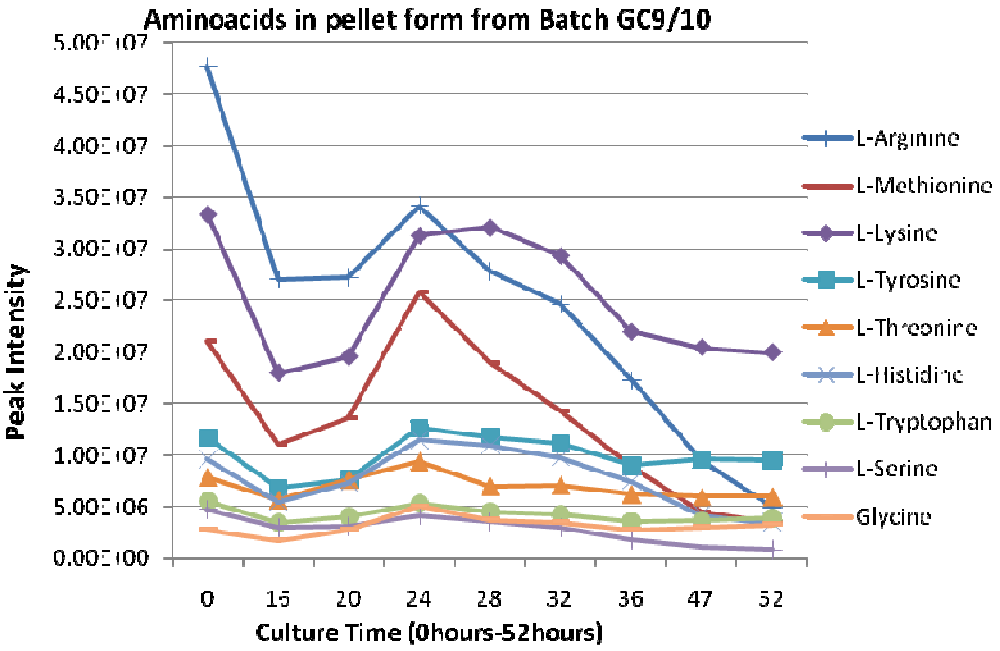
Fig.27. Variation in the levels of amino acid in the supernatant from batches GC7 and GC8

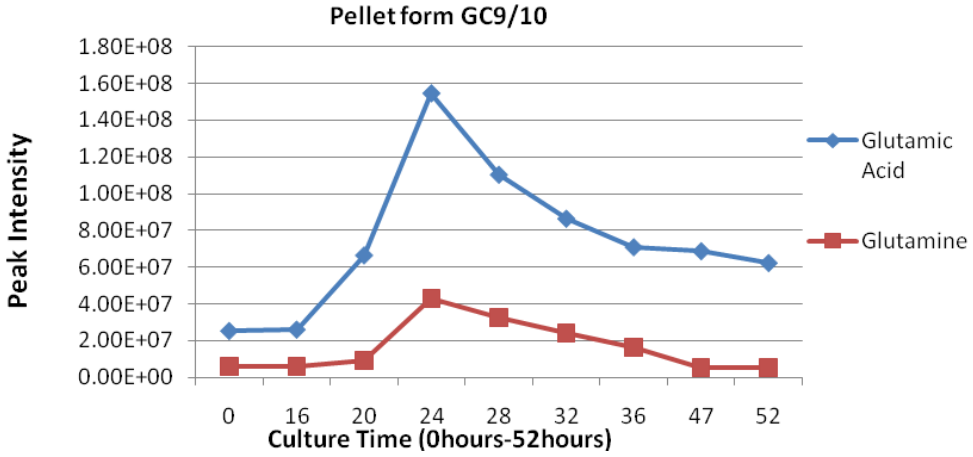
The samples that were collected for batches GC9-10 were collected at 0, 16, 20, 24 , 28 , 32 , 36 ,47 and 52 hours. The difference from the rest samples that were analysed was that the last two time points differed by two hours .

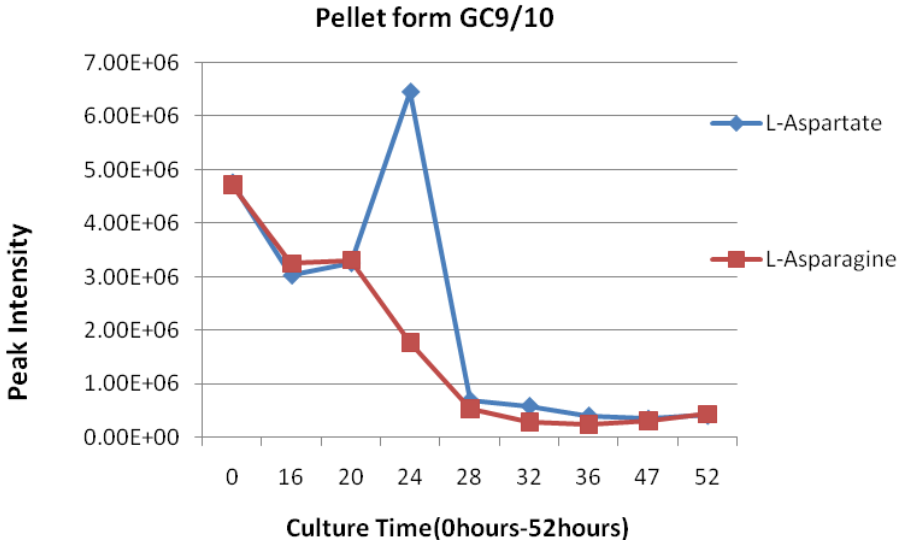
The usual procedure was followed and the results are shown in table 9 and the corresponding plots in figure 28.The amino acids in the supernatant were also monitored in this case and are shown in table 10 and the corresponding plots are shown in figures 29.

Amino acids	**0**	**16**	**20**	**24**	**28**	**32**	**36**	**47**
L-Valine	5.01E+07	3.62E+07	6.75E+07	1.37E+08	1.54E+08	1.19E+08	8.78E+07	6.97E+07
Glutamic Acid	2.50E+07	2.57E+07	6.62E+07	1.54E+08	1.10E+08	8.61E+07	7.06E+07	6.84E+07
L-Leucine	1.70E+08	9.87E+07	1.22E+08	1.93E+08	1.48E+08	1.27E+08	9.15E+07	5.23E+07
L-Phenylalanine	7.03E+07	3.78E+07	4.59E+07	7.71E+07	6.23E+07	5.22E+07	3.90E+07	2.17E+07
L-Proline	2.20E+07	1.26E+07	1.96E+07	4.09E+07	2.83E+07	2.43E+07	1.83E+07	1.42E+07
Glutamine	6.10E+06	5.78E+06	9.25E+06	4.27E+07	3.22E+07	2.39E+07	1.63E+07	5.28E+06
L-Arginine	4.76E+07	2.70E+07	2.72E+07	3.41E+07	2.78E+07	2.46E+07	1.73E+07	9.51E+06
L-Methionine	2.10E+07	1.10E+07	1.36E+07	2.58E+07	1.89E+07	1.42E+07	8.91E+06	4.46E+06
Alanine	3.02E+07	1.73E+07	2.50E+07	4.03E+07	3.60E+07	2.46E+07	1.75E+07	1.74E+07
L-Lysine	3.34E+07	1.79E+07	1.95E+07	3.13E+07	3.20E+07	2.93E+07	2.19E+07	2.04E+07
L-Tyrosine	1.17E+07	6.76E+06	7.63E+06	1.26E+07	1.17E+07	1.11E+07	8.92E+06	9.56E+06
L-Threonine	7.85E+06	5.66E+06	7.61E+06	9.35E+06	6.97E+06	7.07E+06	6.18E+06	5.95E+06
L-Histidine	9.55E+06	5.38E+06	7.25E+06	1.14E+07	1.09E+07	9.73E+06	7.37E+06	4.18E+06
L-Aspartate	4.76E+06	3.02E+06	3.26E+06	6.45E+06	6.96E+05	5.87E+05	4.06E+05	3.50E+05
L-Tryptophan	5.50E+06	3.42E+06	3.99E+06	5.32E+06	4.42E+06	4.19E+06	3.58E+06	3.69E+06
L-Serine	4.76E+06	2.98E+06	3.08E+06	4.16E+06	3.55E+06	2.96E+06	1.80E+06	1.15E+06
L-Asparagine	4.73E+06	3.25E+06	3.32E+06	1.77E+06	5.35E+05	2.84E+05	2.34E+05	3.02E+05
Glycine	2.82E+06	1.73E+06	2.81E+06	5.10E+06	3.73E+06	3.53E+06	2.77E+06	3.06E+06

Table 9 Levels of amino acids detected in pellets from batches GC9-GC10 with time


Aminoacids in pellet form from Batch GC9/10
Peak Intensity
5.00E+07
4.50E+07
4.00E+07
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
0
16
20
24
28
32
36
47
52
Culture Time (0hours-52hours)
L-Arginine
L-Methionine
L-Lysine
L-Tyrosine
L-Threonine
L-Histidine
L-Tryptophan
L-Serine
Glycine


Pellet form GC9/10
Peak Intensity
1.80E+08
1.60E+08
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
0
16
20
24
28
32
36
47
52
Culture Time (0hours-52hours)
Glutamic Acid
Glutamine


Pellet form GC9/10
Peak Intensity
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
0
16
20
24
28
32
36
47
52
Culture Time(0hours-52hours)
L-Aspartate
L-Asparagine

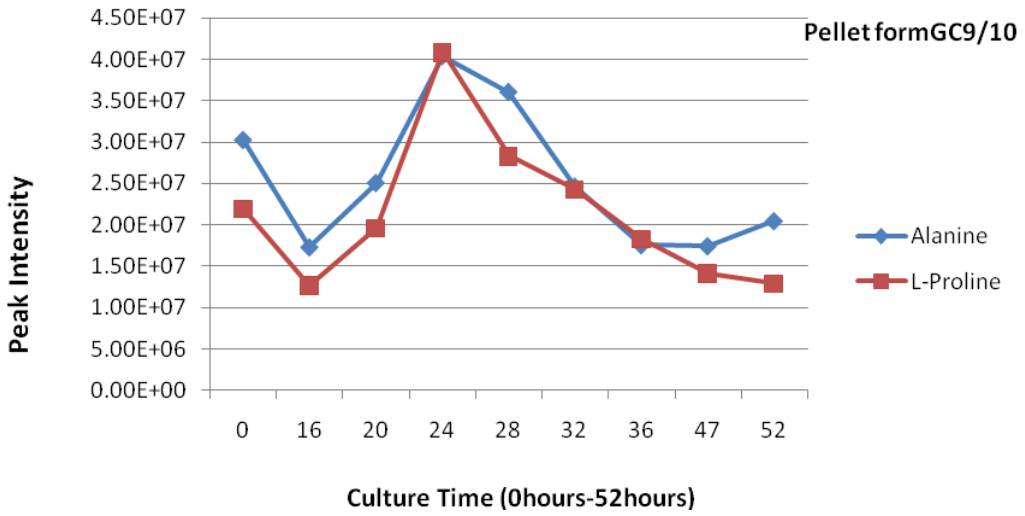
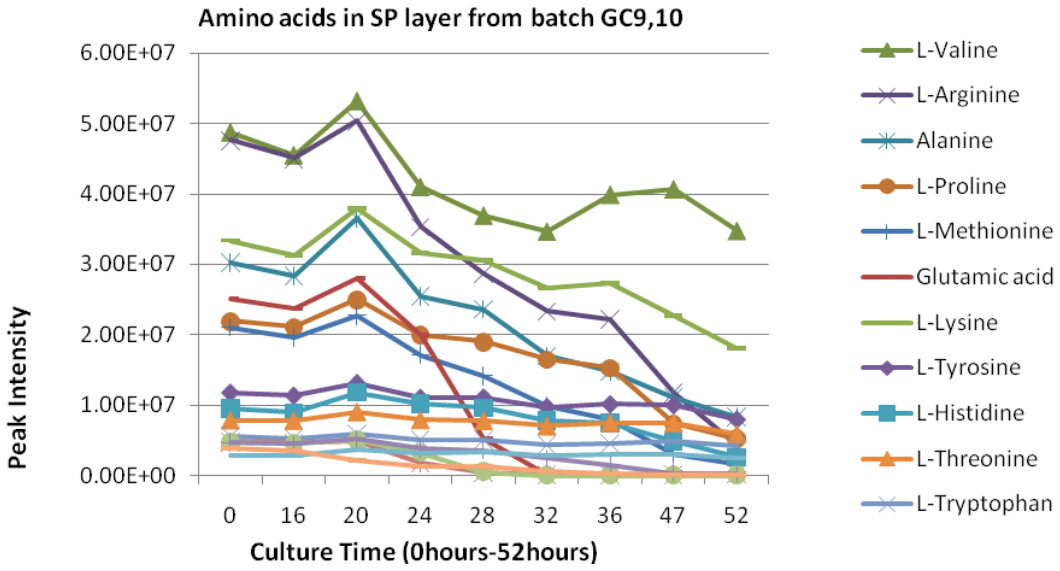


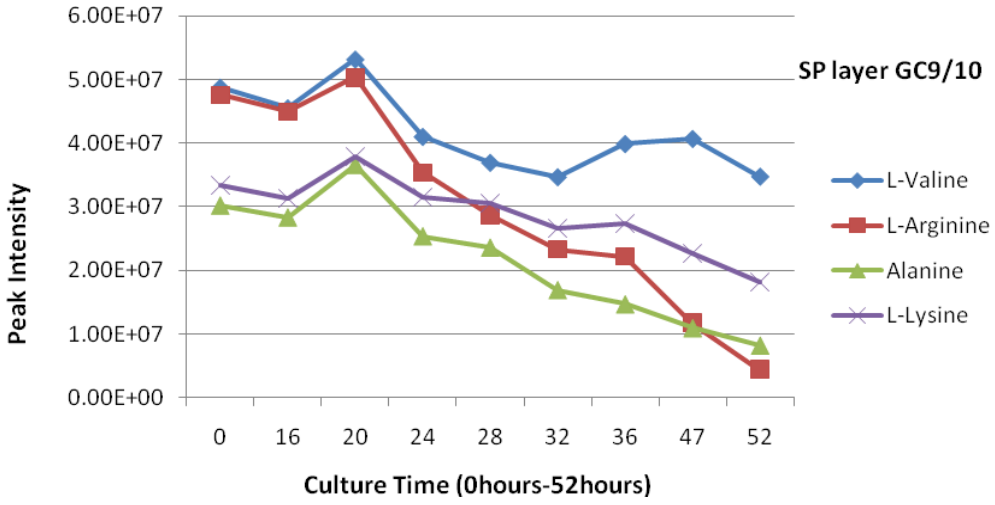
Fig.28 Variation in the levels of amino acid from pellet from batches GC9 and GC10

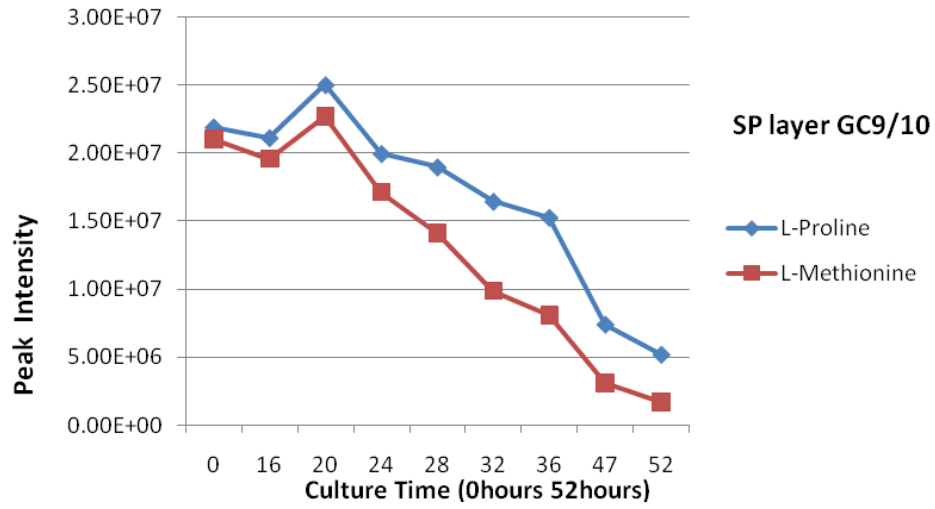
Amino acids	**0h**	**16h**	**20h**	**24h**	**28h**	**32h**	**36h**	**47h**	**52h**
L-Leucine	1.70E +08	1.71E +08	1.96E +08	1.47E +08	1.31E +08	1.04E +08	9.23E +07	5.39E +07	3.20E +07
L-Phenylalanine	7.04E +07	7.00E +07	8.16E +07	6.31E +07	6.02E +07	4.60E +07	4.34E +07	2.39E +07	1.25E +07
L-Valine	4.88E +07	4.55E +07	5.32E +07	4.10E +07	3.70E +07	3.47E +07	3.99E +07	4.07E +07	3.48E +07
L-Arginine	4.76E +07	4.50E +07	5.04E +07	3.53E +07	2.87E +07	2.33E +07	2.22E +07	1.17E +07	4.36E +06
Alanine	3.02E +07	2.84E +07	3.66E +07	2.54E +07	2.36E +07	1.70E +07	1.47E +07	1.10E +07	8.24E +06
L-Proline	2.19E +07	2.11E +07	2.50E +07	2.00E +07	1.90E +07	1.65E +07	1.53E +07	7.41E +06	5.19E +06
L-Methionine	2.10E +07	1.96E +07	2.27E +07	1.71E +07	1.41E +07	9.87E +06	8.10E +06	3.13E +06	1.69E +06
Glutamic acid	2.50E +07	2.37E +07	2.79E +07	1.99E +07	5.22E +06	1.83E +05	2.24E +05	3.51E +05	2.60E +05
L-Lysine	3.34E +07	3.12E +07	3.79E +07	3.15E +07	3.05E +07	2.66E +07	2.74E +07	2.27E +07	1.81E +07
L-Tyrosine	1.17E +07	1.13E +07	1.31E +07	1.10E +07	1.11E +07	9.71E +06	1.01E +07	9.98E +06	7.97E +06
L-Histidine	9.57E +06	8.95E +06	1.18E +07	1.02E +07	9.66E +06	7.84E +06	7.43E +06	4.80E +06	2.59E +06
L-Threonine	7.84E +06	7.79E +06	8.99E +06	7.88E +06	7.76E +06	6.99E +06	7.46E +06	7.41E +06	5.84E +06
L-Tryptophan	5.50E +06	5.30E +06	5.92E +06	5.11E +06	5.09E +06	4.44E +06	4.55E +06	4.95E +06	4.19E +06
L-Asparagine	4.75E +06	4.69E +06	4.81E +06	1.85E +06	3.86E +05	1.30E +05	1.36E +05	2.07E +05	3.40E +05
L-Aspartate	4.76E +06	4.58E +06	5.03E +06	3.23E +06	5.71E +05	1.24E +04	7.77E +03	6.54E +03	4.72E +03

L-Serine	4.76E +06	4.49E +06	5.18E +06	3.81E +06	3.58E +06	2.46E +06	1.50E +06	3.74E +05	3.19E +05
Glycine	2.82E +06	2.90E +06	3.74E +06	3.21E +06	3.33E +06	2.92E +06	3.12E +06	3.03E +06	2.51E +06
Glutamine	3.92E +06	3.66E +06	2.22E +06	1.25E +06	1.37E +06	6.49E +05	3.19E +05	1.06E +05	1.06E +05

Table 10.Levels of amino acid detected in the supernatant layer of batches GC9 and10







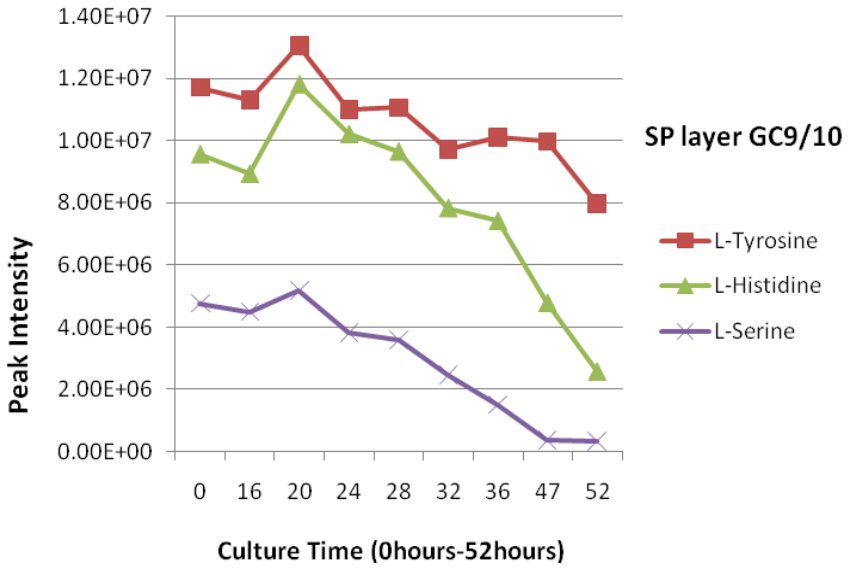


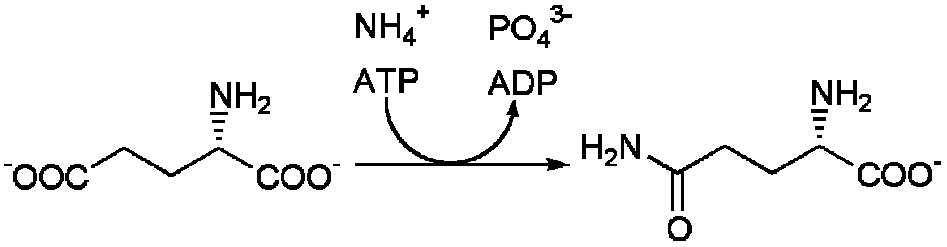
Fig. 29 Amino acid graphs of supernatant layer from batches CG 9 and GC10

3.3 Discussion of Results Obtained for Amino Acid Metabolism in *S.coelicolor*

From the results obtained it is obvious that amino acids were present in the pellet from the beginning of growing time and followed a specific pattern of accumulation which was repeatable in all batches that were analysed, except batches GC 5 and 6 . The amino acids in the medium incubated on its own did

not change appreciably over the time course of the experiments conducted in the presence of *S.coelicolor* .The amino acids reach a peak point at around 27 to 30 hours and then they are consumed to a varying extent. Amino acids are also present because of the culture medium which contains amino acids and peptides which are present in the peptone and yeast extract used in its preparation. The amino acids can be roughly classified into five groups from the patterns observed in the supernatant and cell pellet. Tyrosine, tryptophan, threonine, valine, alanine, lysine and glycine do not decrease markedly during the growth suggesting that the need for these amino acids is met by the culture medium and they are well in excess of the levels required by the cultures. Leucine, histidine and phenylalanine become somewhat depleted in the culture medium during the growth period and this is reflected in lower levels of the amino acids in the pellets. Serine, methionine, proline and arginine become more markedly depleted in either the culture medium or the cell pellet or both during the growth period and this suggests that their requirement by *S.coelicolor* is higher. Aspartate and aspargine become depleted in the culture medium and in the pellet. Glutamate and glutamine are depleted in the culture medium but in the case of glutamate levels not change to the same extent in the cell pellet. The effects on glutamate and glutamine are expected. Glutamate and glutamine are important for recycling ammonia and are the major nitrogen-pool for bacteria growth. All nitrogen metabolism is based on a recycling of ammonia (NH_3) in its neutral or charged form NH_4^+. Ammonia, however, is not a major form of nitrogen instead it has to be replenished to support the growth needs of the culture. Glutamine synthetases (GS), which convert glutamate into glutamine (figure 30) are key enzymes of nitrogen metabolism. Thus although the extracellular levels of glutamine and glutamate become greatly depleted with the

intracellular homeostasis requires that their levels are maintained within the cell pellet. Glutamine is responsible for assimilating ammonia which can then be used in a number of biosynthetic pathways including amino acid biosynthesis and purine biosynthesis. Thus as long as nitrogen is not a limiting nutrient glutamine and glutamate levels are likely to remain at the level required for the turnover of nitrogen.



glutamate glutamine

Fig. 30 Biosynthesis of glutamine from glutamate.

Out of all the amino acids it appears that asparagine and aspartate are limiting nutrients or at least that *S.coelicolor* has a high requirement for them. Asparagine and aspartate are both involved in a number of biosynthetic pathways. Asparagine is required for the biosynthesis of tetracyclines which are nonaketide antibiotics, however, *S.coelicolor* only makes octaketide antibiotics. Aspartate is used directly in *S.coelicolor* in the biosynthesis of ectoine since it initially is converted in L-aspartate-4-semihaldeyde and then it is converted in ectoine(KEGG, pathway map:00260) an osmolyte as part of its metabolic pathway and was detected in the current study in the cultures (table 3).

Of the other amino acids proline is biosynthetically derived from glutamate but also from ornithine ,derivatives of pimelic acid are involved in lysine biosynthesis moreover diaminopimelic acid which also is closely related to lysine, is a characteristic of some cell walls in bacteria and has also been detected in the

cultures(table 3). Methionine is a key intermediate required for many methylation reactions within the cell and participates in phosphatidyl choline lipid biosynthesis and purine and pyrimidine biosynthesis. A major interest in *S.coelicolor* biosynthesis is in the effects of nitrogen limitation since nitrogen depletion triggers off actinorhodin production. Most bacteria contain only one type of glutamine synthetase (GS) enzyme which is encoded by the gene *glnA*. *S. coelicolor*, however is characterized by two functional glutamine synthetases, the *glnA* and the *glnII* which metabolism is mediated by transcriptional and post-translational regulation systems.(Reuther and Wohlleben 2007).

Thus it was of interest to observe the effect of knocking out these genes on the metabolic profile of *S.coelicolor* with time.

3.4 The Effects of Glutamine Synthetase knockouts on *S.coelicolor* Metabolic Profile

The mutant strains that were analysed were the batches GC13-14 and the GC15-16 which had the *glnA* and the *glnII* genes removed respectively.

The samples once more were analysed under the same conditions for both the SP layer and the pellet and in order to be able to exhibit a straightforward comparison of any pattern differences or any alterations in the behavior of their metabolic profile ,they were analysed simultaneously with the GC7-8 batch which was the original bacterial strain.The following tables which were recorded with the results obtained after sample analysis and data processing, are from the analysis of each one mutant stain independently and together with the normal strain. Table 11 shows the results obtained for GC13/14 and figure 31 shows the corresponding plots. Table 12 shows the results obtained for GC15/16 and figure 32 shows the corresponding plots. The corresponding data

for the supernatants is shown in tables 13 and 14 and figures 33 and 34.

Amino acids Mut13,14	0 hours	16 hours	20 hours	24 hours	28 hours	32 hours	36 hours	47 hours	52 hours
L-Leucine	1.80E+08	1.02E+08	8.29E+07	1.17E+08	1.72E+08	1.63E+08	1.84E+08	1.42E+08	1.48E+08
L-Valine	2.69E+07	1.46E+07	1.46E+07	3.74E+07	4.92E+07	7.81E+07	5.82E+07	3.27E+07	3.65E+07
L-Phenylalanine	7.74E+07	4.06E+07	3.15E+07	4.15E+07	6.68E+07	6.36E+07	6.23E+07	5.25E+07	4.51E+07
Glutamic Acid	1.93E+07	1.45E+07	1.92E+07	4.72E+07	5.95E+07	6.63E+07	6.29E+07	6.03E+07	6.10E+07
Alanine	1.94E+07	1.13E+07	9.78E+06	1.80E+07	3.07E+07	3.15E+07	2.39E+07	2.41E+07	2.22E+07
L-Proline	1.40E+07	7.06E+06	6.07E+06	1.20E+07	2.31E+07	2.37E+07	2.05E+07	2.00E+07	2.08E+07
L-Arginine	3.30E+07	1.59E+07	1.07E+07	1.37E+07	2.05E+07	2.05E+07	2.18E+07	1.72E+07	1.48E+07
Glutamine	4.46E+06	2.69E+06	2.36E+06	3.89E+06	1.01E+07	1.65E+07	1.28E+07	7.13E+06	7.07E+06
L-Lysine	2.75E+07	1.20E+07	8.76E+06	1.13E+07	1.71E+07	1.94E+07	2.41E+07	2.77E+07	2.57E+07
L-Methionine	9.61E+06	4.98E+06	4.03E+06	6.02E+06	9.00E+06	7.15E+06	6.07E+06	4.60E+06	3.63E+06
L-Threonine	8.01E+06	4.48E+06	3.58E+06	4.90E+06	5.72E+06	6.52E+06	7.04E+06	7.93E+06	7.50E+06
L-Tryptophan	5.33E+06	3.06E+06	2.60E+06	3.18E+06	3.14E+06	3.01E+06	3.50E+06	3.17E+06	4.00E+06
L-Histidine	6.98E+06	3.39E+06	2.34E+06	3.94E+06	5.69E+06	6.26E+06	6.32E+06	6.33E+06	5.55E+06
L-Aspartate	3.83E+06	2.47E+06	1.97E+06	2.60E+06	4.26E+06	3.50E+06	2.40E+06	1.14E+06	6.45E+05
L-Serine	4.20E+06	2.31E+06	1.88E+06	2.52E+06	3.95E+06	3.90E+06	4.40E+06	4.16E+06	3.61E+06
L-Asparagine	3.55E+06	2.04E+06	1.64E+06	2.19E+06	2.59E+06	1.82E+06	1.23E+06	4.33E+05	3.42E+05
Glycine	1.78E+06	8.71E+05	8.45E+05	1.50E+06	2.76E+06	2.67E+06	2.72E+06	3.24E+06	3.32E+06

Table11. Amino acid in cell pellets from the mutant batches GC13 and GC14

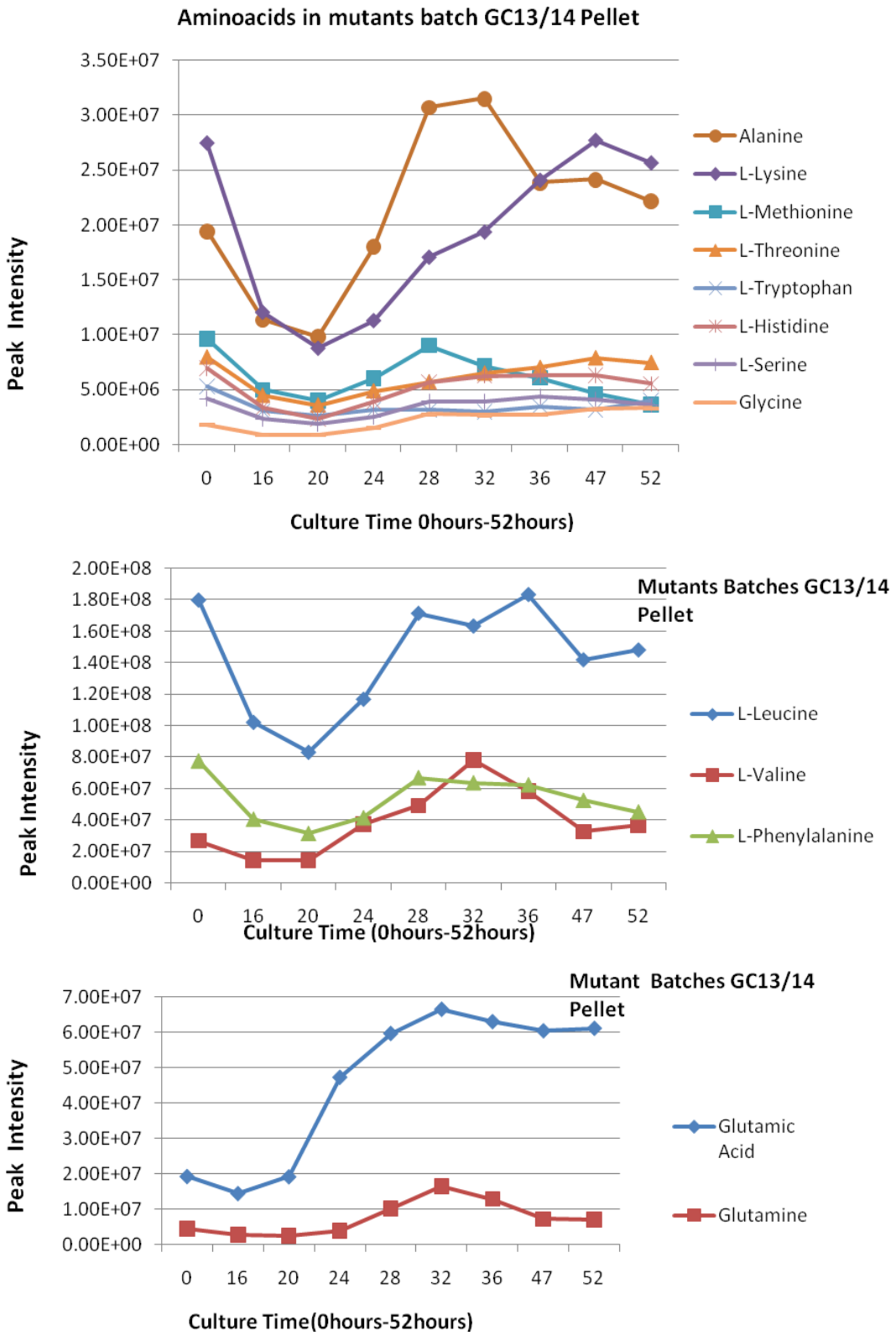
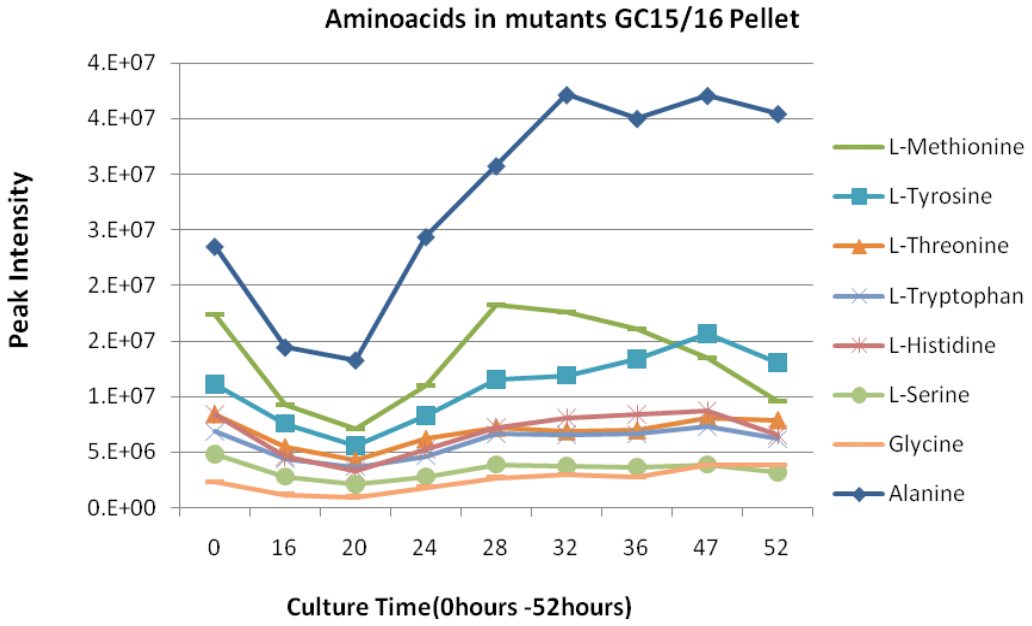
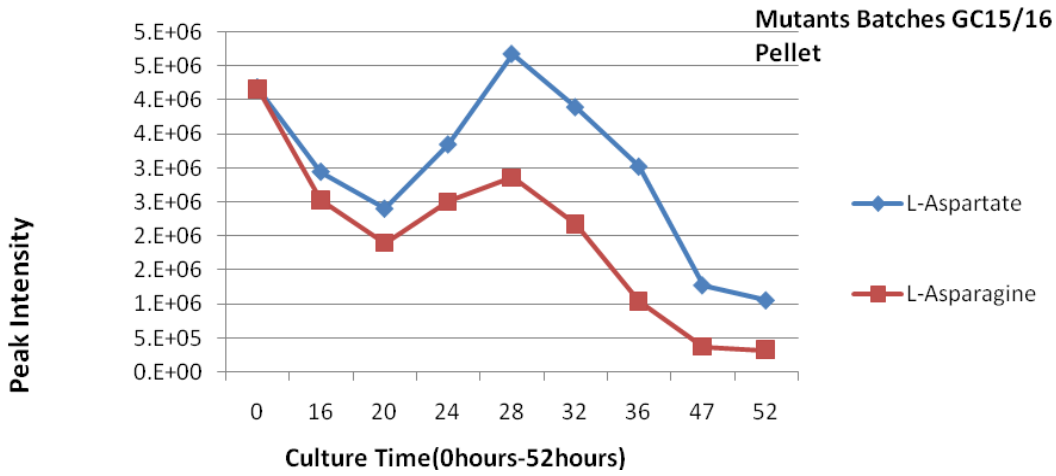


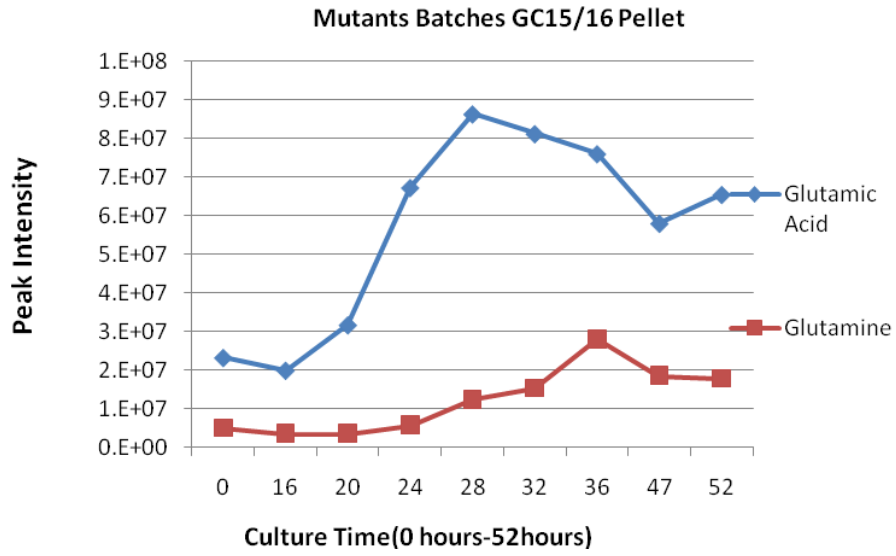
Fig.31 Graph of the amino acid in cell pellets detected in the mutant batches GC13 and14

Aminoacids Mut15,16	**0 hours**	**16 hours**	**20 hours**	**24 hours**	**28 hours**	**32 hours**	**36 hours**	**47 hours**	**52 hours**
L-Leucine	2.12E +08	1.26E +08	1.03E +08	1.47E +08	2.07E +08	1.92E +08	1.91E +08	1.86E +08	1.51E +08
L-Valine	2.93E +07	2.03E +07	2.56E +07	5.88E +07	9.34E +07	9.88E +07	8.90E +07	5.54E +07	5.28E +07
Glutamic Acid	2.31E +07	1.98E +07	3.16E +07	6.72E +07	8.62E +07	8.11E +07	7.59E +07	5.79E +07	6.54E +07
L-Phenylalanine	8.52E +07	4.98E +07	3.71E +07	5.68E +07	7.45E +07	7.65E +07	7.42E +07	7.19E +07	6.28E +07
L-Proline	1.40E +07	8.60E +06	7.83E +06	1.77E +07	2.59E +07	3.37E +07	3.28E +07	3.45E +07	2.83E +07
Alanine	2.35E +07	1.45E +07	1.33E +07	2.44E +07	3.08E +07	3.72E +07	3.50E +07	3.71E +07	3.54E +07
Glutamin	4.99E +06	3.62E +06	3.50E +06	5.79E +06	1.24E +07	1.53E +07	2.81E +07	1.86E +07	1.79E +07
L-Arginine	3.67E +07	1.95E +07	1.27E +07	1.66E +07	2.44E +07	2.20E +07	2.15E +07	2.16E +07	1.36E +07
L-Methionine	1.74E +07	9.24E +06	7.12E +06	1.10E +07	1.82E +07	1.76E +07	1.60E +07	1.35E +07	9.55E +06
L-Lysine	2.87E +07	1.56E +07	1.05E +07	1.43E +07	2.13E +07	2.05E +07	2.45E +07	3.12E +07	2.67E +07
L-Tyrosine	1.11E +07	7.57E +06	5.57E +06	8.30E +06	1.15E +07	1.19E +07	1.34E +07	1.56E +07	1.30E +07
L-Threonine	8.44E +06	5.45E +06	4.23E +06	6.20E +06	7.22E +06	6.88E +06	6.97E +06	8.15E +06	7.90E +06
L-Tryptophan	6.81E +06	4.37E +06	3.67E +06	4.62E +06	6.65E +06	6.56E +06	6.64E +06	7.26E +06	6.19E +06
L-Histidine	8.40E +06	4.56E +06	3.30E +06	5.27E +06	7.27E +06	8.08E +06	8.42E +06	8.74E +06	6.55E +06
L-Serine	4.86E +06	2.82E +06	2.11E +06	2.82E +06	3.91E +06	3.78E +06	3.68E +06	3.92E +06	3.24E +06
L-Aspartate	4.19E +06	2.95E +06	2.40E +06	3.35E +06	4.68E +06	3.89E +06	3.02E +06	1.28E +06	1.06E +06
L-Asparagine	4.17E +06	2.53E +06	1.90E +06	2.50E +06	2.86E +06	2.18E +06	1.04E +06	3.71E +05	3.23E +05
Glycine	2.30E +06	1.18E +06	9.67E +05	1.84E +06	2.68E +06	2.95E +06	2.75E +06	3.81E +06	3.77E +06

Table12. Levels of the amino acid from cell pellets of the mutant batches GC15 and 16


Aminoacids in mutants GC15/16 Pellet
Peak Intensity
4.E+07
4.E+07
3.E+07
3.E+07
2.E+07
2.E+07
1.E+07
5.E+06
0.E+00
0
16
20
24
28
32
36
47
52
Culture Time(0hours -52hours)
L-Methionine
L-Tyrosine
L-Threonine
L-Tryptophan
L-Histidine
L-Serine
Glycine
Alanine


Mutants Batches GC15/16 Pellet
Peak Intensity
5.E+06
5.E+06
4.E+06
4.E+06
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
0
16
20
24
28
32
36
47
52
Culture Time(0hours-52hours)
L-Aspartate
L-Asparagine


Mutants Batches GC15/16 Pellet
Peak Intensity
1.E+08
9.E+07
8.E+07
7.E+07
6.E+07
5.E+07
4.E+07
3.E+07
2.E+07
1.E+07
0.E+00
0
16
20
24
28
32
36
47
52
Culture Time(0 hours-52hours)
Glutamic Acid
Glutamine

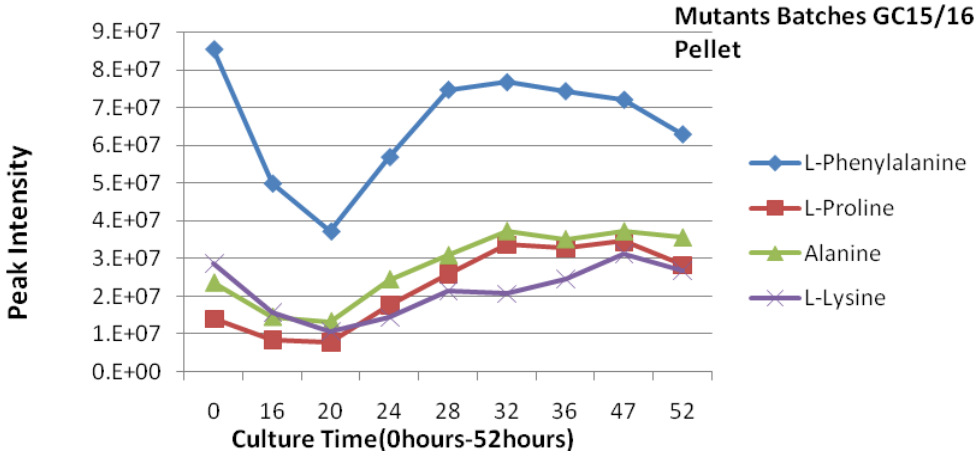


Fig.32 Graph of the aminoacids detected in cell pellets of the mutant batches GC15 and GC16

Amino acids SP mut13,14	**0**	**16**	**20**	**24**	**28**	**32**	**36**	**47**	**52**
L-Leucine	4.69E +08	4.74E +08	4.56E +08	4.93E +08	4.74E +08	4.79E +08	5.36E +08	4.66E +08	4.38E +08
L-Valine	1.20E +08	1.26E +08	1.27E +08	1.32E +08	1.30E +08	1.42E +08	1.41E +08	1.66E +08	1.46E +08
L-Phenylalanine	1.73E +08	1.78E +08	1.73E +08	1.90E +08	1.79E +08	1.89E +08	2.02E +08	1.51E +08	1.38E +08
L-Arginine	1.31E +08	1.31E +08	1.29E +08	1.30E +08	1.16E +08	1.13E +08	1.11E +08	9.29E +07	7.49E +07
Alanine	6.61E +07	6.93E +07	6.83E +07	6.89E +07	6.28E +07	7.07E +07	7.95E +07	7.75E +07	7.37E +07
L-Lysine	1.11E +08	1.17E +08	1.09E +08	1.17E +08	1.14E +08	1.21E +08	1.29E +08	1.22E +08	1.24E +08
L-Proline	4.77E +07	4.92E +07	4.61E +07	4.92E +07	4.85E +07	5.18E +07	5.47E +07	5.73E +07	5.84E +07
Glutamic Acid	5.11E +07	5.35E +07	5.20E +07	5.18E +07	4.78E +07	4.54E +07	4.25E +07	1.90E +06	1.19E +06
L-Methionine	4.56E +07	2.93E +07	2.88E +07	3.03E +07	2.10E +07	2.01E +07	1.98E +07	1.29E +07	1.09E +07
L-Tyrosine	2.44E +07	2.53E +07	2.50E +07	2.64E +07	2.45E +07	2.79E +07	2.73E +07	2.86E +07	2.88E +07
L-Threonine	1.75E +07	1.71E +07	1.78E +07	1.84E +07	1.91E +07	1.92E +07	2.40E +07	2.59E +07	2.93E +07
L-Histidine	2.28E +07	2.09E +07	2.06E +07	2.27E +07	1.95E +07	2.14E +07	2.28E +07	2.00E +07	1.98E +07
L-Tryptophan	1.28E +07	1.13E +07	1.07E +07	1.10E +07	8.15E +06	9.03E +06	9.03E +06	9.02E +06	9.52E +06
L-Serine	1.38E +07	1.42E +07	1.38E +07	1.47E +07	1.29E +07	1.33E +07	1.53E +07	1.36E +07	1.35E +07
Glycine	8.71E +06	8.33E +06	8.14E +06	9.55E +06	9.78E +06	1.15E +07	1.30E +07	1.39E +07	1.65E +07
L-Asparagine	1.24E +07	1.25E +07	1.17E +07	1.22E +07	7.12E +06	5.05E +06	3.72E +06	1.18E +06	8.27E +05
L-Aspartate	1.15E +07	1.10E +07	1.06E +07	1.06E +07	9.92E +06	9.32E +06	6.96E +06	9.95E +04	1.12E +04
Glutamine	8.86E +06	8.43E +06	8.13E +06	5.71E +06	7.04E +05	1.45E +06	3.74E +06	4.42E +06	2.06E +06

Table13. Amino acid from the supernatant layer of the mutant batches GC13/14

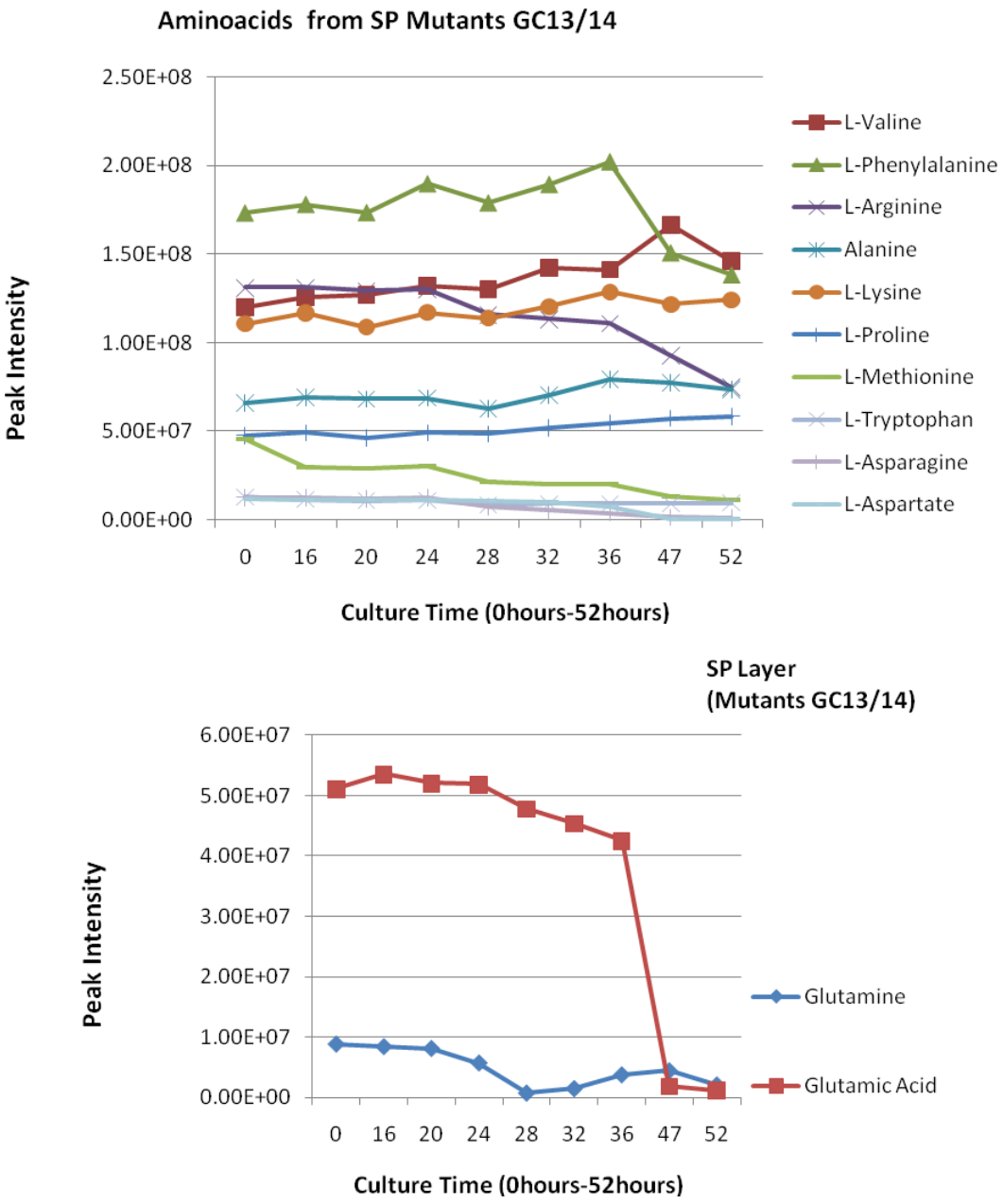
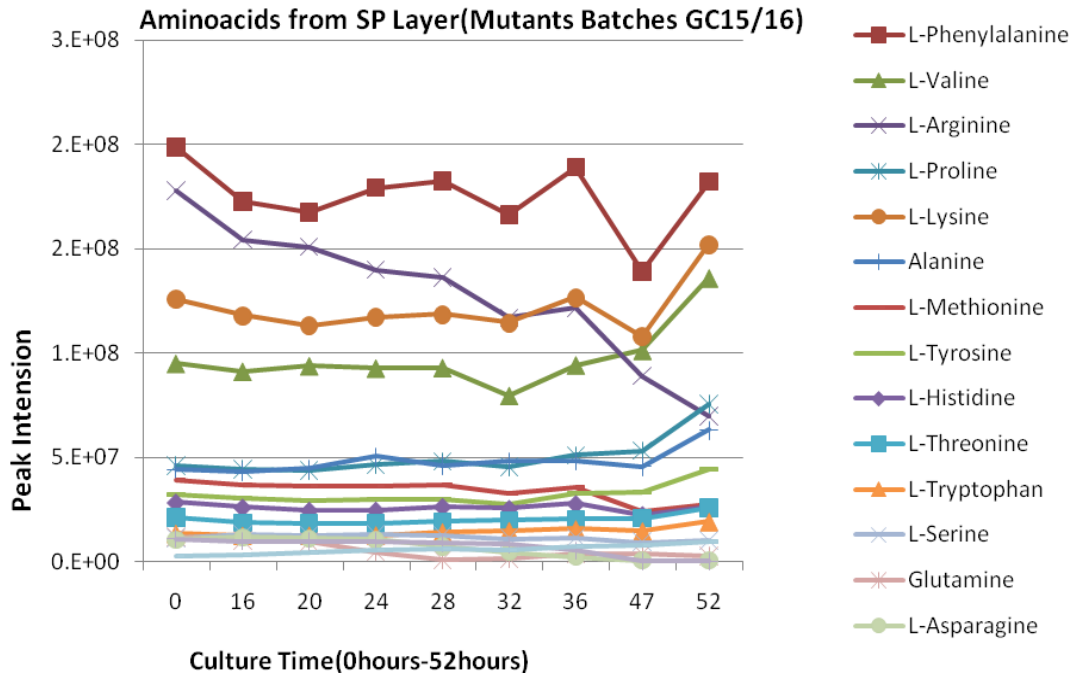
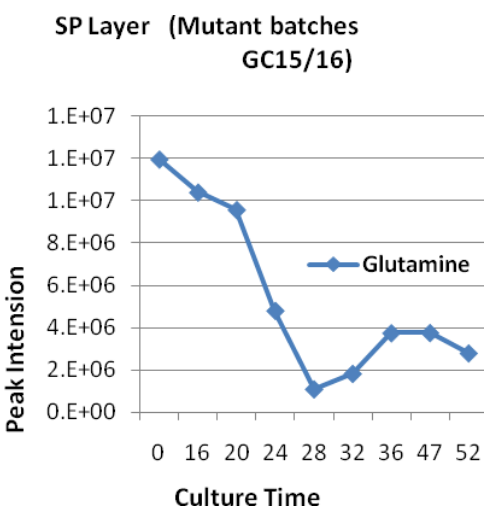


Fig.33 Graphs of amino acid from SP layer of the mutant batch GC13/14

Amino acids SP 15,16	0 hours	16 hours	20 hours	24 hours	28 hours	32 hours	36 hours	47 hours	52 hours
L-Leucine	4.13E +08	3.93E +08	3.99E +08	4.10E +08	4.17E +08	4.06E +08	3.98E +08	3.17E +08	3.85E +08
L-Phenylalanine	1.99E +08	1.72E +08	1.67E +08	1.79E +08	1.82E +08	1.66E +08	1.89E +08	1.39E +08	1.82E +08
L-Valine	9.50E +07	9.13E +07	9.39E +07	9.28E +07	9.31E +07	7.97E +07	9.43E +07	1.01E +08	1.36E +08
L-Arginine	1.78E +08	1.54E +08	1.51E +08	1.40E +08	1.36E +08	1.17E +08	1.22E +08	8.90E +07	6.95E +07
L-Proline	4.59E +07	4.42E +07	4.34E +07	4.61E +07	4.78E +07	4.54E +07	5.09E +07	5.30E +07	7.55E +07
L-Lysine	1.26E +08	1.18E +08	1.13E +08	1.17E +08	1.19E +08	1.15E +08	1.27E +08	1.08E +08	1.52E +08
Alanine	4.42E +07	4.31E +07	4.47E +07	5.03E +07	4.60E +07	4.81E +07	4.81E +07	4.56E +07	6.31E +07
L-Methionine	3.92E +07	3.68E +07	3.62E +07	3.60E +07	3.67E +07	3.25E +07	3.54E +07	2.40E +07	2.72E +07
L-Tyrosine	3.19E +07	3.02E +07	2.95E +07	2.99E +07	2.99E +07	2.75E +07	3.23E +07	3.32E +07	4.40E +07
L-Histidine	2.84E +07	2.60E +07	2.46E +07	2.46E +07	2.64E +07	2.54E +07	2.77E +07	2.25E +07	2.61E +07
L-Threonine	2.12E +07	1.86E +07	1.82E +07	1.81E +07	1.91E +07	1.98E +07	2.03E +07	2.08E +07	2.59E +07
L-Tryptophan	1.35E +07	1.25E +07	1.27E +07	1.28E +07	1.42E +07	1.48E +07	1.56E +07	1.46E +07	1.92E +07
L-Serine	1.12E +07	1.28E +07	1.23E +07	1.30E +07	1.23E +07	1.10E +07	1.15E +07	9.24E +06	1.01E +07
Glutamine	1.20E +07	1.04E +07	9.57E +06	4.80E +06	1.09E +06	1.82E +06	3.75E +06	3.76E +06	2.79E +06
L-Asparagine	1.08E +07	1.17E +07	1.13E +07	1.05E +07	7.37E +06	4.60E +06	2.52E +06	6.19E +05	6.80E +05
L-Aspartate	1.09E +07	9.93E +06	9.74E +06	9.56E +06	9.11E +06	8.26E +06	5.31E +06	9.03E +04	9.86E +04
Glycine	2.47E +06	3.29E +06	4.32E +06	5.53E +06	5.73E +06	5.21E +06	6.83E +06	7.58E +06	9.35E +06

Table14. Amino acid from the supernatant layer of the mutant batches GC15/16





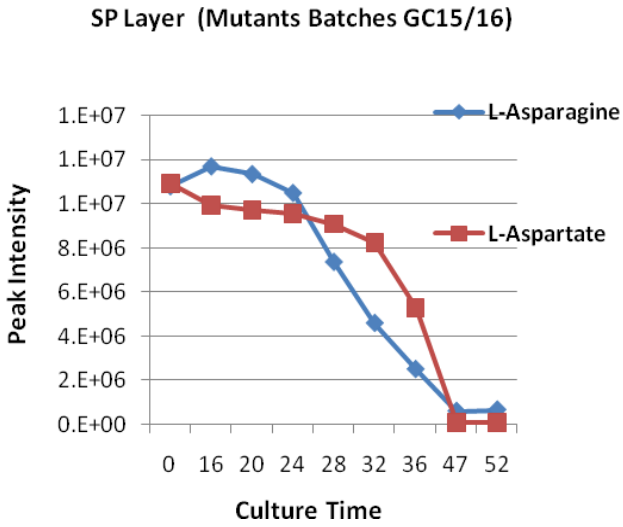


Fig.34 Amino acid of the SP layer of the batches GC15 and 16

3.5 Discussion of the results obtained for the glutamine synthetase gene knockout strains of *S.coelicolor*

The GS-GOGAT pathway is governed by the following reactions:

NH_3 + glutamate + ATP ---> glutamine + ADP + Pi

glutamine + 2-oxoglutarate + NADPH + H^+ ---> 2 glutamate + $NADP^+$

The GS-GOGAT pathway is essential for glutamate synthesis at low ammonium concentrations and for regulation of the glutamine pool, and is used when the cell is not under energy limitation (Hellig1998) since energetically it is of high cost as it consumes 1mole of ATP.

The two strains that had their genes for glutamine synthetase removed exhibited very similar profiles after data processing .The graphical representations of glutamine and glutamate in both batches were very similar both in curve shape and in the time points at which the highest concentration was achieved for them. However, it can be seen in the comparisons made in figure 35 that the intracellular levels of glutamine and glutamate rise towards a plateau level at around 24 hours at which the levels of glutamine and glutamate are about half those in the WT cultures. In comparison in the wild type cultures the levels of glutamate reach a maximum at about 28 hours and then fall to a large extent thereafter. Thus it almost looks as if the GS activity in the mutant cultures is operating at about less than half the efficiency of that in the WT cultures.

However, by the end of the growth period the WT levels of glutamine have fallen to around or below those in the mutant cultures and the levels of glutamate are strongly trending in that direction.

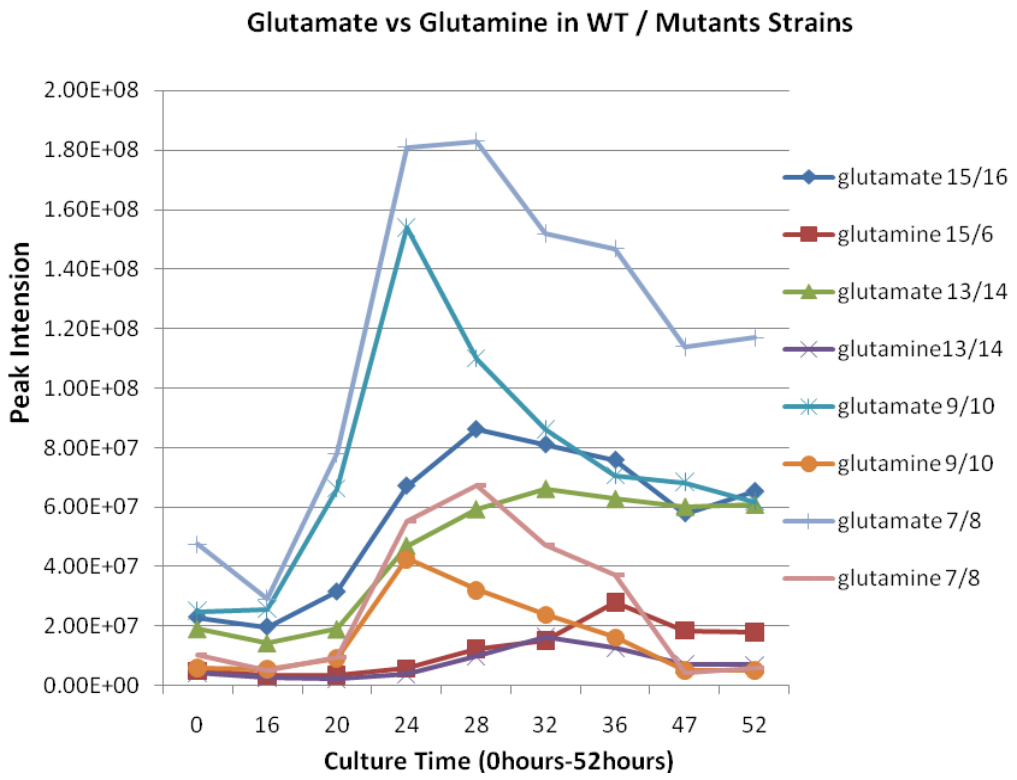


Fig.35 Comparison in glutamate/glutamine behavior in WT and mutants strains

Thus it can be concluded that nitrogen assimilation has been slowed down in the mutant cultures but not prevented. It is possible that both enzymes working together are required for optimal nitrogen assimilation but one enzyme operating on its own can do the job at a slower rate. Where GS genes had been deleted the assimilation of glutamate from the growth medium was much slower although it accelerated rapidly from 36 hours. Thus the question is ,"are any related metabolic systems affected by the gene knockouts"? Glutamine is a key co-factor in the biosynthesis of purines and pyrimidines (figure 36). The levels of some purines are shown in tables 15,16 and 17and the corresponding graphs are shown in figure 37.

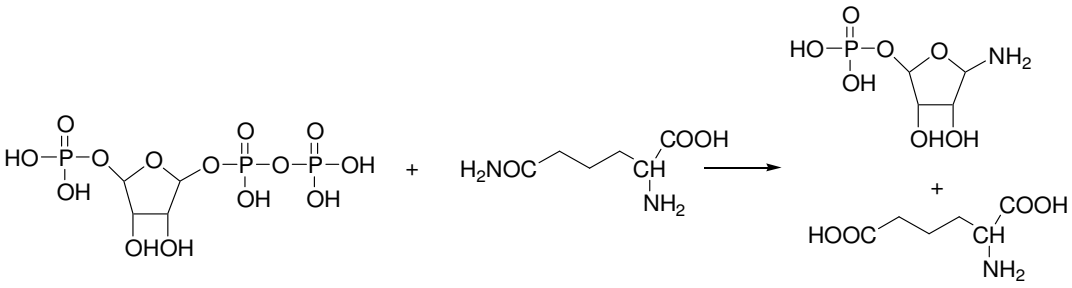


Fig.36 The first glutamine dependent step, in purine biosynthesis.

Purine precursors	**0 hour**	**16 hours**	**20 hours**	**24 hours**	**28 hours**	**32 hours**	**36 hours**	**45 hours**	**52 hou**
Hypoxanthine	1.13E+06	7.14E+05	2.03E+06	2.61E+06	9.50E+06	6.69E+06	4.57E+06	2.86E+06	2.15E+
Guanine	1.28E+06	6.43E+05	1.18E+06	2.38E+06	2.01E+06	1.71E+06	7.26E+05	1.17E+06	1.68E+
Xanthine	1.18E+06	4.46E+05	5.25E+05	1.18E+06	2.98E+06	2.59E+06	2.51E+06	5.90E+05	3.54E+
Urate(	8.58E+04	3.22E+04	3.77E+04	7.25E+04	7.84E+04	5.75E+04	4.66E+04	1.92E+04	1.50E+
Deoxyadenosine	1.40E+05	6.71E+04	8.40E+04	4.34E+05	5.57E+05	3.45E+05	2.86E+05	4.95E+05	3.95E+
Deoxyinosine	3.81E+02	3.87E+02	1.89E+03	5.91E+04	1.34E+05	1.57E+05	1.59E+05	1.93E+05	1.36E+
Adenosine	1.21E+07	4.60E+06	3.85E+06	8.44E+06	7.84E+06	7.73E+06	7.04E+06	9.49E+06	8.07E+
Inosine	2.26E+05	9.58E+04	1.00E+05	3.73E+05	5.95E+06	1.19E+06	6.73E+05	2.19E+06	1.55E+
Guanosine	1.34E+06	5.89E+05	2.79E+05	1.52E+06	1.10E+06	4.47E+05	5.20E+05	4.61E+05	5.50E+
Xanthosine	7.09E+04	2.38E+04	2.80E+04	8.73E+04	1.36E+05	9.32E+04	1.26E+05	2.02E+04	2.06E+
2',3'-Cyclic AMP	1.36E+06	5.58E+05	5.80E+05	2.03E+05	4.85E+03	3.10E+03	4.20E+03	0.00E+00	0.00E+
AMP isomer 2	5.48E+04	9.05E+04	5.11E+05	2.12E+05	7.63E+02	8.39E+02	6.29E+03	2.30E+04	8.69E+
Adenine	4.22E+06	1.41E+06	5.23E+05	3.52E+05	4.83E+05	3.16E+05	4.22E+05	6.01E+05	5.54E+

Table15.Levels of purine precursors in batches GC7 and GC8 with time

Purine precursors	**0 hour**	**16 hours**	**20 hours**	**24 hours**	**28 hours**	**32 hours**	**36 hours**	**47 hours**	**52 hou**
Hypoxanthine	8.43E+05	4.61E+05	7.06E+05	2.33E+06	1.48E+06	1.09E+06	1.52E+06	2.22E+06	1.87E+
Guanine	8.98E+05	5.04E+05	5.02E+05	1.11E+06	8.80E+05	5.71E+05	6.36E+05	6.89E+05	7.18E+
Xanthine	8.84E+05	4.17E+05	3.20E+05	4.98E+05	7.80E+05	5.26E+05	1.06E+06	1.40E+06	9.28E+
Urate	4.27E+04	2.48E+04	1.80E+04	1.54E+04	4.50E+04	2.79E+04	2.54E+04	5.96E+04	1.05E+
Deoxyadenosine	1.14E+05	6.64E+04	6.41E+04	8.79E+04	1.89E+05	1.50E+05	1.45E+05	2.18E+05	1.72E+
Deoxyinosine	3.71E+02	8.20E+01	5.39E+02	4.32E+03	4.39E+04	4.96E+04	6.86E+04	1.09E+05	1.24E+
Adenosine	9.98E+06	4.48E+06	3.49E+06	3.24E+06	3.15E+06	3.08E+06	4.37E+06	6.06E+06	6.34E+

Inosine	1.70E+05	7.89E+04	7.20E+04	1.23E+05	2.99E+05	1.96E+05	2.26E+05	6.66E+05	4.63E+
Guanosine	1.29E+06	7.59E+05	4.75E+05	3.56E+05	3.40E+05	1.83E+05	3.99E+05	2.35E+05	2.47E+
Xanthosine	5.13E+04	2.29E+04	1.70E+04	2.45E+04	3.59E+04	3.06E+04	2.38E+04	3.63E+04	3.27E+
2',3'-Cyclic AMP	9.24E+05	4.42E+05	3.08E+05	4.12E+05	6.40E+05	5.59E+05	3.90E+05	7.82E+03	1.24E+
AMP isomer 2	7.21E+04	6.42E+04	1.22E+05	5.19E+05	1.08E+06	5.24E+05	1.91E+05	1.59E+05	1.09E+
Adenine	3.30E+06	1.56E+06	9.17E+05	4.09E+05	6.90E+04	3.63E+04	9.08E+04	7.16E+04	7.46E+

Table16.Levels of purine precursors in mutant batches GC13 and GC14 with time

Purine precursors	**0 hours**	**16 hours**	**20 hours**	**24 hours**	**28 hours**	**32 hours**	**36 hours**	**47 hours**	**52 hours**
Hypoxanthine	9.57E+05	6.91E+05	1.17E+06	3.34E+06	3.60E+06	1.09E+06	1.79E+06	2.96E+06	2.19E+06
Guanine	1.37E+06	7.98E+05	7.52E+05	1.61E+06	1.90E+06	1.23E+06	1.29E+06	1.55E+06	1.26E+06
Xanthine	9.30E+05	5.17E+05	3.97E+05	7.11E+05	1.03E+06	1.07E+06	1.43E+06	1.69E+06	1.38E+06
Deoxyadenosine	1.29E+05	8.22E+04	8.42E+04	1.49E+05	1.92E+05	1.21E+05	1.12E+05	1.66E+05	1.43E+05
Deoxyinosine	5.13E+02	5.91E+02	1.80E+03	1.13E+04	4.01E+04	5.86E+04	7.36E+04	1.54E+05	1.38E+05
Adenosine	1.03E+07	5.44E+06	3.71E+06	3.35E+06	1.51E+06	1.46E+06	1.60E+06	4.43E+06	4.37E+06
Inosine	1.97E+05	1.03E+05	9.44E+04	1.47E+05	1.51E+05	1.52E+05	1.53E+05	4.12E+05	3.02E+05
Guanosine	1.56E+06	9.45E+05	5.37E+05	3.57E+05	2.83E+05	2.68E+05	2.71E+05	2.48E+05	2.03E+05
2',3'-Cyclic AMP	8.82E+05	4.64E+05	3.20E+05	4.12E+05	3.91E+05	1.39E+05	9.38E+03	3.93E+03	5.41E+03
cGMP	3.16E+05	2.42E+05	1.97E+05	1.99E+05	7.85E+04	3.05E+04	6.99E+02	0.00E+00	0.00E+00
AMP isomer 2	1.09E+05	1.17E+05	2.29E+05	8.48E+05	1.11E+06	9.21E+05	5.03E+05	2.06E+05	2.45E+05
Adenine	3.59E+06	1.80E+06	8.99E+05	2.56E+05	6.04E+04	5.30E+04	6.70E+04	8.35E+04	7.38E+04

Table17.Levels of purine precursors in mutant batches GC15 and GC16 with time

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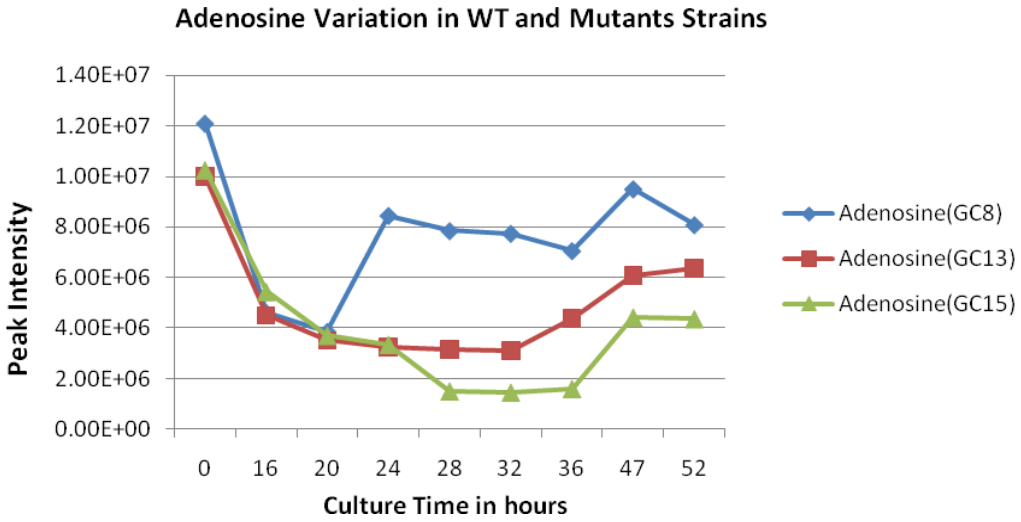


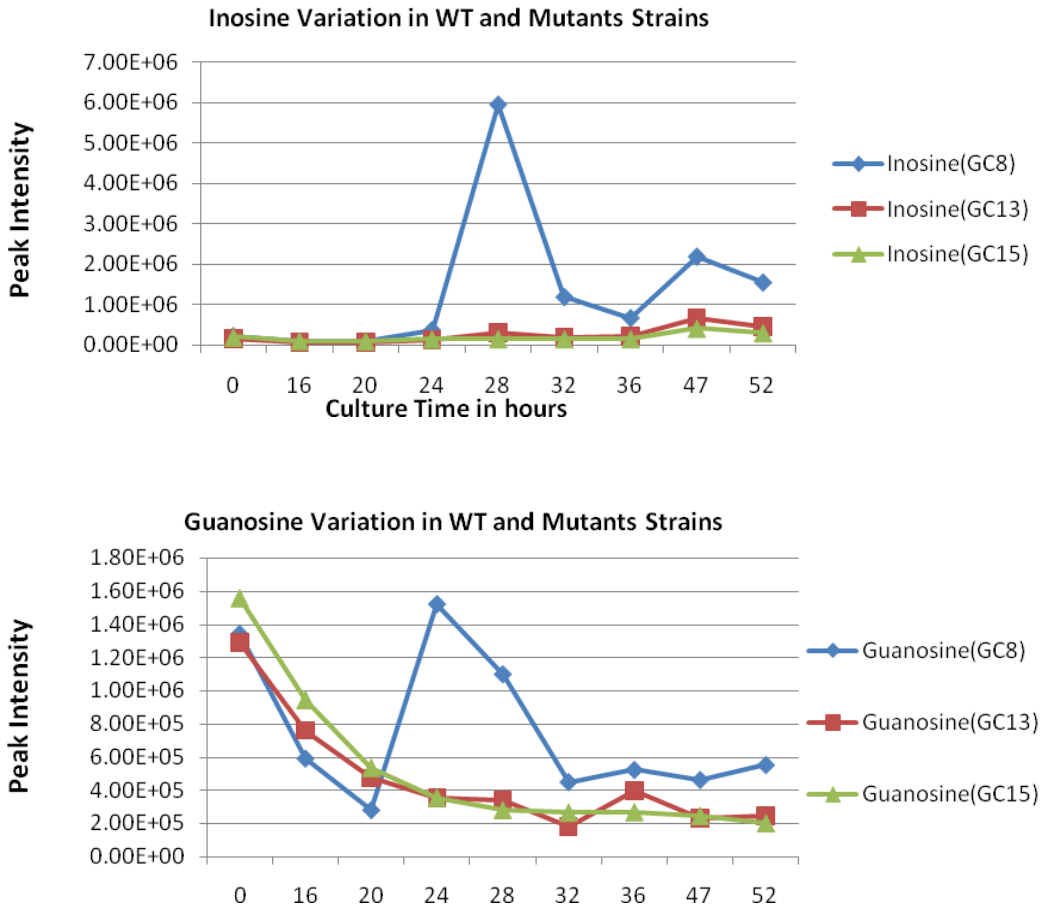


Fig.37 Graphs of adenosine, inosine and guanosine in the WT and the mutant strains of *S.coelicolor.*

As can be seen after comparing the results obtained there was a marked decrease in the knockouts in the levels of inosine, adenosine and guanosine which are all nucleosides involved in the purine biosynthesis pathway. This was also observed for hypoxanthine which is a natural purine derivative and necessary additive in certain cell, bacteria, and parasite cultures as a substrate and nitrogen source and is also a product of adenine deamination.

Adenosine plays an important role in different biochemical processes such as energy transfer ,in the form of ATP and ADP.The amount of adenosine observed in the mutants seems to be about half or one third of that present in the WT and these results are in agreement with those from the

glutamine/glutamate comparison GS activity in the mutant cultures where it appeared to be operating at about less than half the efficiency of that in the WT cultures. The curve shape indicated that initially both the mutants and the WT contain approximately the same amount of these compounds but with time the lack of the enzymes in the mutants reduces their capability for further purine synthesis and the consumption of all the precursors was almost exponential from 0 to 16 hours and in the case of the WT cultures purine biosynthesis was triggered at about 20 hours with inosine, which is the first purine intermediate in the pathway increasing rapidly and reaching a maximum at 24 hours along with adenosine and guanosine which are biosynthetically downstream from inosine. After 24 hours in the WT inosine and guanosine levels fall rapidly but guanosine levels are maintained at high levels. In the mutant cultures there was no marked increase in inosine and guanosine around 24 hours but the cultures do manage to maintain relatively high levels of adenosine indicating that it is possibly a more important metabolite for cell functioning than inosine and guanosine. Considering the results obtained from the glutamine/glutamate comparison along with the results obtained for the purines it appears that the mutants because of decreased glutamate synthetase activity seem to have difficulty in glutamine assimilation moreover they have extreme difficultyin producing all key compounds for the purine biosynthetic pathway .Thus they just consume the purines present in the growth medium without replacing them.The pyrimidine pathway is also affected in the knockouts where the pyrimidine levels are lower in the GS mutants. The results obtained are shown in tables 18 19 and 20 and the corresponding graphs in figure 38. Hence it can be observed that there is similar pattern with the one that already has been observed when purines were

monitored. In this case the levels of pyrimidines fall up to around 20 hours and then the same spike in levels occurs in the WT as was observed for the purines. The most strongly affected metabolite is methylthioadenosine which is another nitrogen rich compound and is formed when it precursor S-adenosyl methionine is used to transfer an aminopropyl group in the biosynthesis of spermidine and spermine. Methylthioadenosine in the mutants reflects the lowered levels of adenosine. S-adenosylmethione which is formed from adenosine and methionine. It was possible to observe that methionine levels were elevated in the mutant cultures reflecting a reduced requirement for it in making S-adenosylmethionine.

Pyrimidines	**0h**	**16h**	**20h**	**24h**	**28h**	**32h**	**36h**	**45h**	**50h**
Cytosine	7.68E+04	3.25E+04	5.98E+04	4.22E+05	1.06E+06	1.30E+06	9.47E+05	1.11E+06	1.12
5,6-Dihydrothymine	7.14E+05	2.97E+05	3.62E+05	6.82E+05	8.68E+05	8.85E+05	7.91E+05	7.78E+05	7.69
Deoxycytidine	2.33E+05	7.12E+04	8.24E+04	1.11E+05	1.19E+05	1.07E+05	9.61E+04	1.02E+05	1.00
Thymidine	1.73E+05	6.26E+04	7.83E+04	4.82E+05	2.91E+05	1.88E+05	1.80E+05	2.15E+05	2.16
Cytidine	2.58E+05	1.04E+05	1.52E+05	8.96E+05	1.92E+06	4.89E+05	6.01E+05	7.65E+05	6.02
Uridine	9.66E+05	3.78E+05	3.93E+05	5.20E+05	2.22E+06	6.76E+05	6.85E+05	7.31E+05	3.91
Uracil	2.40E+05	1.06E+05	7.75E+04	4.37E+05	8.79E+05	1.67E+06	1.40E+06	9.13E+05	3.46
5'-Methylthioadenosine	5.15E+05	2.54E+05	5.43E+05	3.33E+06	2.07E+06	2.33E+06	2.41E+06	2.30E+06	1.85

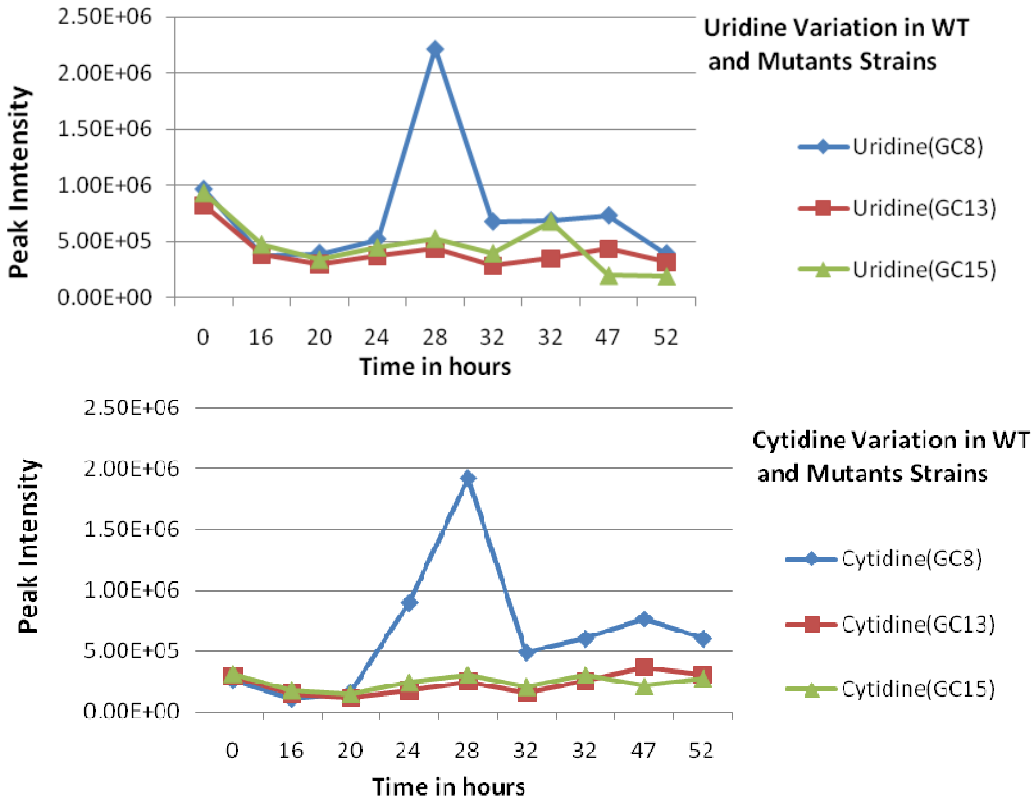
Table18.Levels of pyrimidine precursors in batches GC7 and GC8 with time

Pyrimidines	**0**	**16**	**20**	**24**	**28**	**32**	**32**	**47**	**52**
Cytosine	8.82E+04	4.30E+04	3.71E+04	9.84E+04	2.88E+05	3.97E+05	4.87E+05	8.78E+05	8.79
5,6-Dihydrothymine	6.61E+05	3.21E+05	2.42E+05	3.25E+05	5.68E+05	5.93E+05	6.66E+05	7.60E+05	8.28
Deoxycytidine	1.96E+05	8.03E+04	5.73E+04	8.63E+04	1.45E+05	1.34E+05	1.40E+05	1.60E+05	1.47
Thymidine	1.62E+05	6.59E+04	5.03E+04	7.30E+04	1.29E+05	1.29E+05	1.43E+05	1.97E+05	1.78
Cytidine	2.96E+05	1.47E+05	1.14E+05	1.75E+05	2.50E+05	1.54E+05	2.52E+05	3.68E+05	3.11
Uridine	8.20E+05	3.77E+05	2.97E+05	3.75E+05	4.33E+05	2.84E+05	3.46E+05	4.33E+05	3.16
Uracil	1.81E+05	1.00E+05	7.24E+04	7.46E+04	8.72E+04	7.73E+04	2.40E+05	7.48E+05	7.40
5'-Methylthioadenosine	3.53E+05	1.20E+05	1.10E+05	1.90E+05	4.47E+05	3.46E+05	2.64E+05	2.38E+05	2.48

Table19.Levels of pyrimidine precursors in mutant batches GC13 and GC14 with time

Pyrimidines	0	16	20	24	28	32	36	47	5
Cytosine	8.07E+04	4.83E+04	4.76E+04	1.40E+05	2.53E+05	3.19E+05	4.87E+05	8.24E+05	8
5,6-Dihydrothymine	6.74E+05	3.80E+05	2.83E+05	4.21E+05	5.98E+05	6.11E+05	7.10E+05	9.22E+05	8
Deoxycytidine	2.23E+05	1.10E+05	7.04E+04	1.03E+05	1.48E+05	1.30E+05	1.25E+05	1.29E+05	1
Thymidine	1.61E+05	7.44E+04	5.70E+04	8.48E+04	1.34E+05	1.31E+05	1.42E+05	1.76E+05	1
Cytidine	3.15E+05	1.79E+05	1.44E+05	2.41E+05	3.05E+05	2.06E+05	3.03E+05	2.14E+05	2
Uridine	9.37E+05	4.78E+05	3.41E+05	4.51E+05	5.29E+05	3.99E+05	6.82E+05	2.00E+05	1
2',3'-Cyclic CMP	8.45E+04	7.01E+04	5.27E+04	7.52E+04	1.14E+05	8.97E+04	2.35E+04	4.85E+02	5
2',3'-Cyclic UMP	1.47E+05	7.76E+04	5.09E+04	8.31E+04	1.12E+05	9.03E+04	2.91E+04	0.00E+00	0
Uracil	2.00E+05	1.21E+05	8.14E+04	6.87E+04	8.16E+04	8.40E+04	3.76E+05	8.61E+05	5
uridine monophosphate	3.22E+04	1.28E+04	8.83E+03	2.92E+04	9.43E+04	1.53E+05	1.10E+05	2.22E+03	2
5'-Methylthioadenosine	4.96E+05	2.05E+05	1.80E+05	3.10E+05	3.81E+05	2.87E+05	2.64E+05	1.98E+05	1

Table20.Levels of pyrimidine precursors in mutant batches GC15 and GC16 with time



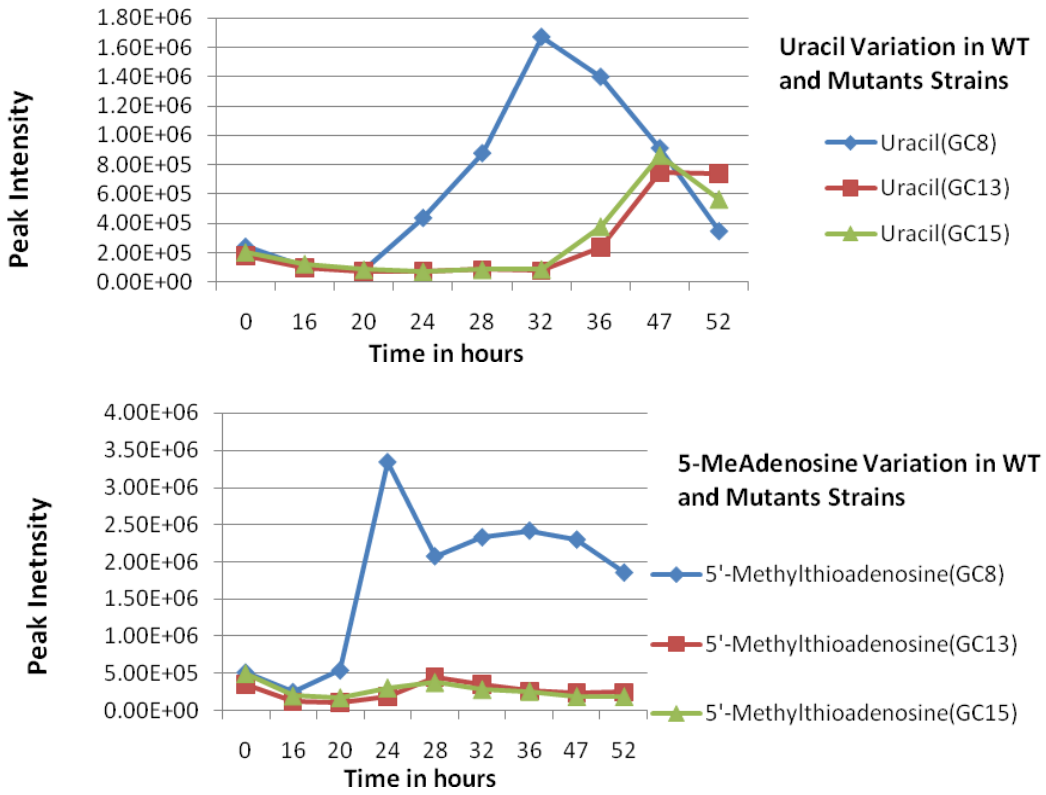


Fig.38 Comparison pyrimidine and methylioadenosine biosynthesis in mutants strains and the WT of *S.coelicolor.*

The other rich nitrogen compounds that were also affected in the knockouts are shown in table 21.

Compound	**MW**	**Rt**	**G13/GC8**	**GC15/GC8**
cytosine	112.0505	15.2	0.72	0.67
uracil	113.0346	8.1	0.68	0.69
cytidine	244.0928	15.8	0.49	0.42
uridine	245.0768	9.4	0.63	0.88
methylthioadenosine	298.0967	9.0	0.12	0.11

Table 21. Other nitrogen rich compounds affected by GS knockouts.

One interesting thing in *S.coelicolor* metabolism is the fact that nitrogen depletion triggers off actinorhodin production. However the antibiotic was not

detected in the samples hence it is assumed that production apparently should have started after the 52 hour time point. However, close inspection of the data indicates that a peak corresponding to the immediate precursor of actinorhodin dihydrokalafungin is present at the 52 h time point of the mutant samples GC 13 (figure 39) and GC15. Figure 40shows a comparison of the GC15 mutant and WT at 52 h. Although the levels of dihydrokalafungin are low in GC15 they are nearly 100 times those in the WT.

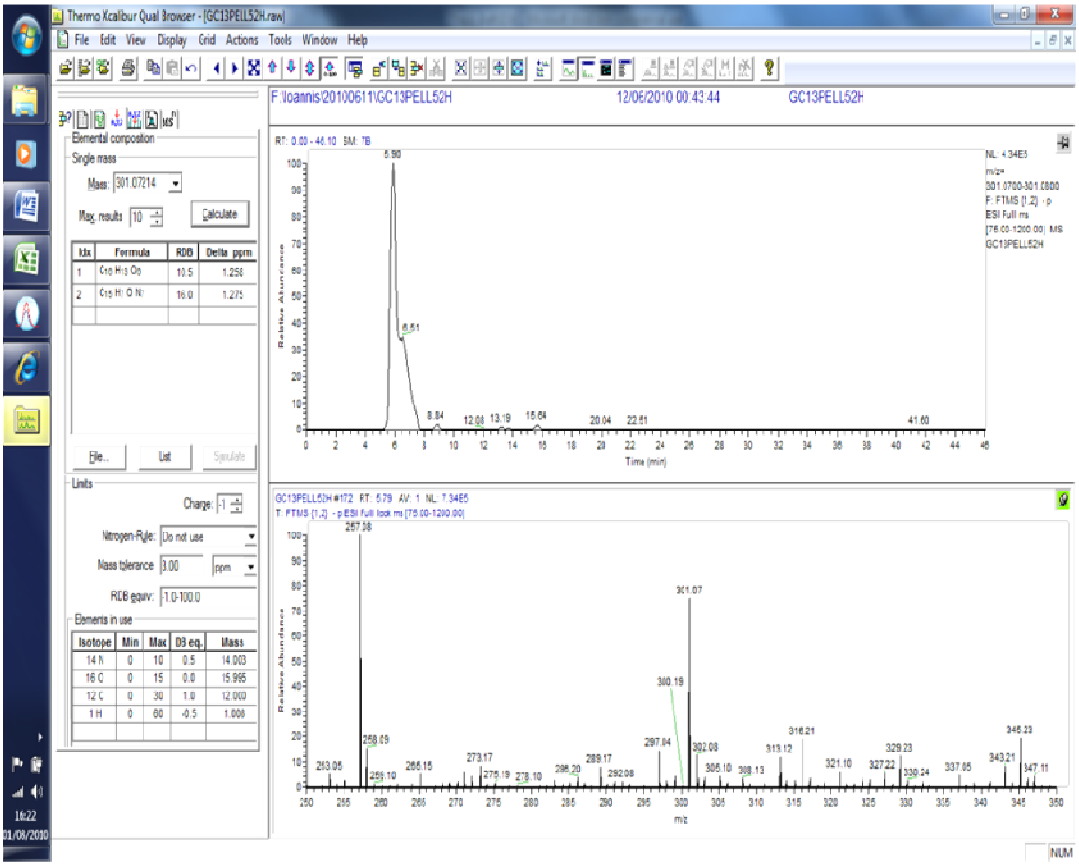
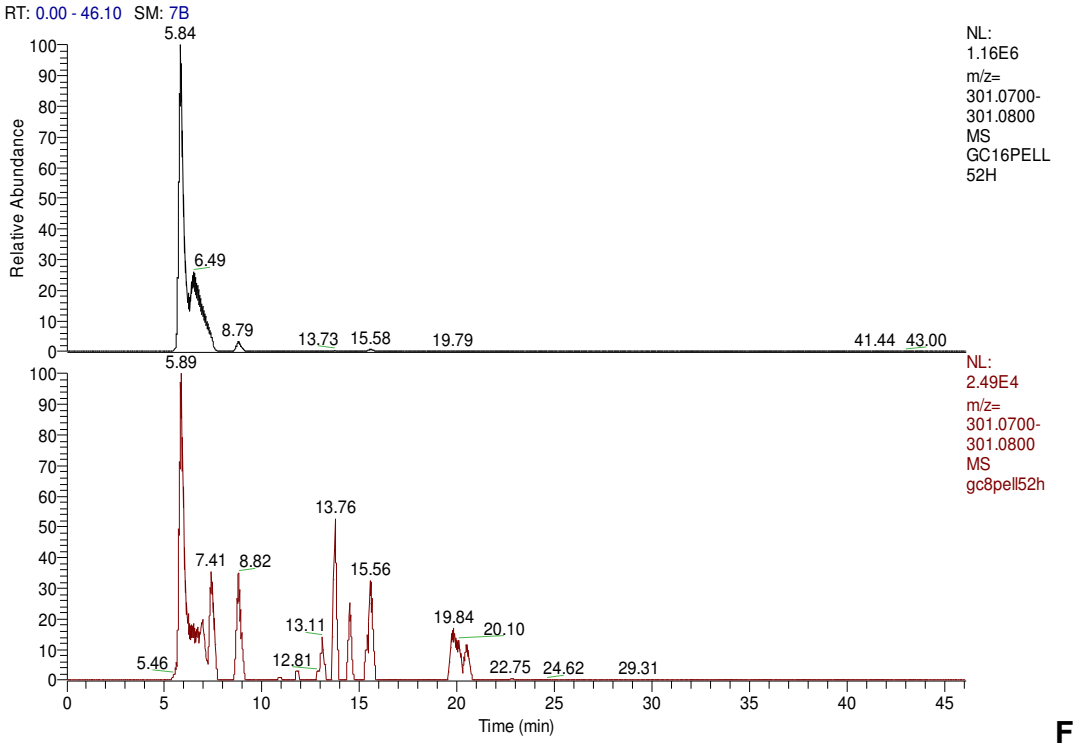


Figure 39 Dihydrokalafungin in mutant GC13 at 52 h



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igure 40 Comparison of dihydrokalafungin in mutant at 52 h vs WT

3.6 Discussion for the results obtained from the analysis of other metabolites of *S.coelicolor*

The sample analysis provided extensive data and there were compounds some of which were not recognized as known compounds ,however they were clearly present in the bacterial culture and were following a pattern from which it was possible to observe their change over time and most of them produced a curve reflecting the growth of the cultures. The software that was used for data processing , produced possible molecular formulas which could be used to seek information in global chemistry data bases like the Pubchem project or the KEGG: Kyoto Encyclopedia of Genes and Genomes.

Ergothioneine, is a naturally occurring metabolite of histidine and its molecular formula is $C_9H_{15}O_2N_3S$ shown in figure 41. In fact it is a thiourea derivative of histidine containing a sulfur atom on the imidazole ring. It is produced in rather a

few microorganisms mainly in Actinomycetes and fungi .It is characterized as an unusual compound since the sulfur atom that is bonded to the imidazole ring is normally more favored to be in the thione form rather than the sulfhydryl form (Hartman, 1990)

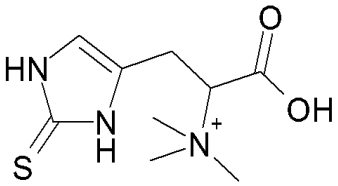
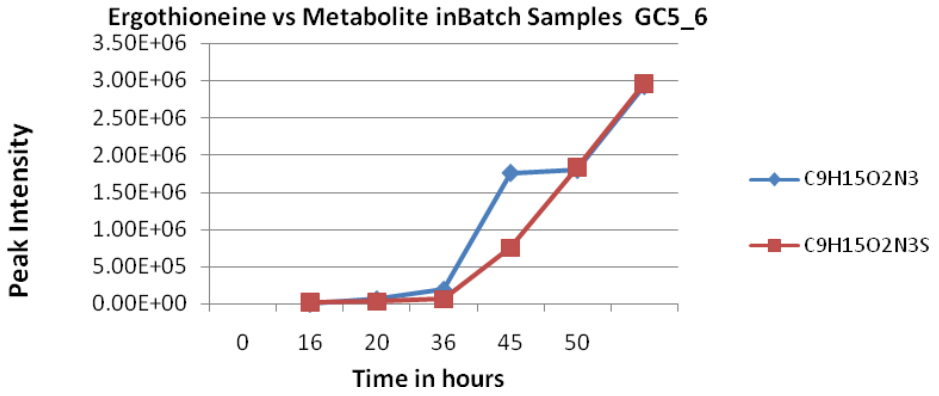


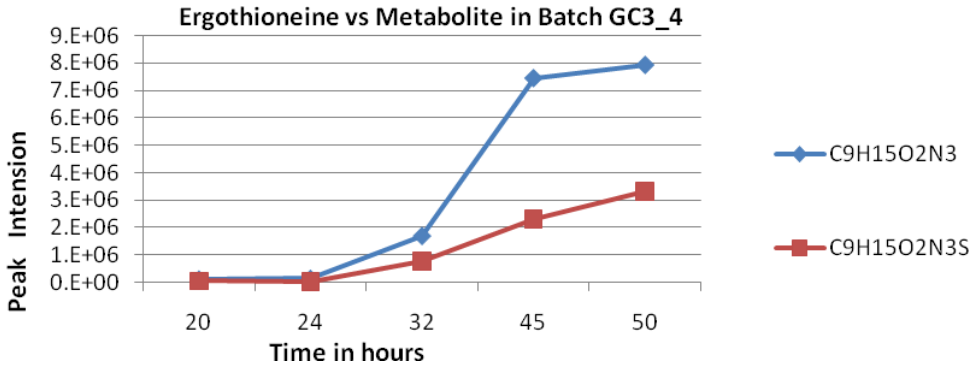
Fig.41 The molecular formula of Ergothioneine

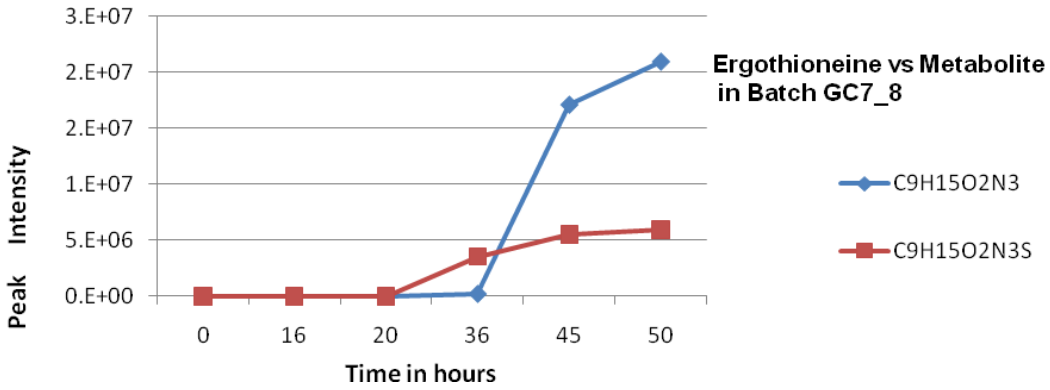
The exact metabolic pathway for its biosynthesis is not yet totally described but it is known that the imidazole ring is supplied by histidine, methylation follows to produce histidine betaine, and then the sulfur atom is incorporated from cysteine. Since it is a thiourea derivative with a thione group rather than a sulfhydryl group the compound is less reactive than other thiols toward alkylating agents and this prevents the compound from oxidizing in air (Ey & Taubert 2007).However, it is known from previous studies that ergothioneine can be slowly oxidized over several days to the disulphide form in acidic solutions and if it does become oxidized, the disulphide is a very strong oxidizing agent, so this will in turn rapidly oxidize other thiols in the cell such as glutathione (Hand *et al* 2005). Moreover Ergothioneine is a compound that cannot be produced in human cells however it is present in tissues in high levels through dietary absorption but there is also the fact that its exact role in human organism still remains unknown (Taubert *et al* 2005).

Ergothioneine was monitored in the *S. coelicolor* extracts since another compound that was differing by a sulphur atom having a potential molecular formula produced by the software of $C_9H_{15}O_2N_3$,was identified in a relatively high concentration thus giving an opportunity of a straightforward comparison

of these two compounds. It was not possible to recover any information for the latter metabolite from any chemical data base whatsoever, however the similarity in their behavior was monitored in all batches as shown in figure 42 and this compound is probably hisitidine betaine. As might be expected these nitrogen rich compounds are at lower levels in the mutant strains.







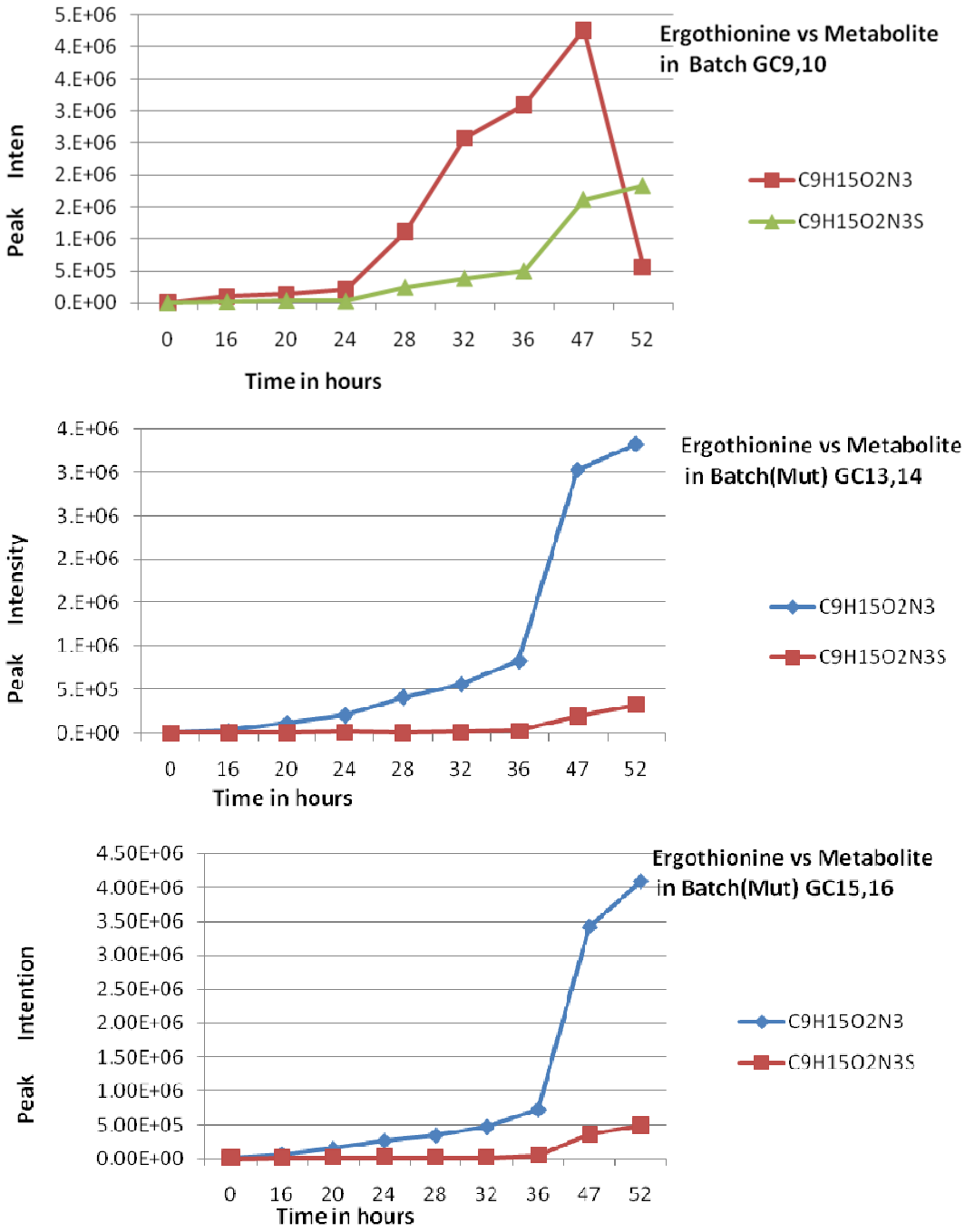


Fig.42 Graphs of Ergothioneine and unknown metabolite in all batches

It is clear that the same pattern was repeated with accuracy in all batches except for the batch GC9,10 where there was a value for the unknown metabolite in the 52 hours time point leading in rapid decrease of its amount but it is very similar. The metabolite appeared to increase rapidly after the 30-35

hours reaching its highest concentration at 50-52 hours .It can also be assumed that the two compounds coincide in the time where they both start rapid accumulation and they both reach their highest amounts at the same time points. There was no further investigation or any other attempt to establish any relationship between these compounds although it is probable that the unknown compound is the immediate precursor of ergothionine histidine betaine before transfer of the sulphur atom to the imidazole ring. Similar attempts in monitoring other potentional biomarkers were made. One compound that was monitored had been identified by having a m/z of 307.095(figure43) which is 1 a.m.u less than the m/z 308.09 which belongs to Glutathione (GSH).

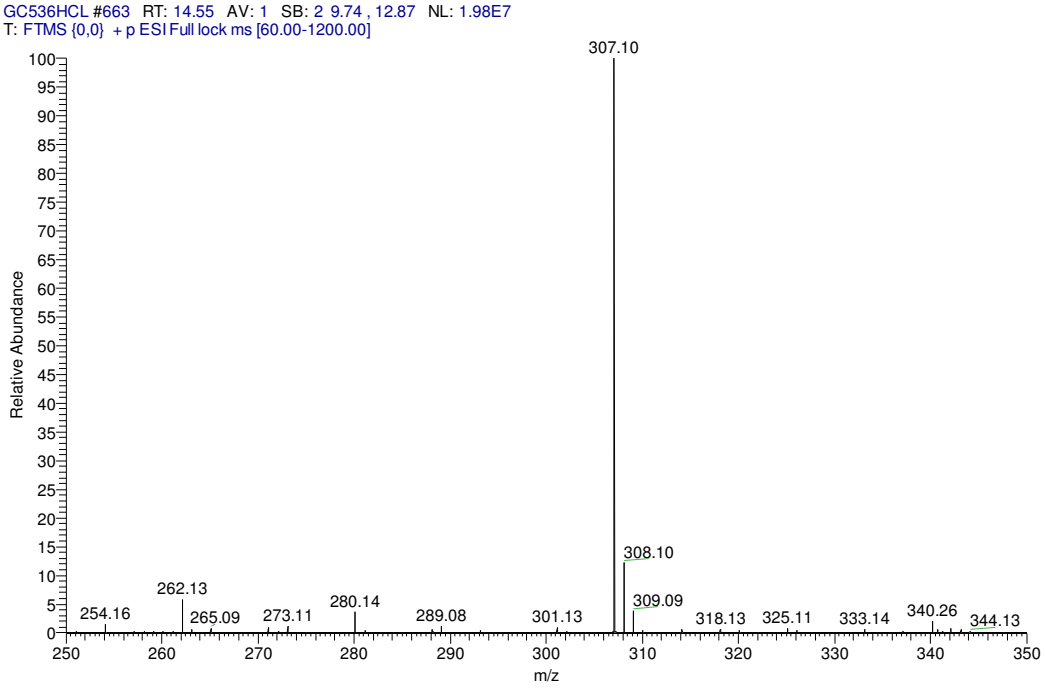


Fig.43 Extracted ion trace of the unknown metabolite molecular ion with m/z 307.10 amu

This metabolite exhibited the same pattern in all sample batches for both normal and mutant strains. The molecular formula of GSH is $C_{10}H_{17}O_6N_3S$ whereas the metabolite's was $C_{11}H_{18}O_6N_2S$.

Plots of the variation of this compound with time are shown in figure 44.

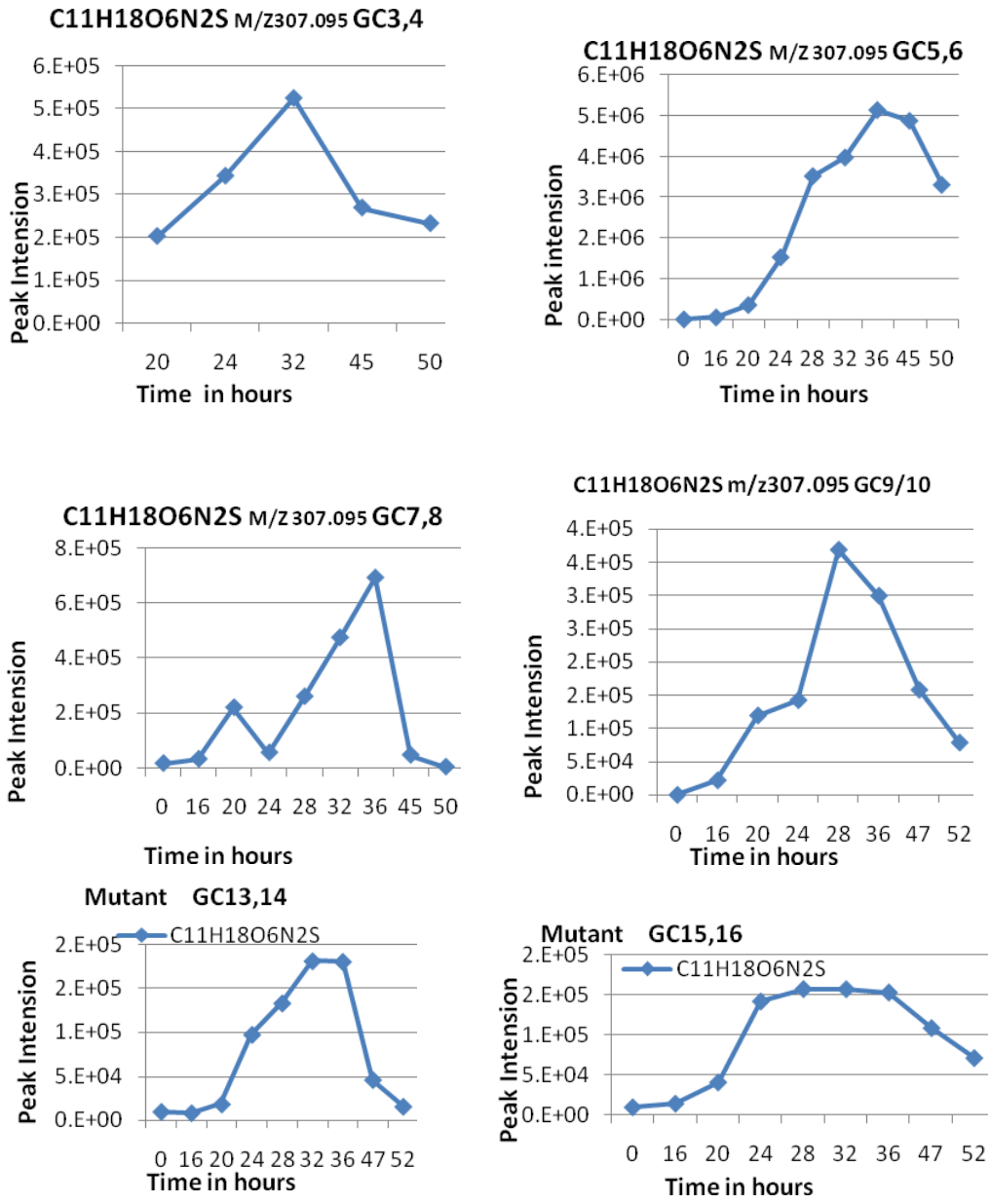


Fig.44 Graphs of the $C_{11}H_{18}O_6N_2S$ metabolite in all batches of samples

Glutathione is a tripeptide, shown in figure 45, synthesized from L-cysteine, L-glutamate and L-glycine. Its biosynthesis is not found in all bacteria .

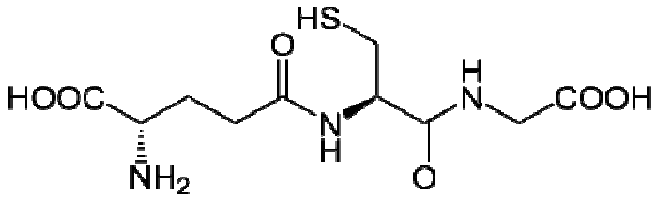


Fig.45 Molecular formula of Glutathione

The interest in this metabolite rose from the fact that only limited information is available on the formation and inter-conversion of sulfur-containing amino acids in Streptomycetes. From previous studies (Kitano *et al* 1985) it is known that thiosulfate is an intermediate in the reduction of sulfate in the reactions that take place in the cysteine biosynthesis. This was also supported by evaluations in *S.coelicolor* and *S. lividans* of the growth requirements of cysteine auxotrophs (Lydiate *et al* 1988).As reported above this thiol containing metabolite had the same behavior in all samples and its pattern indicated that its rapid accumulation started at the 20-22 hour time point while its highest value was achieved at the 32-36 hours. After that time there was a rather acute decrease until the last time point. The only aminoacids that contain sulfur are cysteine and methionine and the former could not be detected in any of the samples. Methionine reached its highest concentration approximately at the 24 hours time points just after the highest concentration value of the metabolite was achieved. The molecular formula that was proposed by the software for this metabolite indicated that it differs from glutathione by having a carbon and hydrogen atom rather than a nitrogen atom. Therefore considering the molecular formula of GSH it is probable for that the terminal amino group has been substituted by a methyl group or the amino group is absent and instead of the glutamate hexandioic acid is bonded one end of the molecule.

There was no further investigation in the metabolite and fragmentation experiments would be required to elucidate its structure further.

4. Conclusions

The results obtained from the samples analysis, gave a good picture of the complexity of the *S. coelicolor* culture. It is obvious that the mutant strains have a serious difficulty in nitrogen assimilation compared to the WT. This was demonstrated by the comparison of the glutamate synthesis in the knockouts and its further conversion to glutamine, in that of the WT where it could approximately be calculated that the efficiency of the glutamine synthetase, which is the enzyme that plays the essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine, was almost half in the mutant strains. Glutamine is responsible for assimilating ammonia which can then be used in a number of biosynthetic pathways including amino acid biosynthesis purine biosynthesis and pyrimidine biosynthesis .Although *S.coelicolor* has two genes that are used as regulators for GS synthesis, *glnA* and the *glnII* ,it was shown that eventually each strain having only one gene managed to synthesize glutamine but it was also shown that both strains were very inadequate in synthesizing rich in nitrogen compounds as it is the case for purines and pyrimidines. It was shown that the difference of the nitrogenous bases between the WT and the mutants was about ten time more for the former. It was shown that *S.coelicolor* strains were exhibited a preference for particular aminoacids like aspartate , asparagine , glutamine and glutamate whereas requirement of some others like glycine , tyrosine and tryptophan were seemed to be met by the aminoacids that were

present in the peptone and yeast of the medium culture.

The analysis that was carried out yielded plenty of data about compounds of secondary metabolism. Many of them were already identified in the library that originated in the Strathclyde MS laboratory but many also were unidentified and further experiments need to be carried out in order to elucidate their structure.

Finally although there was no evidence for accumulation of actinorhodin in the cultures there was an indication that nitrogen depletion in the GS deficient mutants triggered of accumulation of dihydrokalafungin in advance of appreciable levels appearing in WT.

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