Mechanisms of resistance to β-lactam antibiotics in streptomycetes

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DECLARATION

I hereby declare that this Ph.D. Thesis entitled "Mechanisms of resistance to β-lactam antibiotics in *Streptomycetes*" was carried out by me for the degree of Doctor of Philosophy under the guidance and supervision of Dr. Glyn Hobbs at Liverpool John Moores University. This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. No portion of this thesis has been submitted in support of an application for any degree or qualification of Liverpool John Moores University or any other University or professional institution.

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ABSTRACT

The most successful antimicrobial agents in clinical use are of microbial origin and of these the greatest variety has been found in the genus *Streptomyces*. However, the resistance of the pathogenic microbes to the commonly used antibiotics is increasing as a result of the wide-spread and long-term use of these antibiotics. Therefore, understanding the strategies that bacteria use to become resistant is of crucial need. Streptomycetes are Gram positive bacteria, commonly found in soil and are known antibiotic-producers. The focus of this thesis was to underpin the mechanism of resistance to penicillin G in isolated strains of streptomycetes that exhibit elevated resistance to penicillin G and to characterise these organisms. Moreover, to investigate the interaction between penicillin G and PBPs in *Streptomyces* strains and investigate the relationship between growth rate and penicillin G resistance in *Streptomyces in vitro*. Ninety six Streptomycetes were isolated and characterized. Morphological examination and the16s rRNA sequences of these strains indicated that strains belong to the species *Streptomyces*. The MICs and MBCs for penicillin G for the isolated *Streptomyces* strains were measured by plate culture. Some strains showed growth up to 400 µg/ml with penicillin G, which indicate that the strains were highly resistant against penicillin G. Some strains were unable to grow at penicillin concentrations above 200µg/ml. Also, The MICs of penicillin G for isolated *Streptomyces* strains were measured using a novel OxoPlates® system in 96-well culture format employing Mueller-Hinton broth culture. The MICs of all strains ranged from 1-100 µg /ml. Results indicate that the sensitivity of *Streptomyces* strains of penicillin G is not directly related to β-lactamase production in the panel of isolates examined. There was no correlation between the MICs of penicillin G and the growth rate in these isolates. Likewise, there was no association between the position of beta-lactamase producing and non-beta-lactamase producing strains on the phylogenetic tree

and their beta-lactamase activity. Beta-lactamase producing and non-producing strains refers to the same ancestral origin clade. Additionally, the comparative analysis of 16S rRNA gene sequence and phylogenetic relationship of strain (W43) revealed that the isolate clustered with (W76) *Streptomyces lividans* strain *YLA0.*

Bocillin (a penicillin binding protein stain) staining in β-lactamase producing strains showed staining throughout the mycelia whereas in non β-lactamase producing strains staining only occurred in certain parts of the mycelia. Bocillin also revealed that in spores PBPs were located on both poles of the spores.

Streptomyces coelicolor has the ability to grow at high concentrations of penicillin G up to 640 µg/ml in continuous culture. It also has the capacity to grow at very low amounts of dissolved oxygen in continuous culture. Significantly, there was a correlation between the growth rate of *S. coelicolor* and the resistant to penicillin G. *S. Coelicolor* was more sensitive to penicillin G at a high dilution rate.

Furthermore, our strategy of using the Bug-Lab for monitoring the progress of *S. Coelicolor 1147* in continuous culture, even at low concentrations of cells in real time was successful.

ABBREVIATIONS

PCR	Polymerase Chain Reaction amplifications
bp	base pairs
DAPI	4, 6-diamidino-2-phenylindole
dO_2	dissolved oxygen
HMW	high molecular weight
h	hours
L	litre
LMW	low molecular weight
mg	milligram
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
ml	millilitre
mM	millimolar
YEME	yeast extract malt extract medium
NAG	N-acetylglucosamine
NAM	N- acetylmuramic acid
OD	optical density
PBP's	penicillin binding proteins
rpm	revolutions per minute
µg	microgram
µl	microliter

Chapter One

Literature review

1. Streptomycetes Biology and Life cycle

1.1 Characteristics of Streptomycetes

Streptomycetes are the most global among soil bacteria (Hodgson 2000) *Streptomyces* are Gram-positive bacteria, with a high (70%) guanine-plus-cytosine content in their DNA (Stackebrandt and Goebel 1994). *Streptomyces* species are known to contain a linear chromosome and complex morphological differentiation. They undergo filamentous growth similar to fungi and are strictly aerobic and spore forming, widely distributed in soil, water and colonise plants and play an important role in mineralization processes in nature. *Streptomyces* constitute 50% of the total population of soil actinomycetes, with a complex life cycle (Flardh and Buttner 2009). Most *Streptomyces* are distinguished for their distinct earthy odour which results from production of metabolite, geosmin (Madigan and Martinko 2005). *Streptomyces*'s colonies are usually slow-growing, and they develop an aerial mycelium that may appear granular, powdery or smooth, and they produce a wide variety of pigments responsible for the colour of the vegetative and aerial mycelia (Flardh and Buttner 2009, Ambarwati *et al.* 2012). *Streptomyces* are most widely known for their capacity to synthesize and produce several antimicrobial substances such as; antibiotics, examples of which include streptomycin, erythromycin, tetracycline and chloramphenicol (Kieser *et al.* 2000), and β-lactam molecules such as cephamycin and clavulanic acid (Ward and Hodgson 1993). They can also produce antifungal agents including amphotericin B, together with anti-cancer compounds such as migrastatin which is used as an inhibitor of tumor cell migration (Nakae *et al.* 2000).

1.2 The life cycle of *Streptomyces*

Streptomyces have a complex life cycle and are unique among bacteria in that, their colonies are similar to multicellular organisms. The life cycle of the streptomycetes starts once a particular spore encounters conditions favourable for growth, it germinates in response to unidentified signals, which act to generate the influx of water, resulting in an increase in size and reduced phase brightness and produces one or more long filaments Figure 1.1 (Ensign 1979). These filaments grow and branch to forming a vegetative mycelium. Then, the vegetative growth leads to formation of a mycelium consisting of a complicated network of aerial hyphae (Hardisson *et al.* 1978). These aerial hyphae undergo septation and develop into separate pre-spore sections, and metamorphose into chains of grey-pigmented spores, which will separate and start a new life cycle. Differentiation is an important process as it marks the onset of secondary metabolism including antibiotics which are secreted from the vegetative mycelium during the generation of aerial hyphae (McGregor 1954, Chater 1993). Production of pigments and other secondary metabolites is often associated with the beginning of differentiation (Bentley *et al.* 2002).

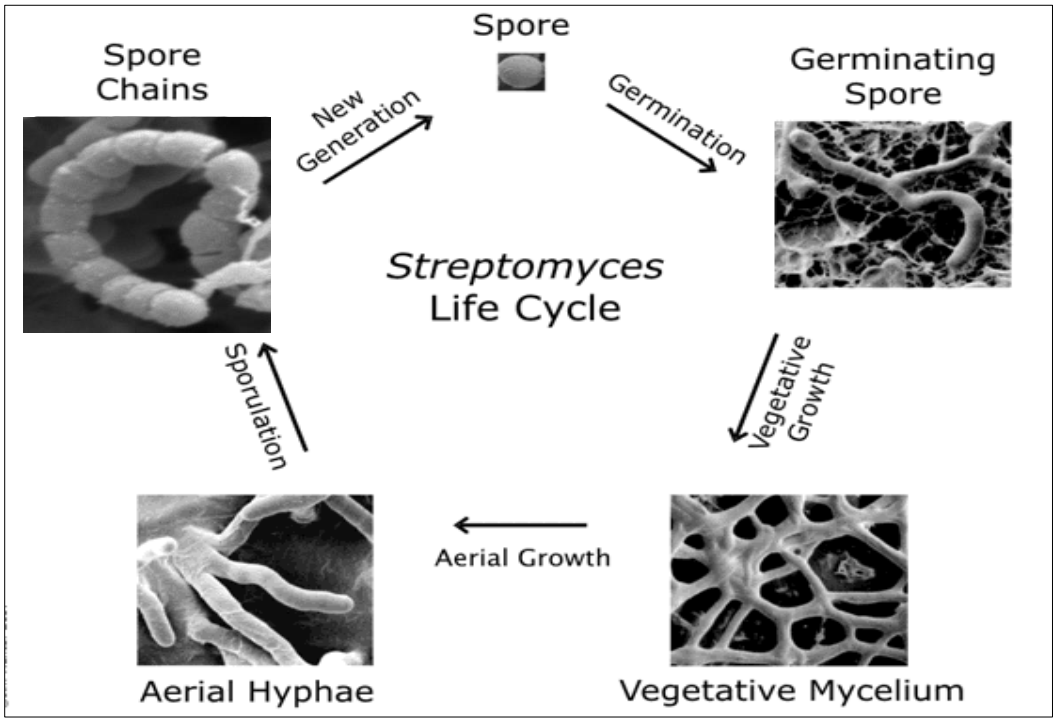


Figure 1-1. The life cycle of *Streptomyces coelicolor*, begin when a single spore germinates a vegetative mycelium, which develop into aerial hyphae. These aerial hyphae undergo into spores, which will separate and start a new cycle (adapted from www.streptomyces.openwetware.org).

1.3 *Streptomyces coelicolor A3 (2)*

Streptomyces coelicolor is the most studied member among the genus *Streptomyces* and the best model organism for most genetics, physiological, biochemical and metabolism studies of streptomycetes (Hopwood 1999). *Streptomyces coelicolor* likes other members of the genus *Streptomyces*; is filamentous, aerobic and Gram-positive bacterium despite the presence of lactate dehydrogenase gene, has a high genomic G-C content and undergoes a complex life cycle of morphological differentiation on solid medium (Borodina *et al.* 2005).

Streptomyces coelicolor has one long linear chromosome contains 8,667,507 base pairs, which containing the largest number of genes (7,825 predicted genes) so far discovered in a bacterium and also has two plasmids; one of them is linear and the other is circular

(Bentley, *et al.* 2002). The linear chromosome has three regions, the core and two different length arms, almost half of the chromosome was found to be in the core region which carried essential genes necessary for the survival of this bacterium such as cell division, transcription, translation and DNA replication. The arms of linear chromosome contains genes coded the non-essential functions such as secondary metabolites production like the production of antibiotics (Bentley, *et al.* 2002). Four chemically distinct known antibiotics produced by *Streptomyces coelicolor*; two are pigmented, undecylprodigiosin (red) and actinorhodin (blue) and the other two are methylenomycin and calcium-dependent antibiotic (Wright and Hopwood 1976, Feitelson and Hopwood 1983, Kieser and Foundation 2000).

1.3.1 Actinorhodin (ACT)

Actinorhodin is a pigmented polyketide antibiotic produced by *Streptomyces coelicolor* and its red at acidic pH and blue at alkaline pH (Brian *et al.* 1996, Bystrykh *et al.* 1996). ACT is made by a type II polyketide synthase-based pathway involving a 22-genecluster (Okamoto *et al.* 2009). It is mainly intracellular actinorhodin and when its exported from the bacterial cell well converted to ϒ-actinorhodin, which is responsible for the blue colour of the culture medium (Bystrykh *et al.* 1996). According to Wright and Hopwood (1976) the excretion of actinorhodin appears to occur exclusively at pH values above 6.7. Whereas, Bystrykhetal. (1996), establish that the excreted pigment is not actinorhodin but its lactone derivative, g-actinorhodin (Wright and Hopwood 1976, Bystrykh *et al.* 1996). Coisne et al (1999) published that the production of actinorhodin appears mainly during the stationary phase in the batch fermentation in the liquid culture (Coisne *et al.* 1999), however, actinorhodin production was growth-associated according to Ozerginulgen and Mavituna (1993) (Ozerginulgen and Mavituna 1993). Also it has been published that the production of actinorhodin was growth associated on solid medium

(Shahab *et al.* 1994). Numerous studies have attempted to explain the effects of growth rate and nutrient feed rate on the production of actinorhodin. The production of actinorhodin in *Streptomyces coelicolor* A3 (2) was quite unaffected to the carbon source concentration, however, it was sensitive to the depletion of nitrogen or phosphate. Or by a decline in the growth rate Wright and Hopwood (1976) reported that actinorhodin was mainly effective against Gram-positive bacteria at high concentration; for instance they found that the minimum inhibitory concentration of this antibiotic against *Staphylococcus aureus* was 25-30 µg/ml.

1.3.2 Undecylprodigiosin (RED)

Undecylprodigiosin is a red hydrophobic tripyrroles (Mo *et al.* 2008), belongs to large family of pigmented oligopyrrole antibiotics called prodiginines, produced by several actinomycetes and other eubacteria including *Streptomyces coelicolor* and *Streptomyces longispororuber* (Wasserman *et al.* 1969, Williamson *et al.* 2006). Prodiginines produced intracellularly by most bacterial producers because they are cell wall-associated antibiotic due to their highly hydrophobic characteristics, which mad by fatty acid synthase-like pathway involving a 22-gene cluster (Kobayashi and Ichikawa 1989, Mo *et al.* 2008). It has a medical potential as immune-suppressants and anti-tumour in addition to antimicrobial activities (Williamson *et al.* 2006). Also undecylprodigiosin suggested as a novel anti breast cancer drug in 2007 (Ho *et al.* 2007).

1.3.3 Calcium-dependent antibiotic (CDA)

The calcium-dependent antibiotic is a non-ribosomal lipopeptide, made by a route involving non ribosomal peptide synthases and specified by a 48-gene cluster produced by *Streptomyces coelicolor* (Hojati *et al.* 2002, Kim *et al.* 2004). CDA act to inhibit the growth of bacterial cells in the presence of calcium ions by making holes in their membranes (Kempter *et al.* 1997).

1.3.4 Methylenomycin

Methylenomycin is acidic cyclopentanone compound produced by *Streptomyces coelicolor* and *Streptomyces violaceus* (Wright and Hopwood 1976). It has anti-microbial effective against a broad range of Gram-positive and some Gram-negative bacteria in specific against *Proteus* species (Corre and Challis 2005). Methylenomycin is the only streptomycetes antibiotic encoded by genes carried on a linear plasmid of 350 kb called SCP1 (Aguilar and Hopwood 1982, Chater and Kinashi 2007). Methylenomycin is produced coinciding with the transition from the exponential to stationary phase in the defined medium (Hobbs *et al.* 1992). It was found that *Streptomyces* species produce two forms of methylenomycin (Challis and Chater 2001). One is methylenomycin A, which is an epoxycyclopentenone made by a pathway encoded by 11 genes located on the SCP1 (O'Rourke *et al*. 2009).

1.4 Morphological differentiation in *Streptomyces coelicolor*

There are two main classes of regulatory genes, identified throughout genetic studies of mutants, included in morphological differentiation in *S. coelicolor* (Chater 1972). The first gene of these is *bld* (bald) genes, which are essential for formation of the aerial hyphae and the colonies of this mutant have a shiny and bald appearance, and the others is the *whi* (White) genes, which are necessary for the formation of spores in the aerial mycelium hyphae. *Whi* mutants produce aerial hyphae but are not able to form the grey spore-associated pigment and therefore have a White colony phenotype. *S. coelicolor* produces a grey polyketide pigmented colour of the spores during sporulation (Davis and Chater 1990). The *whiE* gene group of *Streptomyces coelicolor* is expressed just before sporulation in the aerial mycelium, leading to production of the grey spore pigment (Yu and Hopwood 1995). The *whiE* gene cluster contains eight genes including a minimal type II PKS (Davis and Chater 1990).

1.5 Genes specifically required for formation of aerial hyphe

The genetic characterization of mutants of *S. coelicolor* blocked at the vegetative mycelium stage called *bald* (bld) mutants, which fail to produce the fuzzy aerial mycelium. Some *bld* mutants of *S. coelicolor* such as *bldA, bldB, bldC, bldg* and *bldH* are unable to make pigmented antibiotics (actinorhodin and undecylprodigiosin) in addition to their morphological block.

1.6 Genes specifically required for sporulation

The development of the aerial hyphae into spores can be blocked at different stages by mutating genes. Mutations in genes involved in morphogenesis alter colony appearance for instance modify in the synthesis of the grey polyketide spore pigment leading to form white spores instead of grey spores of *Streptomyces coelicolor,* this called White (*whi*) mutants. Several *whi* mutants have been isolated which form sporulation-defective aerial hyphae including; *whiA, whiB, whiD, whiE, whiG, whiH, whiI, whiJ, whiL, whiM, whiO, sigF, ssgA, ssgB,* and *ssgR* (Chater 1972, Ryding *et al.* 1999, McCormick and Flardh 2012). Such *whi* mutants may be early or late, early mutants failing to achieve full sporulation septation (*whiA, whiB, whiG, whiH, whiI, whiJ,* and *ssgB*) whereas *sigF, ssgA, ssgR,* and *whiD* are late genes (Chater 2001). The biosynthetic genes for a polyketide pigment in spores are establish in the *whiE* gene cluster, and the expression of this cluster depends on the regulatory *whi* genes (Kelemen *et al.* 1998). *whiE* is a complex locus which encodes the enzymes that synthesize the spore pigment itself (Chater 1972, McVittie 1974). Mutations in *whiA, whiB,* and *whiG* mutants are completely block development at early stages and prevent sporulation septation (McVittie 1974). *whiH* mutants are pale grey and produce some sporulation septa. *ssgA mutants* produced aerial hyphae but failed to sporulate and *ssgB* mutant showed defective in the early stage of sporulation septation (Keijser *et al.* 2003). *whiI* mutant produce

some sporulation septa, and it is completely White (Ryding *et al.* 1998). *whiJ* mutant produce low numbers of pale grey normal spore chains (Kelemen *et al.* 1998). *whiD* mutant can make sporulation septa but form spores that highly irregular in size and shape which are extremely variable in spore cell wall deposition (McVittie 1974).

1.7 Antibiotics produced by *Streptomyces*

Streptomyces alone contributes more than half of the naturally occurring secondary metabolites discovered up to date (Berdy 2005). The most important antibiotics produced by *Streptomyces* are streptomycin (*S. griseus)* (Distler *et al.* 1987), neomycin *(S. fradiae)* (Dulmage 1953), chloramphenicol *(S. venezuelae)* (Akagawa *et al.* 1975), fosfomycin *(S.fradiae)* (Woodyer *et al.* 2006), tetracycline *(S. rimosus and S.aureofaciens)* (Nelson *et al.* 2001), puromycin *(S.alboniger)* (Sankaran and Pogell 1975), lincomycin *(S. lincolnensis)* (Peschke *et al.* 1995). There are several secondary metabolites produced by *Streptomyces coelicolor*, the model species, that have antibacterial activity such as; undecylprodigiosin (red pigmented) and actinorhodin (Act) (blue pigmented), methylenomycin and calcium-dependent antibiotic (CDA) (Kieser and Foundation 2000). In addition to the antimicrobial activities, undecylprodigiosin is suggested as a novel anticancer treatment of breast cancer (Ho *et al.* 2007). Recently, it has been published that Warkmycin antibiotic produced by *Streptomyces* species strain Acta 2930 (Helaly *et al.* 2013).

1.8 *Streptomyces* are a potential source of novel antibiotics

The need for new antibiotics, is so urgent to fight pathogens that have developed resistance to common antibiotics, where a large numbers of bacteria are resistant to most if not all useful antibiotics. There is a broad resource and processes that can be taken to discover useful antibiotics and to search for the mechanism of the resistance to these antibiotics. In a screening for potential sources for a new antibacterial activity, including

antibiotic, an actinomycetes in particular those from the genus *Streptomyces*, are very useful source. *Streptomyces* are a unique source of new natural products such as antibiotics. They are responsible for the production of about half of the discovered bioactive secondary metabolites, mainly antibiotics, it produces antibiotics against Gram-positive and Gram- negative bacteria. *Streptomyces* genome sequencing showed that each strain contains genes that encode the enzymes to synthesize 20 or more potential secondary metabolites (Ohnishi *et al.* 2008). It has identified that *Streptomyces flora* of Jordan is a potential source of antibiotics active against antibiotic-resistant Gram-negative bacteria (Saadoun and Gharaibeh 2002). Recently, it has selected *Streptomyces* as broad-spectrum antagonists against soil borne pathogens from arid areas in Egypt (Köberl *et al.* 2013).

1.9 Antibiotics

Antibiotics are defined as natural antimicrobial agents produced by microorganisms and used to kill or inhibit the growth of other organisms. There are three main classes of antibiotics, natural (unmodified from a producing organism), semi-synthetic which are prepared by chemical modification of natural antibiotics and purely synthetic antibiotics that are produced by chemical synthesis (Lancini *et al.* 1995). Antibiotics are secondary metabolites mainly produced by soil bacteria and fungi in nature. This gives the microbe an advantage by killing off competing microbes, when they are competing for food and water and other limited resources in a particular habitat (Williams *et al.* 1989, Angell *et al.* 2006). Usually antibiotics are produced during the late growth phase of microbial culture and they do not have any significant essential role in the growth of producing organisms. The mechanisms by which the bacteria are resistant to their own antibiotics are unknown so far (D'Costa *et al.* 2006). Antibiotics are classified as bacteriostatic or bactericidal. Bacteriostatic antibiotics inhibit the growth of bacteria, but do not kill them

and most bacterial cells resume growth after elimination of the antibiotic. Bactericidal antibiotics kill bacteria (Pankey and Sabath 2004).

Antibiotic resistance may be an inherent or acquired. Inherent resistance appears in some bacteria that naturally resistant to some antibiotics due to their physiological characteristics. Acquired resistance occurs when a sensitive bacterium develops resistance to an antibiotic. There are two major mechanisms by which bacteria can become resistant to antibiotics; spontaneous mutation in the bacterial gene that helps the bacterium survive and acquisition of a resistance gene from a bacterium that is already resistant or from a combination of these two mechanisms (Hawkey 1998). Although, a massive progress has been made to understanding the biosynthesis, mechanisms and the mode of actions of these antibiotics, the question of why bacteria produce antibiotics has not been settled yet.

1.10 Common enzymatic aspect of antibiotic resistance in bacteria

Since the discovery and consequent widespread use of antibiotics, resistance to these antibiotics has been observed, which predictably arises with the use of these compounds. Bacteria develop resistance to specific antibiotics by a variety of mechanisms, and some bacteria have developed specific mechanisms to a different drug. Although the mode of resistance may vary among bacterial species, resistance is generated by some mechanisms (Figure 1.2) including; (1) efflux pumps, which are high-affinity reverse transport systems located in the membrane that transfer the antibiotic out of the cell for instance the mechanism of resistance to tetracycline; (2) Alteration and modification of the antibiotic target when a specific enzyme inactivates the antibiotic for example, alteration of PBP the binding target site of penicillin in penicillin-resistant bacteria; (3) Reduced permeability or uptake of the bacteria due to modifications of the cell surface that limit interactions with the antibiotics or reduce the number of entry channels (Wright

2005); (4) drug inactivation or modification via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics for example, the penicillinases (beta-lactamase enzymes) that cleave the beta lactam ring of the penicillin molecule.

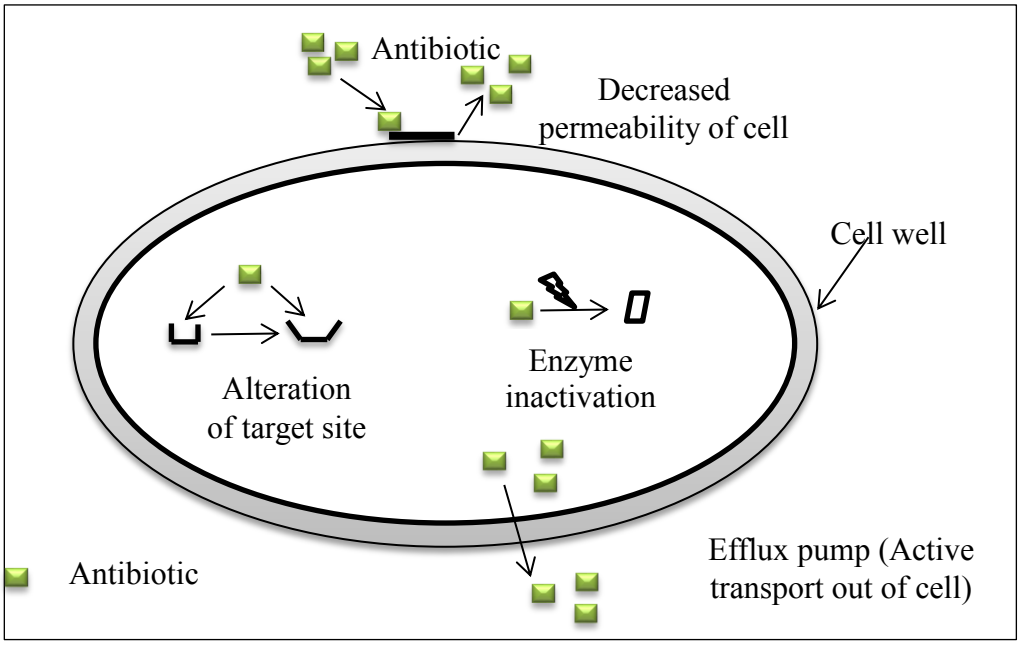


Figure 1-2. Common mechanisms of antibiotic resistance (adapted from (McGraw. 2002)).

1.11 Beta-lactam antibiotics

β-Lactam antibiotics are a broad class of antibiotics that widely used, grouped together based upon a shared structural of β -lactam ring, which is formed of 3 C and 1 N atom in their molecular structures. including penicillin, cephalosporins, monobactams and carbapenems (Holten and Onusko 2000). Penicillin is a group of antibiotics isolated from Penicillium fungi. They include penicillin, procaine penicillin, benzathine penicillin, and penicillin V. Penicillin antibiotics are the first drugs that were effective against many serious diseases such as infections caused by *staphylococci* and *streptococci.* All penicillins are β-lactam antibiotics and are used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms. Although, several types of

bacteria are now resistant to penicillin, penicillin is still useful against some other bacteria and widely used.

1.12 β-Lactam antibiotics and their role in resistance as inhibitors of cell wall synthesis

Beta-lactam antibiotics are bactericidal antibiotics except against *Enterococcus* species (Hollenbeck and Rice 2012). Bacteria often develop resistance to β-lactam antibiotics by many ways such as; (1) the production of β-lactamase enzymes, which is the most common mechanism of resistance in bacteria. β-lactamases react with β-lactam antibiotics to form an acyl-enzyme complex that is rapidly hydrolysed to yield a free enzyme and hydrolysed antibiotic. In some penicillin-resistant bacteria, β-lactamases enzyme deactivates penicillin by breaking a bond in the β-lactam ring of penicillin to deactivate the molecule as revealed in Figure 1.3. Therefore, β-lactam antibiotics are often given with β-lactamase inhibitors such as clavulanic acid which have the ability to bind irreversibly to β-lactamases and, thereby, inhibit their action (Bryan 1984).

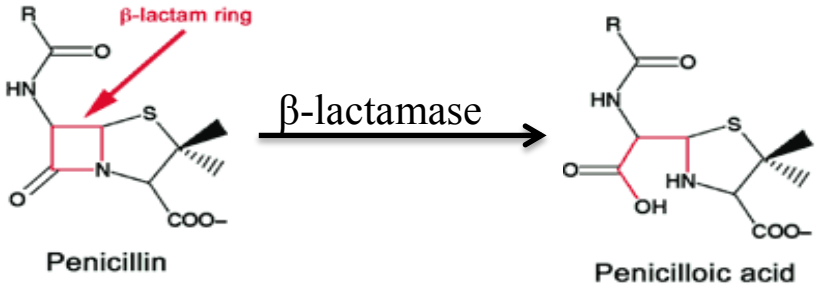


Figure 1-3. β-lactamase enzymes cleave the β-lactam ring of penicillin G to penicilloic acid and inactivate the antibiotic (adapted from (McGraw. 2002)).

Or (2) when the antibiotic fails to bind to an altered penicillin binding protein (PBP) as presented in Figure 1.4. The β-lactam antibiotics are structural analogs of the D-alanyl-D-alanine end of the peptidoglycan strand and binding to the transpeptidase to prevent it from reforming the peptide cross-links between the rows and layers of peptidoglycan monomers in the cell wall (Van Bambeke *et al.* 2004).

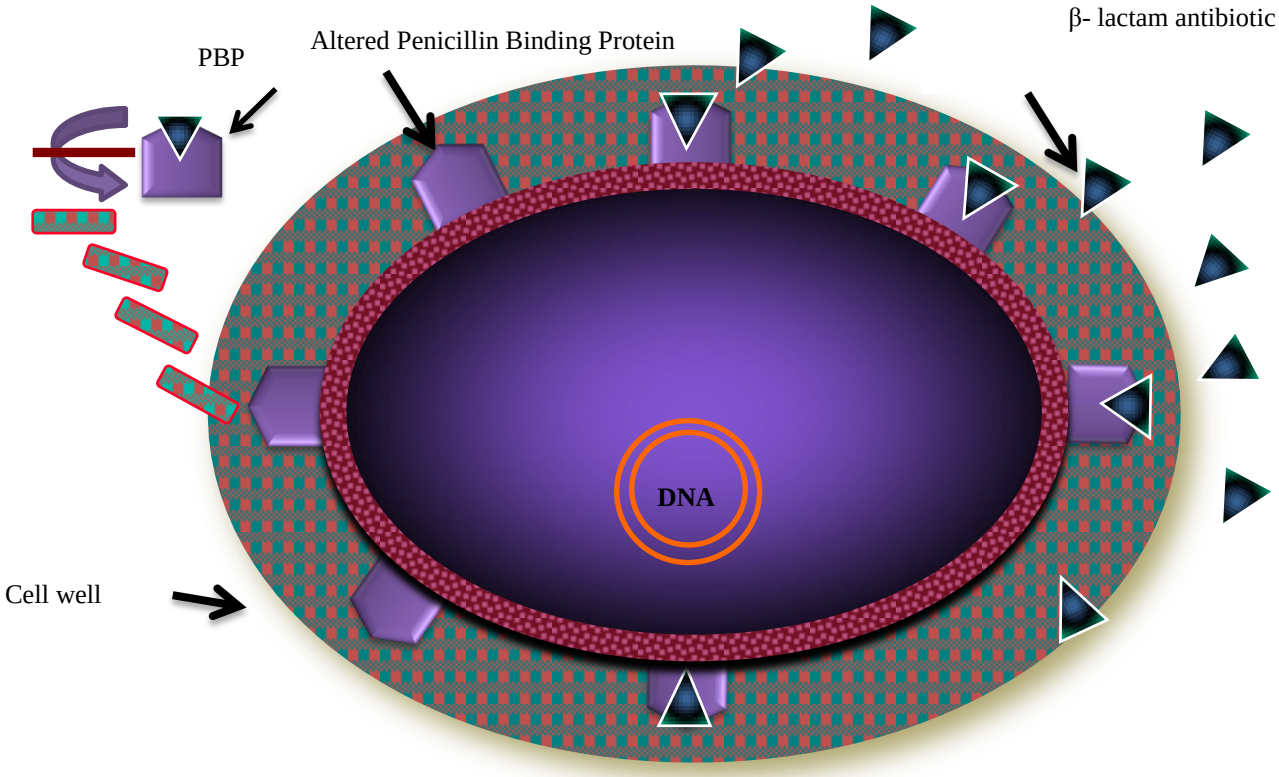


Figure 1-4. β-lactamase enzymes failed to bind to altered PBPs and deactivate the antibioticAdapted from (David and Spach 2014).

1.13 Beta-lactamases and their role in resistance

β-Lactamase are enzyme produced by some bacteria that provide resistance to β-lactam antibiotics like penicillins and cephamycins, they are produced by both Gram-positive and Gram-negative bacteria (Massova and Mobashery 1998). The main function of β-lactamases is inactivating β-lactam antibiotics by hydrolysing the β-lactam ring, that is why β -lactamases are the main causes of β-lactam resistance in many pathogenic bacteria (Richmond and Sykes. 1973). Also, β-lactamase enzymes are produced by non-pathogenic bacteria for instance *Streptomyces* species. Most of the *Streptomyces* species produce β-lactamases constitutively (Ogawara 1975). Beta-lactamases are classified by two different schemes: according to structural homology (Ambler's Classification) or hydrlytic properties (Bush's and Jacoby's classification). According to the Ambler's classification (Ambler 1980), there are 4 groups of beta-lactamases:Group A: which regroups penicillinases (which hydrolyze generally only penicillins and sometimes early-generation cephalosporins), extended-spectrum beta-lactamases (ESBL) which hydrolyze late-generation cephalosporins (such as CTX-M-type) and class A carbapenemases which hydrolyze penicillins, cephalosporins and carbapenems (KPC for Klebsiella penumoniae carbapenemase belongs to this group). These enzymes are inhibited or partially inhibited by class A inhibitors such as Clavulanate or tazobactam. Group C: AmpC or cephalosporinases which exhibits a greater hydrolysis for cephalosporins in comparison to benzylpenicillin. Among representative enzymes, you can found CMY-family AmpC.

Group D: Oxacillinase regroups enzyme able to hydrolyze cloxacillin or oxacillin. It’s a wide group of beta-lactamase and some of them can hydrolyse carbapenem such as e.g. OXA-48 or OXA-23. The last group group B or metallo-beta-lactamase (MBL) differs from the three others by the fact that they possess in the active site metallic ions whereas group A, C and D are serine-active enzymes. This group exhibits a broad-spectrum

hydrolysis including all beta-lactams except aztreonam and these enzymes are not inhibited by clavulanate/tazobactam. β-Lactamases are not essential bacterial proteins in themselves, but are supposed to have developed from the essential PBPs in some β -lactam-producing bacteria such as *Streptomyces* or related bacteria, because these bacteria have to have some self-protective strategies against β -lactam antibiotics (Urbach *and et al* 2008). In *Streptomyces sp* class A β-lactamases are mainly detected in their enzyme activity, over two to five times more classes B and C β-lactamase genes are identified at the whole genomic level (Ogawara 2016). These genes can consequently be transferred to pathogenic bacteria.

1.14 Structure of peptidoglycan in Gram-positive bacteria

The bacterial cell wall synthesis pathway is one of the most important pathways in bacteria and it is a very important target for antibiotics. Since mammalian cells do not synthesize peptidoglycan, this class of antibacterial selectively targets bacteria with no toxic effects toward mammalian cells. Gram-positive bacteria have a thick peptidoglycan (PGN) layer (20 to 80 nanometers) and forms around 90% of the dry weight of Gram-positive bacteria and only 10% of Gram-negative bacteria. Peptidoglycan is a polymer layer, consisting of sugar and amino acid that forms a mesh-like layer outside the plasma membrane of most bacteria forming the cell wall. The sugar component built from alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) as shown in Figure 5. The alternating sugars are connected by a β-(1, 4) -glycosidic bond. A peptide chain consists of three to five amino acids are attached to the N-acetylmuramic acid, each chain containing L-alanine, D-glutamine, L-lysine, and D-alanine with a 5-glycine interbridge between tetrapeptides in the case of *Staphylococcus aureus* (Ryan and Ray 2004). The strong 3-dimensional structure builds up from cross-linking between amino acids in different linear amino sugar chains with the help of the enzyme

transpeptidase (Ryan and Ray 2004). The main functions of peptidoglycan are to maintain the shape of the cell wall, provides strength and rigidity, and to counteract the osmotic pressure of the cytoplasm and also involved in binary fission during bacterial cell reproduction (Nanninga 1998). Peptidoglycan is a well-known target for almost all clinically useful antibiotics that inhibit bacterial cell-wall synthesis. The bacteria lysis when the peptidoglycan is compromised for example, penicillin inhibits the cross-linking of the pentapeptide chains (transpeptidation) by blocking the Insertion of the inter-peptide bridge and significantly weakens the cell wall and finally the bacteria submit to osmotic pressure, resulting in cell death as shown in Figure 1.5.

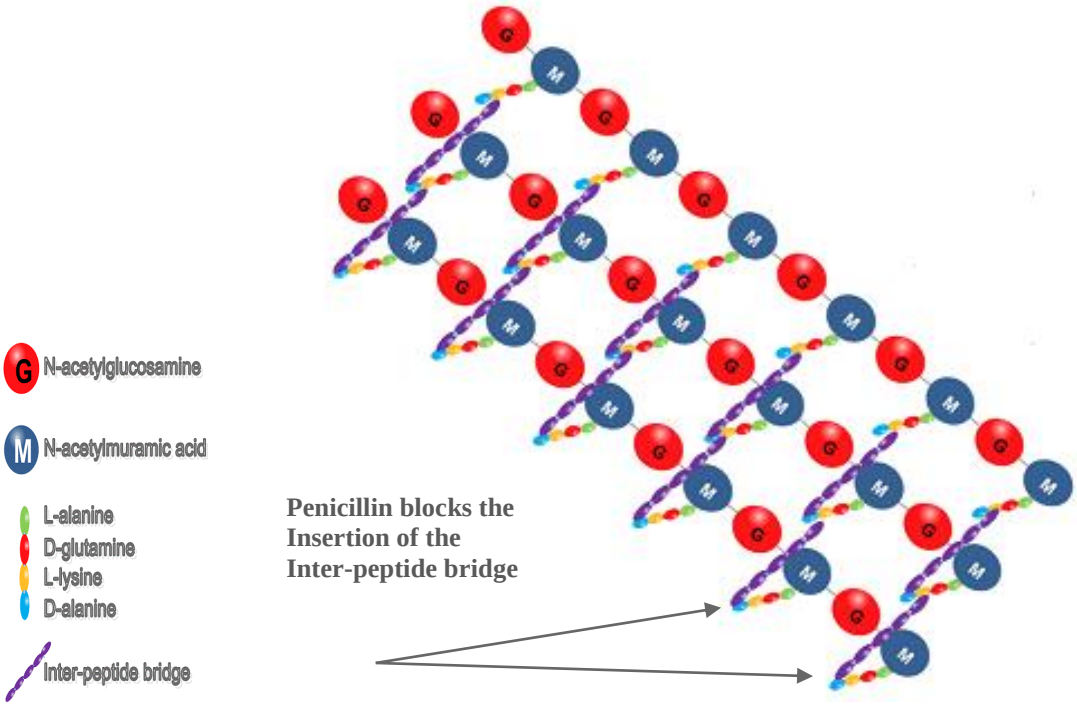


Figure 1-5. Diagrammatic representation of peptidoglycan structure in Gram-positive bacteria (Staphylococcus aureus) illustrate the sugar component (NAG and NAM), amino acid chains, the inter-peptide bridges that join amino acid side chains together and the sites of action of the antibiotic penicillin adapted from (http://www.microbiologybook.org/fox/sa-pep.jpg).

1.15 Peptidoglycan synthesis in Gram-positive bacteria

Peptidoglycan biosynthesis involves multi-stage of enzymatic activities; stage one; starts when the amino acid glutamine gives an amino group to sugar fructose 6-phosphate. This alters fructose 6-phosphate into glucosamine-6-phosphate. Then the amino group on the glucosamine-6-phosphate attach to an acetyl group transferred from acetyl CoA creating N-acetyl-glucosamine-6-phosphate, which change to N-acetyl-glucosamine-1-phosphate by isomerisation (White 2007). The N-acetyl-glucosamine-1-phosphate attacks the pyrimidine nucleotide uridine triphosphate (UTP). UDP-N-acetylglucosamine (2,4) is creating by replacing an inorganic pyrophosphate by the monophosphate. After that, some of the UDP-N-acetylglucosamine (UDP-NAG) is exchanged to UDP-NAM (UDP-N-acetylmuramic acid) by the addition of a lactyl group to the glucosamine.

Stage two; at this stage the peptidoglycan monomers are ongoing to synthesize in the cytoplasmic membrane. The UDP-NAM is modified by the addition of a pentapeptide chain to form a NAM penta. Then, the NAM penta is attached to a lipid carrier called bactoprenol (C_{55}-isoprenyl pyrophosphate) to forming a PP-NAM penta (White 2007). At this time the UDP donate a phosphate group to the bactoprenol carrier when the NAM attaches, which then becomes uridine monophosphate (UMP). This phosphorylation process is crucial for authorizing the transfer of the carrier across the membrane.

Next, a UDP-NAG sugar is transport and attached to form NAG-NAM-pentapeptide unit, which has the ability to transport across the membrane by the bactoprenol carrier and attached to the growing peptidoglycan chain (White 2007). Tranglycosylation, in this reaction will attach the hydroxyl group of the NAG to the NAM in the glycan, and move the lipid-PP from the glycan chain by trans-glycosylase enzyme (White 2007). As the new peptidoglycan monomers are inserted, transpeptidase enzymes

(also known as penicillin-binding proteins) reform the peptide cross-links between the rows and layers of peptidoglycan making the cell wall strong (MT *et al.* 2012). Lipoteichoic acid (LTA) is a main component of the cell wall of Gram-positive bacteria, connect the peptidoglycan layer to the plasma membrane and give the outside of the bacterium a negative charge.

1.16 Penicillin binding proteins (PBPs)

PBPs are a group of proteins that are distinguished by their similarity for and binding of penicillin. They are found in the cytoplasmic membrane. Usually there are many PBPs in each organism. There are two types of PBPs, high-molecular-weight (HMW), that shape the crosslinks between neighbouring peptide side chains and low-molecular-weight (LMW), that have carboxypeptidase and transpeptidase activities (Basu *et al.* 1992). Most β–lactam antibiotics bind to PBPs, which are essential for bacterial cell wall biosynthesis, it is necessary to growth, cell division and maintaining the cellular structure in bacteria, inhibition of PBPs leads to cell death. PBPs in bacteria catalyze many reactions example of which are D-alanine carboxypeptidase, peptidoglycan transpeptidase, and peptidoglycan endopeptidase (Ghuysen 1991) . PBPs have a penicillin-insensitive transglycosylase N-terminal domain, that form the linear glycan strands and a penicillin-sensitive transpeptidase C terminal domain that cross-link the peptide subunits and the serine at the active site is conserved in all members of the PBP family (Basu *et al.* 1992). PBPs and β-lactam antibiotics are similar in their chemical structure to the modular pieces that form the peptidoglycan (Nguyen-Disteche *et al.* 1982). The β-lactam amide bond is ruptured when PBPs bind to penicillin to form a covalent bond with the catalytic serine residue at the PBPs active site and inactivates the enzyme. In *Streptomyces*, LMW PBPs have carboxypeptidase and transpeptidase activity that cleave the terminal D-Ala from petapeptide chains (Hao and Kendrick,

1998). The high molecular weight PBPs are subdivided into two groups, the bifunctional class A high-molecular-weight PBPs possess both transpeptidase and transglycosylase activity, which polymerizes the glycan strands during cell wall synthesis, while class B HMW PBP's are monofunctional transpeptidases (Popham & Setlow, 1994). Both Class A and Class B HMW perform transpeptidase activities, catalyzing the cross-linking of glycan strands (Ghuysen, 1991). While some of HMW PBPs are redundant, others are indispensable. In contrast; the vast majority of LMW PBPs are dispensable for growth (Pogliano *et al*., 1997; Liao and Hancock, 1995; Yanouri *et al*., 1993). Inhibition of high molecular weight PBPs by beta-lactam antibiotics result in cessation of bacterial growth. This implies that the role played by these proteins is vital for the cell's viability (Pechenov *et al*., 2003). Inhibition of some PBPs by β-lactam antibiotics leads to irregularities in bacterial cell wall structure such as elongation and lesions, making the cell wall weak and lysis occurring as consequence of internal osmotic pressure leading to cell death (Strominger & Tipper, 1965)

It has been demonstrated that, there is a relationship between some PBPs and the growth phase of *Streptomyces* species (Nakazawa *et al.* 1981). Previously, it has been shown that β–lactam antibiotics inhibit transpeptidation and prevent germination of spores in *Streptomyces* sp. strain R61 (Dusart *et al.* 1977). Many PBPs of protoplast and sporoplast membranes of *Streptomyces griseus* strains have been identified, but there were no correlations made with any specific function (Baraba´s *et al.* 1988). Moreover, it has showed that the disruption of pcbR gene decreases the resistance to β-lactam antibiotics to less than two fold. The role of PBPs in *Streptomyces* remains to be determined (Baraba´s *et al.* 1988). Because *Streptomyces* has changeable cell morphology during differentiation, several PBPs are likely to be required during the life cycle.

1.17 Penicillin binding proteins in *S. coelicolor*

Bioinformatics analysis using the *S .coelicolor* database DB showed that all penicillin binding proteins of *S. coelicolor* can be classified according to their function to

- Bifunctional (transpeptidase/tansglycosylase). This includes, SCO2897, SCO3580, SCO3901 and SCO5039.
- Transpeptidase (with conserved domains of unknown function, present in other PBP's). This include, SCO1878, SCO3156, SCO3771, SCO3847, SCO4013, SCO5031
- Transpeptidase (with no conserved domains present in other PBP's) SCO3157
- Dimerization domain containing PBP's SCO2608 and SCO2090.
- Peptidase SCO3408 and SCO4439

S.coelicolor has greatest number of penicillin binding proteins. This has been suggested to be attributed to the complex life cycle of *S.coelicolor*.

PBPs share a common dd-peptidase activity, whether a DD-transpeptidase, a DD-carboxypeptidase or aDD-endopeptidase activity (Goffin & Ghuysen, 1998). The crystal structures of *Streptomyces* have not determined yet. However, the crystal structures of *Escherichia coli* PBP5 has been determined (Davies et al., 2001).

The PB domain is associated with a β-strand rich C-terminal domain that ends with an associated membrane amphipathic helix. The PB domain lacks the N-terminal helix and a small loop extends on the top of the active site (green in Figure 1.6.).

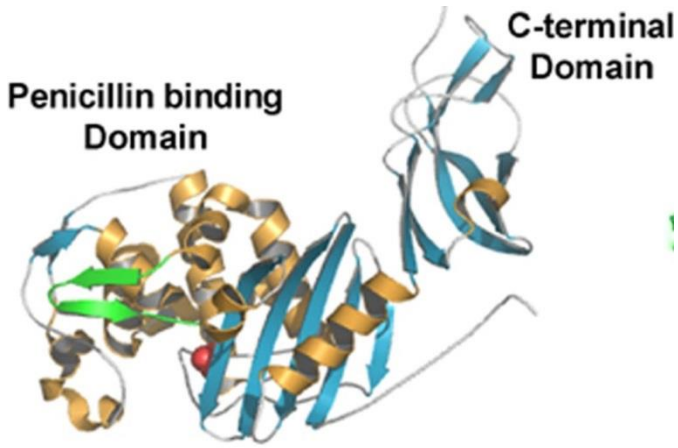


Figure 1-6. Structures of type-5 PBPs (*Escherichia coli* PBP5). Overall view of the structure of *Escherichia coli* PBP5.The catalytic serine of the PB/DD-peptidase domain is shown as a red sphere. On the top active sites, the β-hairpin protuberance is coloured in green.

1.18 Resistance to β-Lactam antibiotics in *Streptomyces*

There are many ways *Streptomyces spp* can develop resistance to β-lactam antibiotics including (1) production of β-lactamase enzyme which inactivates the antibiotics by hydrolyses β-lactam ring. (2) Or by interfering with cell wall synthesis by binding to PBPs. *Streptomyces* have a diverse number of PBPs that can be expressed at different phases of bacterial growth (Sauvage *et al.* 2008). PBPs are membrane-bound enzymes that conduct the final step of bacterial cell wall biosynthesis (transpeptidation) and they are the target for β-lactam antibiotics. Interestingly, there are fewer PBPs in β-lactam-producing *Streptomyces* strains than in β-lactam non-producing strains (Nakazawah 1981).

Many *Streptomyces* species produce β-lactams antibiotics examples of which are penicillin and cephalosporin and they are resistant to moderate to high levels of penicillin

(Ogawara 1981). Moreover, in comparison with PBPs of other Gram-negative and Gram-positive bacteria, these species frequently possess PBPs with low affinities for penicillin, and these PBPs are thought to contribute more to their intrinsic resistance than to β-lactamases (Ogawara 1981). It has also been isolate cephamycin C and clavulanic acid from *Streptomyces clavuligerus* (Reading and Cole 1977). Resistance in β-lactam producers species is also supposed to involve low-affinity PBPs (Ogawara and Horikawa 1980, Nakazawa *et al.* 1981).

Furthermore, there is a complicated relationship between antibiotic production and resistance in *Streptomyces*. It has been found that some strains of *Streptomyces* have genes that are responsible for resistance to the antibiotics that they produce. Examples include vancomycin and streptomycin and these resistance genes can be easily exchanged between streptomycetes and have the ability to transfer from *Streptomyces sp* to other bacterial genera (D'Costa *et al.* 2006, Wright 2007). In addition, it has also been reported that the production and resistance of same antibiotics in *Streptomyces coelicolor* are controlled by genes on plasmids SCP1 (Kirby *et al.* 1975). Interestingly, although the *Streptomyces coelicolor* genome does not contain genes encoding vancomycin the genome does have vancomycin resistance genes (Hong *et al.* 2004). This indicates that antibiotic genes can move freely amongst different species.

1.19 Relationship between β-Lactam Biosynthetic Gene, β-Lactamase and PBP

Some *Streptomyces* species, are β-lactam-producing bacteria, and as such they have to have some self-resistanance mechanism. The β-lactam biosynthetic gene clusters include genes for β-lactamases and penicillin-binding proteins (PBPs), suggesting that these are involved in self-resistance.

The relationship between β-lactamases and the β-lactam biosynthetic gene cluster,

are located in the terminal region of the cephamycin biosynthetic gene cluster and are located within the clavulanic acid biosynthetic gene cluster in *S. clavuligerus* and *S. cattleya*. These two clusters are positioned close to one another and are not positioned close to the β-lactam biosynthetic genes (Ogawara 2014). Therefore, it is suggested strongly that class A β-lactamases and β-lactam biosynthesis are closely related with each other. However, it remains to be clarified whether these β-lactamases are involved in the self-resistance in the *Streptomyces* species. Both sides of the clavulanic acid gene cluster are occupied by PBPs (SCLAV_4180 and SCLAV_4198), indicating that these PBPs behave together with clavulanic acid/cephamycin gene cluster and are involved in the self-resistance of S. *clavuligerus*. The organizations of the clavulanic acid gene clusters of three *Streptomyces* species, *S. clavuligerus, S. flavogirseus, and S. viridis* are similar to each other. However, the relationship between β-lactam biosynthesis, β-lactamases, and PBPs remains to be clarified (Coque, et al.1993, Brakhage, et al 2005).

1.20 Measuring antibiotic resistance in *Streptomyces sp*

The remarkably slow growth rates of *Streptomyces sp* (the doubling time of most *Streptomyces* strains range from 1.45 to 5 hours compare to the doubling time of most wild-type *E.coli* strains that range between 20 to 30 minutes) and long incubation times needed to analyze antibiotic resistance in *Streptomyces sp* represent major problems in evaluation of the effectiveness of the antibiotics against the bacterium. Investigate antibiotic resistance microorganisms are carried out usually by determining the minimum bacteriostatic concentrations (MICs) and minimum bactericidal concentrations (MBCs) of possible antibiotic candidates. There are many techniques used to determine the MICs and MBCs such as broth or agar diffusion methods, disk elution and the OxoPlate®. The OxoPlate® is a useful novel technique in the characterization of antibacterial compounds. Each well of the plate carries two different fluorescent dyes, the first dye is

an oxygen-sensitive indicator dye and the other is a reference dye. The intensity of the fluorescence is relative to the quantity of dissolved oxygen in the medium. Growth of bacterial cells is measured indirectly by the regular reduction of oxygen. The OxoPlate® is a useful method to determine MIC values directly from the oxygen-depletion curves, and able to discriminate bactericidal from bacteriostatic compounds. Oxygen levels of cultures treated with bactericidal compounds drop initially and then increase after the cells have died. In contrast, oxygen levels remaine low when cultures are treated with bacteriostatic compounds as presented in Figure 1.7 (Hutter and John 2004).

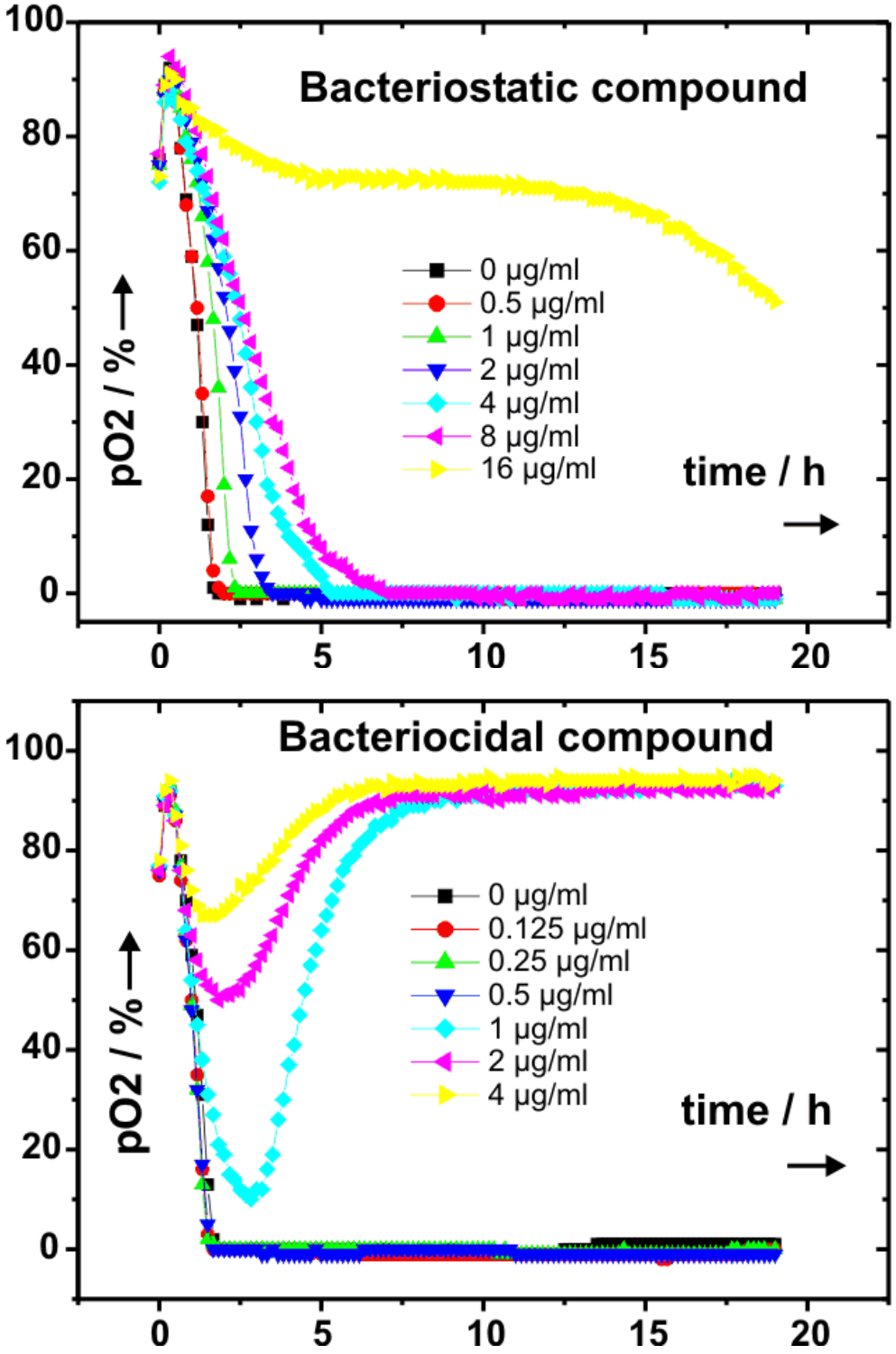


Figure 1-7. Graph shows how to discriminate bactericidal form bacteriostatic compounds using OxoPlate®.(Hutter and John 2004).

1.21 Difficulties in growing *Streptomyces* in continuous culture

Streptomycetes are filamentous soil bacteria that are used in industry for the production of anti-microbial compounds mainly antibiotics. *Streptomyces* species tend to form cell pellets and biofilms during growth in the liquid culture. The pellets are formed of heterogeneous mycelium (different ages at different nutritional and growth stages), which makes control of the culture almost impossible (Doull and Vining 1989). Numerous methods to prevent and aid this problem have been published, for example some researchers used stirring rate of 3000 rpm with a glass blade stirrer (Roth and Noack 1982). others used some glass beads or a high concentration of starch to the culture (Doull and Vining 1989), whereas Hobbs et al. (1989) added polyanions like agar or junlon at low concentrations to the fermentation medium to prevent the development of flocs (Hobbs *et al.* 1989). Furthermore, a method to analyse and sort mycelial pellets using a Complex Object Parametric Analyser and Sorter (COPAS) are other strategies that have been used (Petrus *et al.* 2014).

Furthermore, each of these approaches have different effects on the culture for instance the use of a stirrer rate of 3000 rpm with a glass blade stirrer could lead to extensive cell damage and lysis, and the addition of polyanions to the medium may have an effect on cultures and the antibiotics.

1.22 Project aims and objectives

Antibiotic resistance is a developing problem and is a challenge to human medicine. Infections caused by resistant bacteria usually fail to respond to standard antibiotic treatment, resulting in prolonged illness and greater risk of death. Little is known about the mechanism of resistance of pathogenic bacteria such as *Corynebacterium diphtheriae* and *mycobacterial species* that cause tuberculosis and leprosy to β- lactam antibiotics. This in part is due to the difficulties with working with such dangerous organisms in a laboratory setting.

Recently, it has been established that pamamycins, which are antibiotics isolated from *Streptomyces sp* have potent anti-mycobacterial activity, against *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium smegmatis* (Lefevre 2004). Furthermore, *Streptomyces* species are closely related genetically to these organisms and present themselves as a good safe model to work with and to investigate the mechanisms of action of β-lactam antibiotics and modes of resistance to these antibiotics.

The aims of this study were to identify the mechanisms that underpin the resistance of *Streptomyces* species to β-lactam antibiotics (penicillin G). This was including an investigation into the mode of cell death caused by these antibiotics. The effect of continuous exposure of *Streptomyces coelicolor* to penicillin G was examined.

The objectives of this study are:

(1) Isolated strains of streptomycetes from soil that exhibit elevated resistance to penicillin G and characterise the organisms. These strains examined for their mode of resistance to penicillin G. Part of this work was aimed to identify β -lactamase producing strains.

(2) Establish a novel staining technique to examine the interaction between penicillin G and *Streptomyces* species using a panel of stains including: (A) BOCILLIN FL, a non-radioactive fluorescent derivative of penicillin V, which is a fluorescent β-lactam; (B) Cell Mask™ a deep red plasma membrane stain and (C) DAPI (4',6-diamidino-2-phenylindole) a fluorescent stain that binds strongly to DNA.

(3) This study was designed to investigate the relationship between antibiotic exposure and resistanance to penicillin G in *Streptomyces coelicolor*.

(4) Study the effect of growth rate on antibiotic resistance in *S. coelicolor* using continuous culture.

Chapter Two

Materials and methods

2. Sample collection and isolation of actinomycetes

2.1 Soil samples

Soil samples were collected from different parts of Liverpool, UK. Samples were collected from various depth of the earth surface, ranging from layers just beneath the upper surface to 6 centimeters depth. They were collected in the sterile plastic. Seven soil samples were collected.

2.2 Isolation of pure culture of actinomycetes

Ninety six strains were isolated and obtained as pure culture by using standard microbiological method. From each soil sample, 1 gm of the soil was suspended in 100 ml sterile water and incubated in water bath at 46ºC overnight, then serial dilutions were made up to 10^{-6}. Each time the contents were vortexed to form a uniform suspension. For isolation of actinomycetes an aliquot of 0.1 ml of each dilution was spread evenly over the surface of starch-casein- agar (SCA) plates (g/l: starch 1.0; casein 0.4; potassium nitrate 0.5; dipotassium hydrogen phosphate 0.2; magnesium sulphate 0.1; calcium carbonate 0.1; agar 15; pH 7.2). Plates were incubated at 28ºC and monitored for 7-14 days. The colonies were carefully counted by visual observation. Actinomycetes colonies were purified using a streak plate technique on cultivation medium. The purified actinomycetes were preserved on Manito-Soy Agar (MS) medium (g/l: mannitol 20; soy 20; agar 20) and incubated at 30ºC for 14 days.

2.3 Maintenance of bacteria

Maintenance of isolated strains throughout this work was achieved for short term on MS agar medium plates, and for long terms were achieved on cryobank tubes (CRYOBANK™, Copan Innovation) and were stored in a freezer at -80°C. Cryobank tubes are tubes contain a medium for suspending the bacterial culture and 25 color-coded ceramic beads. The suspending medium comprises tryptone soy broth supplemented with glycerol and sucrose. *Streptomyces coelicolor*1147 was maintained on MS agar after growth for 7-14 days at 30°C and cryobank tubes at -80°C. Nutrient agar was used for the maintenance of *E. coli* after growth for 24 hours at 37°C.

2.3.1 *Streptomyces coelicolor* A3 (2) and mutants

Streptomyces coelicolor A3 (2) strain 1147 "Wild type" was maintained as a frozen suspension at -20˚C. Cryobank™ (Mast Group Ltd) and four mutants (δ2897. δ3580, δ3901 and δ5039) were obtained from a previous PhD study in this laboratory (McCulloch, 2005), where mutants were created by PCR directed gene deletion. The mutants were maintained as a frozen suspension at -20˚C. Cryobank™ (Mast Group Ltd).

2.4 DNA extraction

DNA was extracted using the E.Z.N.A. ® Bacterial DNA Protocol- Spin Protocol (Omega-bio-tek) according to the manufacturer procedure, some colonies were cultured in 100 ml modified yeast extract/ malt extract (YEME) medium (4 g/l yeast extract (Sigma), 10 g/l malt extract (Oxoid) and 4 g/l D- (+)-glucose (Oxoid)) for 2-3 days in a shaking incubator at 30° C, 1 ml of the culture was centrifuged and the pellet was re-suspend using100 µL of Tris-EDTA buffer (TE Buffer). Lysozyme (10 mg/ml) was added and incubating at 37°C for 15 minutes. Glass beads were used to complete disruption and lyse the cells. BTL Buffer (100 µL) and Proteinase K Solution (20 µL)

were added and Incubated at 55°C in a shaking water bath for 2 hours. Then, RNase A (5 µL) was added and the sample incubated at room temperature for 5 mins. The sample was then centrifuged at 14.000 rpm for 1 min. BDL Buffer (220 µL) was added and incubated at 65°C for 10 minutes before adding 220 µL ethanol (96-100%). The sample was then transferred to the HiBind® DNA mini column and centrifuged at 10,000 rpm for 1 minute before 500 µL of HB Buffer was Adding and centrifuge at 10,000 rpm for 1 minute. The column was washed using 700 µL DNA wash buffer. To elute the DNA 30 µL preheated elution buffer (65°C) was added to the HiBind® DNA mini column and incubated for 3 to 5 minutes at room temperature and centrifuged at 10,000 rpm for 1 minute. The DNA was then stored at -20°C.

2.4.1 Polymerase Chain Reaction amplifications (PCR) of DNA

2.4.1. 1 PCR amplification of DNA for sequencing and species identification

The PCR was performed in a 50 µl reaction mixture containing 45 µl Master Mix, 2 µl of Dimethyl sulfoxide (DMSO), 0.5 µl F-primer (27 F), (5'-GAGTTTGATCCTGGCTCAG-3'), specific for bacteria and 0.5 µl universal reverse oligonucleotide primer, R-primer (1492R), (5'-GGTTACCTTGTTACGACTT-3'), 1 µl sterile distillate water and 1.0 µl DNA target were transferred in PCR tubes. The DNA templates were subjected to 35 cycles consisting of denaturing at 95°C, for 1 minute, annealing at 52°C, for 1 minute and elongation at 72°C, for 2 minutes.

2.4.1. 2 PCR amplification of DNA for sequencing and phylogenetic tree

Ten isolated strains that have high MICs from; five beta-lactamase producing *Streptomyces;* (W21), (W51), (W55) (W76) and *(*W60)*;* and five from non-beta-lactamase producing *Streptomyces;* (W32), (W39), (W96), (W79) and (W75)*;* as well as the fragmented strain (W43) were also amplified with nested primer 907R (5′-

CCGTCAATTCMTTTRAGTTT-3′) to get almost completed 16s rRNA gen and to conduct phylogenetic tree.

2.5 The PCR product purification

The PCR product purification was accomplished using a QIAquick PCR purification kit protocol, according to the manufacturer's instructions. Buffer PB (75 ul) was added to 15 µl PCR product and mixed. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 60s before adding 750 µl of PE buffer to the QIAquick column to wash it and this was then centrifuged for 30–60 s. To elute DNA, 30 µl elution Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane and the column was centrifuged for 2 min. The DNA extracted was purified on agarose gel electrophoresis.

2.6 DNA purity was verified by agarose gel electrophoresis

PCR products (5.0 µl) were run at 100 Volts/cm on 1% w/v agarose gel for 60 minutes. In the first well was added 5.0 µl of DNA Hyper ladder buffer. The Tris-acetate-EDTA 50x (TAE) at pH 8.5 was used ((242g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5M EDTA at pH 8 (88 ml distil water, 18.6g of 0.5M EDTA and increase the volume to 100 ml), 750 ml distil water)) in 1L**.**

2.7 Scanning Electron Microscopy (SEM)

Morphology and spore surface ornamentation of *streptomyces* were examined by scanning electron microscopy. The samples were grown on MS agar for 14 days at 30°C. Cells were fixed using glutaraldehyde (EM grade 2.5%) overnight at 4°C, then washed with serial dilution of ethanol (30%, 50%, 70%, 90% and 100) for 1 min at each concentration. The samples were immersed in 100 % acetone before dehydrating by

critical point drying at 35°C at a pressure of 1,200 psi. The samples were coated by gold Sputter Coater Unit (EMITECH K550) before viewing in the SEM (FEI Inspect).

2.8 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of penicillin G to Streptomyces strains

The MIC and MBC of penicillin G for isolated *Streptomyces* strains were measured by two techniques, plate culture in solid medium and OxoPlates® in liquid medium.

In plate culture each strain was grown in MS medium for 14 days and then streaked onto SC plates containing various amounts of penicillin G. The concentrations of penicillin G (Sigma Co.) were 25, 50, 100, 200 and 400 µg/ml. Each plate was incubated at 30°C for 14 days. MICs were obtained from the results of plates without colonies, when full growth was observed on plates without penicillin G.

Moreover, The MICs and MBCs were determined using a novel technique called the OxoPlate® (PreSens, Regensburg, Germany) which is a 96 well plate assay; each well of the plate carrying two different fluorescent dyes; an oxygen-sensitive as guide dye and a reference dye, and the oxygen signal was monitored on-line giving an indication of the cell's metabolic activity. Essentially, this method provides high throughput, real-time, on-line measurements of oxygen uptake by cultures. Using a 96-well plate, The OxoPlate® system has been used in this study to determine the minimum inhibitory concentrations (MICs) for antibiotic and also to monitor the bacteria growth rate. The FLUOstar OPTIMA (BMG LABTECH) microplate reader was set to time resolved fluorescence (TRF) mode with Filter pair 1 (544/650 nm) used to detect fluorescence of the indicator dye. The second filter pair (544/590 nm) measures fluorescence of the reference dye. Fresh spores were harvested from 14 days old plate cultures. Sterile Muller Hinton (MH) medium (5ml) was poured on the surface of the culture and spores were removed off by

gentle agitation with a sterile cotton swab. Then the spore stock was diluted and brought to 10^8 CFU/ml, which corresponded to an optical density 1 at 420 nm. The spore suspension was used to inoculate 50 ml of MH broth.

180 µl of overnight culture was added into each well and 10 µl of penicillin G at different concentration. The plate was covered with a gas permeable adhesive seal (Thermo Scientific) and incubated in a FluoStar Optima plate reader (BMG) at 30 °C for 72 hour. Positive control wells were loaded with MH broth with bacteria added without antibiotic and negative control wells were loaded with just medium. Each strain was assayed in triplicate.

2. 8. 1 Calculation of oxygen concentration

The referenced signal IR was calculated from these two intensities.

$$\text{I R} = \frac{\text{I indicator}}{\text{I reference}}$$

Where I indicator be a symbol of fluorescence intensity dependent on the concentration of oxygen in the sample, while I reference be a symbol of fluorescence intensity independent on the concentration of oxygen in the sample,

The oxygen concentration PO_2 as [%] air saturation is calculated for each measurement point using the following equation $100 * \left(\frac{K_0}{I_R} - 1 \right) / \left(\frac{K_0}{K_{100}} - 1 \right)$

Where K_0 is a constant calculated by taking the average of the signals of IR of at least three wells filled with oxygen-free water (cal $_0$), while K_{100} is a constant calculated by taking the average of the signals of IR of at least three wells filled with air-saturated water (cal 100).

Cal 0 is prepared by dissolving Gram of sodium sulphite in 100 ml water, while Cal 100 was prepared by shaking 100 ml of water in a suitable vessel for two minutes to ensure that water is air-saturated**.**

2.8.2 Determination of Bacterial Growth rate

Growth rates were determined as described by Stitt et al. 2002 using the rate of oxygen uptake as the indices of growth. A typical set of data is shown below.

Values of PO_2 from the Oxoplate® were converted into a natural log (Ln) and then presented on the y axis versus time on the x axis. The doubling time could be determined from Ln (2) divided by the exponential slope (μ).

$$td = \frac{Ln2}{\mu}$$

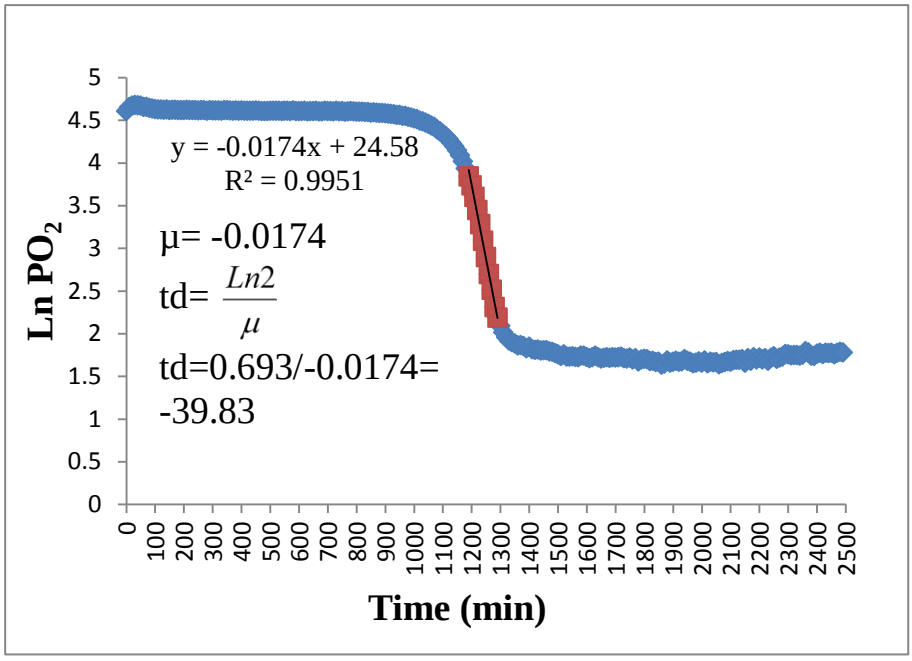


Figure 2-1. Graph represent how the growth rate of organisms was calculated using the OxoPlate® system.

2.9 Screening for β-lactamase activity

Streptomyces strains were grown on a rotary shaking incubator (180 rpm) in 150-ml flasks containing 50 ml of YEME medium for 72 hours at 30°C. After appropriate times of cultivation, 100 ul aliquots were taken out to determined β-lactamase activity using nitrocefin disks (Sigma). *E coil* was used as a positive control as a β-lactamase producing bacteria and water was used as negative control.

Nitrocefin is a chromogenic cephalosporin which changes from yellow to read when the amide bond in beta-lactam ring is hydrolyzed by beta-lactamase**.** Nitrocefin disks test are good, rapid, indicators of ß-lactamase production. However, are less useful to determine the type of ß-lactamase.

2.10 BOCILLIN FL

BOCILLIN FL, fluorescent penicillin, as a labeling reagent for the detection and study of all penicillin-binding proteins (PBPs) (BOCILLIN FL, Molecular Probes) but did not determine the type of PBP that bind to. It is green fluorescent. Fluorescent BOCILLIN was reconstituted in distilled water at a final concentration of 100µg/ml. For solid cultures, spores were mixed with 5µl of the BOCILLIN stain. Samples were kept in the dark at room temperature for 5 min then rinsed with water to remove the excess dye. It fluoresces at 511 nm upon excitation at 504 nm (Molecular Probes, Inc.).

2.11 Chemostat culture experiments

2.11.1 Preparation of inoculum of *Streptomyces coelicolor 1147*

In all chemostat culture experiments, a spore inoculum of *Streptomyces coelicolor* strain 1147 were used and prepared as follows: *Streptomyces coelicolor 1147* was cultivated on 10 plates of MS agar medium for 14 days at 30°C. After full growth and spore formation, 5 ml of sterile distilled water was added to each petri dish and the growth was scraped gently by sterile cotton tip swab sticks to release the spores into the water which were then collected in a sterile bottle.

2.11.2 Chemostat culture conditions

Chemostat cultures were run in a 3L fermenter (Applikon Biotechnology®), constructed from a jacketed pyrex glass vessel with a working volume of 2.8 L with stainless steel lid. The stainless steel lid had several ports usually used for inoculation and feeding, sampling, agitation, aeration, condenser, pH adjustment and antifoam, pH sensor probe, dissolve of oxygen probe, and outlet medium tube. Moreover, four of 250 ml bottles were connected to the fermenter via long tubes and used as supplies of antifoam, inoculum spores, NaoH (1.5 mM) and HCL (1.5 mM). A photograph of 3L fermenter is shown in Figure 2. Applikon chemostat is monitored by an ez-Controller unit, which are linked to a PC workstation loaded with expert software that enables data acquisition and analysis (Applikon Biotechnology®). Chemostat is organized to be operated with Bug Lab's optical density reading system with bug Lab Sensor and FerMac 368 gas analyser ((FerMac 368).

2.11.2.1 Temperature: The temperature of the vessel was kept constant at 30ºC by using cold fingers connectors for water to and from reactor, and the heating jacket around the vessel.

2.11.2.2 Aeration: The vessel was aerated by passing sterilised air through stainless steel tube with L shape with small holes on it to distribute the air consistently. The tube was immersed into the medium near the bottom of the jar. The incoming air was filtered by air filter with 0.2 µm pore size (scientific laboratory supplies).

2.11.2.3 Agitation: a top driven motor with two blades were used to mix the medium throughout the fermentation period and one blade was used on the top of the medium in order to break down foams.

2.11.2.4 Sampling: samples were gained from the vessel by a long stainless steel tube dipped in the medium close to the bottom of the vessel. This tube was connected via a small tube to a stainless steel adapter to a sterile universal tube.

2.11.2.5 Condensation: a small stainless steel condenser was set into the lid aiming to avoid the evaporation of water from the medium. An air filter of 0.2 µm pore size was connected to the outer side of the condenser via a small tube for the exhaust gas.

2.11.3 Inoculation and fermentation

The four fermenter cultures prepared by inoculate 5% (v/v) f spores inoculum of *Streptomyces coelicolor 1147* using Watson-Marlow peristaltic pump to 2 L of modified glucose limited YEME medium (4 g/l yeast extract (Sigma), 10 g/l malt extract (Sigma) and 2.0 g / l D- (+)-glucose (Sigma)), The 2L YEME medium was autoclaved at 121^{o}C for 15 minutes. Anti-foam was sterilized separately in 250 ml bottles. The temperature was controlled at 30^{o}C. The chemostat was operated with agitation of 1000 rpm to prevent the formation of pellets and an aeration of 2.2 L/min. The pH of the medium was initially adjusted to7.2 and then controlled and kept consistent by connecting two 150 ml bottles of alkaline (NaOH, 1.5 mM) and acidic (HCl, 1.5 nM) supplier to fermenter by a long plastic tube and were introduced using a peristaltic pump when necessary. Foam control was achieved by using autoclaved antifoam (1g of silicon, 1000 ml water) stored

in 250 ml bottles connected to the fermenter. The uniform was introduced into the fermenter by a peristaltic pump when necessary.

The rate of medium flow into the vessel is related to its volume, and is defined by the dilution rate (D) and it calculated from the equation;

$$\mu = D = \frac{f}{v}$$

Where D = dilution rate; f = flow rate ($L.h^{-1}$); v = fermenter volume (L);

μ = growth rate (h^{-1})

Each fermenter runs at different dilution rate (D) 0.04, 0.06, 0.08 and 0.1 h^{-1}.

Dilution rate was constants by using Watson-Marlow peristaltic pump. Different concentration of penicillin G sodium salt (Sigma) was filtered using whatman 0.2 µm pore size filter (scientific laboratory supplies) and introduced to the medium bottle via 0.2 µm pore size filter (scientific laboratory supplies). Every day, a sample of 30 ml was taken for the analyses of growth, glucose consumption, under light microscope and fluorescent microscope, subculture on MS and nutrient agar.

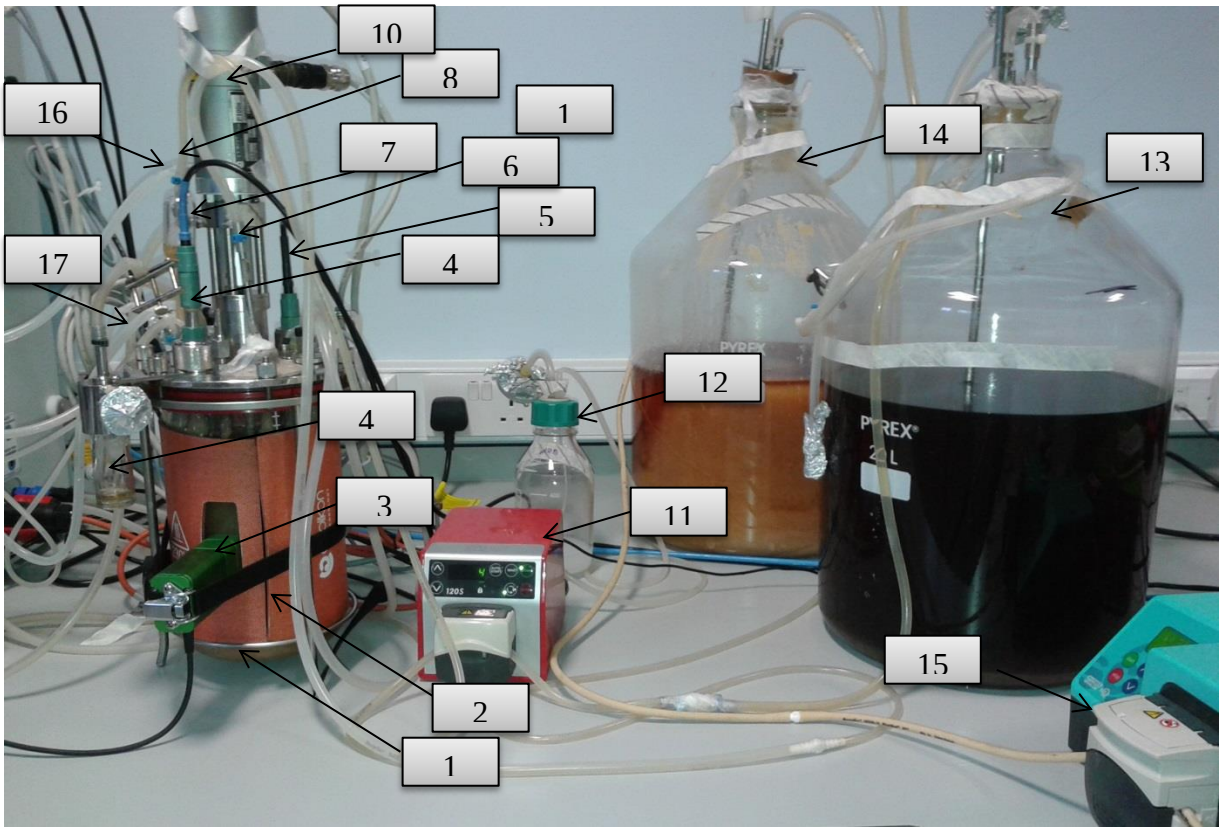


Figure 2-2. Photograph of 3 L bioreactor. Description: 1-Vessel, 2- Jacket, 3- Bug Lab for optical density reading, 4- Sampling bottle, 5- Dissolve of oxygen probe, 6- PH indicator sensor, 7-medium outlet tube, 8- Anti growth back, 9- Feeding tube,10- Stirrer Motor, 11- Peristaltic pump for antifoam feeding, 12- Antifoam reservoir, 13- Medium reservoir, 14- Medium out reservoir, 15- Peristaltic pump for medium feeding, 16- Condenser, 17- Air inlet.

2.11.4 Analytical methods

2.11.4.1 Dry mass determination

The growth of *Streptomyces coelicolor 1147* was measured as the dry weight of cells. 10 ml of the culture was centrifuged at 15000 rpm for 5 min. The pellet was washed three times with distilled water and dried at 85 °C for 7 days before determining the mass of the pellet. In some cases, the procedure was continued until a constant mass was obtained.

2.11.4.2 Gas analysis

The amount of oxygen uptake and carbon dioxide production in the outlet gas from the fermenter were measured using the FerMac 368 gas analyser (FerMac 368).

2.11.4.3 Determination of glucose concentration

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red - violet quinoneimine dye as indicator.

Samples of *Streptomyces coelicolor* culture were filtered through Whatman GF/C filter paper to remove the cells and other solid components that can affect the concentration of the glucose, and then the glucose concentration of the medium was measured using commercial kits for glucose (Randox GL2623). According to manufacturer's procedure for each sample, 1000 µl of reagent was added to three cuvette tubes (scientific laboratory supplies), and 10 µl water was added to first tube in order to act as blank, and 10 µl standard reagent was added to second tube, this was used as standard concentration, and finally 10 µl of sample was added to last cuvette tube. Then the tubes incubated at 37°C for 10 min before the concentrations were measured against the blank at wavelength of 500 nm.

The concentration of glucose was calculated using a standard equation as follows;

Concentration of glucose = standard concentration x A sample (glucose) / A standard (glucose) g/l.

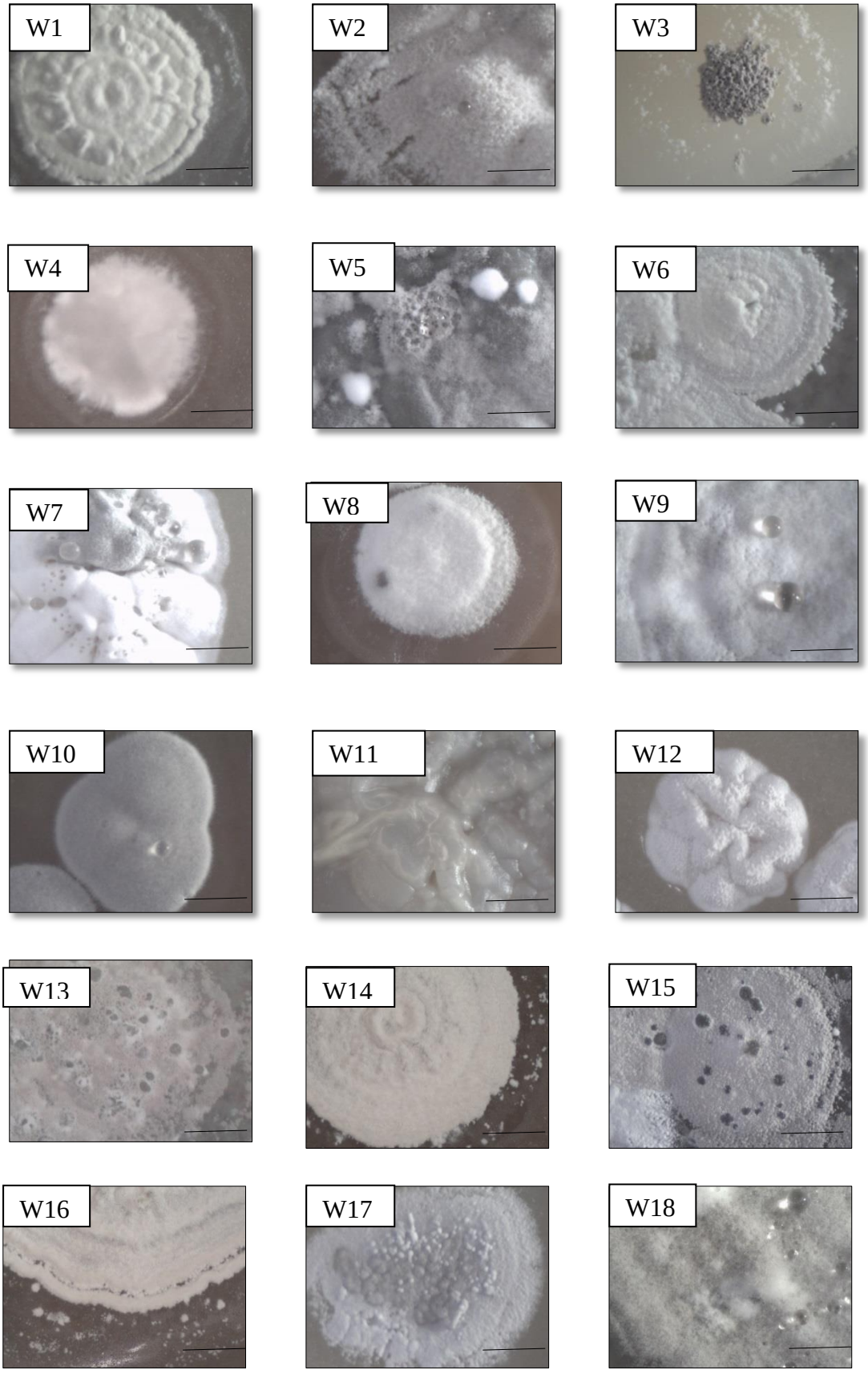
Chapter Three

Results

3.1 Isolation and morphological characteristics of actinomycetes

3.1.1 Growth of *Streptomyces sp* on Solid Media

A total of ninety six isolates of actinomycetes were isolated and purified from seven soil samples collected from different places in Liverpool, UK. The growth of the isolates was tested on various media, solid (MS, YEME, MHA and SC) and liquid media (MHB and YEME); the strains grew well on all of the defined and synthetic media tested. The strain showed maximum growth when cultivated at a temperature of 30°C for 14 days. Typically, most of the colonies were elevated, self-limiting, and covered with white and grey aerial mycelia and in some strain spores could be seen in MS medium. Diffused melanin pigments were sometimes observed. Culture characteristics of strains were derived on the basis of observations made after 14 days of incubation on MS media as shown in Figure 3.1. According to the cultural characteristics, all isolates exhibited a range of colony colours (dark grey, grey, dark brown, brownish, whitish and yellowish). The microscopic examination of the colony of the isolates revealed that aerial mycelia were morphologically branched with a grey and White surface appearance and some strains formed spores. The mycelia growth as well as development of spore chains was studied under a light microscope as presented in Table 3.1. The formation of the spores and aerial mycelia give a colony of *Streptomyces* its characteristic fuzzy, powdery appearance. Moreover, some strains produced secondary metabolites such as W2, W5, W7, W9, W10, W15, W44, W51, W52, W61, W63 and W81 as displayed in Figure 3.1.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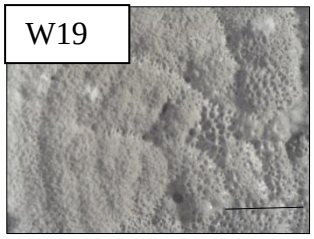
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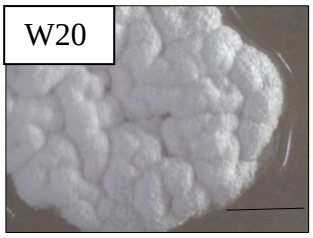
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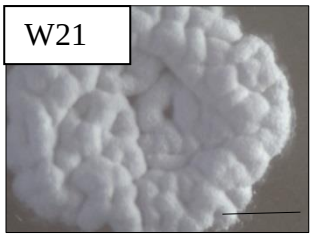
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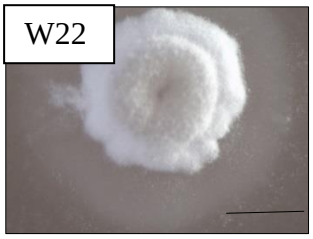
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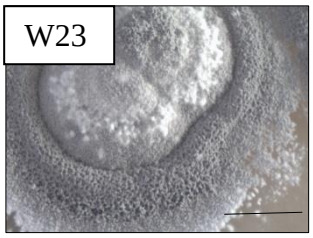
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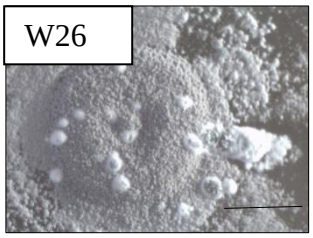
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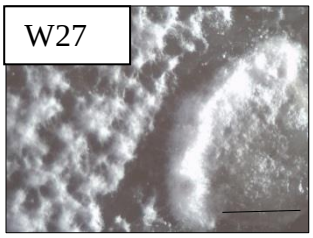
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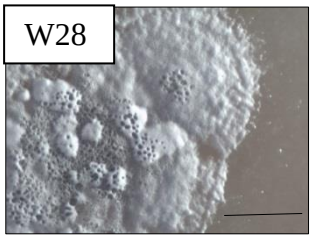
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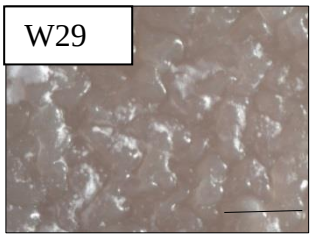
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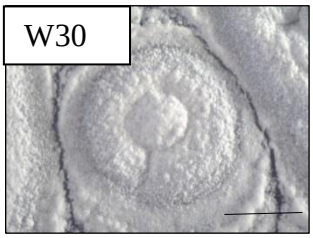
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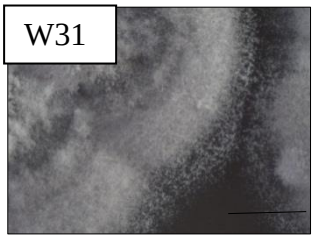
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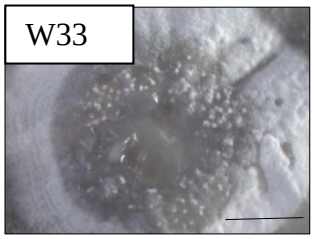
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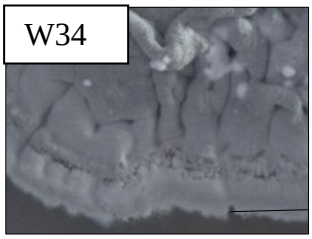
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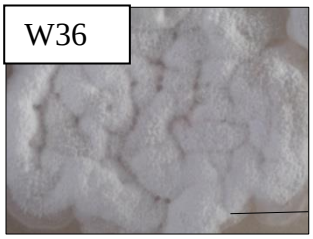
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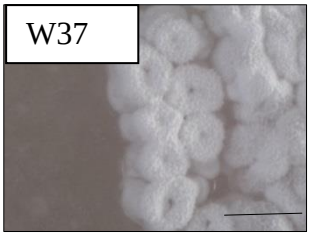
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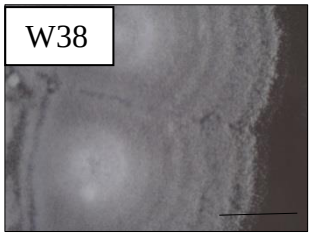
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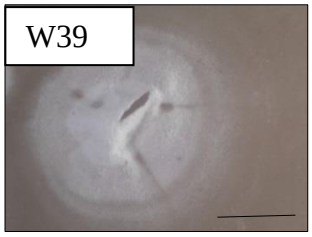
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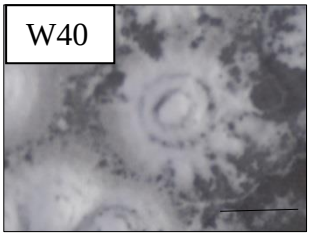
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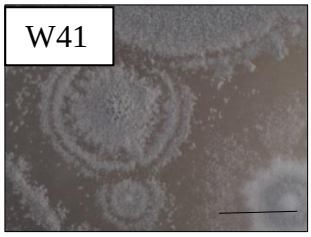
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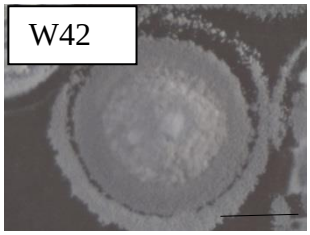
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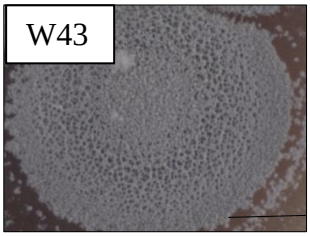
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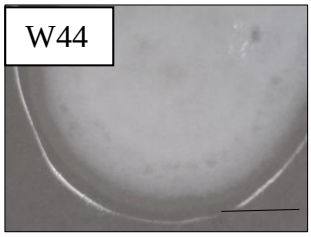
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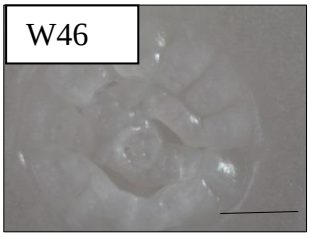
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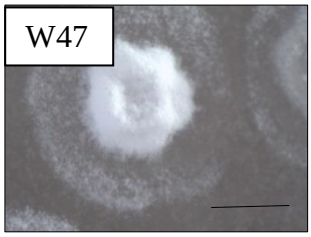
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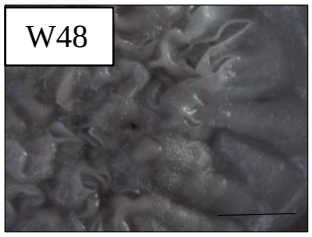
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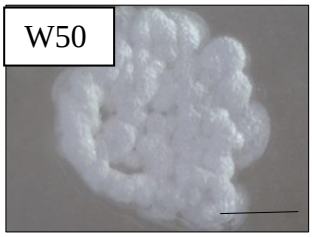
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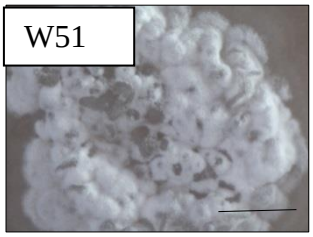
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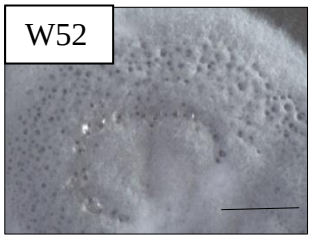
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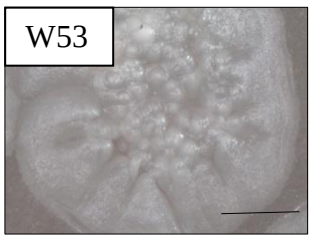
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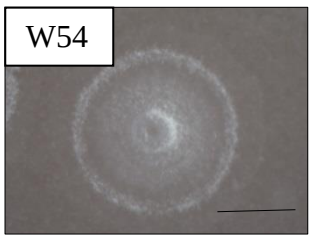
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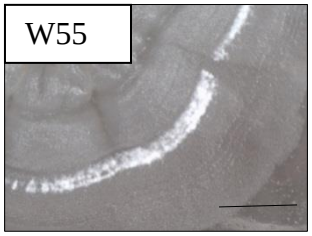
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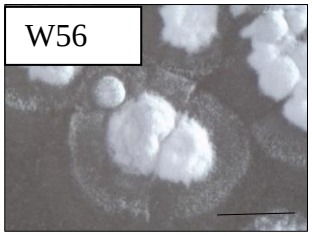
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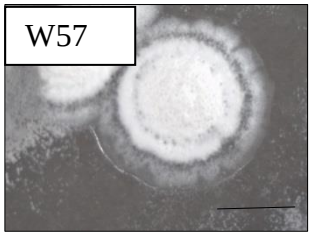
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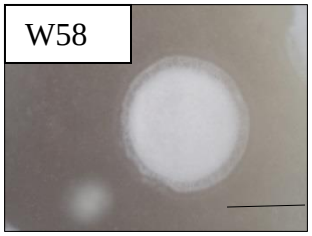
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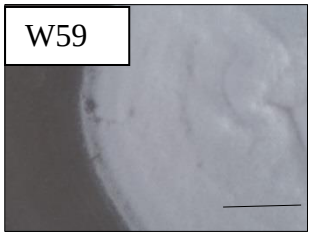
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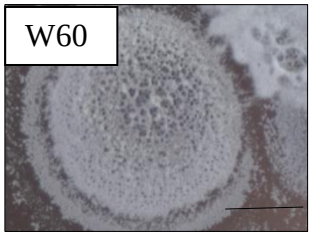
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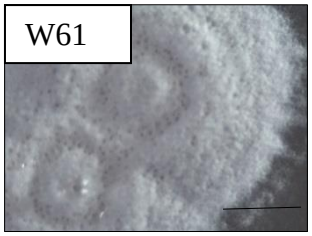
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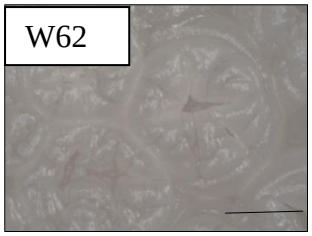
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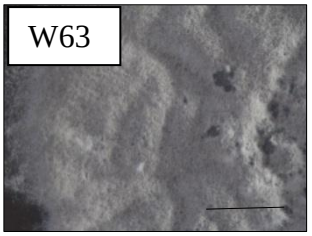
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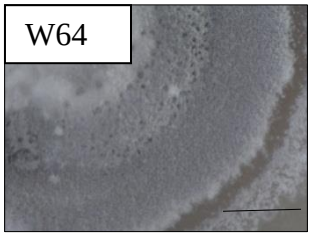
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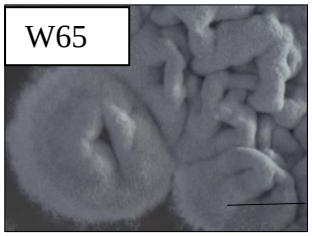
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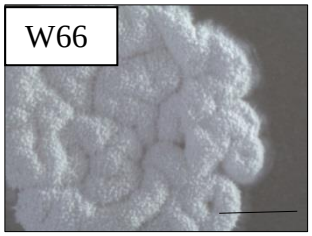
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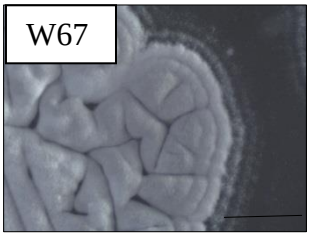
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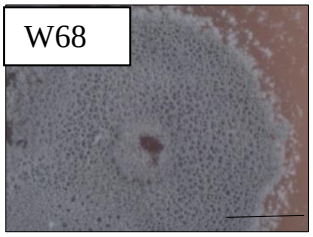
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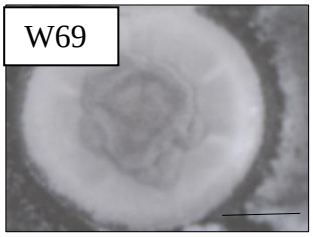
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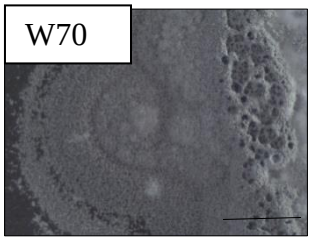
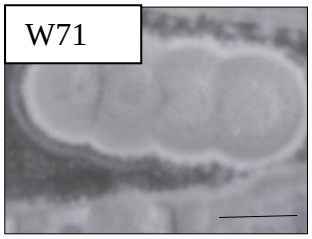

W70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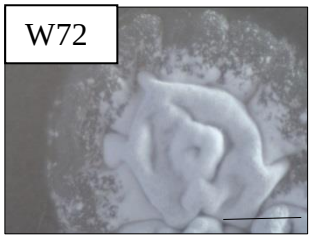
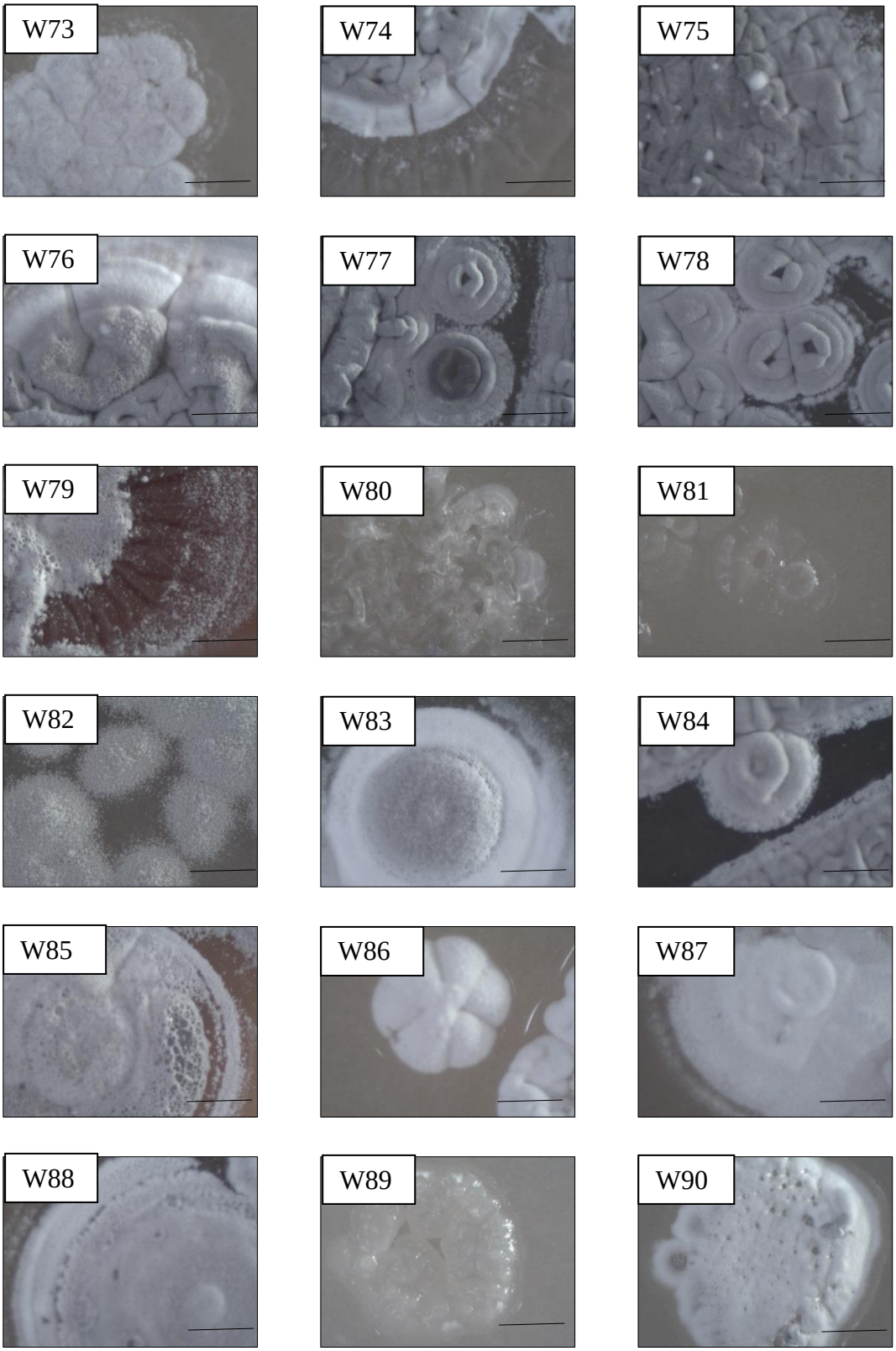

W72


W73
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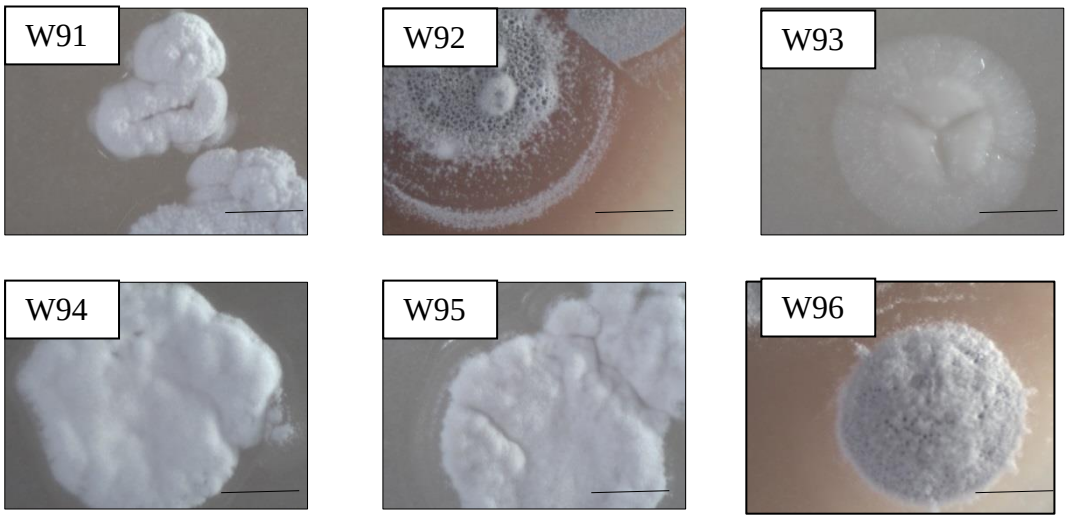


Figure 3-1. Morphology of colonies of the isolated *Streptomyces* strains grows on MS agar for 14 days at 30°C. In most cases the colonies are covered with white or with grey aerial mycelia. Cultivation medium is coloured with different diffusible pigments. Scale bar 2.50 mm.

The presence of spores was confirmed using an impression amount taken from the surface of the colony under a light microscope (Normaski optics) as illustrate in Table 3.1. All strains formed spores excluding eight strains (W11, W29, W46, W62, W80, W81, W89 and W93). The aerial hyphae of these strains appears to be wrinkled and lack any visible signs of speculation.

Strains name	**Aerial mycelium**	**Substrate mycelium**	**Spores**	**Diffusible pigment**
W1	Yellow-White	Grey	+	Grey
W2	White	Grey-White	+	Grey-White
W3	Dark Grey	Grey	+	Whitish-Grey
W4	White	Dark brown	+	Grey
W5	White- Grey	Grey	+	Grey-White
W6	White	Grey	+	Grey-White
W7	White- Grey	Grey	+	light Grey
W8	White	Grey	+	red
W9	White- Grey	Grey	+	Grey-White
W10	Grey	Grey	+	Grey-White
W11	Yellow	Grey	Bold	Creamish
W12	White- Grey	Grey	+	Light Grey
W13	Grey-White	Grey	+	Grey-White
W14	White	Grey	+	Grey-White
W15	Grey-White	Grey	+	Light Grey
W16	White	Grey	+	Grey-White

Strains name	Aerial mycelium	Substrate mycelium	Spores	Diffusible pigment
W17	Grey-White	Grey	+	Grey-White
W18	Grey-White	Grey	+	Light brown
W19	Grey-White	Grey	+	Light Grey
W20	White	Grey	+	Dark Grey
W21	White	Grey	+	Dark Grey
W22	Grey-White	Grey	+	Dark Grey
W23	Grey-White	Grey	+	Dark Grey
W24	White	Grey	+	Dark Grey
W25	Grey-White	Grey	+	Grey-White
W26	Grey-White	Grey	+	Grey-White
W27	Grey-White	Grey	+	Dark Grey
W28	Grey-White	Grey	+	Dark Grey
W29	yellow	Grey	Bold	Creamish
W30	White-Grey	Grey	+	Grey-White
W31	Grey-White	Grey	+	Grey-White
W32	White	Grey	+	Grey-White
W33	White	Grey	+	Grey-White
W34	Grey-White	Grey	+	Grey-White
W35	White	Grey	+	Grey-White
W36	White	Grey	+	Dark Grey
W37	White	Grey	+	Dark Grey
W38	Grey-White	Grey	+	Dark Grey

Strains name	Aerial mycelium	Substrate mycelium	Spores	Diffusible pigment
W39	White	Grey	+	Dark Grey
W40	White	Grey	+	Dark Grey
W41	Dark Grey	Grey	+	Dark Grey
W42	Dark Grey	Grey	+	Dark Grey
W43	Dark Grey	Grey	+	Dark Grey
W44	White	Grey	+	Dark Grey
W45	Grey-White	Grey	+	Dark Grey
W46	Yellow	Grey	Bold	Creamish
W47	Yellow	Grey	+	Dark Grey
W48	White	Grey	+	Dark Grey
W49	White	Grey	+	Dark Grey
W50	White	Grey	+	Dark Grey
W51	Grey-White	Grey	+	Dark Grey
W52	yellow	Grey	+	Dark Grey
W53	Grey-White	Grey	+	Dark Grey
W54	Grey-White	Grey	+	Dark Grey
W55	White	Grey	+	Dark Grey
W56	White	Grey	+	Dark Grey
W57	White	Grey	+	Dark Grey
W58	White	Grey	+	Dark Grey
W59	Grey-White	Grey	+	Dark Grey
W60	Grey-White	Grey	+	Dark Grey

Strains name	Aerial mycelium	Substrate mycelium	Spores	Diffusible pigment
W61	Yellow	Grey	+	Dark Grey
W62	Grey	Grey	Bold	Dark Grey
W63	Dark Grey	Grey	+	Dark Grey
W64	Grey	Grey	+	Dark Grey
W65	Dark Grey	Grey	+	Dark Grey
W66	Grey-White	Grey	+	Dark Grey
W67	Grey	Grey	+	Dark Grey
W68	Dark Grey	Grey	+	Dark Grey
W69	Grey	Grey	+	Dark Grey
W70	Dark Grey	Grey	+	Dark Grey
W71	Grey-White	Grey	+	Dark Grey
W72	Grey	Grey	+	Dark Grey
W73	Grey-White	Light Grey	+	Dark Grey
W74	White	Light Grey	+	Dark Grey
W75	White	Light Grey	+	Dark Grey
W76	Grey-White	Light Grey	+	Dark Grey
W77	Dark Grey	Grey	+	Dark Grey
W78	Dark Grey	Grey	+	Dark Grey
W79	Grey	Grey	+	Dark Grey
W80	Yellow	Grey	Bold	Dark Grey
W81	Yellow	Yellow	Bold	Dark Grey
W82	Grey	Grey	+	Dark Grey

Strains name	Aerial mycelium	Substrate mycelium	Spores	Diffusible pigment
W83	Grey-White	Grey	+	Dark Grey
W84	Grey	Grey	+	Dark Grey
W85	Grey-White	Light Grey	+	Red
W86	Grey-White	Light Grey	+	Dark Grey
W87	Grey-White	Light Grey	+	Dark Grey
W88	Grey-White	Grey	+	Dark Grey
W89	Yellow	Grey	Bold	Creamish
W90	Grey-White	Grey	+	Dark Grey
W91	White	Ivory	+	Grey
W92	Grey- White	Red	+	Red
W93	yellow	yellow	Bold	Creamish-Yellow
W94	Grey-White	Light Grey	+	Grey
W95	Grey-White	Grey	+	Grey
W96	Dark Grey	Red	+	Red

Table 3.1. Cultural characteristics of isolated *Streptomyces* grow on MS agar.

3.1.2 Growth of *Streptomyces* in Liquid Media

It is well known that Liquid-grown *Streptomyces* cultures are characterized by mycelial pellets that are heterogeneous in size. The morphology of mycelia of *Streptomyces* strains was examined under a light microscope at 1000 X magnification from 72 hours age, culture on YEME broth example for each presented in Figure 3.2. None of the cultures produced spores in these conditions. Growth resulted in pelleting in all of these strains except strain W43, which was fragmented in this condition as appeared in Figure 3.3. This was confirmed using fluorescent microscope, the strain was stained with cyto9 stain, and electron microscope, the strain was incubated in YEME for 72h before viewing under electron microscope as shown in Figure 3.3.

In the solid medium (YEME), coverslip technique was used to test the fragments of mycelium of W43 strain. W43 was streaked on the edge around the coverslip and incubated for 7 days at 30 °C.

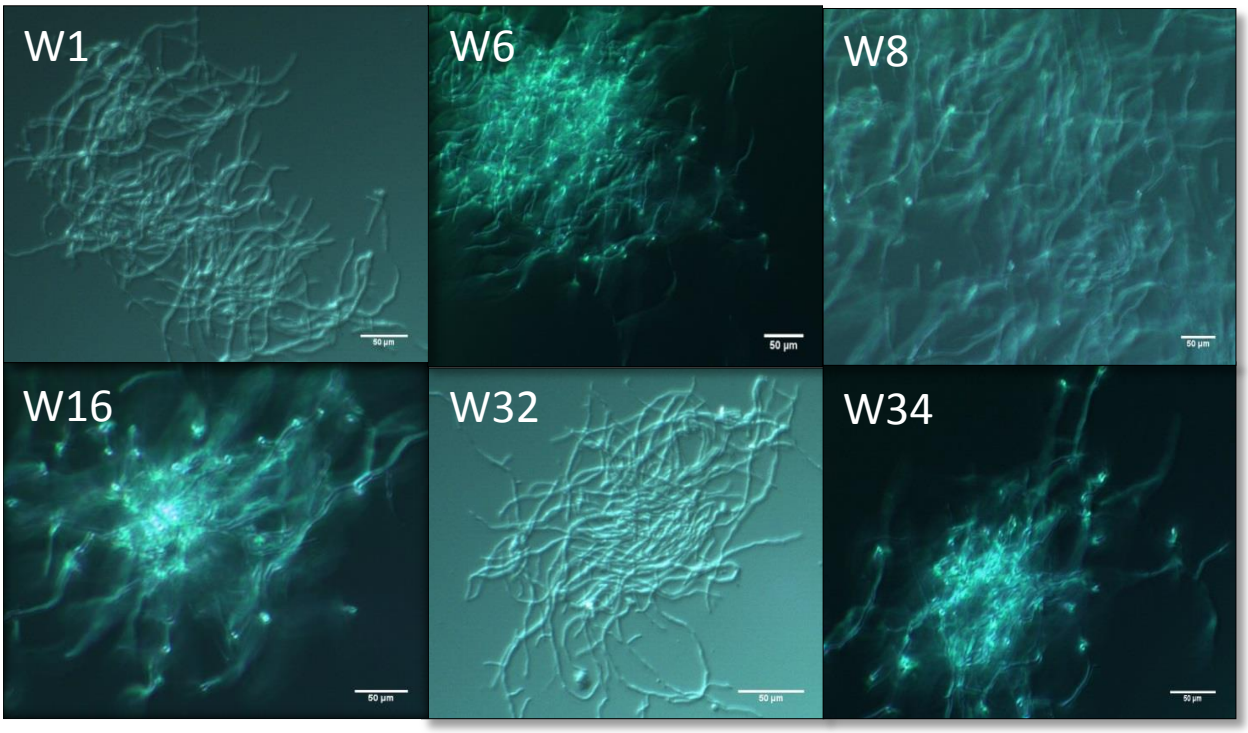


Figure 3-2. Morphology of aerial mycelia of isolated *Streptomyces* strains W1, W6, W8, W16, W32 and W34 at 1000 X under a light microscope using Normaski optics.

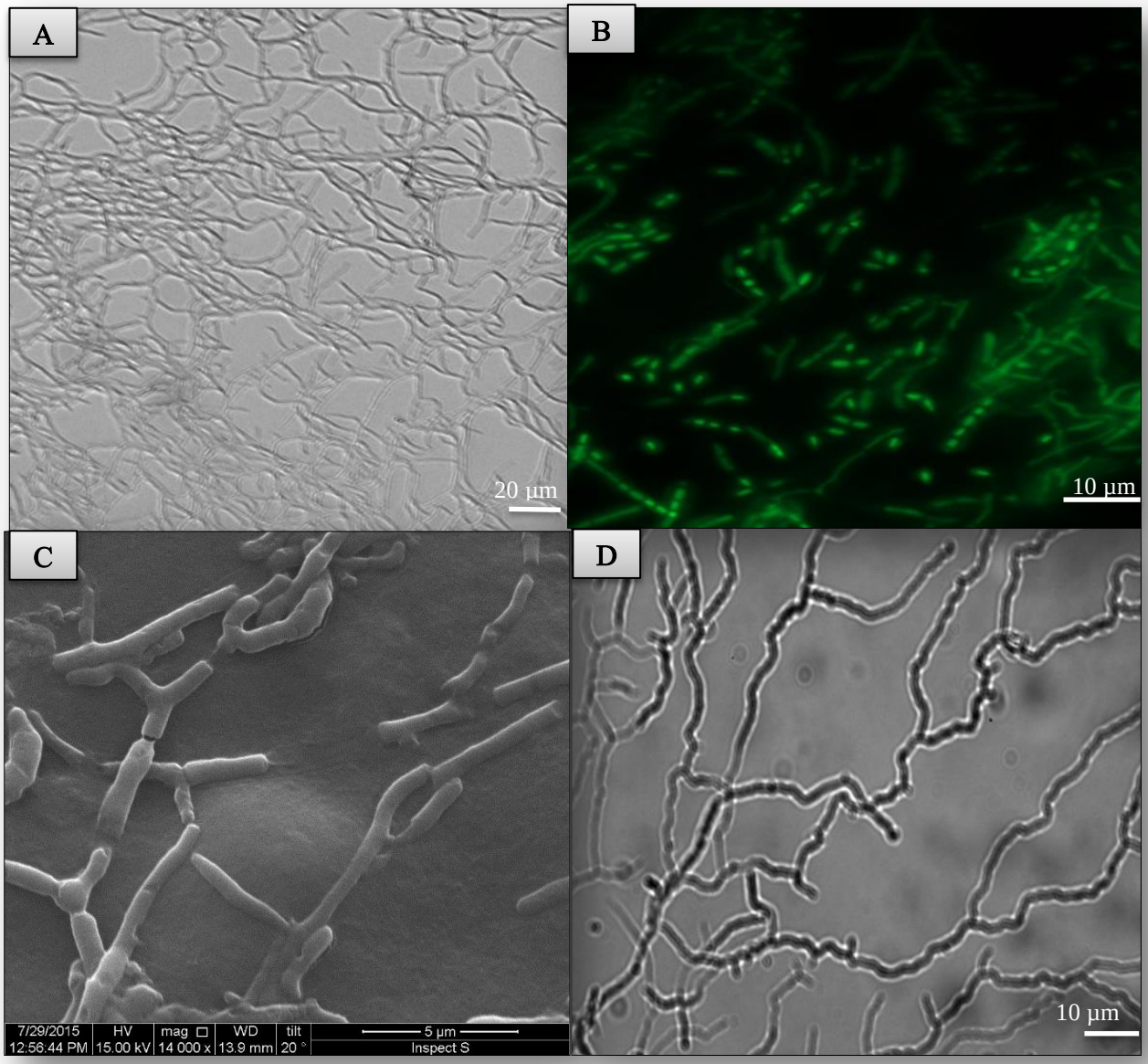


Figure 3-3. Morphology of aerial mycelia of the isolated *Streptomyces* strain W43, (A): Under light microscope on liquid YEME medium. (B): Stained with cyto 9 under a fluorescent microscope on liquid YEME medium. (C): Under electronic microscope on MS agar. (D): Under a light microscope using the coverslip technique on YEME agar medium.

3.2 Identification of the actinomycetes

Ninety six of the isolates were identified using their 16S rRNA sequence. The results obtained from the direct sequencing of purified PCR products showed that all the actinomycetes belong to the genus *Streptomyces*. Performing of PCR reactions using primer pair 27F/1492R of DNA from the 96 soils isolates produced a single ~1500 bp band in all tested isolates as illustrated in Figure 3.4. The sequence of the 16S rRNA gene of each isolate was analyzed using Basic Local Alignment Search Tool (BLAST) (Altschul *etal* 1990) on the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST/. The BLAST analysis of 16S rRNA gene sequences of the isolates strains presented alignments of these sequences with state 16S rRNA genes in the gene bank. The highest similarities found for each strain with different species of the genus *Streptomyces* are summarized in Table 3.2.

16S rRNA-based analysis resulted in the classification of the isolates into three categories. A total of 35 of 96 isolates (36.45%) possessed a 16S rRNA sequence with ≥97% similarity to that of a previously characterized *Streptomyces* species. A total of 45 of the 96 strains (46.87%) possessed a 16S rRNA sequence with ≥90% similarity to *Streptomyces* species. A total of 16 of 96 (16.66%) had a 16S rRNA sequence with <90% similarity to *Streptomyces* species.

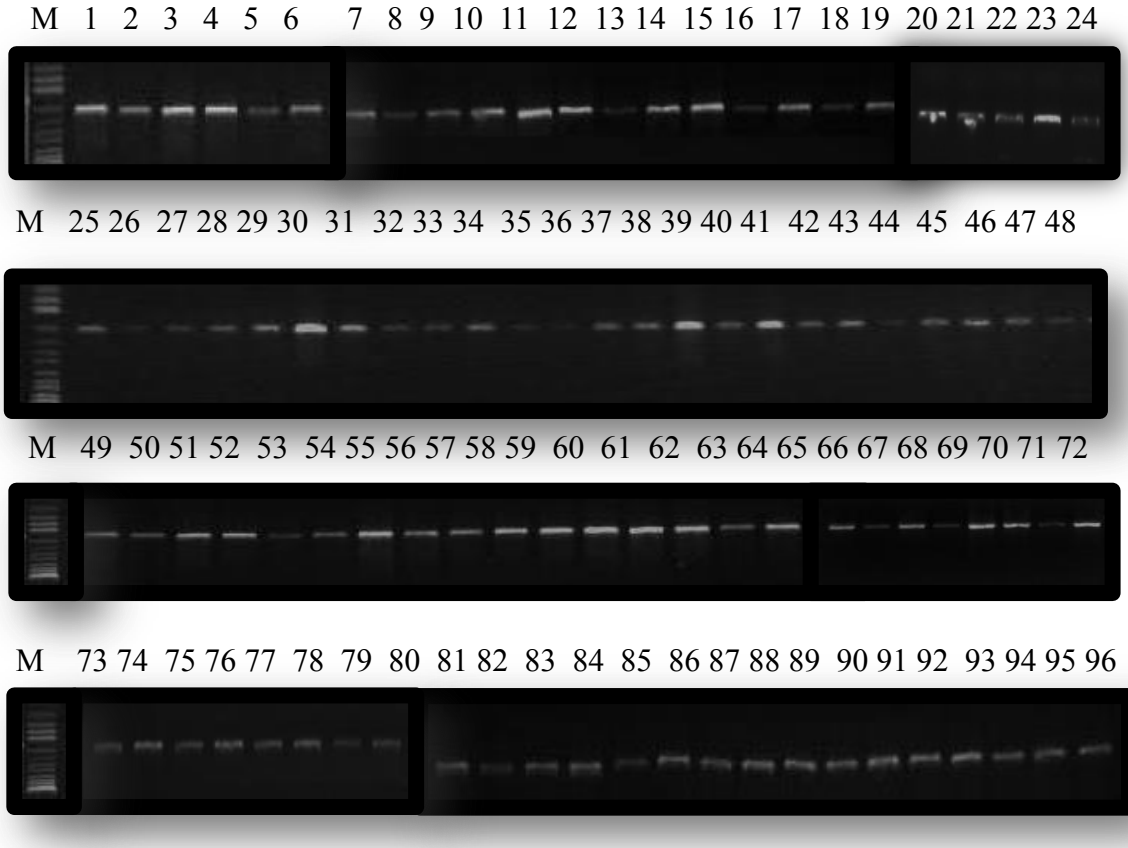


Figure 3-4. Agarose gel electrophoresis of PCR products from amplification of DNA isolated from 96 *Streptomyces* species with the primer pairs 27F/1492R. The symbols in PCR lanes represent M: hyper leader; 1-96 indicated genomic DNA from W1-W96 respectively.

Strains	Similar to	%
W1	*Streptomyces albidoflavus strain BB5*	96
W2	*Streptomyces olivochromogenes strain GG34*	98
W3	*Streptomyces griseorubens strain HBUM174527*	93
W4	*Streptomyces spiroverticillatus strain CB3Z4*	96
W5	*Streptomyces olivochromogenes strain CB1Z3*	99
W6	*Streptomyces albidoflavus strain BB5*	98
W7	*Streptomyces atrovirens strain 100*	95
W8	*Streptomyces olivochromogenes*	97
W9	*Streptomyces rishiriensis strain TSR41*	97
W10	*Streptomyces aureus strain 1H2*	95
W11	*Streptomyces prasinopilosus strain NRRL B-2711*	75
W12	*Streptomyces ambofaciens strain F3*	72
W13	*Streptomyces tricolor strain HBUM174995*	83
W14	*Streptomyces daghestanicus strain 7-5*	97
W15	*Streptomyces griseorubens strain 12-6*	96
W16	*Streptomyces somaliensis strain 5-8*	97
W17	*Streptomyces yanii strain GHI1*	98
W18	*Streptomyces atratus strain DST73*	97
W19	*Uncultured Streptomyces sp. clone 15661*	96
W20	*Streptomyces flavofungini strain BB1*	98
W21	*Streptomyces coelicoflavus strain BB6*	93
W22	*Streptomyces pluricolorescens strain Nr_42*	78
W23	*Streptomyces parvulus strain CFA-9*	97
W24	*Streptomyces filamentosus strain Act50*	83

Strains	Similar to	%
W25	*Streptomyces griseorubens strain MMH 9*	96
W26	*Streptomyces griseorubens strain S6-SC3*	95
W27	*Streptomyces marokkonensis strain 174443*	97
W28	*Streptomyces yanii strain GHI1*	98
W29	*Streptomyces yokosukanensis strain 3g3*	96
W30	*Streptomyces griseorubens strain S6-SC3*	96
W31	*Streptomyces sp. MJM10631*	96
W32	*Streptomyces sp. 1_C7_48*	94
W33	*Streptomyces vinaceusdrappus strain D704*	94
W34	*Streptomyces sp. 764G2*	99
W35	*Streptomyces sp. LK1332.2*	97
W36	*Streptomyces lienomycini strain 173762*	74
W37	*Streptomyces lavendulae subsp. lavendulae strain DSM40713*	99
W38	*Streptomyces sp. S6n14*	68
W39	*Streptomyces marokkonensis strain 174443*	97
W40	*Streptomyces rishiriensis strain LuP30*	90
W41	*Streptomyces coelescens strain BS31*	82
W42	*Streptomyces rubrogriseus strain 173513*	96
W43	*Streptomyces violaceoruber strain BUCBT-23*	98
W44	*Streptomyces sp. PsTaAH89*	97
W45	*Streptomyces atratus strain 173748*	98
W46	*Streptomyces lienomycini strain 173894*	97
W47	*Streptomyces tricolor strain ICN14*	93
W48	*Streptomyces bungoensis*	71

W49	*Streptomyces natalensis strain 20-4*	91
W50	*Streptomyces sp. 764G2*	99
W51	*Streptomyces laceyi gene*	99
W52	*Streptomyces griseoaurantiacus strain HQ-1-53*	71
W53	*Streptomyces pluricolorescens strain Nr_42*	79
W54	*Streptomyces sp. 764G2*	99
W55	*Streptomyces tricolor strain HBUM82677*	92
W56	*Streptomyces tricolor strain HBUM82677*	86
W57	*Streptomyces coelescens strain OAct533*	87
W58	*Streptomyces marokkonensis strain 174443*	97
W59	*Streptomyces rubrogriseus strain 173513*	96
W60	*Streptomyces jietaisiensis strain FXJ46*	96
W61	*Streptomyces rishiriensis strain 5W3*	96
W62	*Streptomyces violaceoruber strain Z12-1*	97
W63	*Streptomyces flavogriseus strain MD12-638-1-A*	70
W64	*Streptomyces parvulus strain S2-SC26*	96
W65	*Streptomyces prunicolor strain AHS1*	96
W66	*Streptomyces violaceoruber strain Z12-1*	96
W67	*Streptomyces prunicolor strain AHS1*	97
W68	*Streptomyces griseoaurantiacus strain NBRC 15440*	96
W69	*Streptomyces sp. G11*	96
W70	*Streptomyces sp. FXJ7.076*	97
W71	*Streptomyces rubrogriseus strain 173513*	97
W72	*Streptomyces parvulus strain PFS10*	95
W73	*Streptomyces coelescens strain BS31*	96

W74	*Streptomyces lividans strain Gs-2*	93
W75	*Streptomyces coelescens strain BS31*	95
W76	*Streptomyces sp. CBH04*	98
W77	*Streptomyces violaceoruber strain NEAE-99*	96
W78	*Streptomyces tricolor strain ICN14*	96
W79	*Streptomyces coelescens*	94
W80	*Streptomyces griseoaurantiacus strain NBRC 15440*	97
W81	*Streptomyces yanii strain GHI1*	90
W82	*Streptomyces atratus strain 174498*	96
W83	*Streptomyces sp. SOK7/16-05*	99
W84	*Streptomyces caesius*	97
W85	*Streptomyces coelescens strain OAct533*	96
W86	*Streptomyces coelescens strain OAct533*	98
W87	*Streptomyces coelescens strain BS31*	87
W88	*Streptomyces sp. CNRD05*	98
W89	*Streptomyces griseoaurantiacus strain BQAB-05d*	95
W90	*Streptomyces tricolor strain ICN14*	97
W91	*Streptomyces sp. 1_C7_48*	97
W92	*Streptomyces laceyi gene*	96
W93	*Streptomyces spiralis strain S6SS1*	96
W94	*Streptomyces tricolor strain ICN14*	96
W95	*Streptomyces lividans*	96
W96	*Uncultured Streptomyces sp. clone 15661*	96

Table 3.2. The highest similarity of each isolates strains (W1-W96) with different *Streptomyces species*.

3.3. The minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of penicillin G for isolated *Streptomyces* strains

The MICs of penicillin G for isolated *Streptomyces* strains were measured by two techniques, plate culture and OxoPlate®. In plate culture each strain streaked onto SC plate containing various amounts of penicillin G. The concentrations of penicillin G (Sigma Co.) were (0, 25, 50, 100, 200 and 400 µg/ml). Each plate was incubated at 30°C for 14 days. The MICs were obtained from the results of plates without colonies, when full growth was observed on plates without penicillin as indicate in Table 3.3. Some strains showed growth in all the concentrations of penicillin G (0, 25, 50, 100, 200 and 400 µg/ml) which indicate that the strains were highly resistant against penicillin G. Some strains were un-able to grow at penicillin G concentration above 200 µg/ml.

Also, the MICs of penicillin G for *Streptomyces* strains were measured using OxoPlates® system. The MICs and MBCs were determined directly from the oxygen-depletion curves. Oxygen levels of cultures treated with bactericidal compounds dropped initially and then increased after the cells had died. In contrast, oxygen levels remained low when cultures were treated with bacteriostatic compounds as demonstrated in Figure 3.5. The MICs and MBCs of penicillin G for the isolated strains of *Streptomyces* were summarised in Table 3.4. The minimum inhibitory concentrations of all strains ranged from 1- 100 µg/ml.

Strains	MICs (µg/ml)	Strains	MICs (µg/ml)
W1	100	W49	50
W2	200	W50	400
W3	25	W51	> 400
W 4	200	W52	50
W5	25	W53	100
W6	200	W54	100
W7	50	W55	> 400
W8	25	W56	400
W9	25	W57	25
W10	200	W58	200
W11	100	W59	25
W12	25	W60	> 400
W13	50	W61	200
W14	25	W62	25
W15	< 25	W63	25
W16	400	W64	200
W17	25	W65	200
W18	100	W66	25
W19	200	W67	25
W20	200	W68	50
W21	> 400	W69	200
W22	50	W70	100
W23	100	W71	100
W24	100	W72	25
W25	200	W73	< 25
W26	100	W74	400
W27	< 25	W75	200
W28	< 25	W76	> 400
W29	100	W77	100
W30	400	W78	25
W31	200	W79	200
W32	> 400	W80	200
W33	50	W81	100
W34	200	W82	> 400
W35	25	W83	50
W36	200	W84	50
W37	25	W85	50
W38	100	W86	25
W39	400	W87	400
W40	50	W88	25
W41	50	W89	< 25
W42	50	W90	50
W43	100	W91	50
W44	25	W92	< 25
W45	25	W93	200
W46	25	W94	50
W47	50	W95	50
W48	50	W96	100

Table 3-3. The MICs of penicillin G for isolated *Streptomyces* strains (W1-W96) from plate dilution culture. The strains were grown in SC medium for 14 days.

Strains	MICs (µg/ml)	MBCs (µg/ml)	Strains	MICs (µg/ml)	MBCs (µg/ml)
W1	10	80	W49	5	40
W2	40	160	W50	50	400
W3	5	20	W51	80	320
W 4	10	40	W52	10	20
W5	5	20	W53	20	40
W6	40	160	W54	5	40
W7	10	160	W55	80	160
W8	5	20	W56	50	400
W9	5	40	W57	5	160
W10	40	160	W58	40	80
W11	10	40	W59	5	80
W12	5	20	W60	100	400
W13	10	40	W61	5	10
W14	5	20	W62	5	10
W15	4	32	W63	5	40
W16	50	400	W64	10	160
W17	5	10	W65	10	320
W18	5	20	W66	5	160
W19	64	128	W67	5	160
W20	40	160	W68	10	320
W21	80	160	W69	40	460
W22	10	80	W70	20	80
W23	32	64	W71	20	160
W24	20	40	W72	5	80
W25	40	80	W73	4	8
W26	10	320	W74	50	200
W27	1	2	W75	40	80
W28	4	8	W76	80	160
W29	20	80	W77	20	80
W30	50	200	W78	5	80
W31	40	80	W79	40	160
W32	80	640	W80	40	160
W33	10	20	W81	5	160
W34	40	80	W82	20	160
W35	5	80	W83	10	40
W36	20	80	W84	10	40
W37	5	40	W85	10	80
W38	20	80	W86	5	20
W39	50	400	W87	40	320
W40	10	80	W88	5	160
W41	5	40	W89	2	16
W42	5	20	W90	10	80
W43	10	20	W91	5	160
W44	5	80	W92	8	32
W45	5	20	W93	40	160
W46	5	160	W94	10	80
W47	5	80	W95	5	40
W48	5	40	W96	20	320

Table 3-4. The MICs and MBCs of penicillin G for isolated *Streptomyces* strains (W1-W96) from OxoPlate® system. The strains grown in MHB medium for 48h.

3.4. β-lactamase activity of isolated *Streptomyces* strains

β-lactamase activity was determined using nitrocefin disk (Sigma). 51% of isolated strains do not produce β-lactamase, and of the isolated strains 49 % of produced β-lactamase activity as demonstrated by a colour change of yellow to red as shown in Figure 3.5. There was no correlation between the production of β-lactamase and the MICs of these isolated strains. For instance, although strain W32 does not produce β-lactamase, it has high MIC and MBC and strain W8 has lower MIC and MBC yet it exhibits β-lactamase activity. The plot of the MICs and MBCs of β-lactamase producing strains were shown in Figure 3.6 and the MICs and MBCs of non-β-lactamase producing strains presented in Figure 3.7.

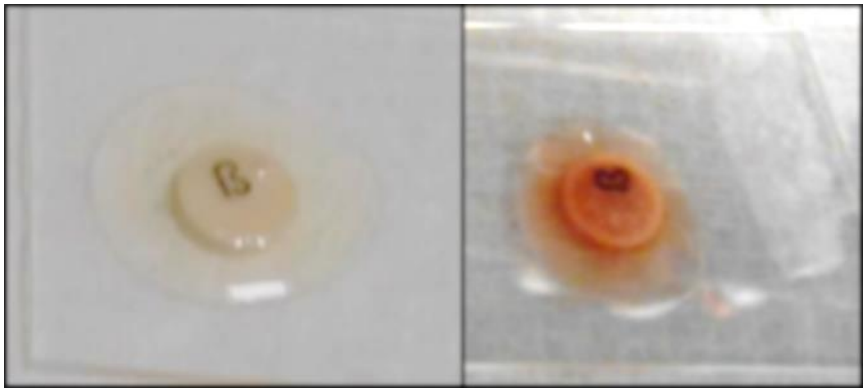


Figure 3-5. Monitoring of β-lactamase activity of *E coil* by nitrocefin disk, the colour change of yellow to red in the present of β-lactamase.

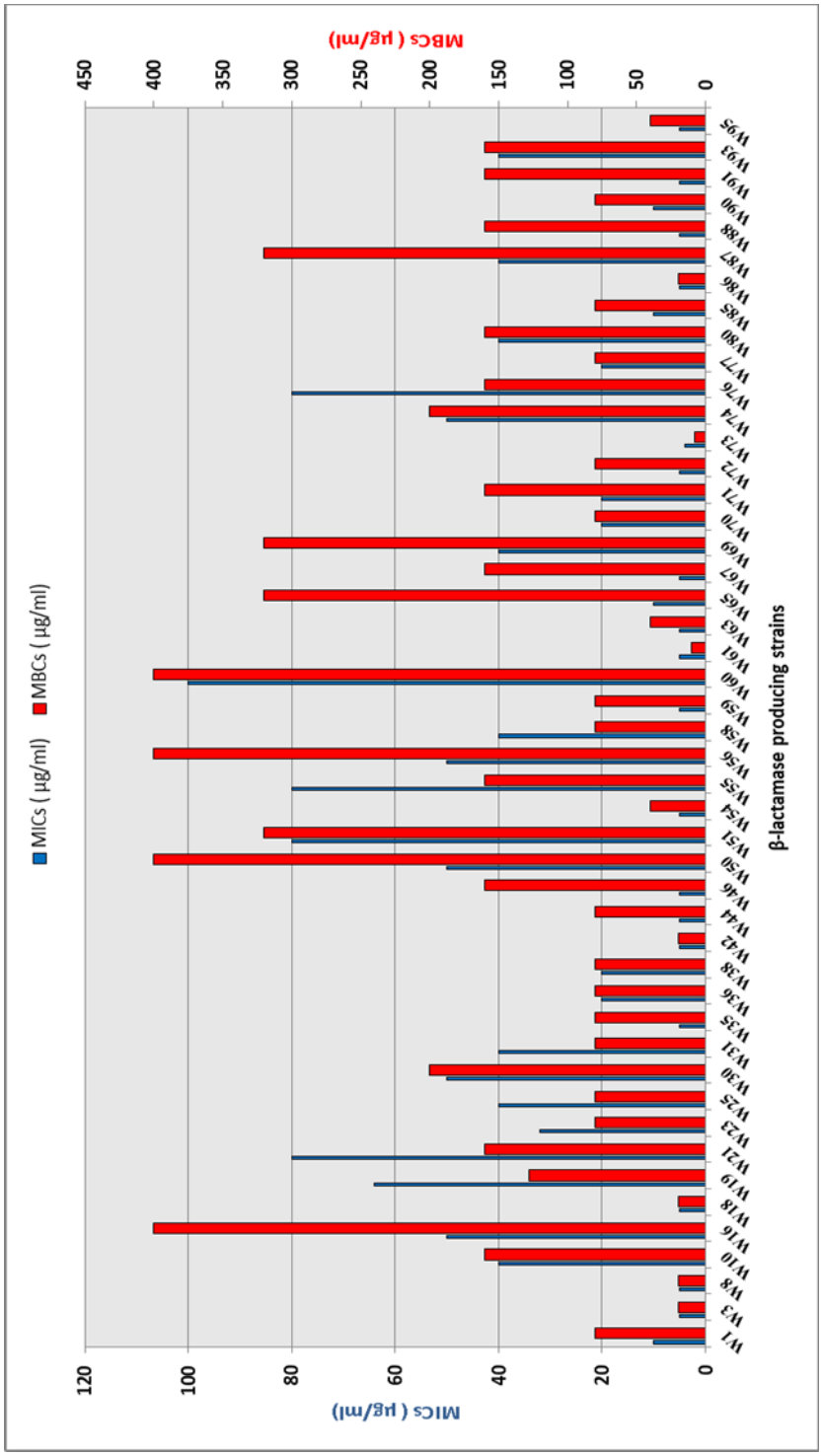


Figure 3-6. Graph shows the MICs and MBCs of β-lactamase producing *Streptomyces* strains. MICs and MBCs plot in different y-axis.

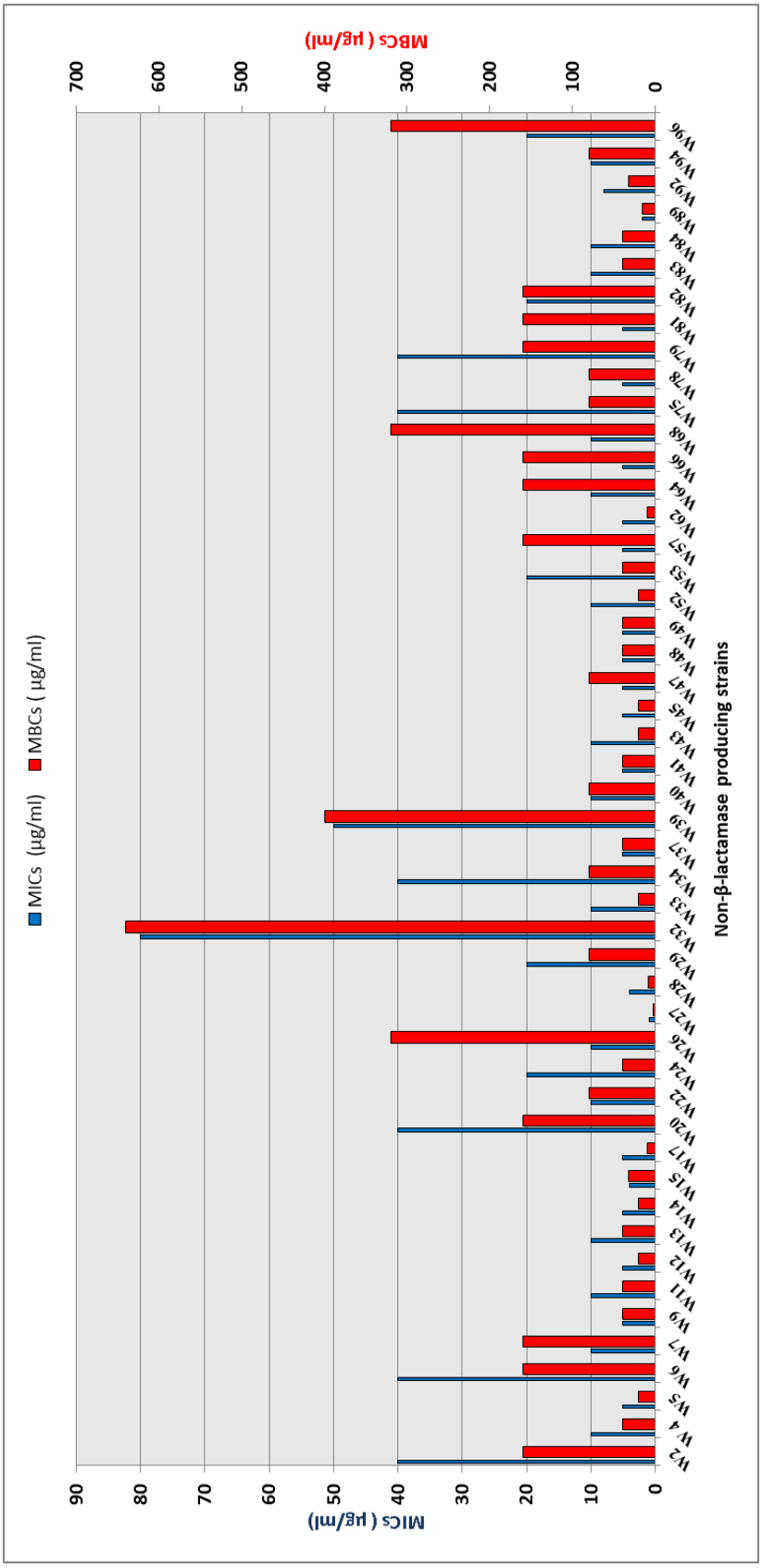


Figure 3-7. Graph shows the MICs and MBCs of Non-β-lactamase producing *Streptomyces* strains. MICs and MBCs plot in different y-axis.

T test was performed to test if this variation between beta lactamase producing and non-beta lactamase producing strains is significantly different from each other. The P value (P=0.00334) was < 0.05 which means they are specifically different.

To sum up these results, the isolation and morphological characteristics of *Streptomyces* presented in this study indicate that ninety six isolates of actinomycetes were isolated from an environmental soil samples. The microscopic examination of the colony of the isolates revealed that aerial mycelia were morphologically branched with grey and white surface and some strains formed spores. The identification of the actinomycetes by the 16S rRNA sequencing of purified PCR products showed that all the actinomycetes isolates belong to the genus *Streptomyces*. The minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of penicillin G for isolated *Streptomyces* strains were varied in their MICs and MBCs to penicillin G. The MICs were different when they were cultured in liquid and on solid media. In MH broth medium the minimum inhibitory concentrations of all strains ranged from 1-100 µg/ml. Furthermore, the result indicates that there is no correlation between the MICs of penicillin G and the growth rates in the isolated strain and the sensitivity of isolated *Streptomyces* strains to penicillin G is not directly related to β-lactamase production.

3.5 16S rRNA gene and phylogenetic analysis

After PCR amplification from 11 isolated strains; five beta-lactamase producing *Streptomyces;* (W21) *Streptomyces olivochromogenes strain NBRC 3178,* (W51) *Streptomyces griseoaurantiacus strain A17,* (W55) *Streptomyces albidoflavus strain JP* (W76) *Streptomyces lividans strain YLA0 and* (W60) *Streptomyces limosus strain Act64,*and five non-beta-lactamase producing *Streptomyces;* (W32) *Streptomyces sp. 1_C7_48,* (W39) *Streptomyces atratus strain PY-1,* (W96) *Streptomyces coeruleofuscus,* (W79) *Streptomyces sp. HB243* and (W75) *Streptomyces aureus strain Fist3*. One fragmented strain (W43) *Streptomyces violaceoruber BUCBT-23,* the partial 16S rRNA genes of the 11 strains were sequenced, and the sequences were aligned using the CLUSTALW multiple alignment program.

To determine the phylogenetic relationship between beta-lactamase producing and non-beta-lactamase producing *Streptomyces,* and the position and phylogenetic relationship between strain W43 *Streptomyces violaceoruber BUCBT-23* and its closest relatives *S. colicoler*, 16S rRNA gene sequence of the isolated strains were aligned using the CLUSTAL W software program (Larkin *et al.* 2007). The aligned sequences were manually checked for Ns and adjusted using Finch TV Sequence Alignment Editor, version 1.5 (Geospiza Inc., Seattle, WA, USA) before constructing the phylogenetic tree. Phylogenetic tree were conducted in MEGA6 (Rzhetsky and Nei 1992) method of MEGA software (Tamura *et al.* 2013). The tree was constructed using the neighbour-joining method (Saitou and Nei 1987). *Mycobacterium tuberculosis* was used as an out-group. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates (Felsenstein 1985).

The phylogenetic relationship between beta-lactamase producing strains and non-beta-lactamase producing strains showed that there was no association between the position of

the strains on the phylogenetic tree and their beta-lactamase activity. Beta-lactamase producing and non-producing strains refers to the same ancestral origin clade as displayed in figure 3.8. A phylogenetic tree was constructed based on 16S rRNA gene sequences to show the comparative relationship between (W43) *Streptomyces violaceoruber BUCBT-23* and other related *Streptomyces sp*. The comparative analysis of 16S rRNA gene sequence and phylogenetic relationship of strain (W43) revealed that the isolate formed a separate phyletic line and clustered with (W76) *Streptomyces lividans strain YLA0,* while other related species i.e. *Streptomyces coelicolor* formed a separate subclade.

Growth rates of isolated strains were determined using the rate of oxygen uptake from the Oxoplate® as shown in Table 3.5. The correlation between the growth rates of the isolated strains and their MICs were studied. There was no significant correlation between the growth rates and the MICs of isolated strains as shown in Figure 3.9.

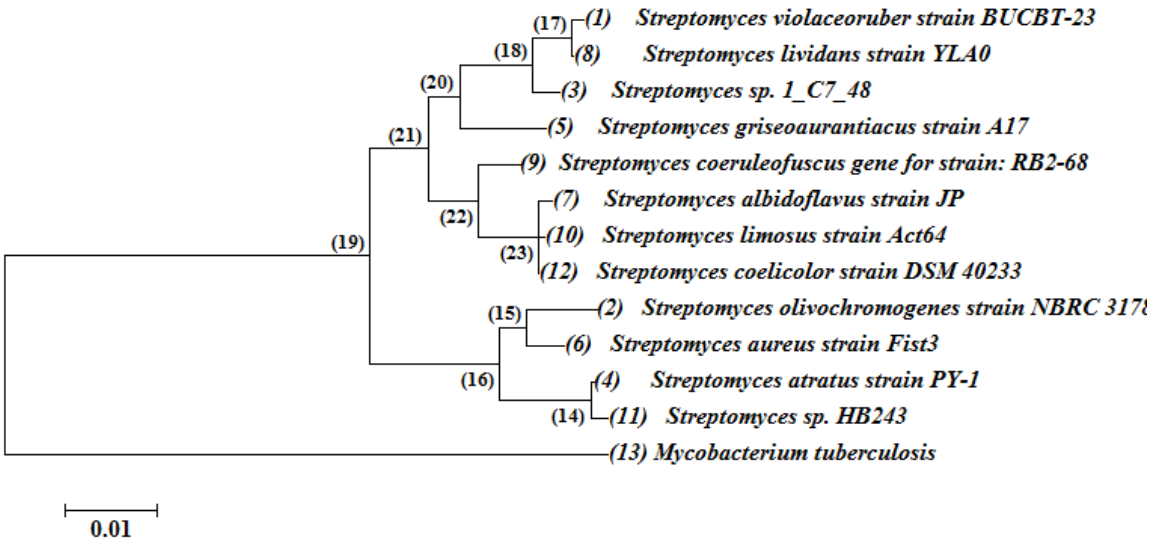


Figure 3-8. The phylogenetic tree of isolated *Streptomyces* based on partial nucleotide sequences of 16S rRNA showing the phylogenetic relationship between beta-lactamase producing *Streptomyces* (W21) *Streptomyces olivochromogenes strain NBRC 3178,* (W51) *Streptomyces griseoaurantiacus strain A17,* (W55) *Streptomyces albidoflavus strain JP* (W76) *Streptomyces lividans strain YLA0,* (W60) *Streptomyces limosus strain Act64* and Non-beta-lactamase producing *Streptomyces;* (W32) *Streptomyces sp. 1_C7_48,* (W39) *Streptomyces atratus strain PY-1*, (W96) *Streptomyces coeruleofuscus,* (W79) *Streptomyces sp. HB243,* and (W75) *Streptomyces aureus strain Fist3*. Also, showing the position and phylogenetic relationship between strain (W43) *Streptomyces violaceoruber BUCBT-23* and related members of the genus *Streptomyces*. The tree was constructed using the neighbour-joining method with MEGA 6.0 proGram (www.megasoftware.net). The Bootstrap values presented as percentages of 1000 replications. *Mycobacterium tuberculosis* was used as out-group.

Strains	Dt (h)	Strains	Dt (h)
W1	5	W49	6
W2	8	W50	1
W3	1	W51	1
W 4	4	W52	3.5
W5	4.5	W53	4.5
W6	4.5	W54	4.5
W7	1	W55	6
W8	4.5	W56	2
W9	4	W57	5.5
W10	5	W58	6.5
W11	3.5	W59	2
W12	16	W60	2.5
W13	4.5	W61	1
W14	13	W62	1.5
W15	3	W63	1
W16	5	W64	7
W17	13	W65	7
W18	10	W66	7.5
W19	6	W67	7
W20	16	W68	6.5
W21	3	W69	7
W22	4.5	W70	1.25
W23	1	W71	1
W24	1.5	W72	1.25
W25	1.25	W73	14
W26	2.5	W74	14
W27	4.5	W75	3.5
W28	4.5	W76	1
W29	4.5	W77	1
W30	3.5	W78	1
W31	2	W79	2.5
W32	5	W80	7.5
W33	3.5	W81	1
W34	4.5	W82	4
W35	3.5	W83	4.25
W36	3	W84	1
W37	6	W85	1.25
W38	6	W86	2.6
W39	5	W87	4
W40	1	W88	4.5
W41	1	W89	3
W42	1.25	W90	2.5
W43	1	W91	2
W44	1	W92	4
W45	2.5	W93	1
W46	4.5	W94	3
W47	1	W95	2
W48	6.5	W96	5.5

Table 3-5. Growth rates of isolated *Streptomyces* strains (W1-W96) obtained from the rate of oxygen uptake from the Oxoplate®.

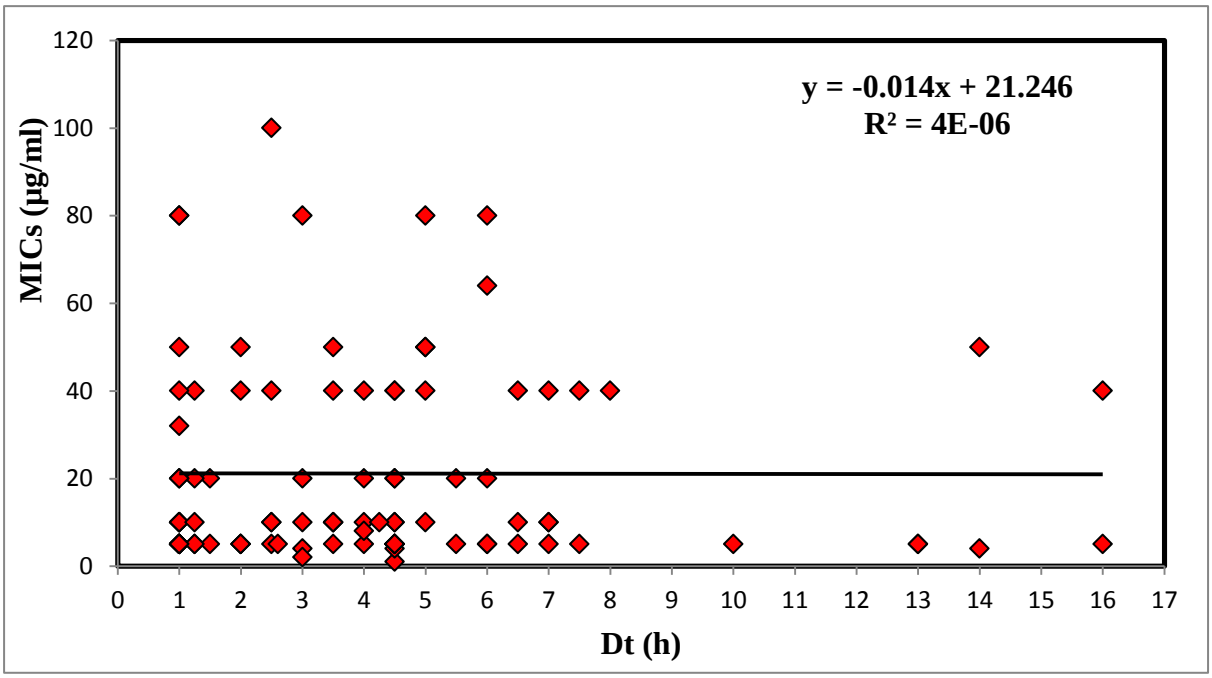


Figure 3-9. The correlation between the growth rates and the MICs of isolated *Streptomyces* strains.

In conclusions, the result indicates that there is no correlation between the MICs of penicillin G and the growth rates in the isolated strain. Moreover, there was no association between the position of the strains on the phylogenetic tree and their beta-lactamase activity. Beta-lactamase and non- lactamase producing strains refers to the same ancestral origin. The comparative analysis of 16S rRNA gene sequence and phylogenetic relationship of strain (W43) revealed that the isolate formed a separate phyletic line and clustered with (W76) *Streptomyces lividans strain YLA0.*

3.6. Investigate the interaction between penicillin G and PBPs in isolated *Streptomyces* strains

To investigate the interaction between penicillin G and PBPs in isolated *Streptomyces* strains, selectively producing and non-producing β-lactamase strains were lined on YEME media around coverslips and incubated at 30 °C for 24-48 h. PBPs were visualising using Bocillin fluorescent stain (green) and live cell membrane was stained with Cell Mask™ stain (red) and DAPI stain was used as nucleotide stain (blue). Bocillin fluorescent stain in β-lactamase producing *Streptomyces* strains (W1, W8, and W16) was detected in all aerial mycelium as shown in Figure 3.10, whereas in non β-lactamase producing strains (W6, W34, W36) was located in certain parts on the cell membrane of *Streptomyces* (Figure 3.11).

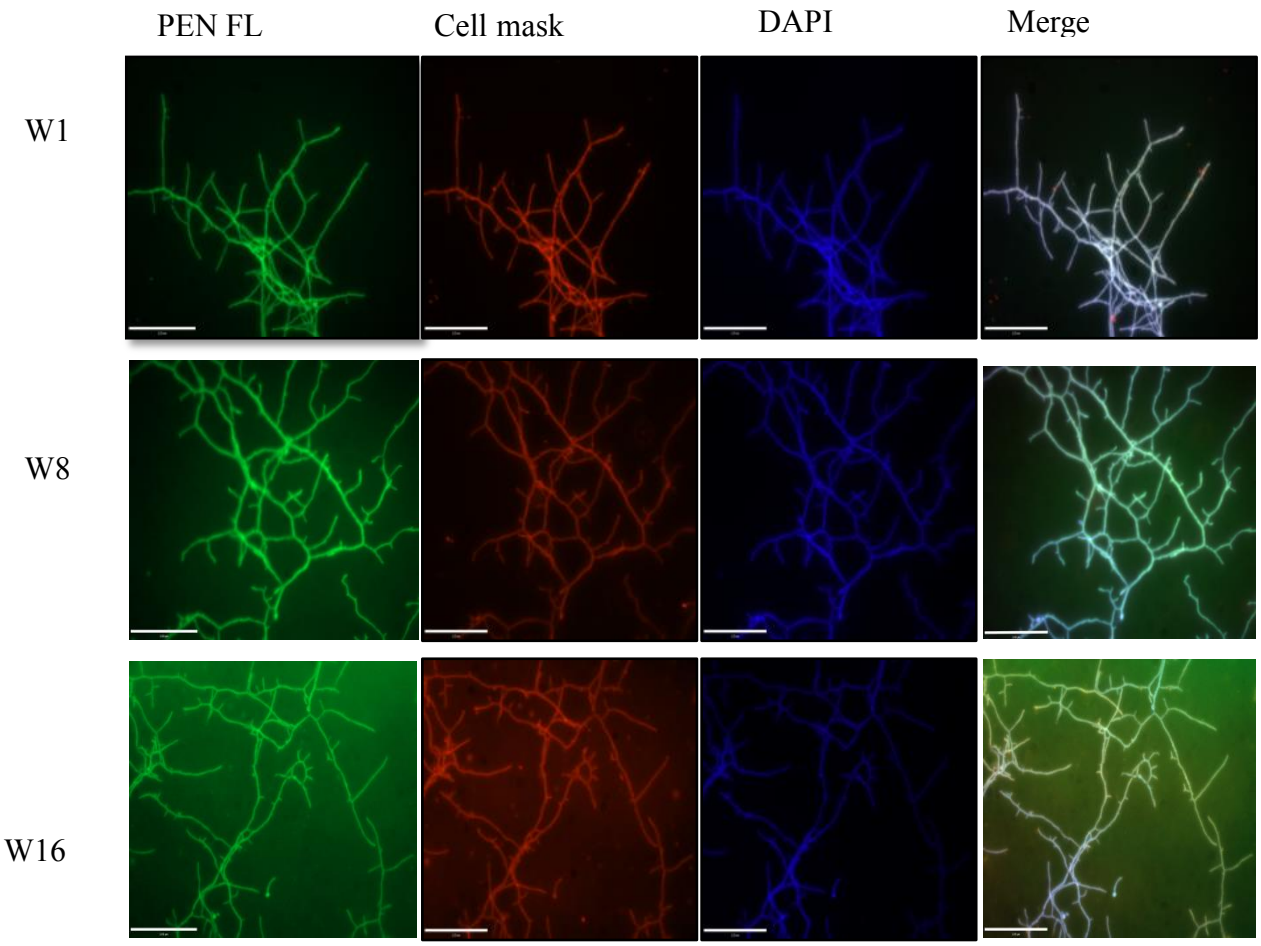


Figure 3-10. Localisation of PBPs in β-lactamase producing *Streptomyces* (W1, W8 and W16), PEN FL (Bocillin stain (green)), cell mask stain (red), DAPI stain (blue) and merge are a combined of the three stains. PBPs are located all over the mycelium. Scale barn is 10 µm.

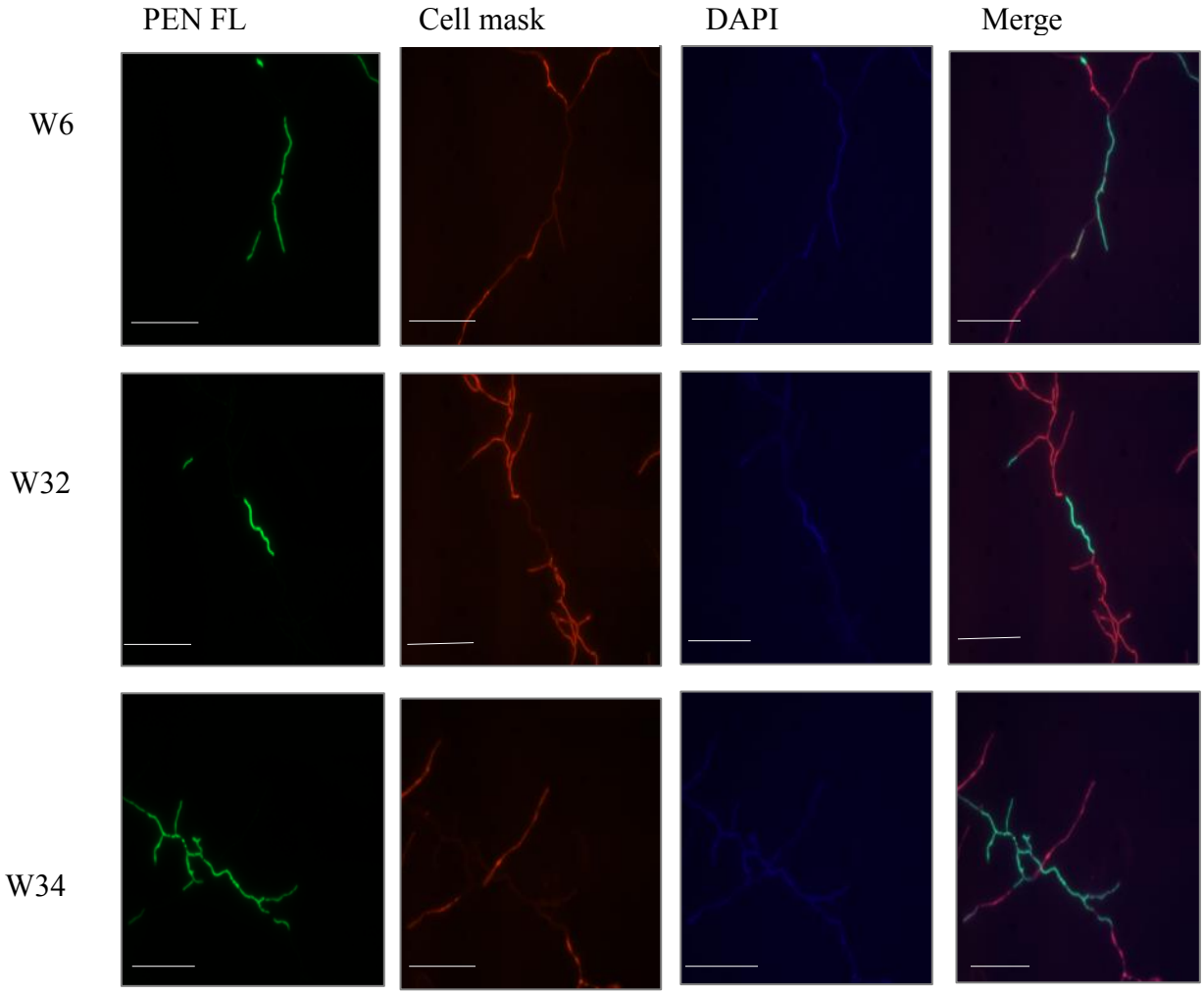


Figure 3.11. Localisation of PBPs in non β-lactamase producing *Streptomyces* (W6, W32 and W34), PEN FL Bocillin fluorescent stain (green), cell mask stain (red), DAPI stain (blue) and merge is a combined of the three stains. PBPs are located in small parts of mycelium. Scale barn is 10 µm.

In order to establish the type of PBP, four knockout mutant's defective in the class A high molecular weight penicillin binding proteins were stained with Bocilin and visualized under a fluorescent microscope (SCO2897, SCO3580, SCO3901 and SCO5039) as illustrated in Figure 3.12. The four PBPs mutants are stained with Bocillin under a fluorescent microscope, which indicated that they are not PBP that was detected in isolated strains.

Examined if this PBP are remaining in sporulation, pressure a mounted coverslip of spores of each; β-lactamase producing *Streptomyces* strain (W6) and non β-lactamase producing *Streptomyces* strain (W1) were used to stain with Bocillin fluoresced stain as shown in Figure 3.13. PBPs were stained in both strains and located on the poles of the spores.

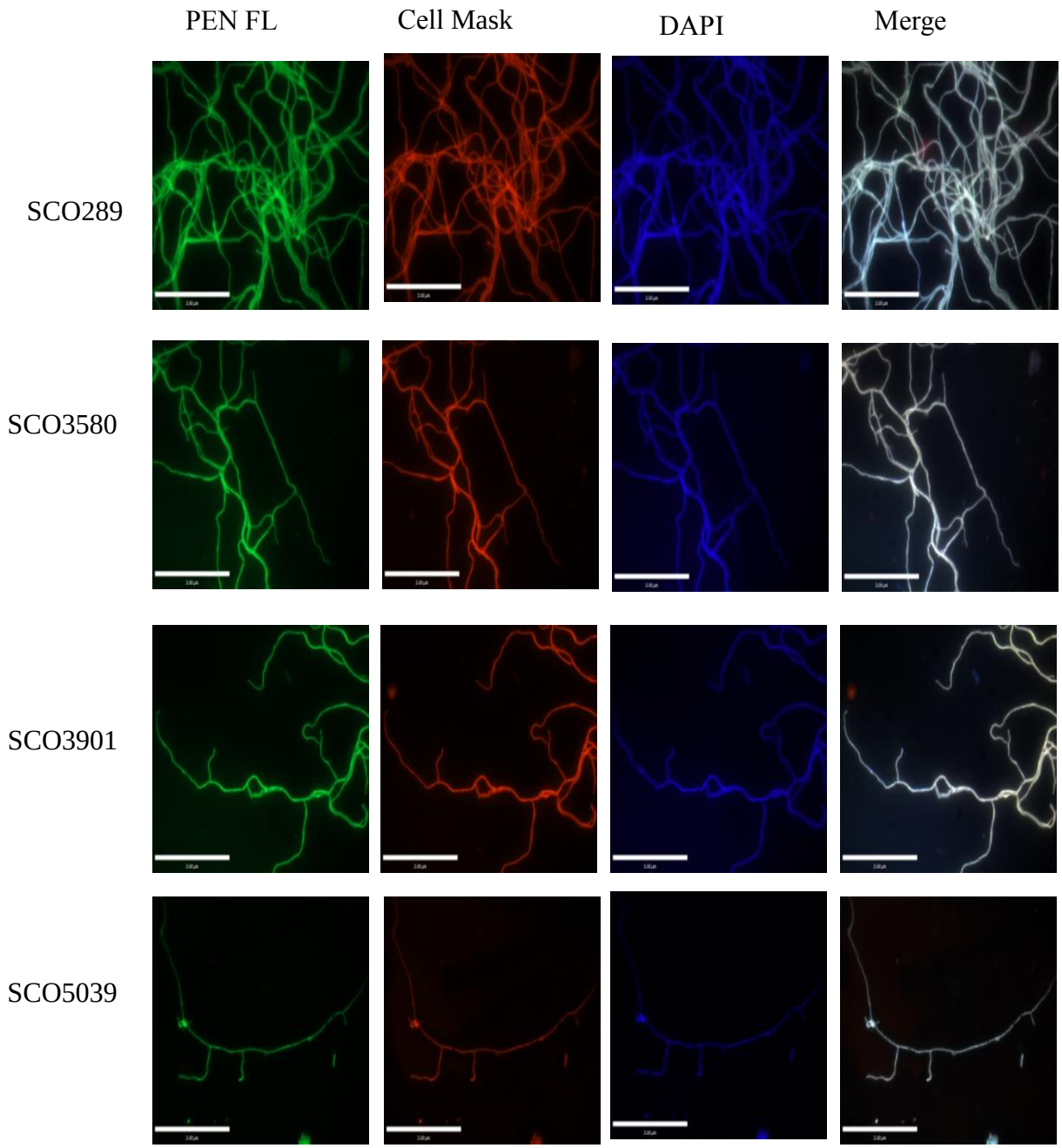
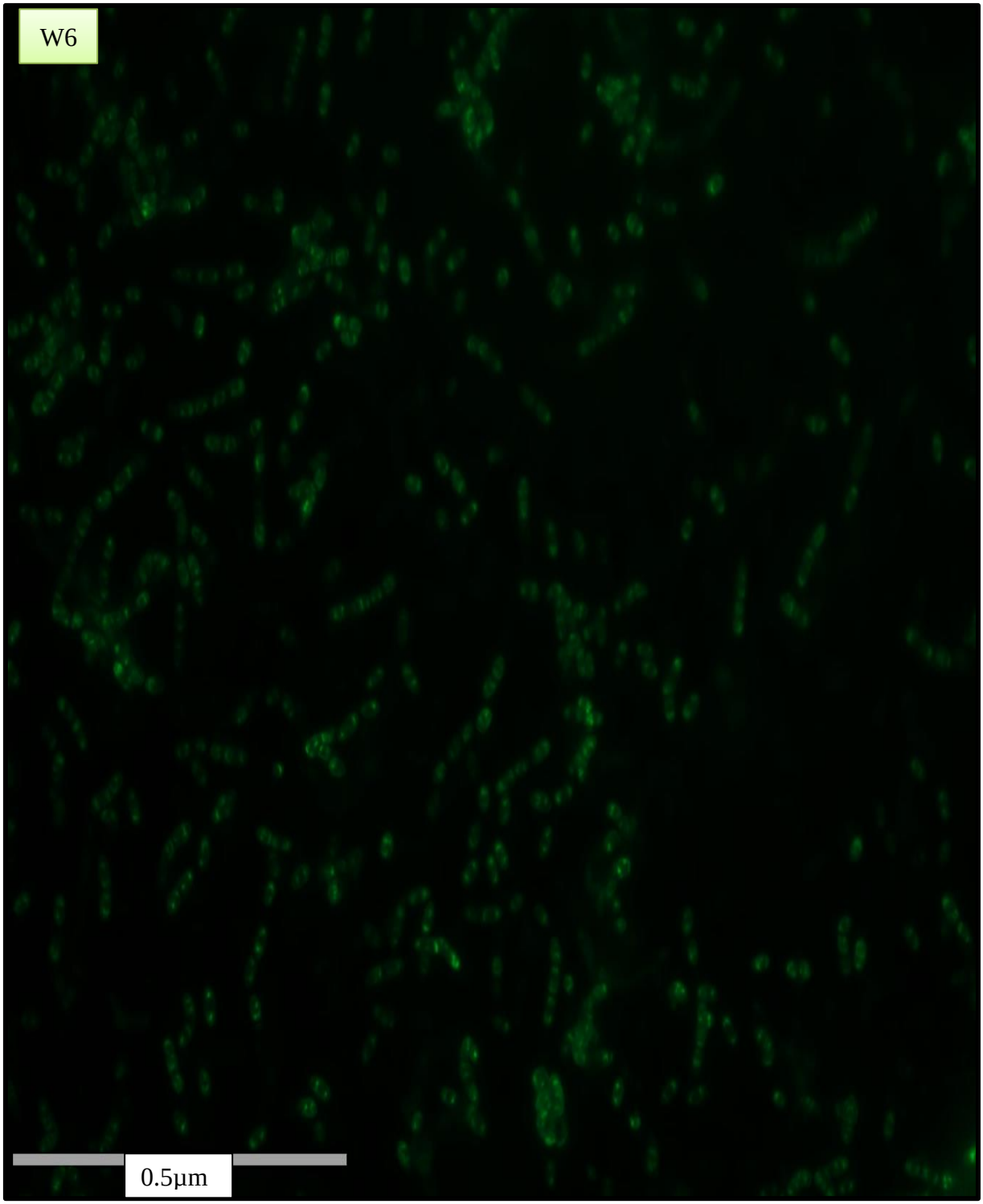


Figure 3-12. Localisation of PBPs mutants (SCO2897, SCO3580, SCO3901 and SCO5039) under fluorescent microscope. PEN FL Bocillin stain (green), cell mask stain (red), DAPI stain (blue) and merge is a combined of the three stains. None of them are PBP was detected in isolated strains. Scale barn is 10 µm.


W6
0.5µm

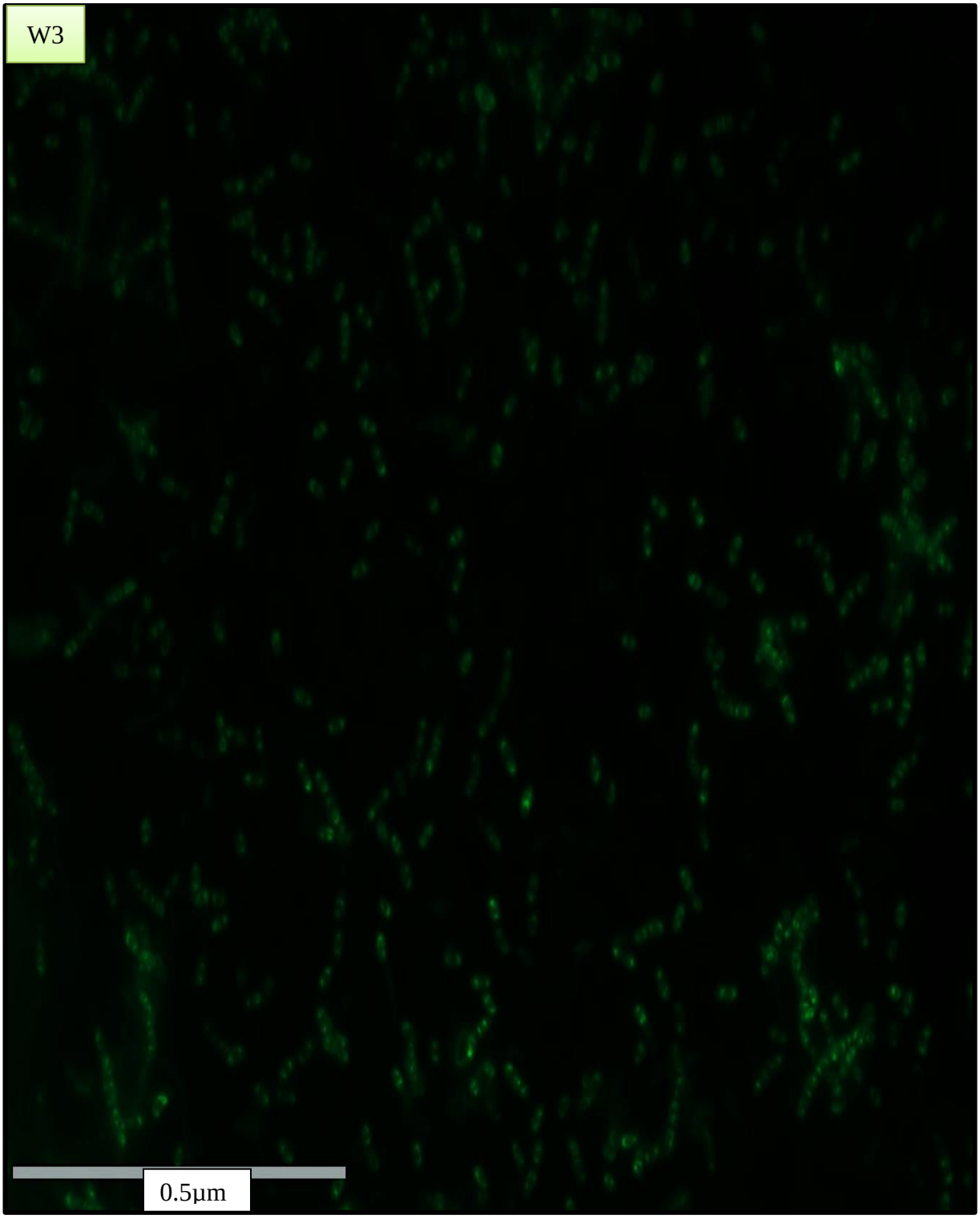


Figure 3-13. Images of β-lactamase producing *Streptomyces* (W6) and non β-lactamase producing *Streptomyces* (W1) spores stained with Bocillin fluorescent stain under fluorescent microscope. PBPs were stained in both strains and located at the poles of the spores.

In conclusion the interaction between penicillin G and PBPs in isolated *Streptomyces* species showed a variation between β-lactamase producing and non β-lactamase producing strains. The Bocillin staining in β-lactamase producing strains showed staining throughout the mycelia, whereas in non β-lactamase producing strains staining detected in only certain parts of the mycelia. PBPs were located at poles of the spores in both β-lactamase producing and non β-lactamase producing strains.

Chapter Four

4. Continuous culture (Fermentation) studies of *S. coelicolor 1147*

4.1 Fermentation Conditions

The study undertaken was focused on *Streptomyces sp* because of their economic importance as antibiotic producers. Although the advantages of continuous culture in the study of bacterial product formation have been known for many years, no studies on the effects of a wide range of antibiotics on *S. coelicolor 1147* have been reported.

To quantify the effect of growth rate of *S. coelicolor 1147* on resistance to β-lactam antibiotics (penicillin G), *Streptomyces coelicolor 1147* was grown in chemostat cultures at various dilution rates, and also in batch culture.

There are two main factors impacting on the productivity of batch growth and antibiotic production by *Streptomyces* species; one is the nature of the inoculum used and the other is permitting the growth as dispersed filaments (Hobbs et al. 1989). In all experiments, a spore inoculum was used and in order to obtain a dispersed growth, the chemostat was operated with agitation of 1000 rpm to prevent the formation of pellets and two Rushton impellers were used to mix the medium throughout the fermentation period. The quality and quantity of the nutritional elements used in the medium usually influence the growths of bacteria. In this work, a complex YEME medium was used for *Streptomyces coelicolor* (Kieser *et al.* 2000) . The pH of this medium was initially set to 7.2. During the fermentation process, the temperature was kept constant at 30°C which is the optimal for growth of this bacterium. As an aerobic bacterium, *Streptomyces coelicolor* needs a sufficient supply of oxygen during fermentations, sterile air was introduced into the medium via a stainless steel tube with small holes on it to distribute the air consistently. Prevent building-up of *Streptomyces* growth on the walls of outlet tube of the medium

and preventing blockages of the outlet tube, a stainless U shape tube was designed to solve this problem as shown in Figure 4-1. Foam was controlled automatically by sterile antifoam silicone 414 (Rhodorsil® Antifoam 414) using an automatic foam control system.

Reliable biomass measurements are critical for efficient monitoring of fermentation processes. It allows the determination of cultural variables such as the specific cell growth and substrate consumption rates (Elmer and Gaden 1959). The most common methods to determine biomass concentration are either chemical (e.g. DNA or RNA assays) or physical (e.g. Spectrophotometric determinations).

Monitoring of filamentous organism growth by spectrophotometry is generally considered not viable because of the noisy output signal due to the filamentous nature of the cells. Near infra-red spectroscopy (NIRS) is usually employed for the measurement of analytes and biomass in single celled fermentations (Tiwari *et al.* 2013).

Several measuring methods have been investigated for on-line measurement of biomass such as the on-line monitoring biomass concentration in mycelial fed-batch cultivations of *Streptomyces clavuligerus* with an *in-situ* capacitance probe fitted to an industrial pilot-plant tank (Neves *et al.* 2001) .

In this study, we used a new non-invasive optical sensor (BugEye® 100) for the real time monitoring of biomass of *Streptomyces coelicolor*. A non-invasive optical sensor and a monitor are part of the BugEye® 100. The sensor contains a group of lasers emitting at 850 nm and detectors manufactured to detect the light reflected from the cells in the vessel at multiple laser-detector distances (Debreczeny and Davies 2012).

The monitor controls the lasers, reads the signals from the detectors, and analyses the signals that are generated by each of the laser-detector pairs. The result is then presented

to the user. Real-time display and calibration of the Bug Lab units into dry weight is completed through the Bug Free software.

The study shows an optical device that can be used to monitor the progress of a filamentous fermentation that is prone to pollution. In our hands the use of the Bug Lab has proved useful to monitor the progress of a filamentous fermentation and it is reliable, sensitive to low concentrations of cells and highly reliable (Nakouti and Hobbs 2015).

4.2 Culture Purity and Stability

Every 24 h the culture was checked microscopically for infection and a sample of the culture fluid was plated onto MS agar and nutrient agar. There was no indication of infection in all fermentations. At the end of each fermentation the DNA sequencing was performed to confirm the purity of the culture.

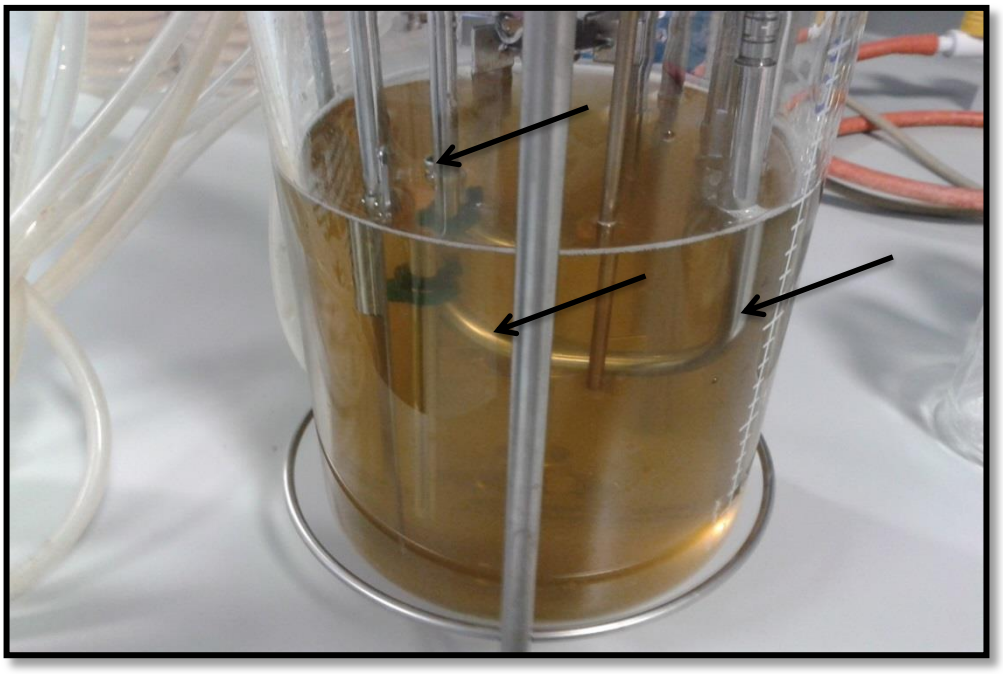


Figure 4-1. Chemostat vessel exhibited a stainless U shape tube as indicated by arrows.

4.3 Batch culture of Streptomyces coelicolor 1147

Batch experiment was performed to investigate patterns of growth of *Streptomyces coelicolor 1147* in the liquid complex medium over 48 hours. The growth (dry weight) and glucose consumption by *Streptomyces coelicolor 1147* are shown in Figure 4-2. There was no significant increase in the biomass concentrations of *Streptomyces coelicolor 1147* cells during the first 9 hours (lag phase) as a result of the adaptation of *Streptomyces coelicolor 1147* cells to the new environment and also, in which germination of the spores takes place. Glucose started to be consumed slightly throughout this phase. The log phase began where biomass levels increased rapidly over the period 9–23 h until it reached its maximum of 39.89 units at 23 h (2.99 g dry weight/ L at 23 h) of incubation (Figure 4.2 and 4.3). This was accompanied by the decline of dissolved oxygen and oxygen off gas evolution as well as the increase of CO_2 production (Figure 4.2). During the active growth phase, the glucose concentration dropped from 2 to 1.5 g/L. Moreover, there was a second biomass accumulation phase was evident (22–23h). This might be due to the switching of the organism to another source of carbon, which in this case could be the maltose from malt extract. CO_2 concentrations reached its maximum at 16 h of fermentation.

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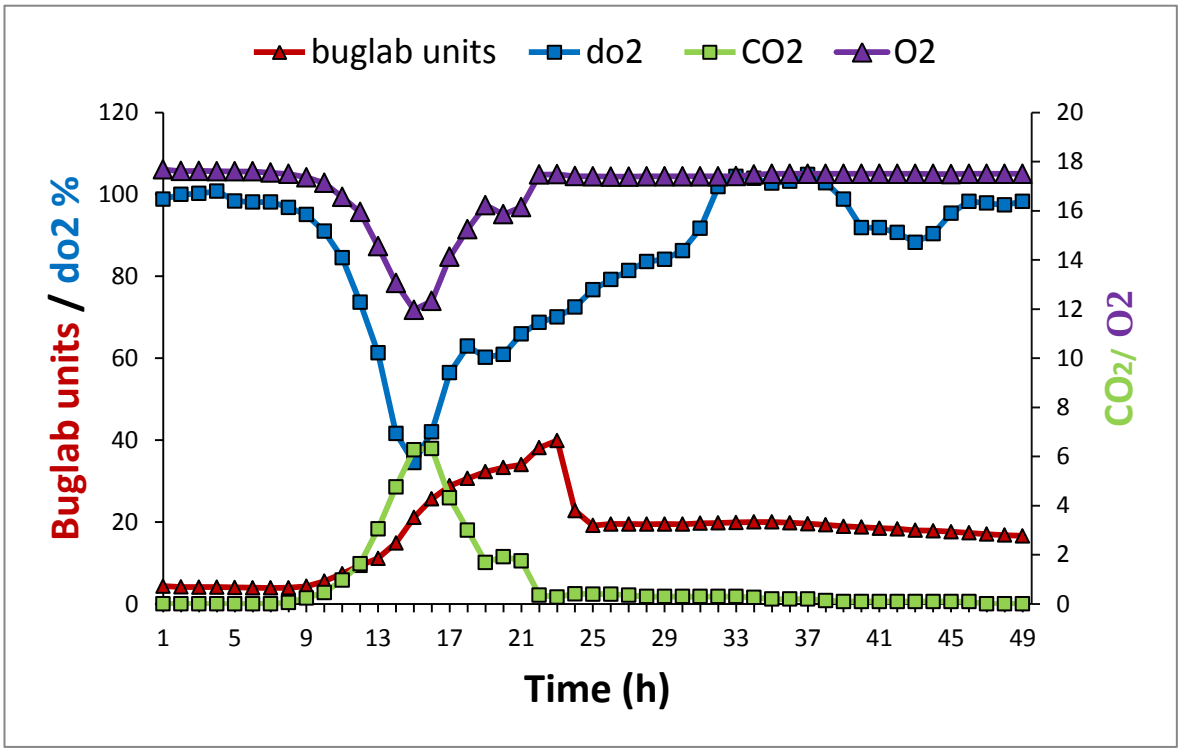

Figure 4-2. Fermentation profile of S. *coelicolor 1147* growing in modified YEME medium. This includes changes in dissolved oxygen and bug Lab biomass, oxygen evolution and CO_2 production measurements during growth over 48 h.

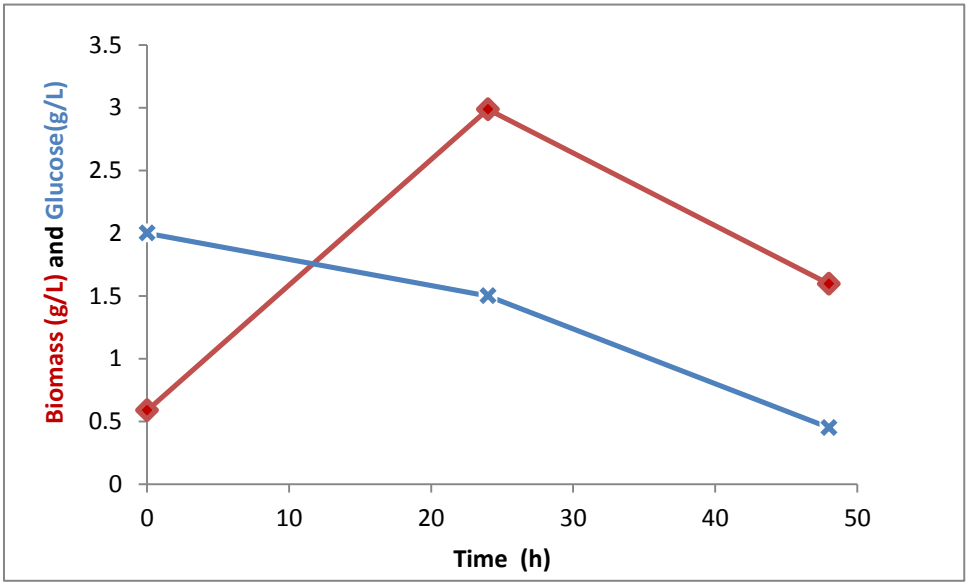

Figure 4-3. Growth of *S. coelicolor 1147* (dry weight) and glucose consumption in batch culture over 48h.

The consumption of glucose was 1.5g at 2.99 g of the biomass, the yield defined as the biomass divided on the consumption of glucose, therefore the yield equal to 1.99, which indicate that the bacteria switched to another source of carbon.

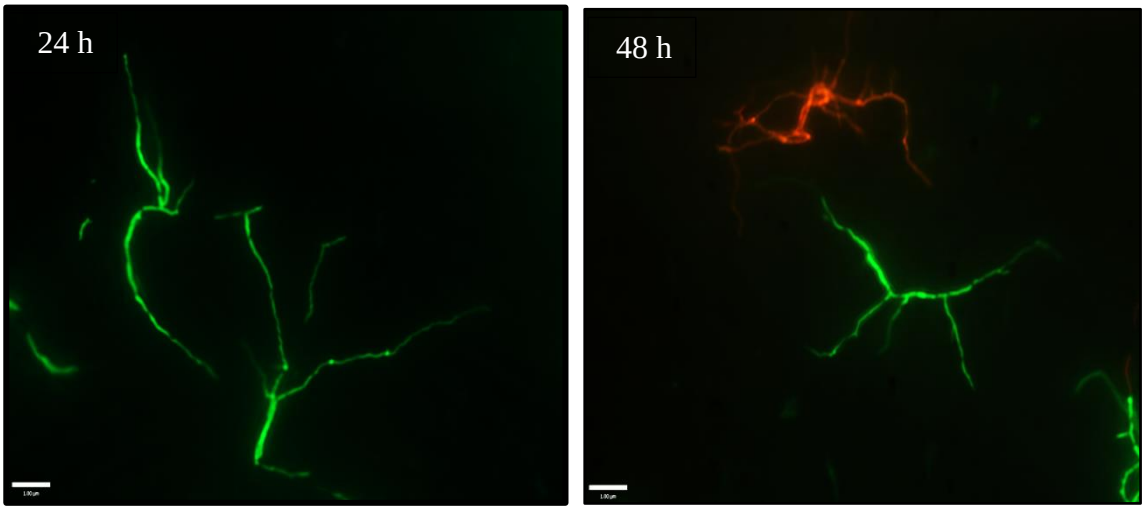

Figure 4-4. Fluorescent microscopy pictures of *S.coelicolor1147* growing in modified YEME medium in batch culture over 48 hours. The mycelia were stained with the fluorescent dye BacLight™, which stains all cells green, and stain those with compromised membranes red.

Throughout the early days of incubation, specifically on the second day of inoculation the colour of the medium changed slightly red as a result of production of undecylprodigiosin as shown in Figure 4.5.

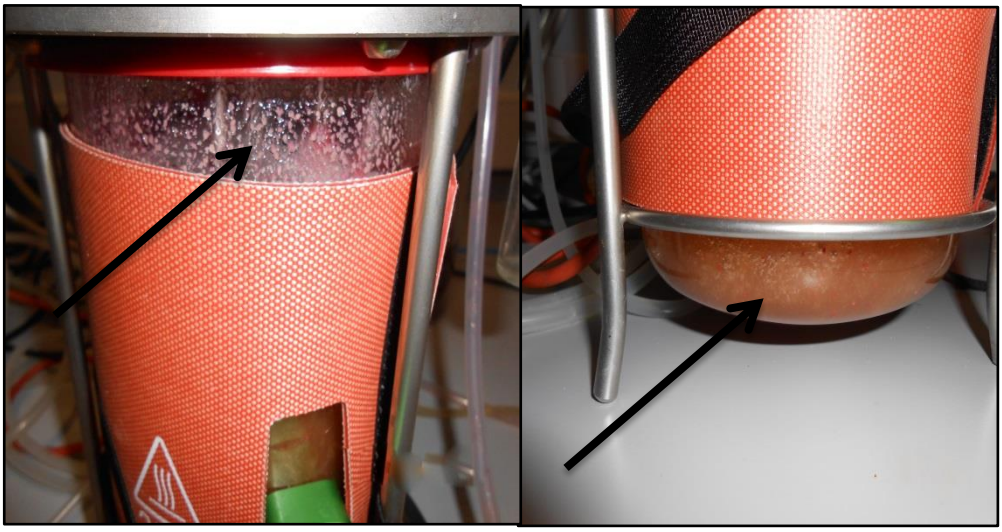

Figure 4-5. Image of *Streptomyces coelicolor 1147* grown in a 3L chemostat culture using a complex YEME medium at 48 hours of the inoculation, the colour of the medium changed red as a result of production of undecylprodigiosin.

The specific growth rate of *Streptomyces coelicolor 1147* grown in batch culture using YEME medium and 5% (v/v) of spore inoculum was calculated using MS Excel from the bug Lab unit's value as shown in Figure 4. 6. The growth rate of *Streptomyces coelicolor 1147* was equal to 0.1825 h^{-1} and the doubling time is 3. 79 h.

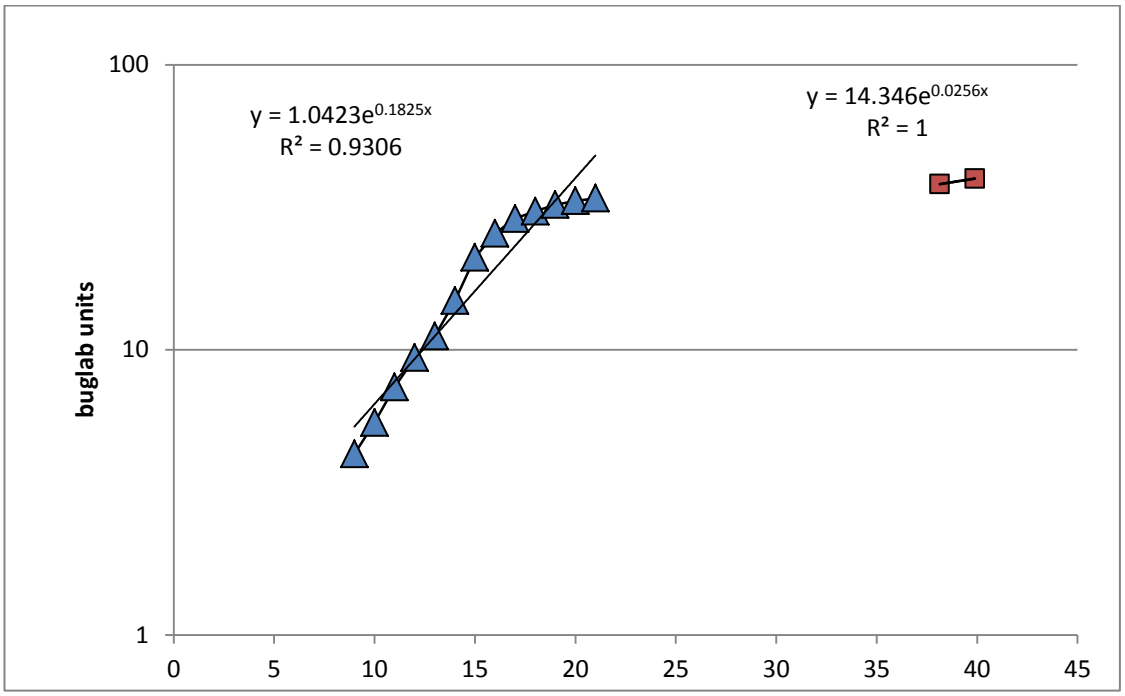

Figure 4-6. Graph represents the growth rate of *Streptomyces coelicolor 1147* grown in a complex medium in batch culture.

Sample from batch culture was sub-culture on MS medium and incubated at 30°C for seven days, an appropriate time to sporulation and the spores were removed and counted as mentioned previously in the material and method. Then the suspension of the spores on YEME mediumwas used to measure the MIC of *S. coelicolor 1147*. The minimum inhibitory concentration for *S. coelicolor 1147* strain was carried out using Oxoplate in 96 well plate as illustrated in Figure 4.7. The MIC of *S. coelicolor 1147* was 40 µg/ml and the MBC was 320 µg/ml.

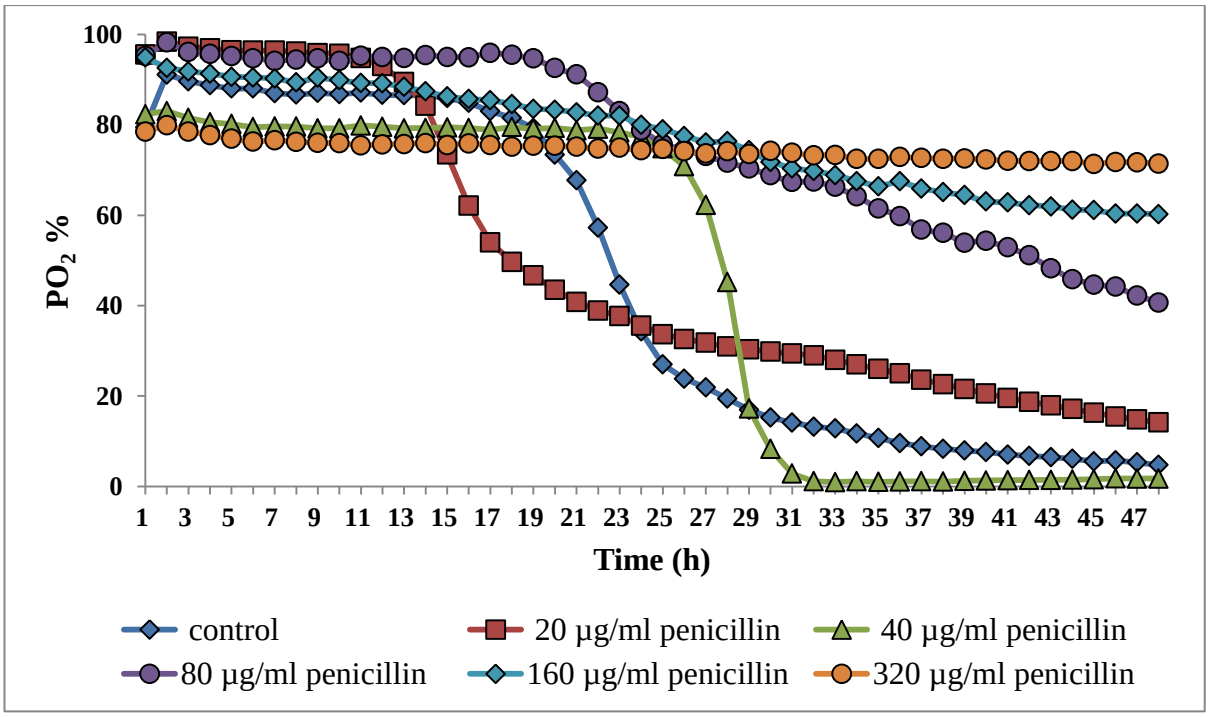

Figure 4-7. Oxygen consumption of *S. coelicolor* 1147 grown in YEME medium after treatment with different concentrations of penicillin G as indicated in the key. Values obtained are the mean of triplicate samples.

4.4 Growth of *S. coelicolor 1147* in continuous culture

Continuous culture was utilized to investigate whether there is any relationship between the growth rate and β-lactam antibiotic resistance in *Streptomyces.* Continuous exposure of *S. coelicolor 1147* to sub bactericidal concentrations of penicillin G was applied using a chemostat. The response of the organism to the antibiotic under a number of concentrations ranging from 80 to 640 µg/ml was investigated. On this basis *S. coelicolor 1147* was grown in four continuous cultures under different growth rates 0.04 h^{-1}, 0.06 h^{-1}, 0.08 h^{-1} and 0.10 h^{-1} over a period of 16 - 21 days. Penicillin G was pumped into vessel culture after 4 days (the time that cultures reached steady state) via the medium feed reservoir. Samples were taken every 24 hours for the following determinations: viability, colony morphology, purity and to measure the MIC. O_2 depletions and CO_2 were measured online.

Dilution rate is simply defined as the volumetric flow rate of nutrient supplied to the chemostat divided by the volume of the culture (unit: $time^{-1}$). In general, raising the dilution rate will raise the growth of the cells. On the other hand, the dilution rate has to be controlled linked to the specific growth rate to avoid wash-out of the cells. The dilution rates were set up to be less than the specific growth rate of *S. coelicolor 1147* measured from the previous batch culture *0.1825* $h^{-1,}$ because the specific growth rate equals the dilution rate at steady state when, the flow rate, temperature, pH, the number of cells in the vessel all remain constant.

4.4.1 Dilution rate 0.04 h^{-1}

The feed rate was adjusted to 0.04 h^{-1} at the doubling time of 17.325 h: over 29.09 generations. After 24 hours of inoculation the feeding without penicillin G was applied at this dilution rate. The colour of the medium changed slightly red as a result of production of undecylprodigiosin. At each recognised steady state that was set at around 96 h, penicillin G was pumped to the feed reservoir at different concentration ranging from 40 to 640 µg/ml at regulated hours 96, 168, 264 and 336 respectively. The result obtained from this fermentation (Figure 4.8) showed that *S. coelicolor 1147* has the ability to adapt to increasing concentration of β-lactam antibiotic (penicillin G). There were no changes in morphology of the colonies and the MIC with the increasing concentrations of penicillin G up to 160 µg/ml. The biomass levels increased over the period 168-216 h, where the concentration of penicillin G pumped into the medium was 80 µg/ml, this was accompanied by the decrease of dissolved oxygen and the increase of CO_2 production. After that the biomass level and CO_2 production decreased and dissolved oxygen increased.

At the concentration 160 µg/ml, there was some decrease in dissolved oxygen and increase of CO_2 production, the biomass was slightly changed at the concentration of 320 µg/ml. Moreover, the organism lost the ability to form spores at this concentration at the generation time of 19.39 as presented in Figure 4.9, the ability to form spores return after 4 subcultures on MS medium Figure 4.9 C. However, *S. coelicolor 1147* did not lose the ability to form an aerial mycelium and this was confirmed using SEM as shown in Figure 4.10. At the concentration 640 µg/ml there was rapidly decreasing in dissolved oxygen and biomass level accompanied by the increase of CO_2 production.

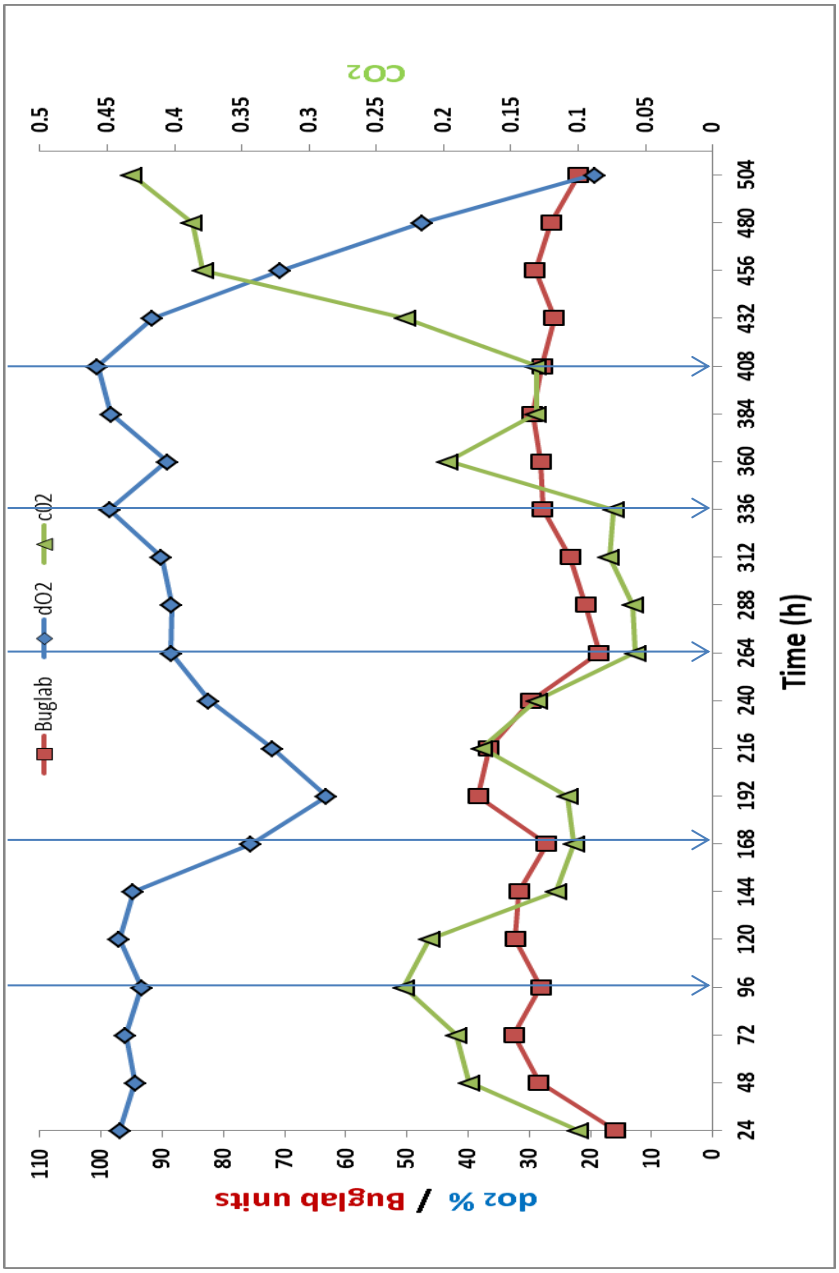

Figure 4-8. Fermentation profile of *S. coelicolor 1147* grown in a complex YEME medium in continuous culture feeding with different concentrations of penicillin G over 504 hours at dilution rat 0.04 h^{-1}. This includes changes in dissolved oxygen and bug Lab measurements and production of CO_2 during growth. Blue arrows indicated the time when penicillin G was added. The concentration of penicillin G ranging from 40 to 640 µg/ml respectively.

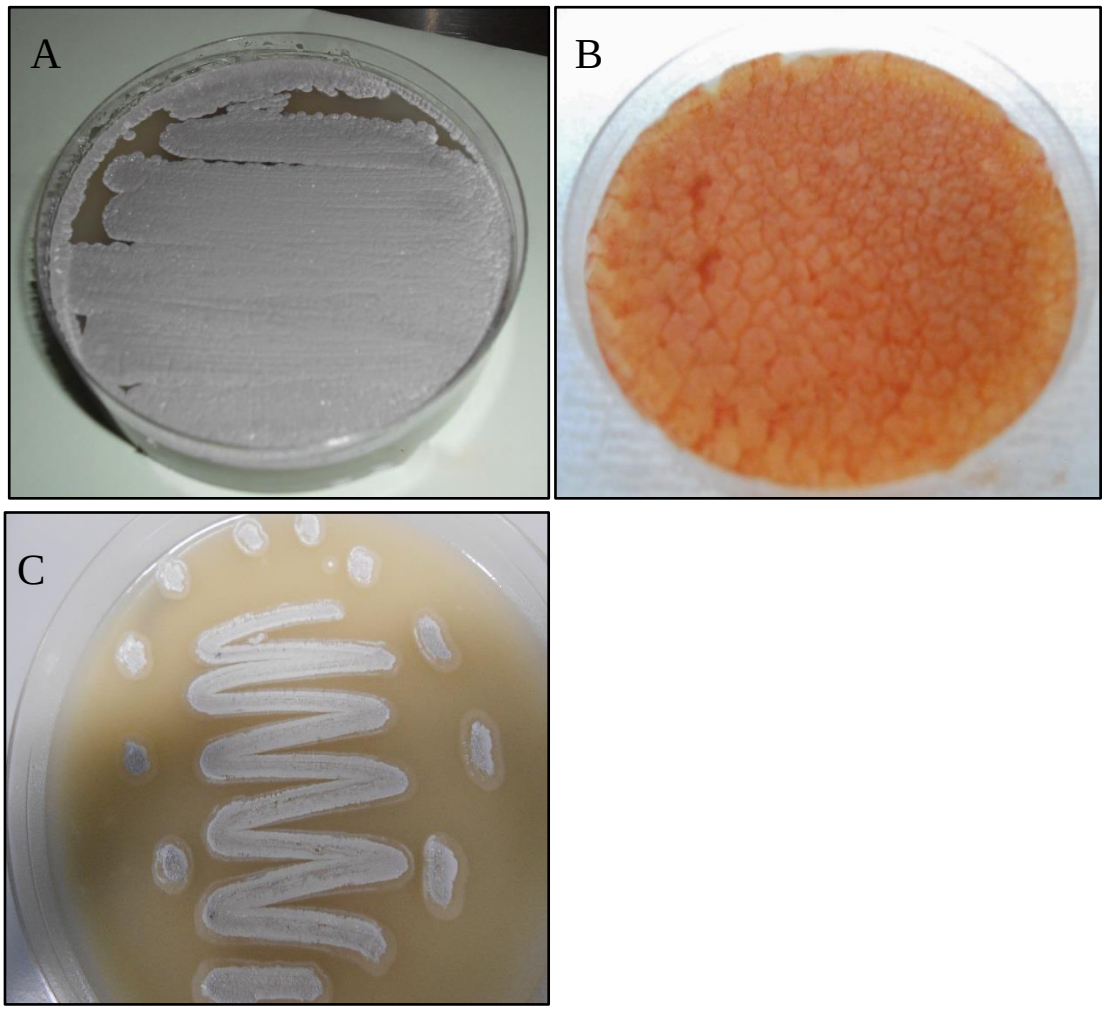

Figure 4-9. Growth of *S. coelicolor 1147* on MS media (A): after seven days of incubation without antibiotic. (B) After incubation with increasing concentration of penicillin G up to 320 µg/ml in continues culture at dilution rate 0.04 h^{-1} and at generation time 19.39. Red colour indicates undecylprodigiosin production. (C): after 4 subculture on MS agar without antibiotic present and each subculture was incubated for 7 days.

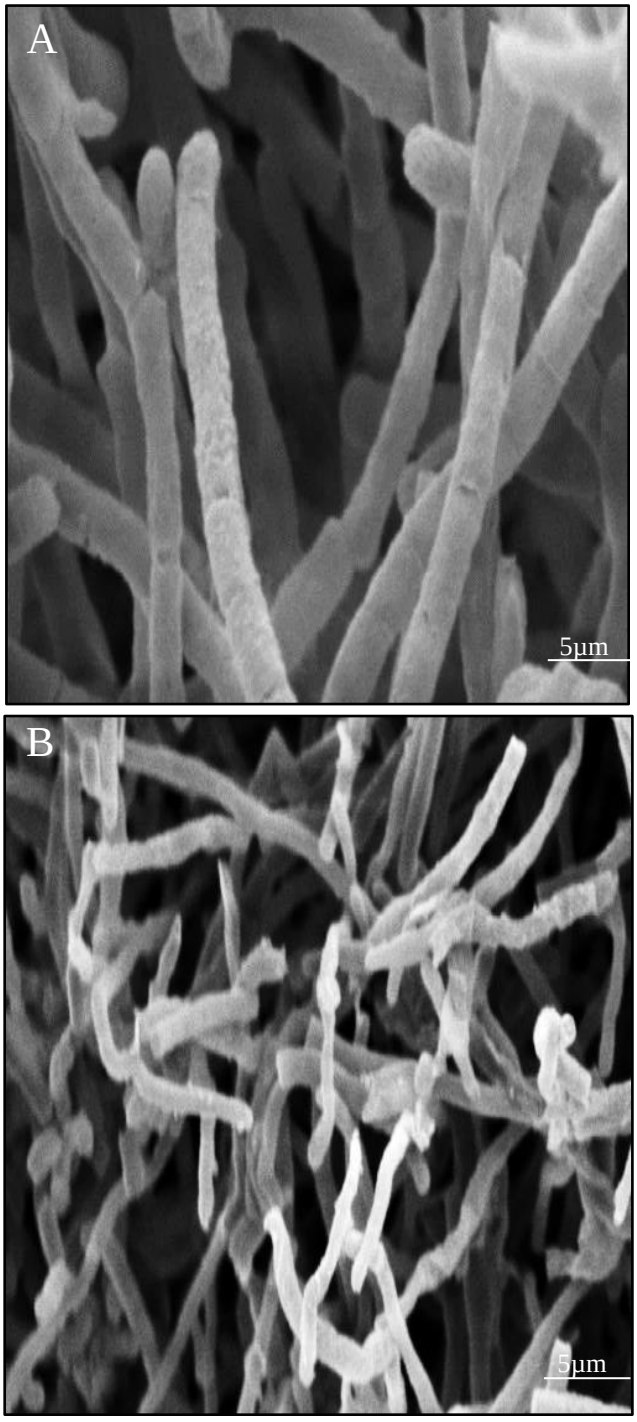

Figure 4-10. Cryo electron microscope image showing the spore morphology of *S. coelicolor 1147* grown on MS medium without antibiotics at 30°C for 7 days; (A) Incubation without antibiotic, (B) After incubation with increasing concentration of penicillin G up to 320 µg/ml in continuous culture at dilution rate 0.04 h^{-1}a and generation time 19.39.

The effect of increasing concentration of penicillin G on the viability of the cells was examined using LIVE/DEAD® *Bac*Light™ fluorescent stain and viewed under fluorescent microscope as presented in Figure 4.11. Live parts with complete cell membrane fluoresce green and membrane-compromised parts fluoresce red. The β-lactam antibiotic (penicillin G) affects not only the tips of mycelium as indicated by arrows in Figure 4.11. Moreover, S. *coelicolor 1147* was able to grow at high concentration of penicillin G up to 640 µg/ml.

Furthermore, the result obtained from Oxoplate indicates that, the MIC of *S. coelicolor 1147* increased from 40 µg/ml to 160 µg/ml after inoculation with different concentration of penicillin G up to 640 µg/ml as shown in Figure 4.12.

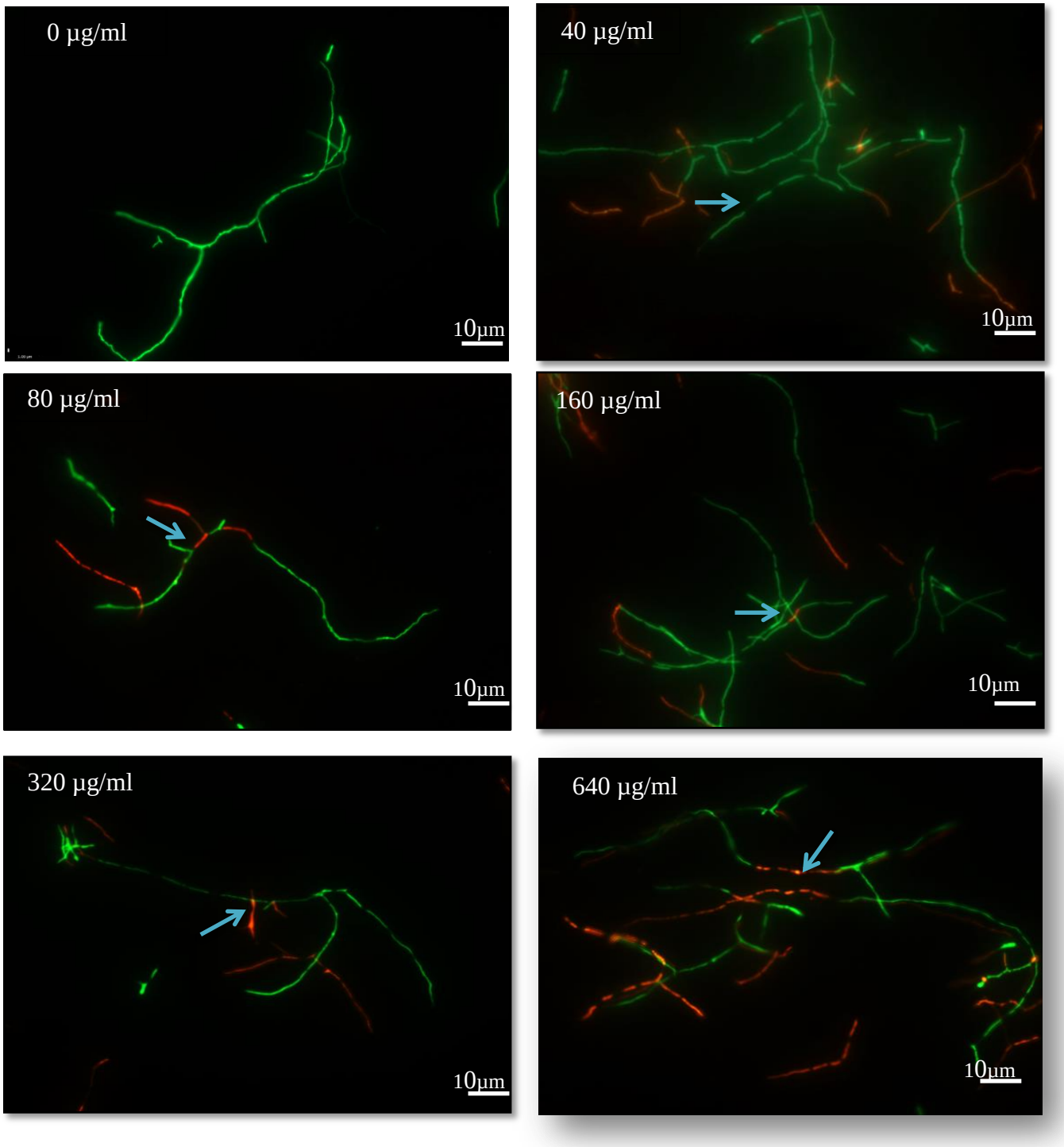

Figure 4-11. Images of *Streptomyces coelicolor 1147* grown in a 3L chemostat culture using a complex medium and 5% (v/v) of spore inoculum with different concentrations of penicillin G as indicated in each panel (0, 40, 80, 160, 320 and 640 µg/ml) at dilution rate 0.04 h^{-1}. Samples stained with LIVE/DEAD® *Bac*Light™ fluorescent stain. Live parts with whole cell membrane fluoresce green and membrane-compromised parts fluoresce red.

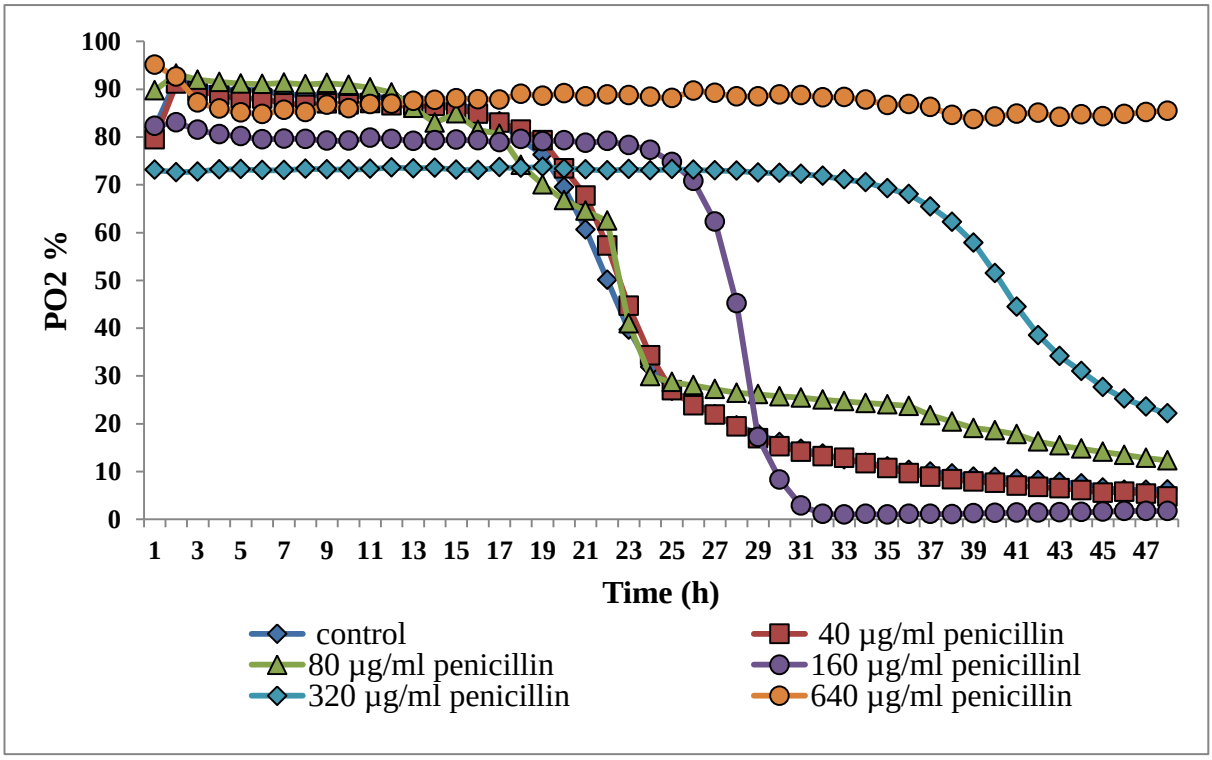

Figure 4-12. Oxygen consumption of *S. coelicolor* 1147 grown in YEME medium after treatment with different concentrations of penicillin G as indicated in the key. Values obtained are the mean of triplicate.

4.4.2Dilution rate 0.06 h^{-1}

In this fermentation the feed rate was adjusted to 0.06 h^{-1} and the doubling time was 11.55 h over a generation time of 35.3. The colour of the medium has not changed at this dilution rate. As mentioned before at the recognised steady, penicillin G was pumped to the feed reservoir at different concentration ranging from 80 to 460 µg/ml at regulated at 96, 168, 264 and 336 hours respectively. The result obtained from this fermentation (Figure 4.13) indicated that there were no changes in morphology of the colonies and the MIC with the increasing penicillin G up to 160 µg/ml. However, during this period the biomass level rapidly decreased, which was accompanied by a slight decrease of dissolved oxygen and increase of CO_2 production. At the concentrations 320 µg/ml and 460 µg/ml, there was a change of the morphology of the colonies as well as the MICs. Similar to the result obtained from a previous fermentation (dilution rate 0.04 h^{-1}), the bacterium failed to form spores, but it was also able to form aerial mycelium at generation time 29. 09 as exhibited in Figure 4.14. The capacity to procedure spore resumed after 4 times of sub culturing the organism on MS medium and incubated at 30 °C for seven days, which indicate that the change was a phenotypic change not mutating. Also, it lost the ability to produce the red pigments on MS medium.

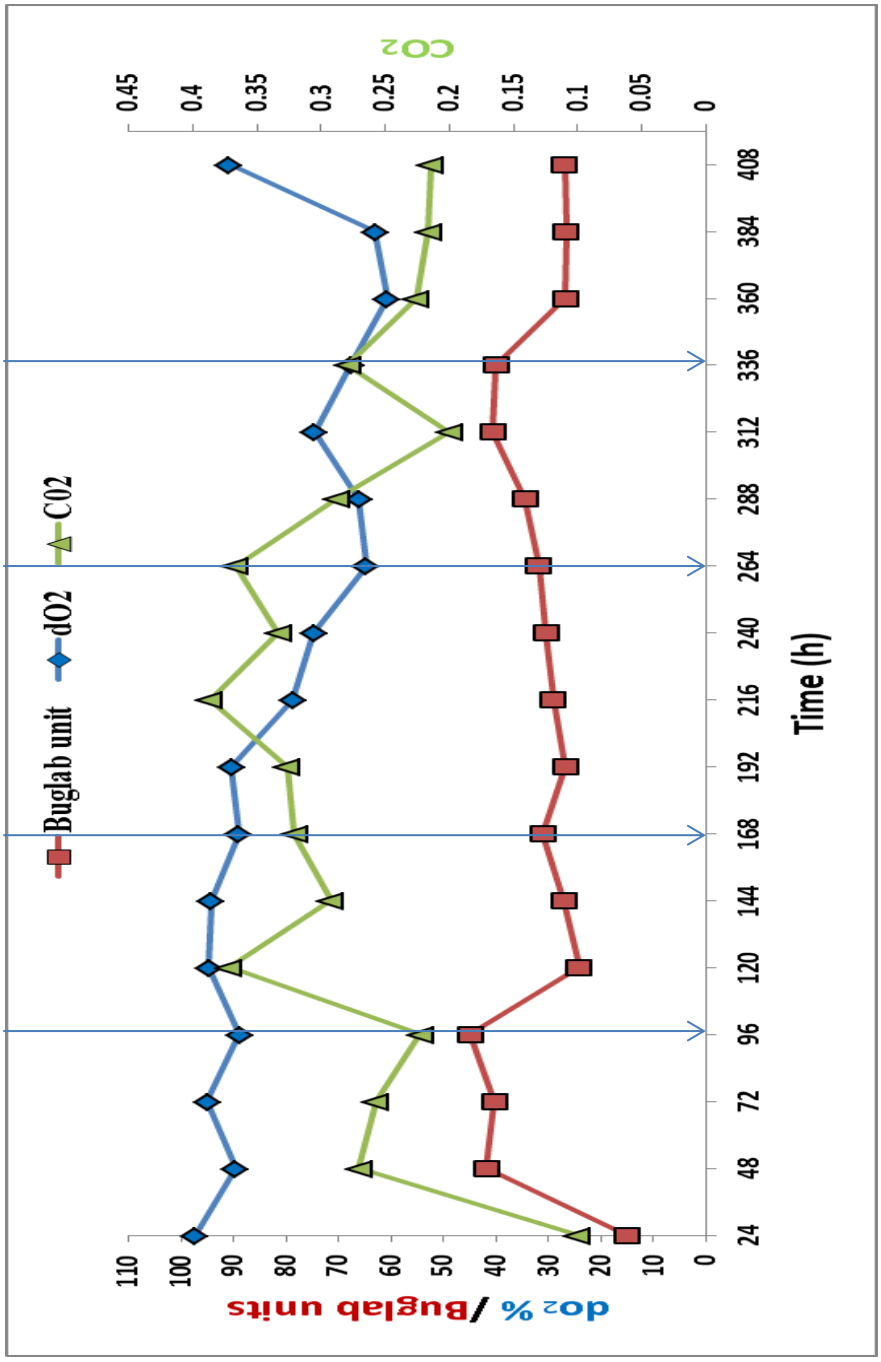

Figure 4.13. Fermentation profile of *S. coelicolor 1147* grown in modified YEME medium in continuous culture feed with different concentrations of penicillin G over 408 hours at dilution rat 0.06 h^{-1}. This includes changes in dissolved oxygen and bug Lab units and production of Co_2 during growth. Blue arrows indicated the time when penicillin G was added. The concentration of penicillin G ranging from 80 to 640 µg/ml respectively.

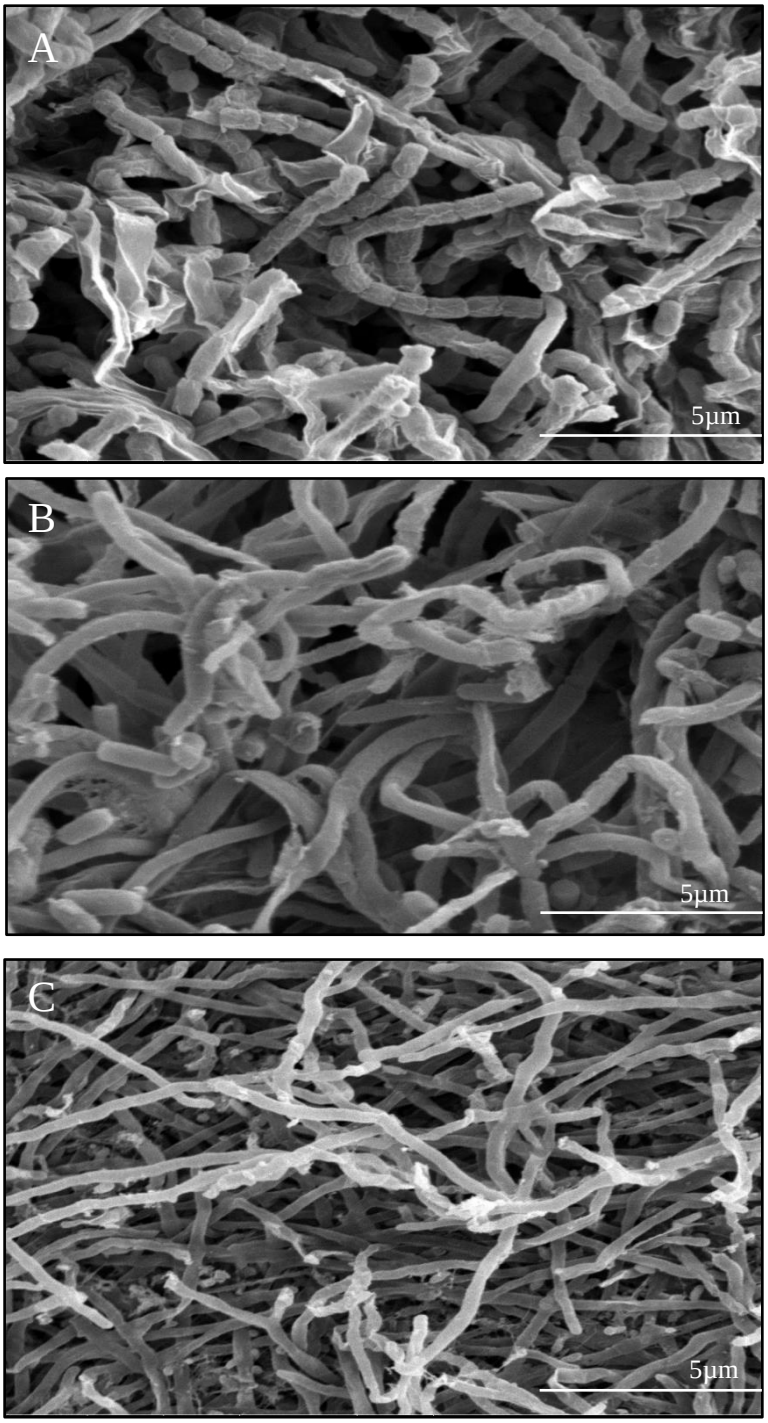

Figure 4-14. Cryo electron microscope images showing the spore morphology of *S. coelicolor 1147* grown in MS medium without antibiotics at 30 °C after 7 days; (A) Incubation without antibiotic, (B) After incubation with increasing concentration of penicillin G up to 320 µg/ml at generation time 29. 09 (C) After incubation with increasing concentration of penicillin G up to 640 µg/ml at generation time 35.32. *S. coelicolor 1147* grown in a 3L chemostat using a complex medium at dilution rate 0.06 h^{-1}.

The viability of the cells was examined using LIVE/DEAD® *Bac*Light™ fluorescent stain and viewed under a fluorescent microscope (Figure 4.15). Live parts with complete cell membrane fluoresce green and membrane-compromised parts fluoresce red. The result showed that the organism has the ability to grow at high concentration of penicillin G up-to 640 µg/ml at this dilution rate (0.06 h^{-1}).

Additionally, the MIC of *S. coelicolor 1147* increased (Figure 4.16) at this dilution rate also (0.06 h^{-1}), however, it increased just one fold (80 µg/ml) compared to the lowest dilution rate in this study which increased two fold (160 µg/ml). The MBC was same in both dilution rates (640 µg/ml).

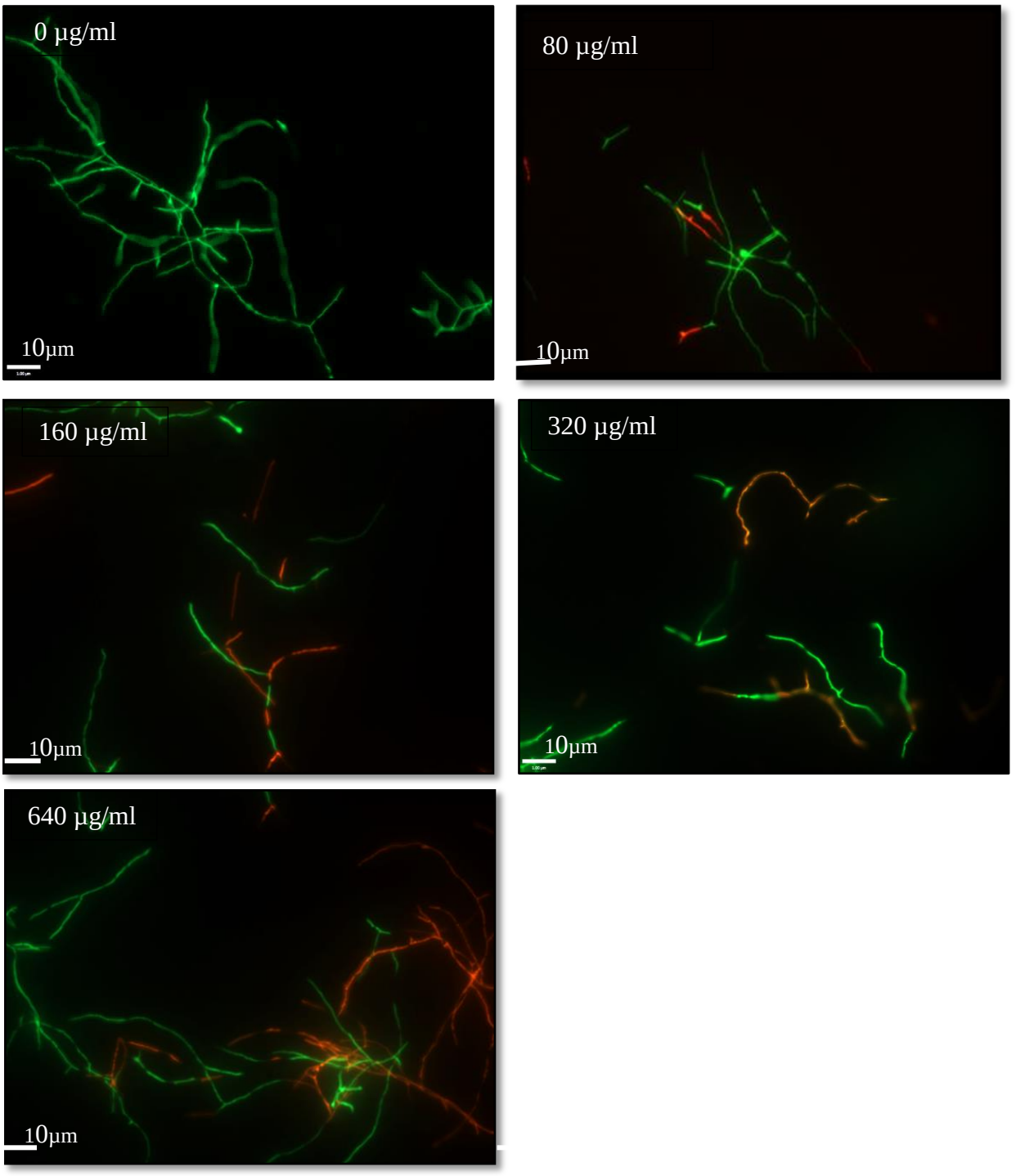

Figure 4-15. Images of *Streptomyces coelicolor 1147* grown in a 3L chemostat culture using a complex medium and 5% (v/v) of spore inoculum with different concentrations of penicillin G as indicated in each panel (0, 80, 160,320 and 640 µg/ml) at dilution rate 0.06 h^{-1} over 408 h. Samples stained with LIVE/DEAD® *Bac*klight™ fluorescent stain. Live parts with whole cell membrane fluoresce green and membrane-compromised parts fluoresce red.

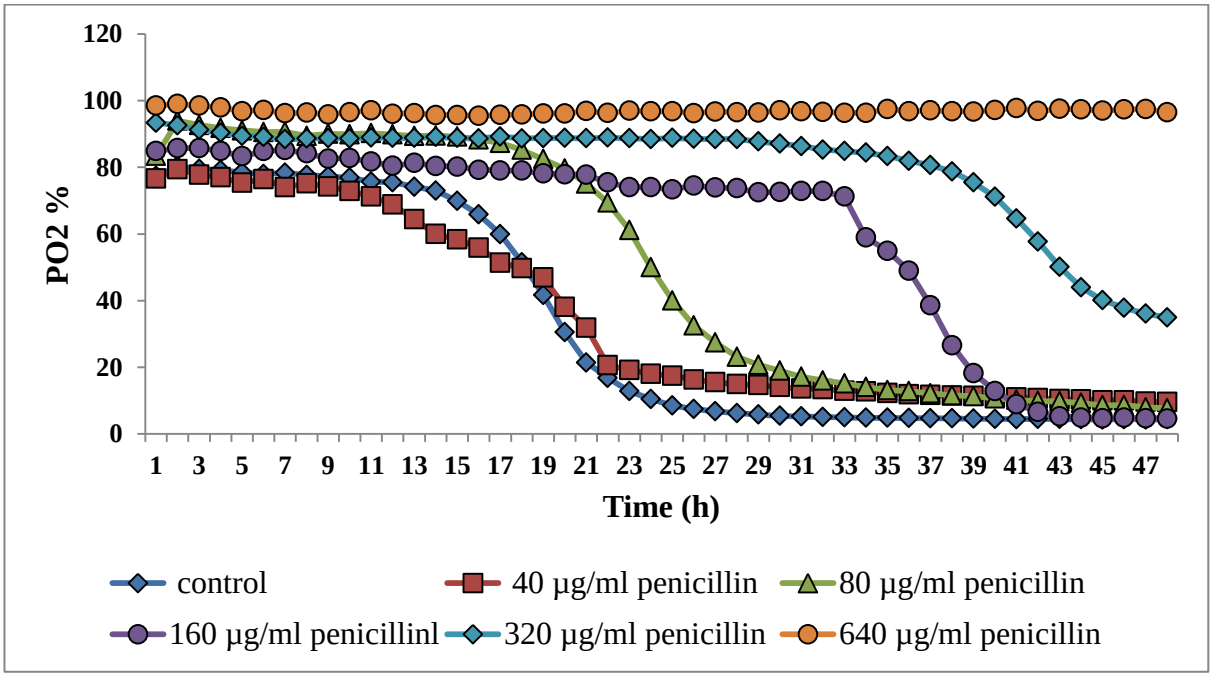

Figure 4-16. Oxygen consumption of *S. coelicolor 1147* grown in YEME medium treatment with different concentrations of penicillin G up to 640 µg/ml. Values obtained are the mean of triplicate samples. The penicillin G concentration is indicated in the key.

4.4.3 Dilution rate 0.08 h^{-1}

Fermentation profile of *S. coelicolor 1147* at this dilution rate 0.08 h^{-1} and doubling time 8.66 hours over 52. 65 generation presented in Figure 4.17. The biomass level and dissolved oxygen declined rapidly when the organism feeding with the different concentration of penicillin G up to 640 µg/ml. Interesting, dissolved oxygen dropped to less than 2% and *S. coelicolor 1147* was able to grow at this condition. Also, there was a change in the spore morphology produced by *S. coelicolor 1147* treated with increasing concentration of penicillin G up to 460 µg/ml at this dilution rate, the wild type strain produced long spore chains, spore surfaces are coated with a proteinaceous fibrous sheath, whereas, treated *S. coelicolor 1147* exhibited short aerial hyphae with few irregular spores without coating with a proteinaceous fibrous sheath (Figure 4.18).

The viability of the cells was examined using LIVE/DEAD® *Bac*Light™ fluorescent stain and viewed under a fluorescent microscope (Figure 4.19). Live parts with complete cell membrane fluoresce green and membrane-compromised parts fluoresce red. The result showed that the organism has the ability to grow at high concentration of penicillin G up to 640 µg/ml and with very low amount of dissolved oxygen. Furthermore, the MIC of *S. coelicolor 1147* at this dilution rate (0.08 h^{-1}) was unchangeable and equal to the MIC of wild type strain (Figure 4.20), however, there was an increase in the MBC (640 µg/ml) which indicate that the organism became less sensitive to penicillin G.

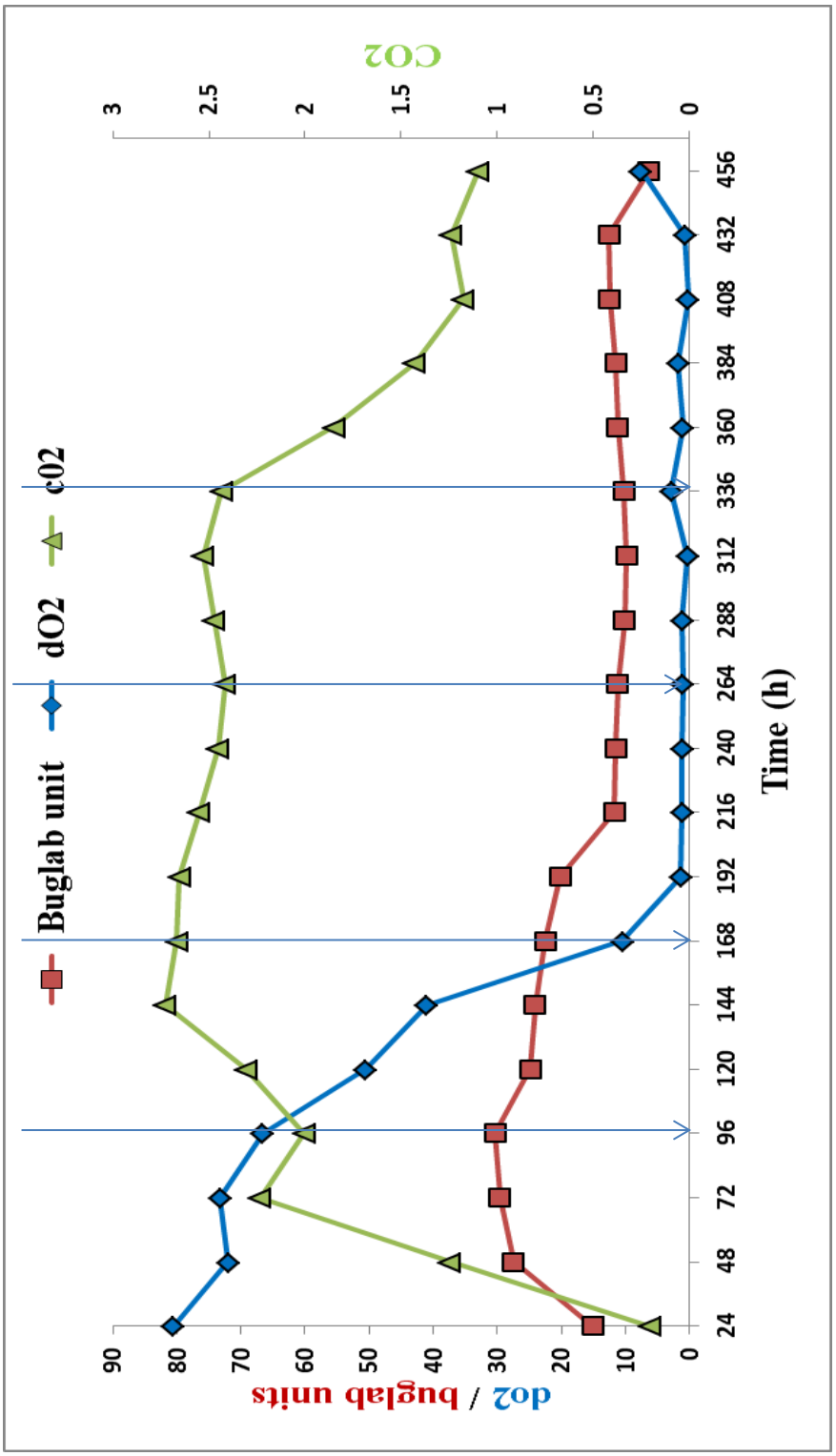

Figure 4-17. Fermentation profile of *S. coelicolor 1147*grown in modified YEME medium in continuous culture feeding with different concentrations of penicillin G over 456 hours at dilution rat 0.08 h^{-1}. This includes changes in dissolved oxygen and bug Lab biomass and production of Co_2 during growth. Blue arrows indicated the time when penicillin G was added. The concentration of penicillin G ranging from 80 to 640 µg/ml respectively.

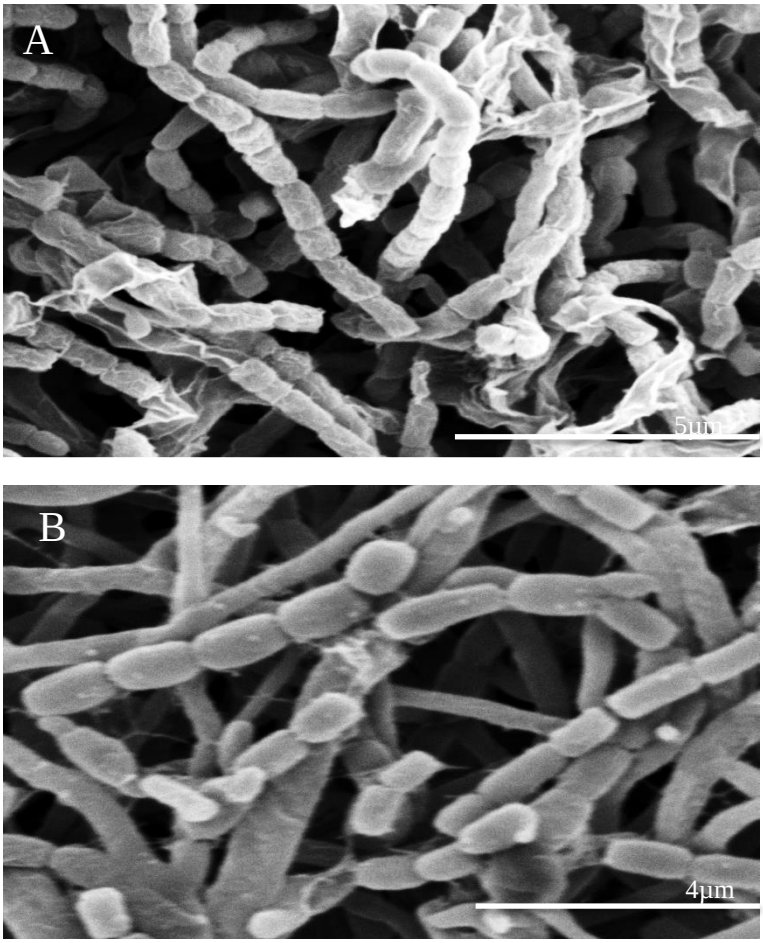

Figure 4-18. Cryo electron microscope images showing the spore morphology of *S. coelicolor 1147*grown in MS medium without antibiotics at 30 °C after 7 days; (A) Incubation without antibiotic, (B) After incubation with increasing concentration of penicillin G up to 320 µg/ml in a 3L chemostat using a complex medium at dilution rate 0.08 h^{-1} and at generation time 42.26.

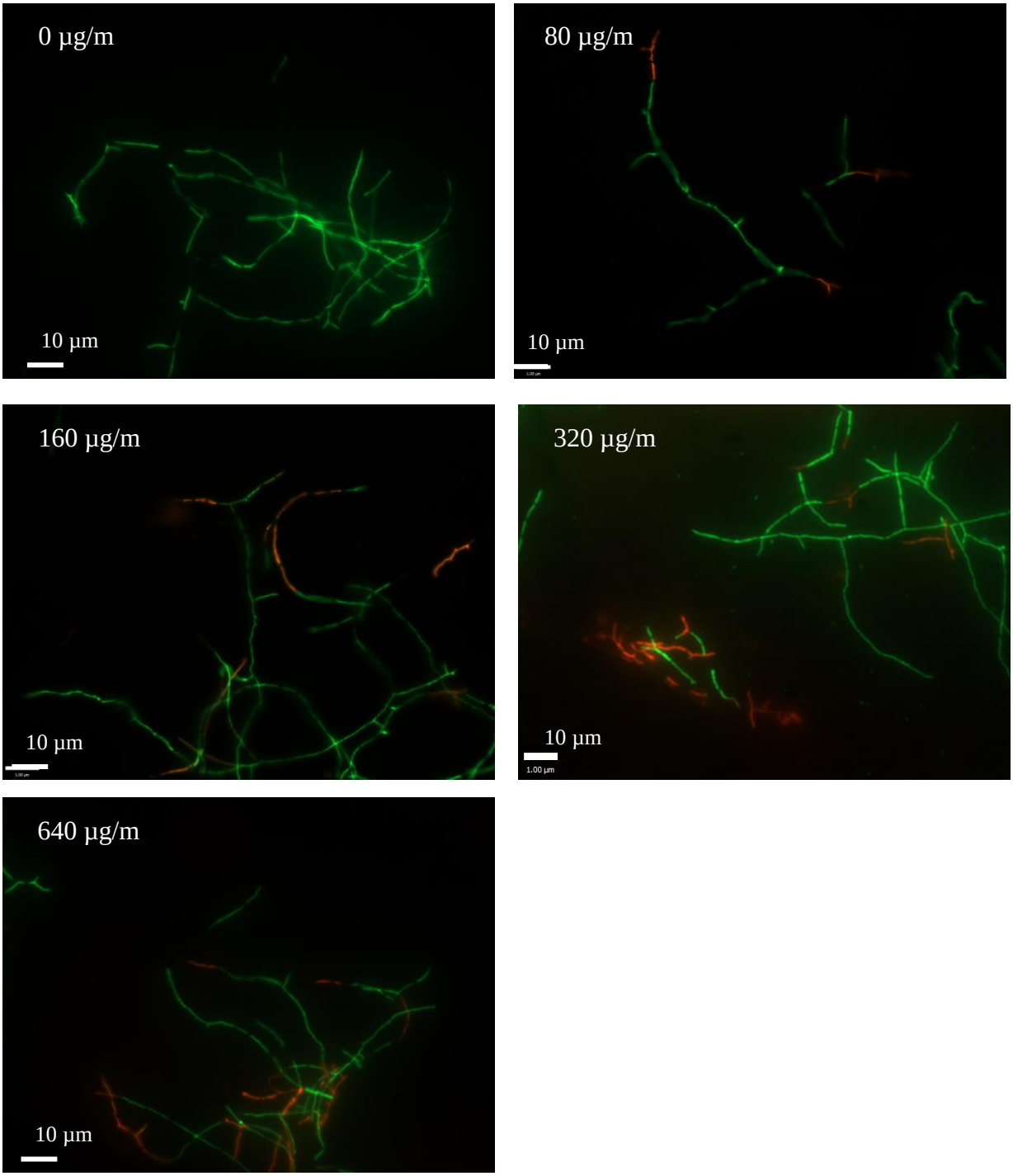

Figure 4-19: Images of *Streptomyces coelicolor 1147* grown in a 3L chemostat culture using a complex medium and 5% (v/v) spore's inoculum with different concentrations of penicillin G as indicated in each panel (0, 80, 160, 320 and 640 µg/ml) at dilution rate $0.08h^{-1}$ over 456 h. Samples stained with LIVE/DEAD® *Bac*Light™ fluorescent stain and viewed using fluorescence microscopy. Live parts with whole cell membrane fluoresce green and membrane-compromised parts fluoresce red.

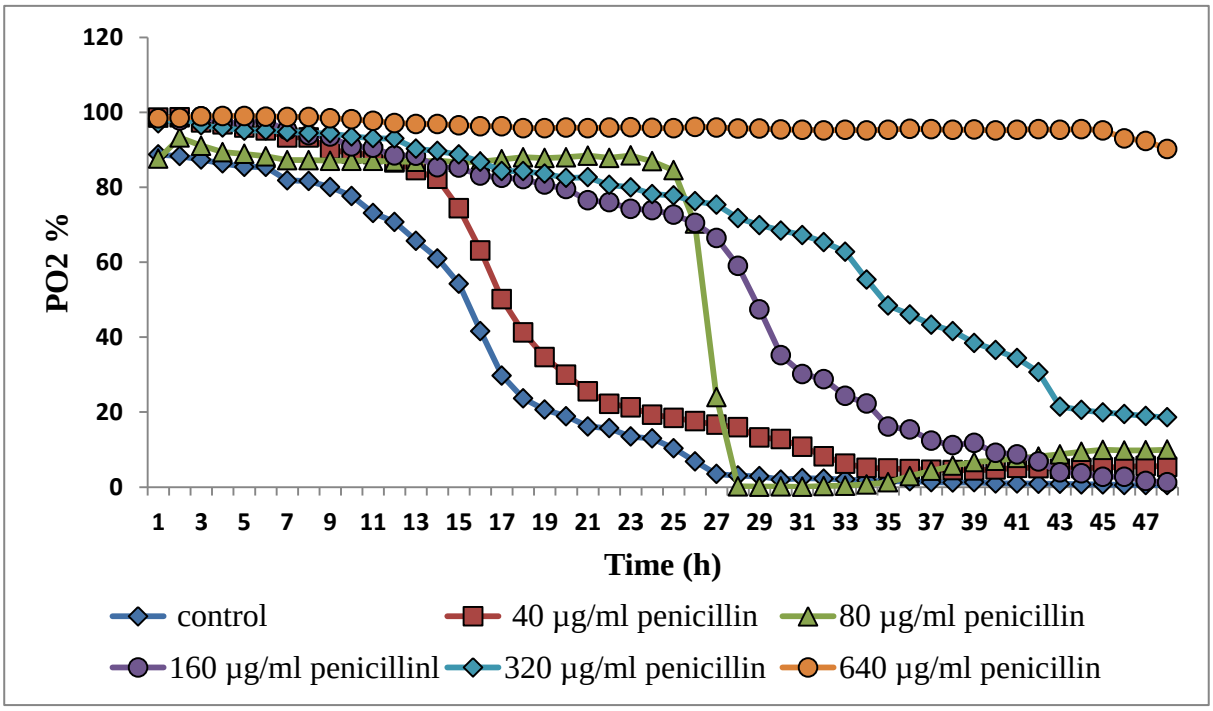

Figure 4-20. Oxygen consumption of *S. coelicolor 1147* grown in modified YEME media in continues culture at dilution rate 0.08 h^{-1}, treatment with different concentrations of penicillin G up to 640 µg/ml. Values obtained are the mean of triplicate samples. The penicillin G concentration is indicated in the key.

4.4.4. Dilution rate 0.1 h^{-1}

Fermentation result of *S. coelicolor 1147* grown in continues culture feeding with different concentrations of penicillin G over 55 generating at dilution rat 0.1 h^{-1} and doubling time of 6. 93 h illustrated in Figure 4-21. Overall, there was a dramatic increase of the biomass and CO_2 production complemented with the decrease of dissolved oxygen. The CO_2 production reaches its maximum at 336h and the dissolved oxygen decreased to less than 2% at 216 h.

The cell viability was assessed using LIVE/DEAD® *Bac*Light™ fluorescent stain and observed under a fluorescent microscope (Figure 4-22). As mentioned previously in the fermentation (0.08 h^{-1}) the organism has the talent to grow at high concentration of penicillin G up to 640 µg/ml and also with very low amount of dissolved oxygen.

Besides, there was no change of spore morphology under SEM. The MIC of *S. coelicolor 1147* at this dilution rate (0.1 h^{-1}) was unchangeable and equal to the MIC of wild type strain (Figure 4-23), however, there was an increase in the MBC (640 µg/ml) which indicate that the organism became less sensitive to penicillin G.

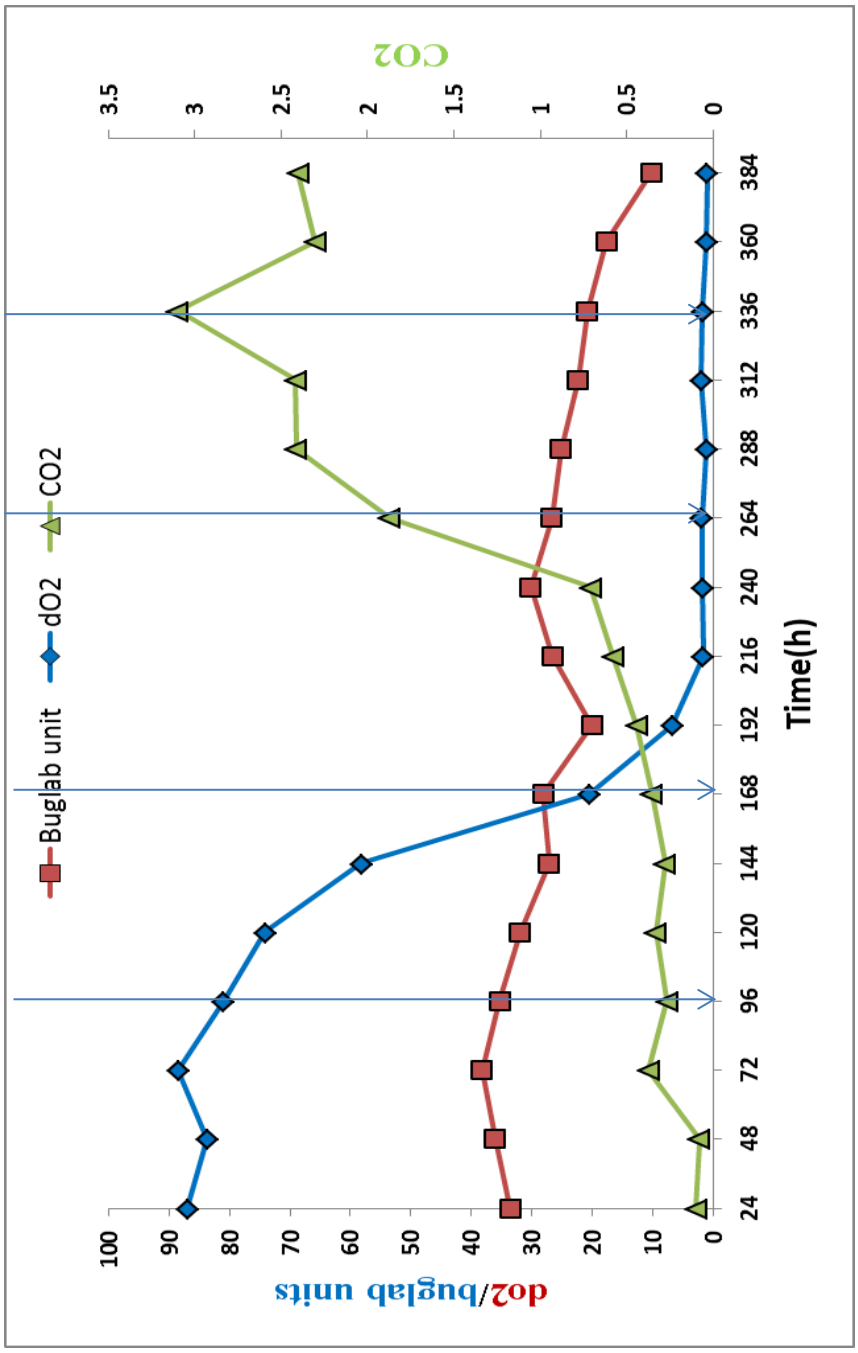

Figure 4-21. Fermentation profile of S. *coelicolor 1147* grown in modified YEME medium in continues culture feeding with different concentrations of penicillin G over 384 hours at dilution rat 0.1 h^{-1}. This includes changes in dissolved oxygen and bug Lab biomass measurements and production of Co_2 during growth. Blue arrows indicated the time when penicillin G was added. The concentration of penicillin G ranging from 80 to 640 µg/ml respectively.

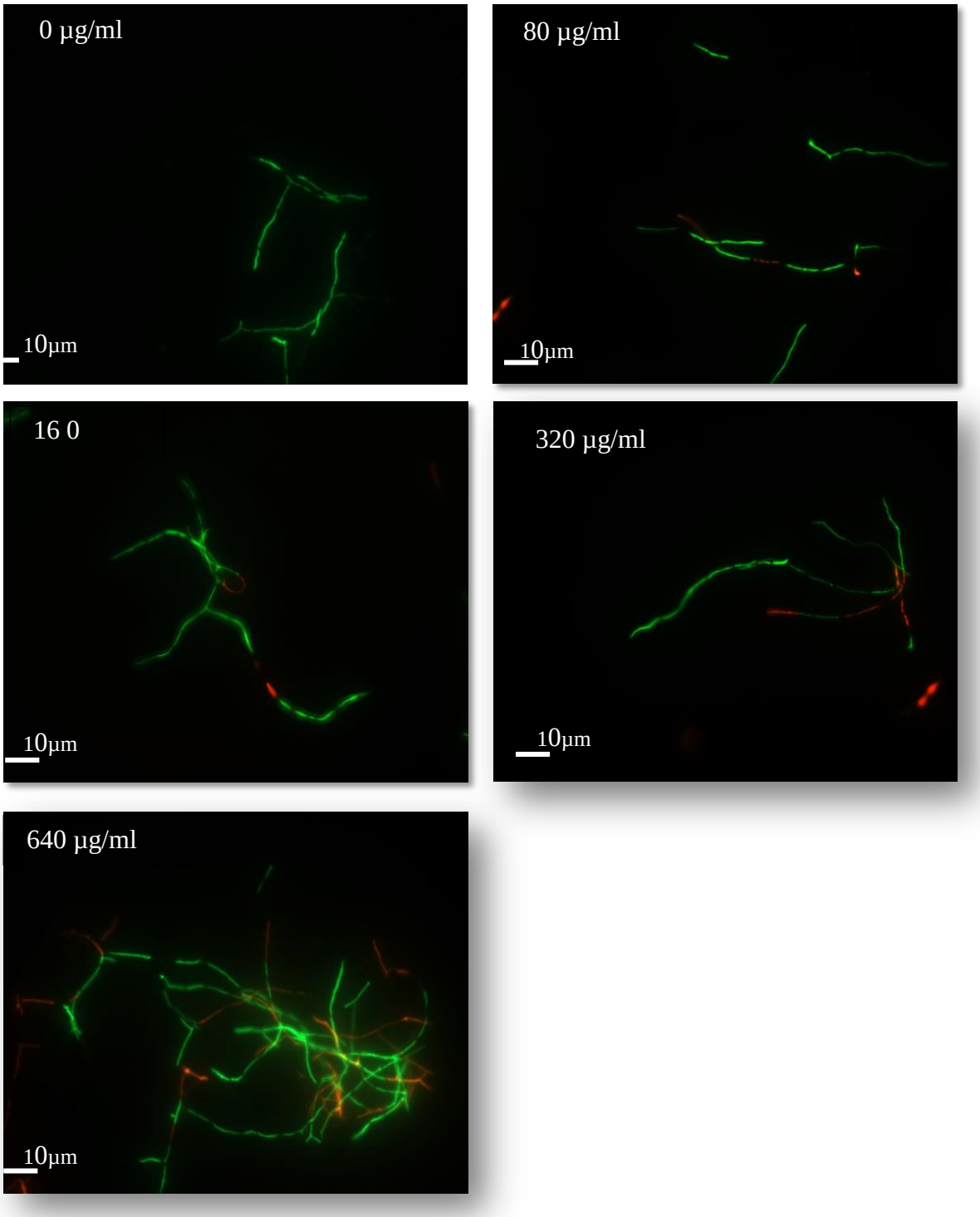

Figure 4-22. Images of *Streptomyces coelicolor 1147* grown in a 3L chemostat culture using a complex medium and 5% (v/v) spore's inoculum with different concentrations of penicillin G as indicated in each panal (0, 80, 160, 320 and 640 µg/ml) at dilution rate $0.1h^{-1}$ over 384h. Samples stained with LIVE/DEAD® *Bac*Light™ fluorescent stain. Live parts with whole cell membrane fluoresce green and membrane-compromised parts fluoresce red.

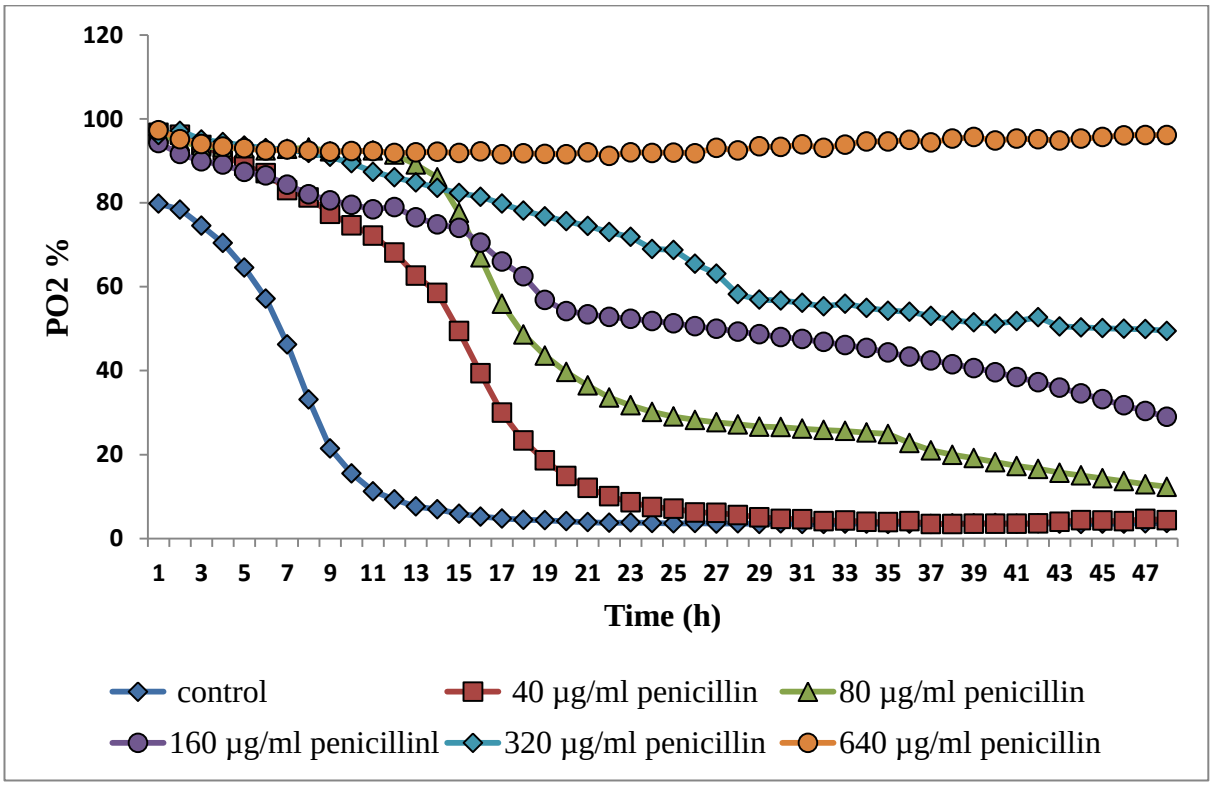

Figure 4-23. Oxygen consumption of *S. coelicolor 1147* grown in modified YEME medium treated with different concentrations of penicillin G up to 640 µg/ml. Values obtained are the mean of triplicate samples. The penicillin G concentration is indicated in the key.

The effects of different concentration of penicillin G on the biomass during the four different dilution rate fermentations are shown on Figure 4.24. There was a decrease in the biomass when the four fermentations were fed with 80 µg/ml. the biomass was diverge with the other concentrations of penicillin G.

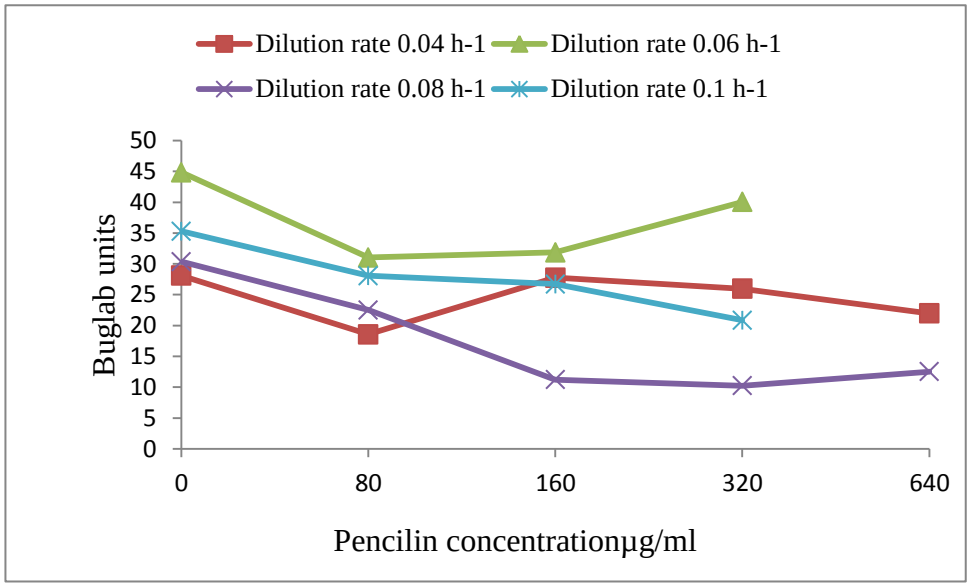

Figure 4-24. The effect of different concentration of penicillin G (80, 160, 320 and 640 µg/ml) on the biomass in continues fermentation at different dilution rates.

The results obtained from three fermentations (0.04, 0.06 and 0.08 h^{-1}) signify that there is a correlation between the growth rates of *S. coelicolor 1147* and the resistant to penicillin G, as illustrated in Figure 4.25. Additionally, the result indicated that the cells became more resistant to penicillin G as the length of exposure to penicillin G and the concentration was increased.

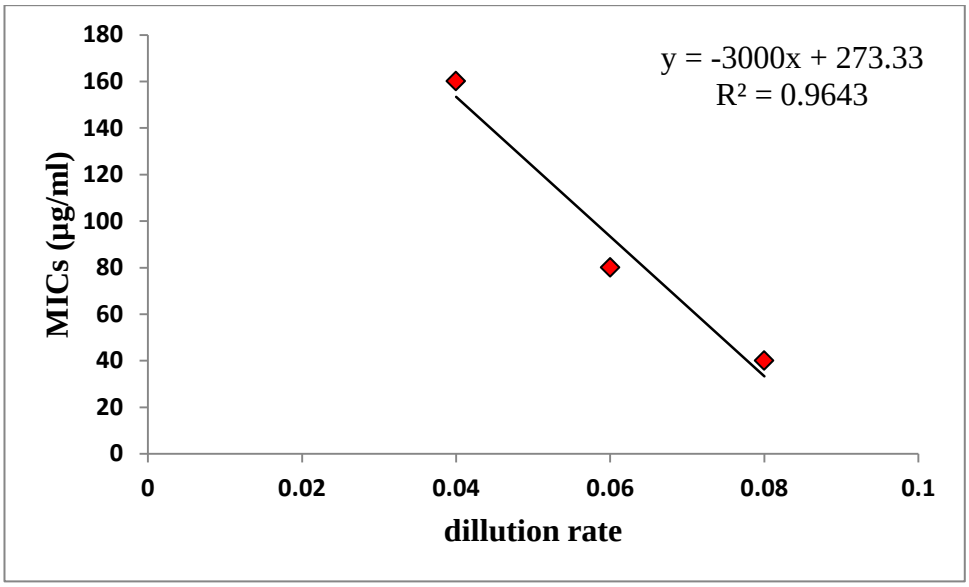

Figure 4-25. Correlation between the growth rate of *S. coelicolor 1147* and the resistance to penicillin G. *S. coelicolor 1147 grown* in modified YEME medium in continues culture at different dilution rates (0.04, 0.06 and 0. 08 h^{-1}) with different concentrations of penicillin G.

Chapter Five

5. DISCUSSION

5.1 Isolation and morphological characteristics of *Streptomyces*

Streptomyces are soil bacteria responsible for producing over half of the world's naturally produced antibiotics. Our results show that different species of *Streptomyces* were isolated from environmental soil samples. The morphological study of these strains in liquid and solid media showed that they were Gram–positive, slow-growing, aerobic, and contain both aerial and substrate mycelia with a variety of colours, which indicates that they belong to the *Streptomyces* genus. Previous studies of morphology of strains of *Streptomyces* showed similar branching patterns as seen in the results of this study (Tresner *et al.* 1967). Some strains have exhibited the pigment formation; few of them have shown the formation of melanin. The identification of strains regard to the partial 16S rRNA gene sequence of the isolated strains showed that some strains had high homology (>96%) with the various *Streptomyces* species recorded in the GenBank database. The lack of high similarity of the other strains to other *streptomycetes.sp* is probably related to the well-recognized divergence in *Actinomycetes* between whole genome DNA–DNA homologies and similarity to 16S rRNA (Ward and Goodfellow 2004). Or It might be they are a novel strains and they need more investigation studies.

16S rRNA gene sequences are not always sufficient to discriminate between closely related species (Girard *et al.* 2013). It has been established that the 16S rRNA gene may be not appropriate for *S. coelicolor 1147* because *S. coelicolor 1147*A3 has many 16S rRNA genes in the genome (Bentley, 2002), and the measured transcripts of 16S rRNA is the amount of all homologs; and also the transcript abundance of 16S rRNA is usually much higher than that of the target genes (Vandesompele *et al.* 2002), which makes it difficult to subtract the baseline value accurately during data analysis.

The isolates varied in their MICs and MBCs to penicillin G. The MICs were different when they were cultured in liquid and on solid media. The deference of the MICs might be due to the composition of the culture medium and the incubation time. The growth of these organisms on solid media could also be considered as a biofilm. It is well known that biofilms are more resistant to antimicrobials than their planktonic counterparts

(Lamber, 2002) Also, the cultivation on solid media and liquid media might lead to production of different secondary metabolites (Robinson *et al.* 2001).

Minimum inhibitory concentration of penicillin G for *Streptomyces* strains in liquid medium were conducted using the OxoPlate® technique, all strains have an MIC in the range between 1-100 µg/ml. This was within the range of MICs of penicillin G for some other streptomycetes (Ogawara 1975).

Streptomyces species are one of the largest groups of organisms in soil and produce a great many antibiotics, including penicillins and cephalosporins (Nagarajan 1972). In the present study, we classified the isolated strains according to the production of β - lactamases, and their relation to resistance to penicillin G. β–lactamase enzyme activity of the isolated strains was tested using a nitrocefin based assay, the β-lactamases essentially hydrolyse the endocyclic amide bond of the β-lactam ring, resulting in inactive antibiotics.

51 % of the isolated strains do not produce active β-lactamase enzymes. On the other hand 48 % were β-lactamase producer's strains. This is a little surprising, since β - lactamases in many Gram-positive bacteria are known to be inducible not constitutive. The high percentage of β-lactamase producer's strains might be due to selection in the natural environment, where penicillin and cephalosporin may be produced by fungi and *Streptomyces*. However, our results indicate that the sensitivity of *Streptomyces* strains to penicillin G is not directly related to β-lactamase production.

5.2 16S rRNA gene and phylogenetic analysis

There was no association between the position of the strains on the phylogenetic tree and their beta-lactamase activity. Beta-lactamase and non-beta-lactamase producing strains raises to the same ancestral origin. 16S rRNA gene sequence and phylogenetic relationship of strain (W43) *Streptomyces violaceoruber BUCBT-23* and related members of the genus *Streptomyces*, revealed that the strain was clustered with (W76) *Streptomyces lividans strain YLA0*. 16S rRNA gene sequences are a powerful tools to infer inter- and intragenic relationships, but are too short to be useful in inferring the phylogenetic relationships between closely related organisms and among strains belonging to a species because of the evolutionary conservation of 16S rRNA (Stackebrandt *et al.* 1997).

5.3 Visualization of penicillin binding proteins of *Streptomyces*

β-Lactam antibiotics are bactericidal antibiotics and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. It is generally considered that penicillin interferes with cell wall biosynthesis, causes a mechanically weakened cell wall to be generated resulting in cell death and lysis. The effect of penicillin in the cell wall of *Streptomyces* strains was determined on each of; beta lactamase producing *Streptomyces sp.*, non-beta lactamase producing *Streptomyces sp.* and mutants defective in class A HMW PBPs. The bifunctional class A HMW PBPs act as both transpeptidases and transglycosylases (Ghuysen 1991).

PBPs are usually identified by tagging bacterial membrane preparations with ^{3}H-, ^{14}C-, or ^{125}I-labeled penicillin G and then separating the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and imaging the gels using X-ray films (Preston *et al.* 1990). In this study, we used a rapid and non-radioactive technique using BOCILLIN FL, using a commercially available fluorescent penicillin, as a labelling reagent (Molecular Probes, Inc., Eugene, Oreg.). The BOCILLIN FL-labelled PBPs were then viewed in *vivo* under a fluorescent microscope.

We examined the PBPs of vegetative mycelium as well as those of the spores. Our result showed that Bocillin fluorescent stain in β-lactamase producing strains were stained all aerial mycelium, whereas in the non β-lactamase producing strains they were located in only some parts of the cell membrane of *Streptomyces*. The difference of the distribution of PBPs in beta lactamase producer strains and non-beta lactamase producer strains might be because in β-lactamase producing strains the Bocillin fluorescent stain both beta lactamase enzymes and PBPs.

In Gram-negative bacteria, β-lactamases are periplasmic and act in combination with altered outer membrane permeability (Nikaido and Normark 1987). In Gram-positive bacteria, they are exocellular, although they are probably associated with the cell wall through electrostatic interactions (Sara and Sleytr 1987). The PBP was presented in the early log phase of the life cycle as well as in the spores. Interestingly, the location of PBP in both β-lactamase producer strains and in non β-lactamase producer strains was at the poles of the spores.

Our results suggest that PBP present in early stage of the life cycle of these strains may be reflecting certain stages of the life cycle. The appearance of the PBP in the spore may be associated with the sporulation process. In bacterial strains like *Bacillus subtilis* and some *Streptomyces* strains, PBP patterns are dependent on the stage of the growth cycle (Buchanan and Sowell 1983).

S. coelicolor 1147 actively grows by cell wall extension at hyphal tips. One of the essential proteins involved in hyphal tip growth is DivIVA (Flardh 2003). Similar to *E coli*, fluorescence microscopy showed that the PBP is recruited to the division site in isolated *Streptomyces* strains. The high-molecular-weight PBP 2B is one of the few division proteins, which catalyse the final stages of peptidoglycan synthesis (Ghuysen 1991). PBP 2B seems to be specific for the formation of septal peptidoglycan because; in *E. coli* at least, pbpB mutations prevent septation but not continued synthesis of the cylindrical part of the cell wall (Spratt 1977, Pogliano *et al.* 1997).

Bocillin stained the four mutants (δ2897), (δ3580), (δ5039) and mutant (δ3901), which indicated that none of them is the PBP was presented in vegetative mycelium and the spores.

It has been shown that *Bacillus subtilis* has more than one class A PBP, and many PBPs of the same class within a species often display redundant function (McPherson *et al.* 2001). According to its complex life cycle *S. coelicolor* has been shown to have highest number of penicillin binding proteins.

5.4 Fermentations studies of *Streptomyces coelicolor 1147*

Streptomyces coelicolor 1147 is a filamentous bacterium displaying a complicated range of morphologies when grown in liquid culture. This study was designed to investigate the relationship between antibiotic exposure and resistance to penicillin G in *Streptomyces coelicolor*. Our results reported in chapter four showed the successful use of continuous culture to study the effect of prolonged exposure of *Streptomyces coelicolor 1147* to the antibiotic penicillin G and the correlation between the growth rate and resistant to the β-lactam antibiotic .

The quantification of small cell amounts of filamentous microorganisms such as *Streptomyces coelicolor*, is methodologically limited (Hopwood 2006). Optical

density is usually used to determine bacterial density, nonetheless it is limited to unicellular microorganisms (Dalgaard *et al.* 1994). Commonly, evaluation of growth of filamentous microorganisms is determined by dry-weight (Mallette 1969). However, it remains a challenge to quantify small cell amounts of *Streptomyces* under laboratory conditions. A recent study showed the utility of methylene blue to visualize small differences in the amount of *S. coelicolor 1147* mycelium growing in minimal medium (Fischer *et al.* 2012). Measuring the growth of *Streptomyces* and other filamentous microorganisms by methylene blue adsorption-desorption (Fischer and Sawers 2013)

Some quantifying methods have been studied for on-line measurement of biomass in filamentous microorganism like the on-line monitoring of biomass concentration in mycelial fed-batch cultivations of *Streptomyces clavuligerus* with an *in-situ* capacitance probe fitted to an industrial pilot-plant tank (Neves *et al.* 2001) this technology is very expensive and does not lend itself to everyday research. In this study, we used a new non-invasive optical sensor (Bug Eye® 100, Debreczeny 2012) for the real time monitoring of biomass of *Streptomyces coelicolor*. The study shows an optical device that can be used successfully to monitor the progress of a filamentous microorganism in fermentation even at low concentrations of cells.

Our method for growing cells in a dispersed state is relatively simple and gentle. It is probable that the uses of spore inoculate and at the same time the agitation of 1000 rpm with two Rushton impellers avoids the formation of pellets. Microscopic observation indicated that these filaments were spread out and there was no evidence of lysed cells.

A batch experiment was conducted to examine outlines of growth of *Streptomyces coelicolor 1147* in YEME medium. The study revealed that there is no significant increase in the biomass concentration of *Streptomyces coelicolor 1147*cells during the lag phase. This is might be as a result of the adaptation of *Streptomyces coelicolor 1147*cells to the new medium and also, it is the time for spores to germinate. The biomass levels increased rapidly during the log phase and was complemented by the decline of dissolved oxygen and the increase of CO_2 production (Figure 4.2). There was a second biomass accumulation phase evident, and the level of glucose did not decline to zero during the active phase. The amount of glucose consumed by the organism was 1.5 g/L, yet biomass levels reached 2. 99 g/L, suggesting that glucose was not the only carbon source being utilized (Figure 4.3).

Since the medium used contains nitrogenous compounds and maltose (YEME), this might be due to the switching of the organism to other sources of carbon, which in this case could be the maltose from malt extract and also it is possible that organism used amino acids rather than glucose. This was in agreements with previous studies on actinomycetes, where amino acids often function as the primary carbon source even when simple sugars are present, and also it has proven that glucose is not always the preferred carbon source (Karandikar *et al.* 1997, Hoskisson *et al.* 2003). In *Amycolatopsis mediterranei* specific amino acids were found to function as the unique carbon source (Bapat *et al.* 2006).

The production of undecylprodigiosin is present during the early days of incubation and the colour of the medium changed slightly red as a result of the production. The production of undecylprodigiosin and actinorhodin by *Streptomyces coelicolor 1147* is considerably affected by the growth rate (Kang *et al.* 1998). The growth rate of *Streptomyces coelicolor 1147* in batch culture was 0.1825 h^{-1} and the doubling time is 3. 79 h.

Continuous fermentations were performed to investigate the relationship between growth rate and resistance to penicillin G in *Streptomyces coelicolor 1147* using four dilution rates. The result obtained from the four dilution rates of this study (Figure 4.25) showed that *S. coelicolor 1147* has the ability to adapt to increasing concentrations of β-lactam antibiotic up to 640 µg/ml. The effect of different concentration of penicillin G on the biomass during the four different dilution rates indicated that a decrease in the biomass when the four fermentations were fed with 80 µg/ml. There was no change in the morphology of colonies and the MICs with the increasing concentrations of penicillin G up to 160 µg/ml in all dilution rates. The data indicated that the cells became more resistant to the antibiotic as the length of exposure to penicillin G and the concentration was increased.

At dilution rates 0.04 h^{-1} and 0.06 h^{-1} (generation time 19.39 and 29. 09, respectively) the organism lost the ability to form spores at concentration of 320 µg/ml of penicillin G. On the other hand, it did not lose the ability to form aerial mycelium and the ability to form spores returned after 4 subcultures on MS medium Figure 4-10. The possible explanation for the return of the ability to form spores when sub-cultured without antibiotic is that the loss of the ability to form spores is associated with the exposure to

the antibiotic. This return to the sporulating phenotype on subculture certainly suggests that the loss of sporulation was not due to a mutation.

FtsZ mutants in *S. coelicolor* were able to produce aerial hyphae, but failed to convert aerial hyphae into chains of spores (McCormick *et al.* 1994).

At dilution rate 0.08 h^{-1} and generation time 42.26 the organism did not lose the ability to form spores, but images taken from the electron microscopy revealed irregular spores and a difference in spore size was also seen between wild type and cultures treated with penicillin G.

Viability investigations were carried out using the fluorescent bacterial viability stain *Bac*Light™. Images showed that cells have the ability to grow at high concentration of the antibiotic in all dilution rates and the effect of antibiotic was observed not just at the hyphal tips even with high concentration of penicillin G.

Hyphal extension in *Streptomyces coelicolor 1147A3 (2)* was shown to occur by addition of newly synthesized wall material in an apical extension zone(GRAY *et al.* 1990). The images taken in this study would suggest that wall growth was not limited to tip regions and that these tips are not the primary site of action of penicillin G.

The dissolved oxygen concentration was initially set at 100% saturation in all fermentations. However, when biomass concentration increased, the dissolved oxygen concentration dropped to 60% saturation at the lowest two dilution rates in this study 0.04 and 0.06 h^{-1}. Furthermore, at the highest two dilution rate of this study the dissolved oxygen concentration dropped to less than 2% saturation. *S. coelicolor 1147*is able to survive in the absence of oxygen for long periods of time (van Keulen *et al.* 2007). This current study would suggest that *S. coelicolor* can actually grow at low oxygen tensions.

Streptomyces coelicolor 1147 is an aerobic bacterium that needs a sufficient supply of oxygen during fermentation. A shake flask usually has sufficient volume of air for growth by using a shaking incubator to generate turbulence inside the flasks in order to increase the dissolved oxygen concentration; however, it is the concentration of the dissolved oxygen in the fermentation medium that matters (Donovan *et al.* 1995).Thus in the experiments carried out in the chemostat, sterile air was introduced into the medium to increase the rate of delivery of oxygen to the organism.

The result obtained from the growth at dilution rates 0.04, 0.06 and0.08 h^{-1} indicates that there is a correlation between the growth rate of *S. coelicolor 1147* and the resistance to penicillin G. Additionally, if the similarity in the culture conditions such as pH, temperature, inoculum, agitation and aeration in all fermentations is taken into consideration, under the conditions used, it can be concluded that the environmental conditions are not the reason for the change observed in the MICs and MBCs in *Streptomyces coelicolor.*

Chapter six

6.1 Conclusions and Future Work

The overall aim of this work was to investigate the mechanisms that underpin the resistance of *Streptomyces* species to β-lactam antibiotics. Our investigations were performed using three different techniques; Oxoplate, fluorescent microscope and continuous culture. In view of the results obtained in this work, the following conclusions can be drawn:

1. **Isolation and morphological characteristics of *Streptomyces*.** The results presented in this study indicate that ninety six isolates of actinomycetes were isolated from an environmental soil samples. The microscopic examination of the colony of the isolates revealed that aerial mycelia were morphologically branched with grey and white surface and some strains formed spores.

2. **Identification of the actinomycetes.** The results obtained from the partial 16S rRNA sequencing of purified PCR products showed that all the actinomycetes isolates belong to the genus *Streptomyces*.

3. **Determine the minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of penicillin G for isolated *Streptomyces* strains.** The isolates were varied in their MICs and MBCs to penicillin G. The MICs were different when they were cultured in liquid and on solid media. In MH broth medium the minimum inhibitory concentrations of all strains ranged from 1 - 100 µg/ml.

4. **Study the association between the growth rates and resistant in *Streptomyces sp.*** Our result indicates that there is no correlation between the MICs of penicillin G and the growth rates in the isolated strain.

5. **Identified β-lactamase production in the isolated *Streptomyces* strains and there relation to the MICs.** 51 % of the isolated strains do not produce active β-lactamase enzymes. The sensitivity of isolated *Streptomyces* strains to penicillin G is not directly related to β-lactamase production.

6. **The phylogenetic relationship between beta-lactamase producing and non-beta-lactamase producing strains**. There was no association between the position of the strains on the phylogenetic tree and their beta-lactamase activity. Beta-lactamase and non- lactamase producing strains refers to the same ancestral origin. The comparative analysis of 16S rRNA gene sequence and phylogenetic relationship of strain (W43) revealed that the isolate formed a separate phyletic line and clustered with (W76) *Streptomyces lividans strain YLA0.*

7. **Investigate the interaction between penicillin G and PBPs in isolated *Streptomyces* species.** Bocillin staining in β-lactamase producing strains showed staining throughout the mycelia. Non β-lactamase producing strains revealed staining in only certain parts of the mycelia. PBPs were located at poles of the spores.
8. **The use of Bug-Lab for monitoring the growth of a filamentous microorganism in continues culture**. It can be said that, our strategy of using the Bug-Lab for monitoring the progress of *S. coelicolor 1147* in fermentation even at low concentrations of cells in real time was successful.
9. ***Streptomyces coelicolor 1147* has the ability to grow at high concentration of penicillin G.** Viability investigations were carried out using the fluorescent bacterial viability stain *Bac*Light™. The images showed that cells have the ability to grow at high concentration of the antibiotic and the effect of antibiotic was observed not just at the hyphal tips even with high concentration of penicillin G.
10. **Investigate the relationship between growth rate and penicillin G resistance in *Streptomyces in vitro.*** There was a correlation between the growth rates of *S. coelicolor 1147* grown in modified YEME media in continues culture with the resistant to penicillin G. *S. Coelicolor 1147* was more sensitive to penicillin G at high dilution rate.

On the other hand, the findings of this project highlight our lack of understanding of the mechanism of resistance to antibiotics and therefore provide the following suggestions for the future work.

6.2 Further work

1. One of the findings of this study is that Bocillin fluorescent bind to PBPs in the cell membrane of *Streptomyces* and also stain the pole of the spores. We have eliminated some of high molecular weight of PBPs. Future research should concentrate on identifying the type of PBP that bind to Bocillin in *Streptomyces* and their functional in resistant.

2. One of the results obtained in this thesis indicate that the sensitivity of isolated *Streptomyces* strains of penicillin G is not directly related to β-lactamase production. Therefore, further experimental investigations are needed to elucidate the expression and the function of the β-lactamase genes in *Streptomyces sp*. This would help us to find β-lactamase enzymes that bind to the cell membrane and its role in cell wall metabolism of *Streptomyces*. The use of analytical methods such as DNA microarrays would be useful in this study.

3. One of the findings of this study is that *Streptomyces coelicolor 1147*can grow at low concentration of oxygen when grown in continuous culture at different dilution rates under the conditions used in this study. *Streptomyces sp* are considered to be strict aerobes, yet this study does not support this. Further work on the physiology of these bacteria at low oxygen tensions is required.

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